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Original Article

Calcitriol inhibits arsenic-promoted tumorigenesis through regulation of arsenic-uptake in a human keratinocyte cell line

Ichiro Yajima[1,2], Akira Tazaki[2,4], Nobutaka Ohgami[2,3,4], Masashi Kato[2,3,4]

[1]Unit of Molecular and Cellular Toxicology, Department of Bioscience and Engineering, College of Systems Engineering and Science, Shibaura Institute of Technology, Fukasaku 307, Minuma-ku, Saitama 337-8570, Japan; [2]Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan; [3]Unit of Environmental Health Sciences, Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai, Aichi 487-8501, Japan; [4]Voluntary Body for International Health Care in Universities, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

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Abstract: Chronic arsenic exposure from drinking water causes a variety of diseases and it is now recognized that at least 140 million people in 50 countries have been drinking water containing arsenic at levels above the WHO provisional guideline value of 10 μg/L. Long-term exposure to arsenic is associated with various types of cancers in humans including skin cancers. However, there is limited information on key molecules regulating arsenic-promoted carcinogenesis, and methods for the prevention and therapy of arsenic-promoted carcinogenesis have not yet been fully developed. Our *in vitro* study in human nontumorigenic HaCaT skin keratinocytes showed that calcitriol (activated vitamin D3, 1,25$(OH)_2D_3$) inhibited arsenic-mediated anchorage-independent growth with downregulations of cancer-related activation of MEK, ERK1/2 and AKT and activity of cell cycle. Moreover, calcitriol significantly repressed arsenic uptake in HaCaT cells with inhibition of expressions of aquaporin genes (AQP7, 9 and 10) which were modified by arsenic exposure. VDR, a vitamin D receptor, expression was significantly increased by arsenic exposure whereas calcitriol had no effect on its expression. These results suggest that treatment of calcitriol inhibits arsenic uptake via suppressions of aquaglyceroporin gene expressions resulting in inhibition of arsenic-promoted tumorigenesis in keratinocytes.

Keywords: Calcitriol, vitamin D3, arsenic, skin cancer, aquaporins

Introduction

Inorganic arsenic, a carcinogen in humans, is one of the major world-wide contaminants of drinking water [1-4]. There are millions of arsenicosis patients who are drinking arsenic-polluted well water in a variety of countries including Bangladesh [2, 3, 5-7]. Chronic exposure of arsenic causes lung, skin, and urinary bladder cancers and, as such, arsenic is classified as a Group I carcinogen by the World Health Organization (WHO) [2-5, 8-10]. Cutaneous squamous cell carcinoma (SCC) is a serious cancer developed by arsenic chronic exposure [5, 11] and several kinds of molecules including Placental Growth Factor (PlGF), Hypoxia Inducible Factor 1-Alpha (HIF-1α) and GLI transcription factor-3 (GLI3) have been reported to induce arsenic-promoted tumorigenesis [7, 12, 13]. The arsenic toxicity is closely related to the uptake of arsenic into the cells. Aquaporin (AQP) 7, 9 and 10 proteins, which are encoded by aquaglyceroporin genes, have been known to be transporters for water, glycerol and urea [14], and AQP7 and 9 have been reported to play arsenic transporters in mammalian cells [15, 16], suggesting that aquaglyceroporins may have effects on arsenic-mediated disease. Clinically available molecular targets for the prediction, prevention, and treatment of arsenic-promoted cancer have not been well established.

To analyze arsenic-mediated diseases at the molecular level, appropriate cell lines are required. The HaCaT keratinocyte cell line was a naturally immortalized-nontumorigenic cell line established from human skin [17]. HaCaT cells are often used to elucidate mechanisms of tumorigenesis induced by environmental factors such as ultraviolet light and heavy metals including arsenic [6, 7]. Analysis of the anchorage-independent growth activity of arsenic-treated HaCaT cells is often used to elucidate the activity of arsenic-promoted tumorigenesis *in vitro* [6-8].

Calcitriol (activated vitamin D3, $1,25(OH)_2D_3$) is rendered from vitamin D3 by several enzymes expressed in the liver and kidney, and bind to the vitamin D3 receptor (VDR) protein, one of the 48 members of the ligand-activated transcription factor superfamily of nuclear hormone receptors [18]. The binding of calcitriol with a specific nuclear vitamin D3 receptor (VDR), which is present in all target tissues, starts compounding necessary proteins to initiate gene transcription. Vitamin D3 influences expressions of the hundreds of genes through the activation of VDR. VDR activation by calcitriol negatively and/or positively regulates several signaling pathways including the MEK/ERK pathway and PI3K/AKT pathway [19-22], which are strongly associated with tumor development.

Several kinds of studies have been performed to elucidate correlations between calcitriol and the tumor development, indeed, some types of tumor development have been reported to be correlated with vitamin D3 [23]. In many types of cancer cells, calcitriol treatments induce the downregulation of cyclin-dependent kinases including Cyclin D1 and upregulation of cyclin-dependent kinase inhibitors including $p21^{CIP1}$ and $p27^{KIP1}$, resulting in cell cycle arrest [24, 25].

In this study, we analyzed suppressive activities of calcitriol on arsenic-mediated anchorage-independent growth, a characteristic phenotype of cancer formation, focusing on cancer-related molecules and mechanism of arsenic uptake to clarify the therapeutic potential of calcitriol against arsenic-promoted skin cancer.

Materials and methods

Reagents

Sodium arsenite (SIGMA) and calcitriol (1,25-Dihydroxyvitamin D3, Wako) were used in this study. Rabbit polyclonal antibodies against ERK1/2 (Cell Signaling), phpspho-ERK1/2 (Cell Signaling), AKT (Cell Signaling), phospho-AKT (Cell Signaling), and mouse monoclonal antibodies against MEK (Cell Signaling Technology), phosphor-MEK (Cell Signaling Technology), Cyclin D1 (Cell Signaling), $p21^{CIP1}$ (Cell Signaling), and alpha-TUBULIN (SIGMA) were used as primary antibodies for immunoblotting.

Cell culture

A human nontumorigenic skin keratinocyte cell line (HaCaT), supplied from Cell Line Service (Germany) and a human normal lung epithelial cell line (Beas-2b), supplied from Health Science Research Resources Bank (Japan) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640, respectively, supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (WAKO) at 37°C in 5% CO_2. The level of arsenic was undetectably low in DMEM with 10% FBS by ICP-MS.

We examined the phosphorylation levels of MEK and ERK1/2 during the time course of arsenic exposure (Figure S1) to decide the better timing of arsenic exposure for analysis of the intracellular signaling pathway. After 24, 48, and 72 hours of arsenic exposure, p-MEK levels showed an increase at 24- and 48-hours post-exposure and a decrease at 72-hours post-exposure. On the other hand, an increase in p-ERK levels was observed from 24 hours post-exposure and remained high levels at 48 and 72 hours. Based on these results, we conclude that arsenic exposure for 48 hours is better to verify the intracellular signaling pathway. According to our conclusion, levels of phosphorylation and expression levels of proteins and genes in cells treated with arsenic after 48 hours were measured.

Anchorage-independent growth assay

Cells (2×10^4) were added to 1 ml of 1.5% methylcellulose/DMEM and treated or not treated

Table 1. Sequences of genes for PCR

Gene name	Type	Forward	Reverse
AQP7	Primer for PCR	TCCAAAATGGTCTCCTGGTC	GAAGCCAAAACCCAAGTTGA
AQP9	Primer for PCR	GGTGGAAAACTGCTGATCGT	AATGCGTTCGCCAGAGATAG
AQP10	Primer for PCR	ACTCATGCTCCTCACCCAAG	AGCACAGAAAGCAGACAGCA
VDR	Primer for PCR	TGACCCTGGAGACTTTGACC	GTTGAAGGGGCAGGTGAATA
TBP	Primer for PCR	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC

with arsenic in 24-well culture plates with Ultra-low attachment surfaces (Corning). After incubation for 7 days, colony size was determined by analysis of microscopic images [8]. All colonies exceeding 50 μm diameter in wells were counted and presented as an activity of anchorage-independent growth according to a previous paper [7, 26, 27].

Immunoblotting

Cells were washed twice with ice-cold PBS and lysed in 0.3 ml of lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 12.5 mM b-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 20 mM aprotinin, and 0.5% Triton X-100). Whole cell lysates were resolved on SDS-PAGE and transferred to Hybond-P membranes (GE Health Sciences). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG (Calbiochem) using an ImmunoStar Zeta and ImmunoStar LD (WAKO). Densitometric evaluation was performed using the software program WinROOF (MITANI Corporation).

Measurements of arsenic in HaCaT cells

Levels of arsenic in HaCaT cells were measured by an inductively coupled plasma-mass spectrophotometer (ICP-MS; 7500cx, Agilent Technologies, Inc.) following the method previously described [28]. In brief, HaCaT cells were collected 1.5 ml tubes by centrifuge, and then the samples were incubated with 100 μl of nitric acid (61%) at 80°C for 3 h, followed by cooling for 1 h to room temperature. After cooling, 100 μl of hydrogen peroxide (30%) was added to each tube, followed by incubation at 80°C for 3 h. After suitable dilution of the digested materials with water, arsenic level was determined by ICP-MS.

Realtime-PCR

Total RNA was prepared from culture cells using a High Pure RNA Purification Kit (Roche) according to the manufacturers' protocol. cDNA was then synthesized by reverse transcription of total RNA using Supercript III reverse transcriptase included in the RT enzyme mix and RT reaction mix (Invitrogen) according to the manufacturers' protocol. Real-time quantitative RT-PCR with SYBR green was performed using Thermal Cycler Dice Real Time System (TaKaRa). The expression levels of human AQP7, AQP9, AQP10 and VDR transcripts measured by quantitative RT-PCR (real-time PCR) were adjusted through the transcript expression level of human TATA-box-binding protein (TBP). Sequences of primers for these genes were described in **Table 1**.

Statistics

The JMP Pro (version 16.0) software package (JMP Statistical Discovery) was used for statistical analyses by Student's t-test and Tukey-Kramer test, and the significance level was set at $P<0.05$.

Results

Inhibition of arsenic-promoted tumorigenesis of calcitriol-treated HaCaT cells

To elucidate effects of calcitriol on arsenic-promoted tumorigenesis, anchorage-independent growth assays [7] were performed in HaCaT cells (**Figure 1**), whereas arsenic strongly induced anchorage-independent growth of HaCaT without calcitriol (**Figure 1A**, **1D**), 10 and 100 nM calcitriol inhibited arsenic-mediated anchorage-independent growth (**Figure 1B**, **1C**, **1E**, **1F**). Arsenic-mediated anchorage-independent growth of calcitriol-treated HaCaT cells was suppressed by 49.3-73.1% (**Figure 1G**), suggesting that calcitriol may regulate arsenic-promoted cancer development.

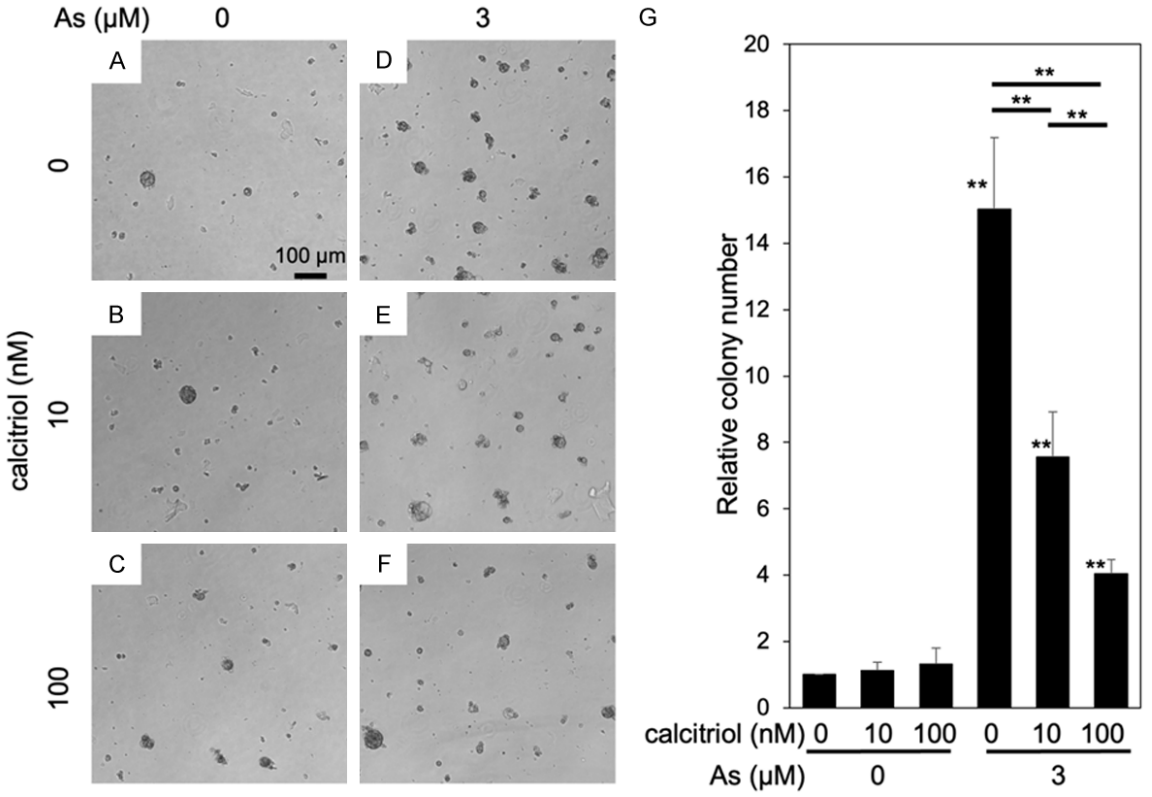

Figure 1. Inhibition of arsenic-mediated anchorage-dependent growth of calcitriol-treated HaCaT cells. A-F. Colonies derived from arsenic-exposed HaCaT cells untreated or treated with calcitriol. Original magnification, ×20. Scale bar: 100 μm. G. Number of colonies per well derived from HaCaT cells untreated or treated with arsenic and/or calcitriol are presented as ratios (mean ± SD; n=5) relative to untreated HaCaT cells. Significantly different from untreated HaCaT cells (**, P<0.01) by Tukey-Kramer test.

Inhibition of cancer-related signal transductions of calcitriol-treated HaCaT cells

As previous studies revealed that mitogen-activated protein kinase (MAPK) signal transduction including MEK/ERK and/or PI3K/AKT pathways are aberrantly activated in most of all kinds of human cancers [29-32], we next examined the influence of calcitriol on phosphorylated levels of MEK, ERK1/2 and AKT in arsenic-promoted tumorigenesis of HaCaT cells (**Figure 2**). Arsenic-induced phosphorylation of MEK, ERK1/2 and AKT was suppressed in calcitriol-treated HaCaT cells, suggesting that calcitriol inhibits arsenic-mediated cancer-related signaling pathways in HaCaT cells (**Figure 2**).

Effects of calcitriol on arsenic-mediated regulations of cell cycle-related molecules in HaCaT cells

We next examined the effects of calcitriol on arsenic-mediated regulations of cell cycle-related molecules (**Figures 3** and **4**). Previous studies have shown that the expression of cell cycle-related positive regulators such as Cyclin D1 are upregulated whereas negative regulators such as $p21^{CIP1}$ are downregulated in various types of cancers including arsenic-promoted skin cancer [33-35]. Expressions of *Cyclin D1* and *$p21^{CIP1}$* mRNAs were significantly upregulated and downregulated in arsenic-treated HaCaT cells, respectively, whereas those were significantly downregulated and upregulated in calcitriol-treated HaCaT cells, respectively (**Figure 3**). Protein levels of Cyclin D1 and $p21^{CIP1}$ were also similarly regulated by calcitriol on the dose-dependent manner (**Figure 4**).

Effects of calcitriol on arsenic uptake in HaCaT cells

As our results provided evidence that calcitriol-mediated inhibition of cancer-related molecules could be an important mechanism on inhibition of arsenic-promoted tumorigenesis,

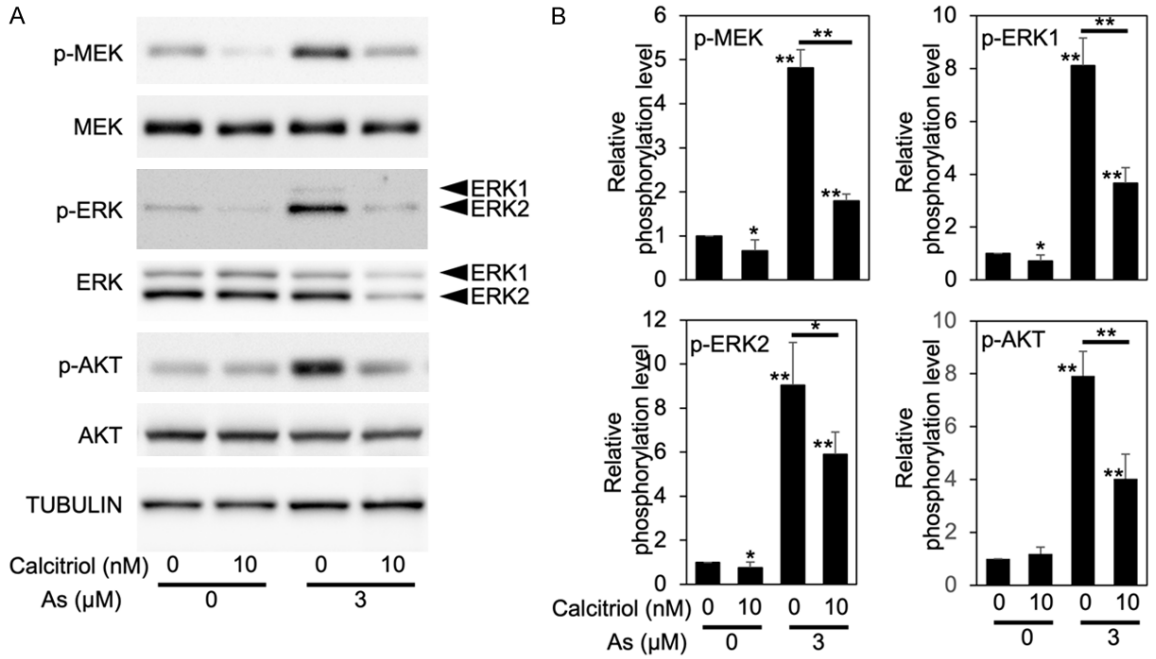

Figure 2. Calcitriol-induced modification of tumorigenesis-related signaling activities mediated by arsenic. A. Phosphorylation and protein levels of MEK and ERK in HaCaT cells untreated or treated with arsenic and/or calcitriol for 48 h are presented. B. Intensities of bands are presented as percentages (mean ± SD; n=4) relative to untreated HaCaT cells. * and **, Significantly different (*, P<0.05; **, P<0.01) by Tukey-Kramer test.

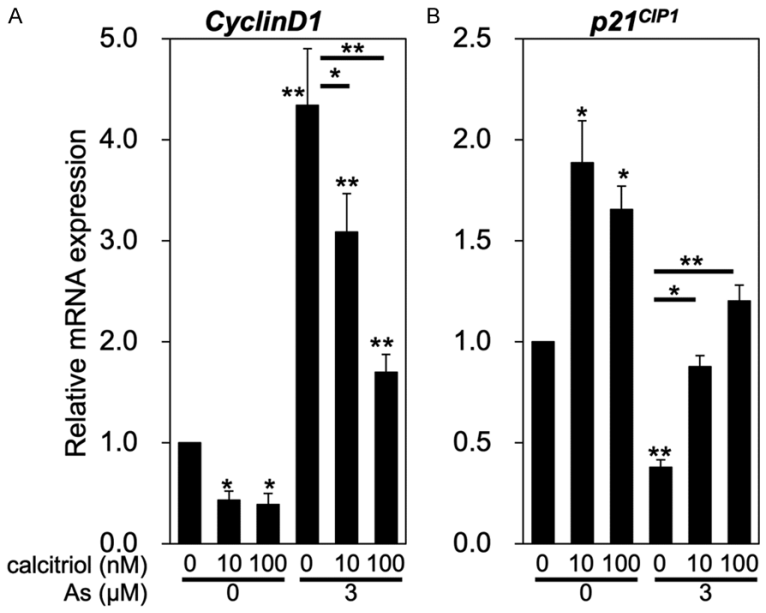

Figure 3. mRNA levels of cell cycle-related molecules in arsenic-exposed HaCaT cells treated with calcitriol. A. Expression levels of Cyclin D1 mRNAs evaluated by real-time PCR in HaCaT cells untreated or treated with arsenic and/or calcitriol for 48 h are presented. The results are presented as ratios (mean ± SD; n=4) of Cyclin D1 mRNA relative to non-exposed HaCaT cells without calcitriol. B. Expression levels of $p21^{CIP1}$ messenger RNA (mRNA) evaluated by real-time PCR in arsenic-exposed HaCaT cells untreated or treated with calcitriol are presented. The results are presented as ratios (mean ± SD; n=4) of $p21^{CIP1}$ mRNA relative to untreated HaCaT cells. * and **, Significantly different (*, P<0.05; **, P<0.01) by Tukey-Kramer test.

we investigated the point of action of calcitriol in arsenic-promoted tumorigenesis of HaCaT cells. The arsenic toxicity is closely related to arsenic uptake, which is regulated by several kinds of channels including the aquaporin family [36]. Aquaporin 7 and 9 (AQP7 and AQP9), members of the aquaglyceroporin family, have been reported to play arsenic transporters in mammalian cells, resulting in regulating cellular toxicity [15, 16, 37]. To elucidate the relationship between arsenic uptake and calcitriol treatment, arsenic levels in HaCaT cells treated with calcitriol were measured by an inductively coupled plasma-mass spectrophotometer (ICP-MS). Arsenic levels in HaCaT cells cultured with arsenic were significantly decreased by calcitriol treatment with the dose-dependent manner, whereas there

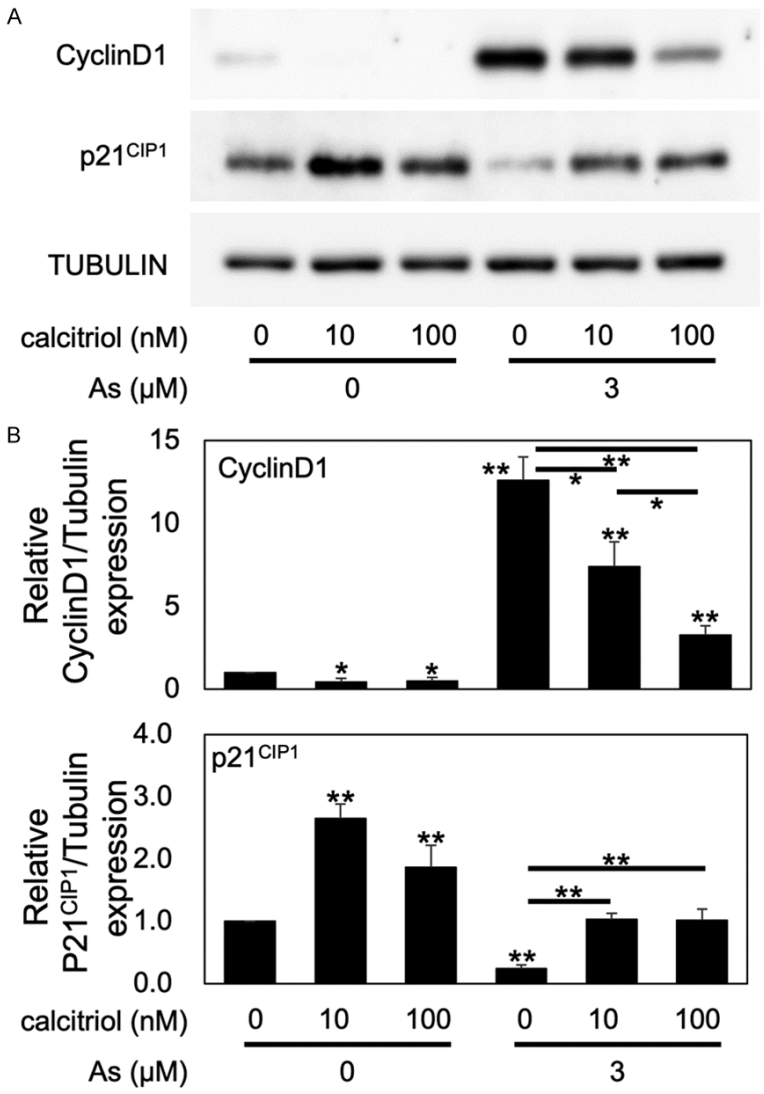

Figure 4. Protein levels of cell cycle-related molecules in arsenic-exposed HaCaT cells treated with calcitriol. A. Expression levels of Cyclin D1 and $p21^{CIP1}$ proteins in HaCaT cells untreated or treated with arsenic and/or calcitriol for 48 h are presented. The results are presented as ratios (mean ± SD; n=4) of these proteins relative to non-exposed HaCaT cells without calcitriol. B. Intensities of bands are presented as percentages (mean ± SD; n=4) relative to untreated HaCaT cells. * and **, Significantly different (*, P<0.05; **, P<0.01) by Tukey-Kramer test.

was no detection of arsenic in HaCaT cells without arsenic (**Figure 5**). These results suggest that calcitriol inhibits arsenic uptake, resulting in inhibition of arsenic-mediated cell toxicity.

Expression levels of aquaporins and VDR genes in HaCaT cells treated with arsenic and calcitriol

To address mechanisms of calcitriol-mediated inhibition of arsenic uptake, expression levels of *AQP7, AQP10* and *AQP9* genes were analyzed in calcitriol-treated HaCaT cells exposed to arsenic (**Figure 6**). Expression levels of all *aquaglyceroporin* genes in calcitriol-treated HaCaT cells were significantly lower than those in non-treated HaCaT cells (**Figure 6A-C**), suggesting that activated vitamin D3 usually represses all *aquaglyceroporin* genes expression. Expression levels of *AQP7* and *AQP9* genes in HaCaT cells exposed to arsenic were significantly higher than those in control HaCaT cells (**Figure 6A**, **6B**) whereas expression levels of *AQP10* gene in calcitriol-treated HaCaT cells were significantly lower than those in non-treated HaCaT cells (**Figure 6C**). Interestingly, arsenic strongly increased the expression level of VDR, a calcitriol receptor whereas calcitriol treatment showed no effect on that in HaCaT cells (**Figure 6D**). These results suggest that arsenic exposure regulates expression patterns of *aquaporin* genes with dependent on gene types in the family, and activated vitamin D3, such as calcitriol, strongly inhibit all *aquaporin* genes expression to regulate arsenic uptake.

Discussion

In this study, our anchorage-independent growth assays demonstrated calcitriol-induced inhibition of arsenic-mediated anchorage-independent growth of HaCaT cells. We also demonstrated the molecular mechanism by which calcitriol may regulate expression patterns of *aquaglyceroporin* genes in keratinocyte cells, resulting in inhibition of arsenic uptake and arsenic-mediated cellular proliferation via MEK/ERK and/or AKT signaling pathways and cell cycle-related molecules including Cyclin D1 and $p21^{CIP1}$. Our results suggest that calcitriol treatment pre-

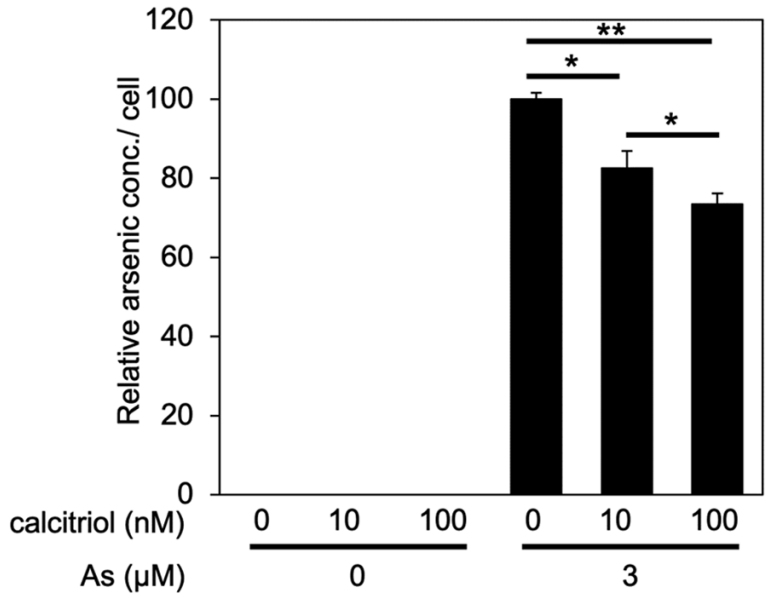

Figure 5. Calcitriol-mediated decrease of arsenic levels in HaCaT cells. Concentrations of arsenic in HaCaT cells untreated or treated with arsenic and/or calcitriol are presented (n=5). HaCaT cells were treated with arsenic for 120 minutes after treatment of calcitriol for 3 days. The results are presented as ratios (mean ± SD; n=4) of arsenic relative to untreated HaCaT cells. * and **, Significantly different (*, $P<0.05$; **, $P<0.01$) by Tukey-Kramer test.

vent arsenic-induced skin cancer development through inhibition of arsenic uptake.

The anchorage-independent growth assay is useful for analysis of arsenic-promoted tumorigenesis and has been used to identify and analyze molecules related to arsenic-promoted tumorigenesis, including Placental growth factor (PlGF) [7, 8]. Strong correlations between calcitriol and the tumor development have been reported in several types of tumors [23], and calcitriol treatments induce cell cycle arrest in many types of cancer cells [24, 25]. Our results, the inhibition of anchorage-independent growth by calcitriol treatment, suggest that calcitriol may be effective in the prevention and treatment of arsenic-promoted tumorigenesis.

We further analyzed the molecular mechanism of calcitriol-induced inhibition for arsenic-mediated cancer promotion in HaCaT cells. Since previous studies showed calcitriol-mediated decrease of phosphorylation levels of ERK1/2 and our study showed those of MEK and ERK1/2 in HaCaT cells, calcitriol may inhibit upper stream molecules, such as MEK in MEK/ERK signaling pathway. Previous studies also showed that arsenic exposure activates phosphorylation of ERK1/2 and AKT to induce cell proliferation through calcitriol decreases phosphorylation levels of AKT and our study showed that calcitriol inhibited arsenic-induced increase of AKT phosphorylation levels, suggesting that calcitriol inhibits arsenic-mediated increase of AKT phosphorylation levels.

Since we found that calcitriol decreased activities of ERK1/2 and AKT, calcitriol may suppress signaling pathways associated with ERK1/2 and AKT. The developmental process of various cancers including skin cancer caused by chronic exposure to arsenic is triggered by various molecular mechanisms such as activation of ERK and AKT [38]. Arsenic-mediated activation of ERK and/or AKT signaling pathways induces increase of Cyclin D1 expression and decrease of $p21^{CIP1}$ expression [38-40]. Therefore, we next examined whether calcitriol affects expression levels of Cyclin D1 and $p21^{CIP1}$. The results showed that Cyclin D1 expression was decreased and $p21^{CIP1}$ expression was increased in HaCaT cells treated with calcitriol on both mRNA and protein levels. Our results suggest that calcitriol suppresses cell cycle through regulation of ERK and AKT signaling pathways resulting in inhibition of tumorigenesis.

We finally analyzed relationships between calcitriol and arsenic uptake. The arsenic toxicity is closely related to the uptake of arsenic into the cells. Arsenic uptake is directly involved in cell tumorigenesis and is regulated by a variety of arsenic transporters, including aquaglyceroporins [41]. AQP7 and 9, members of aquaglyceroporins have been reported to be associated with arsenic uptake [15, 16]. Therefore, we assumed that effect to arsenic-promoted tumorigenesis by calcitriol treatment would be associated with levels of arsenic uptake via aquaglyceroporins. In our results, calcitriol reduced concentrations of arsenic in HaCaT

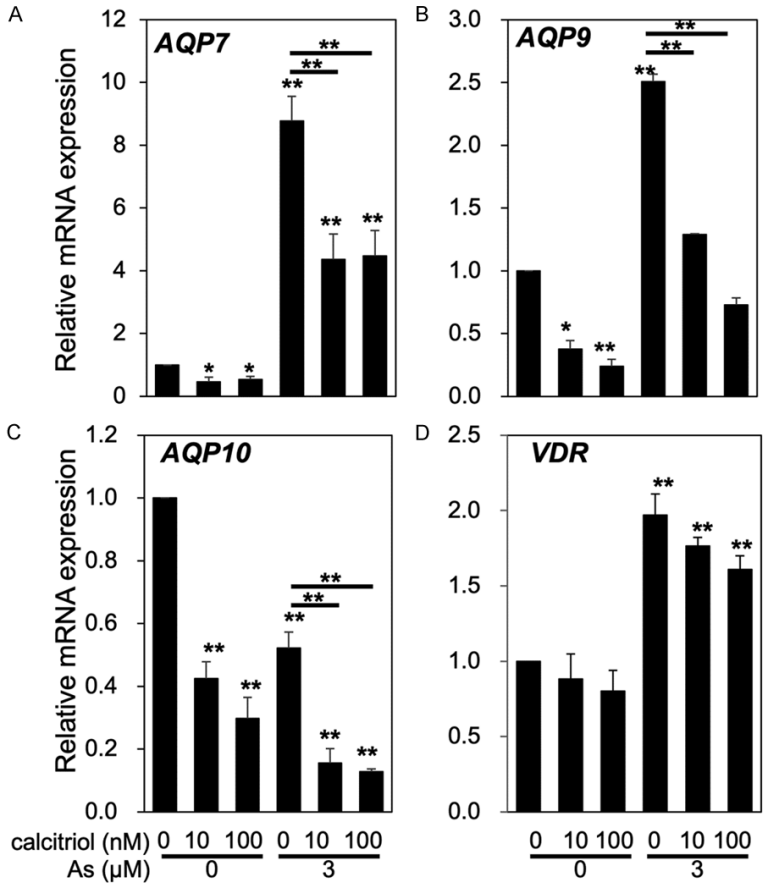

Figure 6. MRNA levels of aquaglyceroporin family genes in arsenic-exposed HaCaT cells treated with calcitriol. Expression levels of AQP7 (A), AQP9 (B), AQP10 (C) and VDR (D) mRNAs evaluated by real-time PCR in HaCaT cells untreated or treated with arsenic and/or calcitriol for 48 h are presented. The results are presented as ratios (mean ± SD; n=4) of these proteins relative to non-exposed HaCaT cells without calcitriol.

cells treated with arsenic. Our results also showed that calcitriol inhibited expressions of aquaglyceroporin genes including *AQP7*, *AQP9*, and *AQP10* in HaCaT cells treated with arsenic. Moreover, arsenic strongly increased the expression level of VDR whereas calcitriol treatment showed no effect on that in HaCaT cells, suggesting that sensitivity to calcitriol may be increased through the VDR overexpression in arsenic-promoted cancer cells. These results suggest that calcitriol prevents arsenic-promoted tumorigenesis by suppressing the activation of cancer-related molecules via inhibiting aquaporin expressions in keratinocytes.

Tumorigenesis induced by chronic arsenic exposure is known to occur not only in the skin but also in other organs such as the lungs and bladder [2-5]. To clarify whether calcitriol has an inhibitory effect on arsenic-induced tumorigenesis in cells other than skin keratinocytes, we performed the anchorage-independent growth assay using a human normal lung epithelial cell line (Beas-2b) (Figure S2). Whereas arsenic strongly induced anchorage-independent growth of Beas-2b cells without calcitriol (Figure S2A, S2D), 10 and 100 nM calcitriol inhibited arsenic-mediated anchorage-independent growth (Figure S2B, S2C, S2E, S2F). Arsenic-mediated anchorage-independent growth of calcitriol-treated Beas-2b cells was suppressed by 21.4-70.0% (Figure S2G), suggesting that calcitriol has the potential to suppress arsenic-induced tumorigenesis not only on keratinocytes, but also on other target cells including lung epithelial cells.

In conclusion, our results indicate that activated vitamin D3, calcitriol, may contribute to the prevention and therapy for arsenic-mediated diseases. Further study should be carried out to clarify the details of calcitriol function and the presence of side effects.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ichiro Yajima, Unit of Molecular and Cellular Toxicology, Department of Bioscience and Engineering, College of Systems Engineering and Science, Shibaura Institute of Technology, Fukasaku 307, Minuma-ku, Saitama 337-8570, Japan. Tel: +81-48-720-6035; Fax: +81-48-720-6011; E-mail: yajimai@shibaura-it.ac.jp

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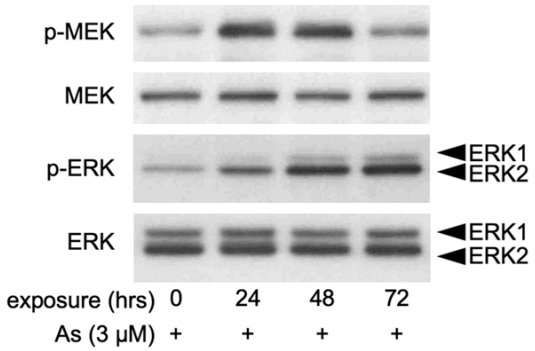

Figure S1. Relationship between arsenic exposure time and MEK/ERK signaling cascade. Phosphorylation and protein levels of MEK and ERK in HaCaT cells treated with arsenic for 0, 24, 48 and 72 h are presented.

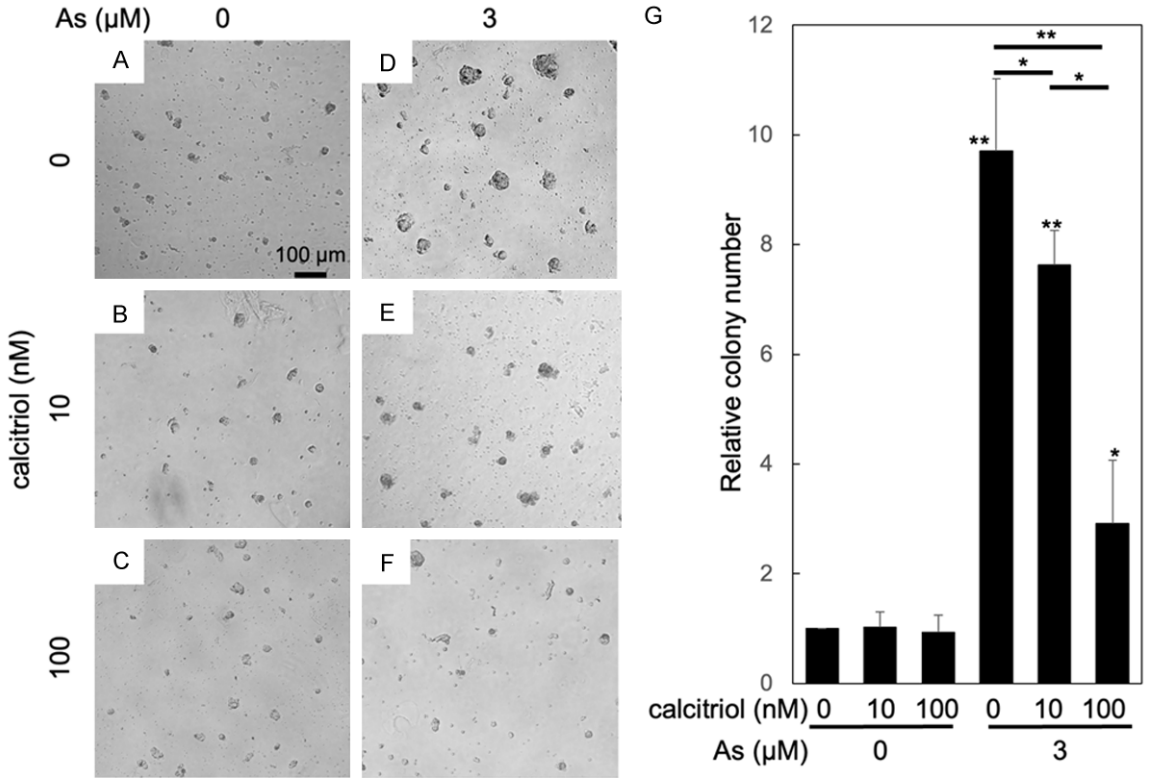

Figure S2. Inhibition of arsenic-mediated anchorage-dependent growth of calcitriol-treated Beas-2b cells. A-F. Colonies derived from arsenic-exposed Beas-2b cells untreated or treated with calcitriol. Original magnification, ×20. Scale bar: 100 μm. G. Number of colonies per well derived from HaCaT cells untreated or treated with arsenic and/or calcitriol are presented as ratios (mean ± SD; n=5) relative to untreated HaCaT cells. Significantly different from untreated HaCaT cells (**, $P<0.01$) by Tukey-Kramer test.