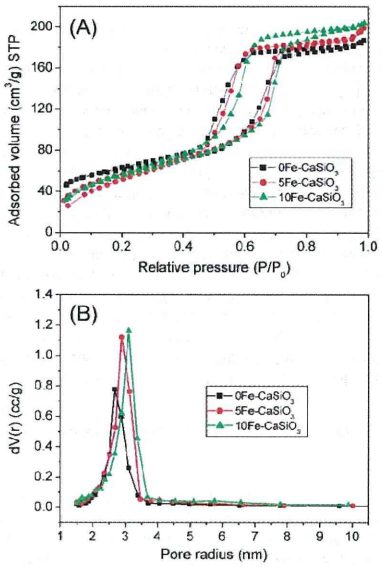


**Figure 4.** $N_2$ adsorption-desorption isotherms (A) and the corresponding pore size distributions (B) of mesoporous Fe-$CaSiO_3$ materials.

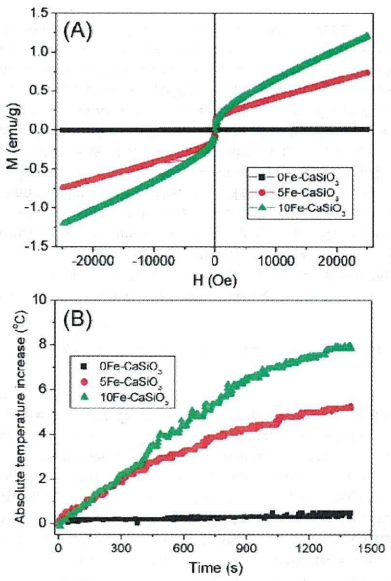


**Figure 5.** (A) Magnetization curves as a function of the applied magnetic field for different mesoporous Fe-$CaSiO_3$ materials at room temperature; (B) magnetic heating curves of different mesoporous Fe-$CaSiO_3$ materials in an alternating magnetic field.

observed that the apatite layer exhibited a small decrease in thickness for mesoporous Fe–$CaSiO_3$ materials with increasing Fe substitution, suggesting a small decrease in the apatite-formation rate. Generally, the release of Ca ions from a biomaterial accelerates the apatite formation due to the increase of Ca concentration in the SBF solution. In this study, the Ca ions were released from the 0Fe–$CaSiO_3$, 5Fe–$CaSiO_3$ and 10Fe–$CaSiO_3$ materials, but the Ca ions release rates showed small decrease with increase in the Fe substitution in mesoporous $CaSiO_3$ materials, which contributed to small decrease in the apatite-formation rate for mesoporous Fe–$CaSiO_3$ materials with increase in the Fe substitution.

### *3.4. Cytotoxicity evaluation of mesoporous Fe–$CaSiO_3$ materials*

To evaluate the cytotoxicity of mesoporous Fe–$CaSiO_3$ materials, the elution cell culture assay (also known as extract dilution) was used in this study. The cytotoxic effect of mesoporous Fe–$CaSiO_3$ extracts on osteoblast-like MC3T3-E1 cells is shown in figure 8. It can be seen that there were no cytotoxic effects on MC3T3-E1 cells for mesoporous Fe–$CaSiO_3$ materials from a low extract concentration (6.25 mg $ml^{-1}$) to a high extract concentration (50 mg $ml^{-1}$) after 7 days. The 5Fe–$CaSiO_3$ and 10Fe–$CaSiO_3$ extracts had comparable cell viability compared to the 0Fe–$CaSiO_3$ extracts. However, the extracts of mesoporous Fe–$CaSiO_3$ materials with concentrations from 6.25 to 25 mg $ml^{-1}$ improved the proliferation of MC3T3-E1 cells compared to the blank control. The corresponding ion concentrations of mesoporous Fe–$CaSiO_3$ extracts for cell culture are listed in table 3. The Ca, Si and Fe ions were released from mesoporous Fe–$CaSiO_3$ materials in culture medium. The Ca and Si ion concentrations respectively ranged from 72.7 to 96.3 mg $ml^{-1}$ and 4.9 to 63.1 mg $ml^{-1}$, while the Fe ion concentrations were lower than 2.8 mg $ml^{-1}$. Previous studies showed that high concentrations of Ca, Si and Fe ions can result in excessive cytotoxicity [25, 50]. In this study, the results indicated that the 0Fe–$CaSiO_3$, 5Fe–$CaSiO_3$ and 10Fe–$CaSiO_3$ extracts were not cytotoxic to MC3T3-E1 cells and could induce osteoblast activity, suggesting that the released Ca, Si and Fe ions from mesoporous Fe–$CaSiO_3$ materials are in the feasible levels and are very useful for bone regeneration.

### *3.5. Gentamicin release from mesoporous Fe–$CaSiO_3$ materials*

Besides the magnetic heating ability and bioactivity, mesoporous Fe–$CaSiO_3$ materials can also efficiently load drugs, such as antibiotics, for local drug delivery, which is very useful for bone regeneration, because implantation always leads to inflammatory responses and, quite often, to infections [51]. In this study, gentamicin, an antibiotic, was used as a model drug to investigate the drug loading and release behavior of mesoporous Fe–$CaSiO_3$ materials. The gentamicin-loading capacities of the 0Fe–$CaSiO_3$, 5Fe–$CaSiO_3$ and 10Fe–$CaSiO_3$ materials were estimated at 155, 125 and 124 mg $g^{-1}$, respectively. Figure 9 shows the accumulative gentamicin release from mesoporous Fe–$CaSiO_3$ materials in SBF at 37 °C. It can be seen that gentamicin in mesoporous Fe–$CaSiO_3$ materials revealed a sustained release in the SBF solution. The 0Fe–$CaSiO_3$, 5Fe–$CaSiO_3$ and 10Fe–$CaSiO_3$ materials exhibited a similar release behavior throughout the whole study period, with an initial fast release followed by a relatively slow release. Furthermore, the gentamicin release rates from the 0Fe–$CaSiO_3$, 5Fe–$CaSiO_3$ and 10Fe–$CaSiO_3$ materials were close to each other, and the accumulative release of gentamicin was around 85% after 1 week. It suggested that mesoporous Fe–$CaSiO_3$ materials maintained the sustained drug delivery property, and the substitution of Fe for Ca in mesoporous $CaSiO_3$ materials did not change the drug release kinetics.

In this study, the mesopore sizes of the 0Fe–$CaSiO_3$, 5Fe–$CaSiO_3$ and 10Fe–$CaSiO_3$ materials were between 5.3 and 6.2 nm, which are much larger than the size of a gentamicin molecule (0.52 × 1.53 nm) [52], indicating that gentamicin molecules could be loaded in the mesoporous channels. Furthermore, studies demonstrated that silicate-based biomaterials can easily form Si–OH groups on the surface of materials, allowing interacting with gentamicin molecules by hydrogen bonding [53]. On the other hand, the gentamicin release was determined by the mesoporous structure and the interaction between gentamicin and the surface of mesoporous Fe–$CaSiO_3$ materials. The 0Fe–$CaSiO_3$, 5Fe–$CaSiO_3$ and 10Fe–$CaSiO_3$ materials exhibited similar mesoporous structure and surface characteristics, which result in similar gentamicin release kinetics. Therefore, the mesoporous structure of mesoporous Fe–$CaSiO_3$ materials and the Si–OH groups on their surface help to adsorb gentamicin and benefit the sustained release, which indicates their potential as a local drug delivery system for bone tissue regeneration.





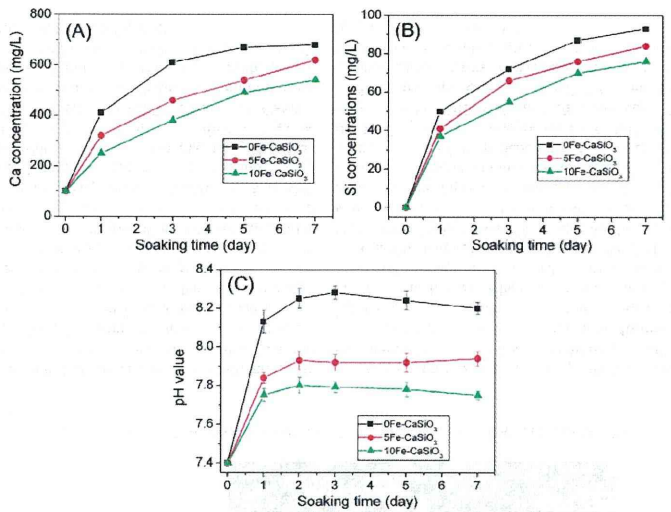


**Figure 6.** Ca (A) and Si (B) ion concentrations in SBF solutions and pH values (C) of SBF solutions after soaking mesoporous Fe-$CaSiO_3$ materials for various time periods.

**Table 3.** The Ca, Si and Fe ion concentrations of mesoporous Fe–$CaSiO_3$ extracts (mg $l^{-1}$).

| | | Extracts concentrations (mg $ml^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| Materials | Ions | Blank | 6.25 | 12.5 | 25 | 50 |
| 0Fe–$CaSiO_3$ | Ca | 72.6 | 73.5 | 78.9 | 86.8 | 96.3 |
| | Si | 0 | 7.8 | 15.8 | 32.0 | 63.1 |
| | Fe | 0 | 0 | 0 | 0 | 0 |
| 5Fe–$CaSiO_3$ | Ca | 72.6 | 73.9 | 76.8 | 80.6 | 89.7 |
| | Si | 0 | 6.2 | 12.2 | 24.5 | 48.6 |
| | Fe | 0 | <1 | <1 | 1.2 | 2.1 |
| 10Fe–$CaSiO_3$ | Ca | 72.6 | 72.7 | 74.5 | 76.4 | 81.1 |
| | Si | 0 | 4.9 | 10.1 | 20.4 | 40.5 |
| | Fe | 0 | <1 | <1 | 1.5 | 2.8 |

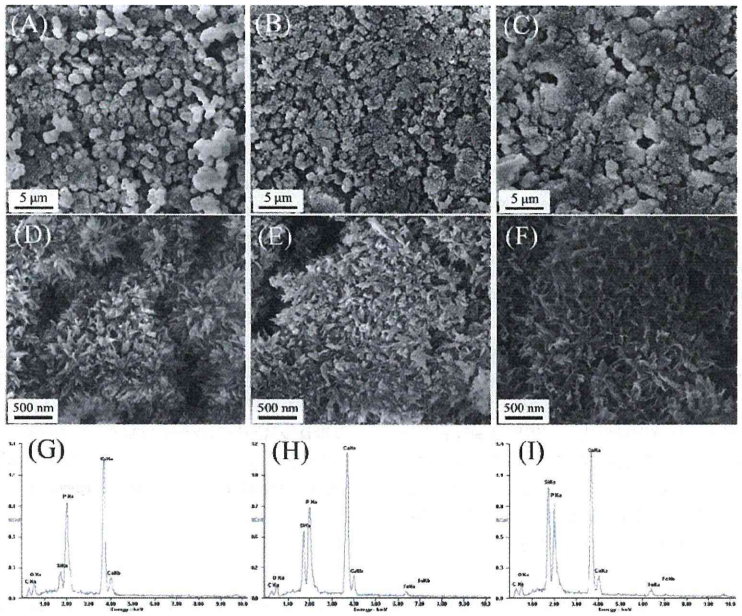


**Figure 7.** SEM images and the corresponding EDS analysis of mesoporous Fe-$CaSiO_3$ materials after soaking in SBF solutions for 3 days ((A), (D) and (G): 0Fe-$CaSiO_3$; (B), (E) and (H): 5Fe-$CaSiO_3$; (C), (F) and (I): 10Fe-$CaSiO_3$).

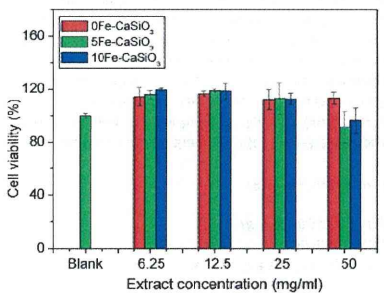


**Figure 8.** The cytotoxic effect of mesoporous Fe-$CaSiO_3$ extracts on MC3T3-E1 cells evaluated by WST-8 assay.

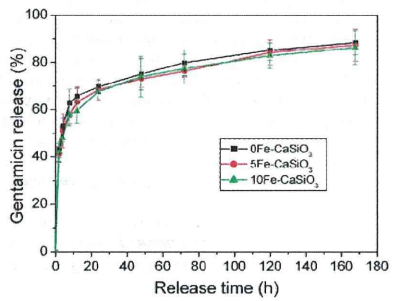


**Figure 9.** Gentamicin release profiles from mesoporous Fe-$CaSiO_3$ materials with different Fe substitution in SBF solution.

## 4. Conclusions

Multifunctional magnetic mesoporous Fe–$CaSiO_3$ materials have been prepared using P123 as a structure-directing agent. The substitution of Fe for Ca in mesoporous $CaSiO_3$ materials did not change their mesoporous structure, but endowed them with magnetic property. The results indicated that mesoporous Fe–$CaSiO_3$ materials exhibited good bioactivity and sustained drug delivery property. Furthermore, magnetic mesoporous Fe–$CaSiO_3$ materials could generate heat in an alternating magnetic field for potential hyperthermia application. Therefore, magnetic mesoporous Fe–$CaSiO_3$ materials have potential for the regeneration of bone defects caused by bone tumors with local drug delivery and magnetic hyperthermia therapy. Further studies will be conducted to investigate how to fabricate mesoporous Fe–$CaSiO_3$ scaffolds with multifunctionality for bone regeneration.





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—Note—

# 乳幼児が誤飲する可能性のある金属製アクセサリーからの有害8元素の溶出

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# Migration of Eight Harmful Elements from Metal Accessories That Infants May Swallow by Mistake

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The International Standard ISO 8124-3:2010 "Safety of toys—Part 3: Migration of certain elements" controls the levels of migrated eight harmful elements (antimony, arsenic, barium, cadmium, chromium, lead, mercury and selenium) from infants toys. Moreover, the Japanese Food Sanitation Law controls the levels of migrated lead from metal accessory toys. However, the levels of migrated harmful elements from metal accessories that are not infants toys are not controlled, since they are not covered by the ISO Standard or the Food Sanitation Law. Therefore, we investigated the level of eight harmful elements migrated from metal accessories that infants may swallow by mistake. The extraction test of ISO 8124-3:2010 was executed in 117 products (total 184 specimens), and the concentration of these eight elements was measured by inductively coupled plasma mass spectroscopy (ICP-MS). As a result, 28 and one products released lead and cadmium beyond the maximum acceptable levels of the ISO standard, respectively. Metal accessories that infants may swallow by mistake should ideally not release harmful elements such as lead and cadmium.

**Key words**——lead; cadmium; metal accessory; ISO 8124-3:2010; inductively coupled plasma mass spectroscopy (ICP-MS)

## 緒　　言

国際標準化機構（International Organization for Standardization, ISO）は，玩具安全規格（ISO 8124-3:2010）を制定し，6歳以下の幼児用玩具を対象として，玩具材料毎にアンチモン，ヒ素，バリウム，カドミウム，クロム，鉛，水銀及びセレンの溶出限度値を定めている（Table 1）.[1] また，わが国の食品衛生法のおもちゃの規格基準では，金属製アクセサリー玩具のうち，乳幼児が飲み込むおそれがあるものについて，鉛の溶出量は90 μg/g以下でなければならないと規定している．しかしながら，乳幼児が飲み込むおそれがある大きさでも，金属製アクセサリー玩具に該当しない金属製アクセサリー等には，鉛その他の有害元素の溶出量に係る基準はない．

平成18年に米国で起きた鉛を高濃度含有するブレスレットを誤飲した幼児が鉛中毒で死亡した事故を受けて，平成18年及び平成19年に金属製アクセサリー類等及びアクセサリー類を除く金属製品を対象にカドミウム及び鉛の含有量及び溶出量が調査された.[2-5] その結果，一部の製品から一定量を超える鉛の溶出が確認されたため，厚生労働省は注意喚起等を行った.[6,7] ところが，再び米国で一部の子供用金属製アクセサリーからカドミウムが溶出することが確認され，平成22年及び平成23年に消費者庁及び国民生活センターが輸入された子供用金属製アクセサリーを対象にカドミウム及び鉛の溶出量を調

Table 1. Maximum Acceptable Element Migration from Toy Materials Required by ISO 8124-3:2010

| Toy material | Element (mg/kg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Sb | As | Ba | Cd | Cr | Pb | Hg | Se |
| Any toy material except modelling clay and finger paint | 60 | 25 | 1000 | 75 | 60 | 90 | 60 | 500 |
| Modelling clay and finger paint | 60 | 25 | 250 | 50 | 25 | 90 | 25 | 500 |

The authors declare no conflict of interest.

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査した．その結果，一部の製品から一定量を超える鉛の溶出が認められ,[8,9] 消費者庁及び国民生活センターは注意喚起を行った.[10-13] また，平成22年度家庭用品等に係る健康被害病院モニター報告によると，小児の誤飲事故のうち，約5%が金属製品によるものであった.[14] さらに，東京都のヒヤリ・ハット調査でも，金属製と推察されるアクセサリー（ヘアピン，指輪など）及び家庭用品（携帯ストラップ，キーホルダーなど）を乳幼児が「誤飲しそうになった」又は「誤飲した」という回答が報告されている.[15] 鉛その他の有害元素を含有した製品を乳幼児が誤飲した場合，健康被害を起こす可能性がある．そこで，乳幼児が誤飲する可能性のある金属製アクセサリー等を対象に，ISO玩具安全規格で規制されている有害8元素（アンチモン，ヒ素，バリウム，カドミウム，クロム，鉛，水銀及びセレン）の溶出量について，フォローアップ調査を実施した．

## 方　　法

**1. 試料**　平成23年8月から11月までに，東京都内の複数の小売店で，乳幼児が誤飲する可能性のある大きさの金属製アクセサリー等117製品を購入した（Table 2）．製品は千円以下で購入可能な安価なものを選び，購入に際して製造国は考慮しなかった．製品の表示から，中国製43製品，韓国製15製品，日本製8製品及び不明51製品であった．また，乳幼児が誤飲する可能性のある大きさは，食品衛生法に基づく「食品，添加物等の規格基準　第4おもちや」で規定する寸法を持つ容器内に圧縮しない状態で置いたときに収まるものとした.[16] 製品又は容易に分離可能な部品を検体とした（計184検体）．

Table 2. Number of Products and Specimens by the Commodity Classification

| Commodity classification | Number of products | Number of specimens |
|---|---|---|
| Straps | 30 | 65 |
| Accessory parts | 21 | 22 |
| Hairpins | 20 | 20 |
| Necklaces | 17 | 42 |
| Charms | 9 | 9 |
| Rings | 6 | 6 |
| Fastener straps | 4 | 4 |
| Bracelets | 3 | 8 |
| Earrings | 3 | 3 |
| Cufflinks | 2 | 2 |
| Pin badges | 1 | 2 |
| Buttons | 1 | 1 |
| Total | 117 | 184 |

**2. 試薬**　有害金属測定用塩酸（和光純薬工業株式会社）を，純水製造装置 Elix UV 5（日本ミリポア株式会社）及び超純水製造装置 Milli-Q Synthesis A10（日本ミリポア株式会社）を用いて製造した超純水で希釈し，溶出試験用の0.07 mol/L塩酸を調製した．アンチモン，ヒ素，バリウム，カドミウム，クロム，鉛，水銀及びセレンの各1000 mg/L標準液（和光純薬工業株式会社）並びに0.07 mol/L塩酸を用いて，当該8元素の10 mg/L混合溶液を調製した．この混合溶液を0.07 mol/L塩酸で段階希釈し，検量線作成用の混合標準液を調製した．イットリウム及びタリウムの1 μg/L溶液（アジレント・テクノロジー株式会社）を内標準溶液とした．

**3. 装置及び測定条件**　誘導結合プラズマ質量分析（ICP-MS）には，Agilent 7500ce ORS ICP-MS（アジレント・テクノロジー株式会社）を使用した．

ICP-MSの測定条件は，高周波出力：1500 W，プラズマガス：Ar 15 L/min，キャリアガス：Ar 0.7 L/min，メイクアップガス：Ar 0.33 L/min，コリジョンガス：He 5 mL/min，サンプリング位置：7.8 mm，スプレーチャンバー温度：2℃，積分時間：0.1 s/element（セレンを除く7元素）及び1 s/element（セレン），測定回数：3 timesとした．測定元素及び内標準元素並びにそれらの測定質量数（$m/z$）をTable 3に示した．

**4. 試験溶液の調製**　検体の質量を量り，内径約40 mmのポリプロピレン製容器に入れ，37℃に加温した溶出試験用0.07 mol/L塩酸を試料が浸漬するまで加えて蓋をし，遮光して37℃で2時間放置した後，ポアサイズ0.45 μmのメンブレンフィルター（ザルトリウス・メカトロニクス・ジャパン株

Table 3. Determined Elements, Internal Standards and Their Mass Numbers

| Determined element | Sb | As | Ba | Cd | Cr | Pb | Hg | Se |
|---|---|---|---|---|---|---|---|---|
| Mass number ($m/z$) | 121 | 75 | 137 | 111 | 53 | 208 | 202 | 82 |
| Internal standard | Y | Y | Y | Y | Y | Tl | Tl | Y |
| Mass number ($m/z$) | 89 | 89 | 89 | 89 | 89 | 205 | 205 | 89 |

式会社）でろ過し，試験溶液とした．なお，検量線の範囲に収まるように，必要に応じて 0.07 mol/L 塩酸を用いて 100 倍から 10000 倍に希釈した．

**5. 定量**　混合標準液の ICP-MS 測定から，内標準法により検量線を作成した．作成した検量線から試験溶液中の各測定元素の濃度を求め，次式により試験に供した検体 1 kg 当たりの溶出量（mg/kg）を算出した．

$$\text{溶出量 (mg/kg)} = \frac{\text{試験溶液中の濃度 (mg/L)} \times \text{試験溶液の容量 (L)}}{\text{検体質量 (kg)}}$$

食品衛生法のおもちゃの規格基準及び ISO 玩具安全規格では，試験法の正確化のため，分析補正値により分析値を補正することが規定されているが，今回の調査は食品衛生法のおもちゃの規格基準及び ISO 玩具安全規格への適否を判定することが目的ではないので，分析補正値は考慮しなかった．また，ISO 玩具安全規格では，溶出限度値の 1/10 を測定下限値としているが，今回は溶出限度値の 1/100 を測定下限値とした．なお，試験溶液の ICP-MS 測定における検出下限値及び定量下限値は，ブランク値の標準偏差のそれぞれ 3 倍及び 10 倍とした．[17]

## 結　　果

**1. ICP-MS 測定の精度**　ICP-MS 測定における各測定元素の検出下限値，定量下限値及びバックグランド相当濃度（BEC）を Table 4 に示した．今回の ICP-MS 測定における検出下限値は，いずれの測定元素についても ISO 玩具安全規格の溶出限度値（Table 1）のおよそ 1/100000 に相当する濃度であり，試験溶液の測定に ICP-MS を用いることは妥当である．

**2. 市販製品の溶出試験**　鉛，カドミウム及びクロムについて，商品分類別の ISO 玩具安全規格の溶出限度値（Table 1）の 1/10 以下の製品数（検体数），溶出限度値の 1/10 を超え溶出限度値以下の製品数（検体数）及び溶出限度値を超えた製品数（検体数）をそれぞれ Table 5–7 に示した．また，測定元素のいずれかが溶出限度値（Table 1）を超えた製品（検体）及びそれらの各測定元素の溶出量をそれぞれ Fig. 1 及び Table 8 に示した．ストラップ，アクセサリーパーツ，チャーム，ヘアピン，ネックレスなど 28 製品（30 検体）が溶出限度値（90 mg/kg）を超える鉛を溶出し，さらに 26 製品（31 検体）が溶出限度値の 1/10 を超え溶出限度値以下の鉛を溶出した（Table 5）．チャーム（No. 16; 569 mg/kg），アクセサリーパーツの飾り（No. 15–1; 523 mg/kg），ストラップの金具（No. 1–2; 501 mg/kg）及びアクセサリーパーツ（No. 14; 461 mg/kg）が溶出限度値（90 mg/kg）の 5 倍を超える鉛を溶出した（Table 8）．また，ブレスレット（No. 26; 160 mg/kg）の 1 製品（1 検体）が溶出限度値（75 mg/kg）を超えるカドミウムを溶出し（Table 8），さらに 5 製品（5 検体）が溶出限度値の 1/10 を超え溶出限度値以下のカドミウムを溶出した（Table 6）．溶出限度値（60 mg/kg）を超えるクロムを溶出した製品はなく，6 製品（7 検体）が溶出限度値の 1/10 を超え溶出限度値以下のクロムを溶出した（Table 7）．なお，溶出限度値の 1/10 を超えるアンチモン，ヒ素，バリウム，水銀及びセレンを溶出した製品（検体）はなかった．

## 考　　察

食品衛生法は，飲食に起因する衛生上の危害の発生の防止を目的とするものであるが，乳幼児玩具には，乳幼児が口に接触することをその本質とするおもちゃが存在すること及び一定年齢の乳幼児は身の周りにあるものを口に入れるという性質があること等の理由から，同法に基づき，乳幼児が接触することによりその健康を損なうおそれがあるものとして厚生労働大臣が指定する玩具（指定おもちゃ）が規定され，必要な規格及び製造基準が設定されている．平成 20 年 3 月 31 日に，食品衛生法に基づく「食品，添加物等の規格基準」の「おもちゃ又はその原材料の規格」が改正され，金属製アクセサリー玩具のうち，乳幼児が飲み込むおそれがあるものについて，鉛の溶出量は 90 μg/g 以下でなければならないという規定が追加された．[18,19] しかし，金属製アクセサリー玩具に該当しない金属製アクセサリー等の金属製品から溶出する鉛その他の有害元素は規制されておらず，乳幼児が誤飲した場合の健康影響が懸念される．

今回調査した乳幼児が飲み込むおそれがある金属製アクセサリー等 117 製品のうち，いずれかの元素が ISO 玩具安全規格の溶出限度値（Table 1）を超えたのは 29 製品あり，検出頻度は 25% であった．特に，カドミウムを溶出した 1 製品を除き，鉛を溶出した製品が多かった．鉛は金属製品以外の家庭用品等に含有したり，それらから溶出したりすることも報告されている．例えば，子供用髪留めの塗膜から食品衛生法の規格基準を超える鉛の溶出が確認され，食品衛生法の対象外の製品であるが，国内販売

Table 4. Detection Limits, Determination Limits and Background Equivalent Concentrations (BEC) in ICP-MS

| Element | Sb | As | Ba | Cd | Cr | Pb | Hg | Se |
|---|---|---|---|---|---|---|---|---|
| Detection limit[a] (ng/L) | 12.8 | 21.3 | 15.4 | 3.08 | 38.8 | 89.3 | 9.65 | 32.2 |
| Determination limit[b] (ng/L) | 42.7 | 71.1 | 51.4 | 10.3 | 129 | 298 | 32.2 | 107 |
| BEC (ng/L) | 77.1 | 14.4 | 52.2 | 8.11 | 197 | 593 | 84.6 | 104 |

[a] Three times of the standard deviation of a blank. [b] Ten times of the standard deviation of a blank.



Table 5. Migrations of Lead by the Commodity Classification

| Commodity classification | Number of products (Number of specimens) | ≤ 9 mg/kg | 9 mg/kg<, ≤ 90 mg/kg | 90 mg/kg[a]< |
|---|---|---|---|---|
| Straps | 30( 65) | 13( 47) | 7( 7) | 10(11) |
| Accessory parts | 21( 22) | 12( 12) | 4( 4) | 5( 6) |
| Hairpins | 20( 20) | 15( 15) | 2( 2) | 3( 3) |
| Necklaces | 17( 42) | 7( 29) | 7(10) | 3( 3) |
| Charms | 9( 9) | 4( 4) | 1( 1) | 4( 4) |
| Rings | 6( 6) | 1( 1) | 4( 4) | 1( 1) |
| Fastener straps | 4( 4) | 4( 4) | 0( 0) | 0( 0) |
| Bracelets | 3( 8) | 1( 4) | 1( 3) | 1( 1) |
| Earrings | 3( 3) | 3( 3) | 0( 0) | 0( 0) |
| Cufflinks | 2( 2) | 2( 2) | 0( 0) | 0( 0) |
| Pin badges | 1( 2) | 1( 2) | 0( 0) | 0( 0) |
| Buttons | 1( 1) | 0( 0) | 0( 0) | 1( 1) |
| Total | 117(184) | 63(123) | 26(31) | 28(30) |
| Frequency (%) | 100(100) | 54( 67) | 22(17) | 24(16) |

[a] Maximum acceptable migration of lead from toy materials required by ISO 8124-3:2010.

Table 6. Migrations of Cadmium by the Commodity Classification

| Commodity classification | Number of products (Number of specimens) | ≤ 7.5 mg/kg | 7.5 mg/kg<, ≤ 75 mg/kg | 75 mg/kg[a]< |
|---|---|---|---|---|
| Straps | 30( 65) | 30( 65) | 0(0) | 0(0) |
| Accessory parts | 21( 22) | 20( 21) | 1(1) | 0(0) |
| Hairpins | 20( 20) | 20( 20) | 0(0) | 0(0) |
| Necklaces | 17( 42) | 14( 39) | 3(3) | 0(0) |
| Charms | 9( 9) | 9( 9) | 0(0) | 0(0) |
| Rings | 6( 6) | 5( 5) | 1(1) | 0(0) |
| Fastener straps | 4( 4) | 4( 4) | 0(0) | 0(0) |
| Bracelets | 3( 8) | 3( 8) | 0(0) | 1(1) |
| Earrings | 3( 3) | 3( 3) | 0(0) | 0(0) |
| Cufflinks | 2( 2) | 2( 2) | 0(0) | 0(0) |
| Pin badges | 1( 2) | 1( 2) | 0(0) | 0(0) |
| Buttons | 1( 1) | 1( 1) | 0(0) | 0(0) |
| Total | 117(184) | 112(179) | 5(5) | 1(1) |
| Frequency (%) | 100(100) | 95( 96) | 4(3) | 1(1) |

[a] Maximum acceptable migration of cadmium from toy materials required by ISO 8124-3:2010.

Table 7. Migrations of Chromium by the Commodity Classification

| Commodity classification | Number of products (Number of specimens) | ≤ 6 mg/kg | 6 mg/kg<, ≤ 60 mg/kg | 60 mg/kg[a]< |
|---|---|---|---|---|
| Straps | 30( 65) | 29( 63) | 1(2) | 0(0) |
| Accessory parts | 21( 22) | 16( 17) | 5(5) | 0(0) |
| Hairpins | 20( 20) | 20( 20) | 0(0) | 0(0) |
| Necklaces | 17( 42) | 17( 42) | 0(0) | 0(0) |
| Charms | 9( 9) | 9( 9) | 0(0) | 0(0) |
| Rings | 6( 6) | 6( 6) | 0(0) | 0(0) |
| Fastener straps | 4( 4) | 4( 4) | 0(0) | 0(0) |
| Bracelets | 3( 8) | 3( 8) | 0(0) | 0(0) |
| Earrings | 3( 3) | 3( 3) | 0(0) | 0(0) |
| Cufflinks | 2( 2) | 2( 2) | 0(0) | 0(0) |
| Pin badges | 1( 2) | 1( 2) | 0(0) | 0(0) |
| Buttons | 1( 1) | 20( 20) | 0(0) | 0(0) |
| Total | 117(184) | 111(177) | 6(7) | 0(0) |
| Frequency (%) | 100(100) | 95( 96) | 5(4) | 0(0) |

[a] Maximum acceptable migration of chromium from toy materials required by ISO 8124-3:2010.

Table 8. Migrations of Eight Elements from Products at Levels More than the Maximum Acceptable Levels of ISO 8124-3:2010

| No. | Product | Specimen | Element (mg/kg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Sb | As | Ba | Cd | Cr | Pb | Hg | Se |
| 1-1 | Strap | decoration | nd[b] | nd | nd | nd | nd | 136[c] | nd | nd |
| 1-2 | | fitting | nd | nd | nd | 0.764 | nd | 501 | nd | nd |
| 2 | Strap | fitting | nd | nd | nd | 1.07 | nd | 418 | nd | nd |
| 3 | Strap | fitting | nd | nd | nd | nd | nd | 428 | nd | nd |
| 4 | Strap | fitting | nd | nd | nd | nd | nd | 125 | nd | nd |
| 5 | Strap | decoration | nd | nd | nd | nd | nd | 167 | nd | nd |
| 6 | Strap | decoration | nd | nd | nd | nd | nd | 368 | nd | nd |
| 7 | Strap | fitting | nd | nd | nd | nd | nd | 104 | nd | nd |
| 8 | Strap | decoration | nd | nd | nd | nd | nd | 218 | nd | nd |
| 9 | Strap | decoration | nd | nd | nd | nd | nd | 252 | nd | nd |
| 10 | Strap | decoration | nd | nd | nd | nd | nd | 145 | nd | nd |
| 11 | Accessory part | —[a] | nd | nd | nd | nd | nd | 123 | nd | nd |
| 12 | Accessory part | — | nd | nd | nd | 21.3 | nd | 241 | nd | nd |
| 13 | Accessory part | — | nd | nd | nd | 1.52 | nd | 129 | nd | nd |
| 14 | Accessory part | — | nd | nd | nd | 1.24 | nd | 461 | nd | nd |
| 15-1 | Accessory part | decoration | nd | nd | nd | 2.23 | nd | 523 | nd | nd |
| 15-2 | | decoration | nd | nd | nd | 1.50 | nd | 312 | nd | nd |
| 16 | Charm | — | nd | nd | nd | nd | nd | 569 | nd | nd |
| 17 | Charm | — | nd | nd | nd | nd | nd | 296 | nd | nd |
| 18 | Charm | — | nd | nd | nd | 2.24 | nd | 296 | nd | nd |
| 19 | Charm | — | nd | nd | nd | 1.44 | nd | 330 | nd | nd |
| 20 | Hairpin | — | nd | nd | nd | nd | nd | 166 | nd | nd |
| 21 | Hairpin | — | nd | nd | nd | nd | nd | 112 | nd | nd |
| 22 | Hairpin | — | nd | nd | nd | nd | nd | 145 | nd | nd |
| 23 | Necklace | chain | nd | nd | nd | nd | nd | 200 | nd | nd |
| 24 | Necklace | decoration | nd | nd | nd | nd | nd | 107 | nd | nd |
| 25 | Necklace | decoration | nd | nd | nd | 1.63 | nd | 187 | nd | nd |
| 26 | Bracelet | decoration | nd | nd | nd | 160 | nd | 0.680 | nd | nd |
| 27 | Bracelet | decoration | nd | nd | nd | 1.14 | nd | 133 | nd | nd |
| 28 | Ring | — | nd | nd | nd | 1.15 | nd | 139 | nd | nd |
| 29 | Button | — | nd | nd | nd | nd | nd | 139 | nd | nd |

[a] Tested the whole product. [b] Less than and equal to 1/100 of the maximum acceptable levels of ISO 8124-3:2010. [c] The underline shows the migrations at levels more than the maximum acceptable levels of ISO 8124-3:2010.




Fig. 1. Products That Released Lead at a Level More than the Maximum Acceptable Level of ISO 8124-3:2010


No. 16 No. 17 No. 18

No. 19 No. 20 No. 21

No. 22 No. 23 No. 24

No. 25 No. 26 No. 27

No. 28 No. 29

Fig. 1. (Continued)



業者は自主的に回収した.[20,21] また，われわれも乳幼児が飲み込むおそれがある家庭用品の塗膜[22]及び合成樹脂製家庭用品[16]の一部から鉛が溶出することを確認している．さらに，一部のレジ袋は最大25000 mg/kg，平均310 mg/kgの鉛を含有することが報告され，幼児がレジ袋を舐めたり飲み込んだりした場合の健康影響が懸念されている.[23]

鉛は中枢神経障害，腎機能障害，生殖機能障害及び造血器障害を生じる有害金属元素の1つであり，特に，乳幼児に対しては，一定レベル以上の血中濃度で，知能や神経の発達に有害な影響を与える可能性がある.[24,25] 米国疾病予防管理センター（Centers for Disease Control and Prevention）は，小児の血中鉛濃度の許容値を10 μg/dLと定めている.[26] さらに，米国消費者製品安全委員会（The U.S. Consumer Product Safety Commission, CPSC）は，急性的暴露によって小児の血中鉛濃度が10 μg/dLを超過するのを避けるため，短期間に175 μgを超える鉛を摂取することがないよう勧告している.[27] 今回調査した117製品（184検体）について，製品（検体）1個当たりの鉛の溶出量を算出した（Table 9）．ストラップ，アクセサリーパーツ，ネックレスなど36製品（41検体）が製品（検体）1個当たり175 μgを超える鉛を溶出し，最大値はアクセサリーパーツ（No. 14）の5904 μgであった．これらを小児が誤飲すると，米国CPSCの勧告（175 μg）を超える量の鉛を摂取する可能性があり，安全性に問題があると考えられる．

平成18年に実施した調査[3]は，米国CPSCが規定した試験法[28]に準じ，鉛含有量が0.06％を超えた製品は鉛溶出量を175 μg以下とする当時の暫定指針[27]に基づいて判定した．その結果，金属製アクセサリー類等140製品のうち，鉛含有量が0.06％を超えたのは90製品であった.[3] さらに，鉛含有量が0.06％を超え，乳幼児が誤飲するおそれのある66製品のうち，鉛溶出量が175 μgを超えたのは39製品であった.[3] 米国CPSC指針の溶出試験とISO玩具安全規格の溶出試験は試験条件が異なり，一般に米国CPSC指針の溶出試験の方が鉛の溶出力が高い傾向にある.[29] これらを考慮すると，わが国で市販されている金属製アクセサリー等において，高濃度の鉛を溶出する製品の割合は平成18年から現在までほとんど改善されていないと考えられる．乳幼児が誤飲する可能性のある金属製アクセサリー等の金属製品は，鉛やカドミウムなどの有害元素を溶出しないことが望ましく，これらの有害元素の溶出量を低減する努力が必要であろう．

米国では，2009年8月までに，子供用製品の鉛含有量を600 mg/kgから300 mg/kgに，子供用製品に使用される塗料及び塗膜の鉛含有量を600 mg/kgから90 mg/kgに，それぞれ低減するように規制が強化された.[30] さらに，2011年8月までに，子供用

Table 9. Migrations of Lead per Product or per Specimen by the Commodity Classification

| Commodity classification | Number of products (Number of specimens) | ≤ 175 μg | 175 μg[a]<, ≤ 1750 μg | 1750 μg< |
|---|---|---|---|---|
| Straps | 30 ( 65) | 19 ( 53) | 11 (12) | 0 (0) |
| Accessory parts | 21 ( 22) | 13 ( 13) | 5 ( 6) | 3 (3) |
| Hairpins | 20 ( 20) | 17 ( 17) | 3 ( 3) | 0 (0) |
| Necklaces | 17 ( 42) | 12 ( 36) | 5 ( 6) | 0 (0) |
| Charms | 9 ( 9) | 5 ( 5) | 4 ( 4) | 0 (0) |
| Rings | 6 ( 6) | 4 ( 4) | 2 ( 2) | 0 (0) |
| Fastener straps | 4 ( 4) | 4 ( 4) | 0 ( 0) | 0 (0) |
| Bracelets | 3 ( 8) | 1 ( 4) | 2 ( 4) | 0 (0) |
| Earrings | 3 ( 3) | 3 ( 3) | 0 ( 0) | 0 (0) |
| Cufflinks | 2 ( 2) | 2 ( 2) | 0 ( 0) | 0 (0) |
| Pin badges | 1 ( 2) | 1 ( 2) | 0 ( 0) | 0 (0) |
| Buttons | 1 ( 1) | 0 ( 0) | 1 ( 1) | 0 (0) |
| Total | 117 (184) | 81 (143) | 33 (38) | 3 (3) |
| Frequency (%) | 100 (100) | 69 ( 77) | 28 (21) | 3 (2) |

[a] Maximum acceptable migration of lead from children's metal jewelries by CPSC policy.

製品の鉛含有量を 100 mg/kg まで低減するように規制がより強化された.[30] わが国では，平成 18 年以降，鉛等を含有する金属製アクセサリー類の安全対策として，消費者等への注意喚起，医療関係者への情報提供及び業者団体への指導を行ってきた.[4-13,25] しかし，乳幼児の誤飲事故は後を絶たず,[14,15] 製品の改善も認められなかったことから，金属製アクセサリー等の誤飲による乳幼児の健康被害を防止するため，わが国でもさらに踏み込んだ対策が望まれる.

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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: 101A: 2573–2585, 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, 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 $1.2 \times 10^4$ 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

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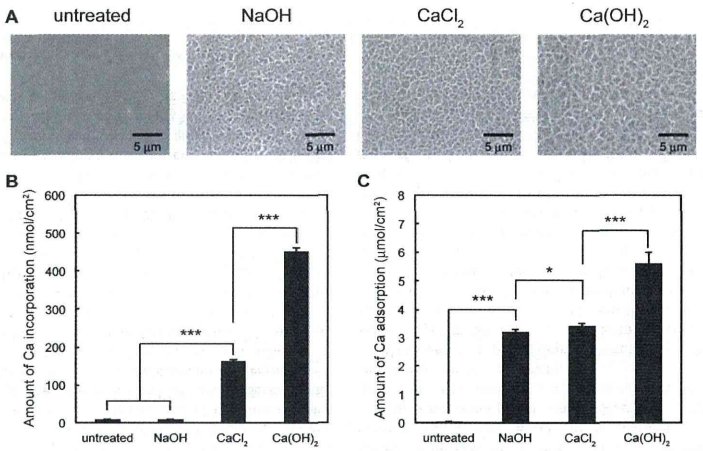


**FIGURE 1.** SEM images of the surface of untreated, NaOH-, NaOH + $CaCl_2$ ($CaCl_2$)-, and NaOH + $Ca(OH)_2$ ($Ca(OH)_2$)-treated titanium disks (A). Images are representative of three independent experiments. The amount of $Ca^{2+}$ incorporation into the chemically modified titanium disks (B; left)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

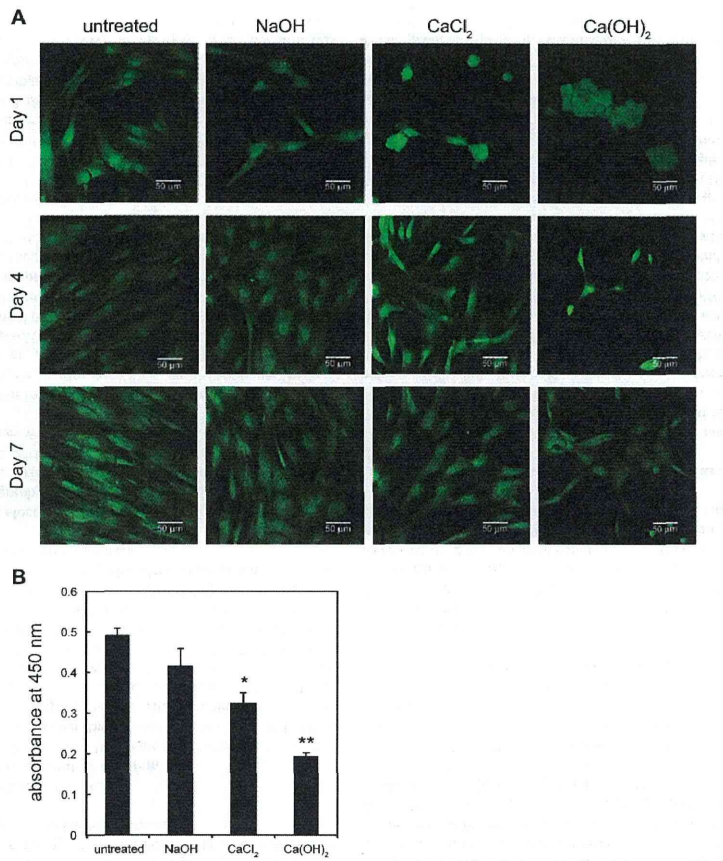


**FIGURE 2.** Confocal microscopy of hMSCs cultured on untreated, NaOH, $CaCl_2$, and $Ca(OH)_2$ treated titanium disks for 1, 4, and 7 days (A). Images are representative of five independent experiments. Cells were stained with CellTrackerTM Green Fluorescent Probe (Lonza). Proliferation of hMSCs for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

### 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)

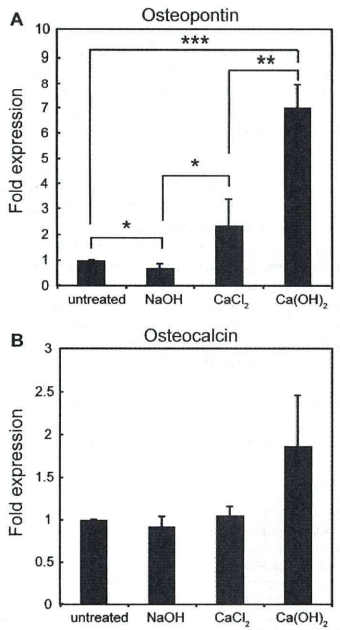


**FIGURE 3.** Relative expression of osteopontin (A) and osteocalcin (B) were analyzed by real-time PCR at 7 days after hMSC seeding ($n = 3$). The value was normalized to GAPDH. Significant differences between groups are shown as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

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



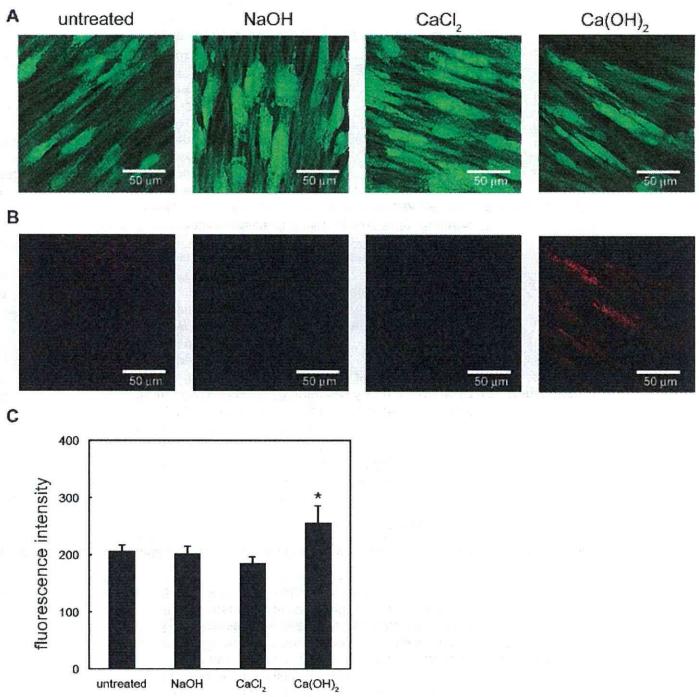


**FIGURE 4.** Confocal microscopy of hMSCs (A) and osteocalcin in hMSCs (B) cultured on chemically modified titanium disks for 28 days. Green: CellTrackerTM Green Fluorescent Probe; red: Alexa Fluor 647 donkey anti-mouse. Images representative of three independent experiments are shown. Fluorescence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