Research Article

Journal of Applied Toxicology

Received: 22 September 2011, Revised: 28 December 2011, Accepted: 28 December 2011 Published online in Wiley Online Library: 21 February 2012

(wileyonlinelibrary.com) DOI 10.1002/jat.2724

Phosphorylation of histone H3 at serine 10 has an essential role in arsenite-induced expression of *FOS*, *EGR1* and *IL8* mRNA in cultured human cell lines

Toshihide Suzuki,* Kayoko Kita and Takafumi Ochi

ABSTRACT: Trivalent inorganic arsenite [iAs(III)] is known to alter the expression of a number of genes associated with transcription and cell proliferation, which was thought to be one of the possible mechanisms of arsenical carcinogenesis. However, the detailed mechanisms underlying iAs(III) induction of changes in gene expression are not fully understood. Here we examine the role of histone H3 phosphorylation at serine 10 (Ser^{10}) in gene regulation when the cells were treated with iAs(III). Among the 34 genes tested, iAs(III) induced mRNA expression of *JUN*, *FOS*, *EGR1*, *HMOX1*, *HSPA1A*, *IL8*, *GADD45A*, *GADD45B* and *GADD153*. Phosphorylation of histone H3 Ser^{10} was induced by iAs(III) in interphase cells, and was effectively blocked by the ERKs pathway inhibitor (U0126). U0126 treatment significantly reduced constitutive mRNA expression of *FOS* and *EGR1*, and dramatically suppressed the induction of *FOS*, *EGR1* and *IL8* mRNA in iAs(III)-treated cells. The other genes, which were induced by iAs(III), were not affected by U0126 treatment. When the histone H3 nonphosphorylatable mutant of serine 10 (S10A) was overexpressed in cells, iAs(III) induction of *FOS*, *EGR1*and *IL8* expression was significantly decreased as compared with wild-type cells. The other genes induced by iAs(III) were not changed in S10A cells nor by U0126 treatment. In addition, S10A cells were more resistant to iAs(III) cytotoxicity. These results indicated that the phosphorylation of histone H3 at Ser^{10} through the ERKs pathway in interphase cells is an important regulatory event for iAs(III)-mediated gene expression. Aberrant gene expression seems to be an important cause of cytotoxicity and may have some relation to iAs (III) carcinogenicity. **Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: arsenite; histone H3; phosphorylation; epigenetics; gene expression

INTRODUCTION

Inorganic arsenics are widely distributed in the environment as natural components, and arsenic contamination of drinking water is a serious problem worldwide (IPCS, 2001). Arsenic is a well-established human carcinogen and ingestion of arsenic causes cancer of the skin, lung and bladder, and is also a suspected cause of kidney, liver and other malignancies (IARC, 2004). Several mechanisms by which arsenicals induce tumorigenesis have been proposed, including oxidative stress (Liu *et al*., 2001b), chromosomal abnormalities (Ochi *et al*., 2004a, 2008), protein binding (Kitchin and Wallace, 2008) and activation of the signal transduction pathway (Dong, 2002). Recent studies have suggested that the epigenetic modification by arsenicals, such as DNA methylation and histone modification, may also mediate the toxicity and carcinogenicity of arsenics (Salnikow and Zhitkovich, 2008; Ren *et al*., 2011).

Arsenic exposure has been shown to alter histone modification, such as histone methylation, acetylation and phosphorylation (Ren *et al*., 2011). Phosphorylation of histone H3 is involved in both cell division and transcription, which are events requiring opposite alteration in the degree of chromatin compaction (Nowak and Corces, 2004). Phosphorylation of histone H3 is critical for proper chromosome condensation and segregation during the mitosis (Wei *et al*., 1999; Ota *et al*., 2002). By contrast, phosphorylation of histone H3 in interphase cells induced chromatin relaxation and then gene expression (Prigent and Dimitrov, 2003; Kouzarides, 2007). We have previously reported that dimethylarsinous acid [DMA(III)] induced phosphorylation of histone H3 Ser^{10} in mitotic cells, and iAs(III) induced phosphorylation of histone H3 Ser^{10} in interphase cells (Suzuki *et al*., 2009). Phosphorylation of histone H3 Ser^{10} in interphase cells by iAs(III) may induce chromatin relaxation and genes expression.

The effects of arsenic compounds on mRNA expression have been widely examined by the microarray technique in cultured cells (Simeonova *et al*., 2000; Yih *et al*., 2002) and in rodents (Liu *et al*., 2001a; Xie *et al*., 2004). Arsenicals induce the expression of a number of genes associated with cell growth, such as *FOS*, *JUN* and *EGR1*, as well as cell arrest genes (*GADD153*, *GADD45*) and stress-responsive genes (*HMOX1*, *HSPA1A*). Although the detailed mechanisms have not been elucidated, phosphorylation of histone H3 may have some role in mRNA induction by iAs(III) exposure. Indeed iAs(III) promoted histone H3 phosphoacetylation at the chromatin of *FOS*, *JUN* and *CASPASE-10*, together with the expression of these genes (Li *et al*., 2002, 2003). CASPASEs are

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key enzyme for cell death in connection with apoptosis, and AP-1, which is heterodimer of *JUN* and *FOS*, mediates many biological effects of tumor promotion and is an important regulator of cell growth. These observations suggest that aberrant gene expression through histone modification is an important cause of cytotoxicity and carcinogenicity of iAs(III). In this study, we investigated whether phosphorylation of histone H3 Ser^{10} is required for the induction of gene expression when the cells are treated with iAs(III).

MATERIALS AND METHODS

Chemicals

Sodium arsenite [iAs(III)] was obtained from Tori Chemical Corp. (Uenohara, Japan). Dimethylarsine iodide, a model compound of trivalent dimethylarsenicals [DMA(III)], was a kind gift from Dr Walter Goessler (Analytical Chemistry, University of Graz, Austria). Anti-phospho-p44/42 ERK MAP kinases antibody, anti-p44/42 ERK MAP kinases antibody and U0126 were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-phosphohistone H3 (ser10) antibody and anti-histone H3 antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), HRP-conjugated anti-rabbit IgG antibody and HRP-conjugated anti-goat IgG from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), and TRITC-labeled anti-rabbit IgG antibody from EY Laboratories Inc. (San Mateo, CA, USA). All other chemicals used were of the highest grade commercially available.

Cell Culture

HepG2 cells, derived from human hepatocarcinoma, were grown in Dulbecco's modified Eagle's medium (D-MEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (MP Biomedicals Inc., Irvine, CA, USA). HeLa cells derived form human cervix carcinomas were grown in Eagle's essential medium (MEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum. The cells were cultured in an incubator in an atmosphere of 5% CO_2 at 37 °C in humidified air.

Treatment of Cells with Arsenicals

Sodium arsenite [iAs(III)] was dissolved in sterilized water as 20 mM of stock solution. Dimethylarsine iodide stored at −20 °C was first diluted to 5 mM solution with ice-cold 50% ethanol and then added to the culture medium immediately before the treatment. These arsenicals were prepared before each experiment.

Cytotoxicity

Cells, seeded at a density of 2×10^4 cells per well in 96-well plate (ASAHI Techno Glass Corp., Chiba, Japan) and preincubated for 24 h, were placed in fresh medium that contained various concentrations of arsenicals and then were incubated for another 24 h. The cytotoxic effects were assayed by the method of WST-8 (Cell Counting Kit-8, Wako Pure Chemical Industries Ltd, Osaka, Japan) as previously described (Ochi *et al*., 2008).

Immunofluorescence

HepG2 cells, seeded at the density of 1×10^4 cells per well in type-I collagen-coated eight-chamber slides (ASAHI TECHNO GLASS Corp.) and preincubated for 24 h, were placed in fresh medium that contained 50 μM of iAs(III) and then incubated for 5 h. After incubation, the cells were fixed with 4% paraformaldehyde in phosphate buffer for 20 min at room temperature then soaked in methanol at −20 °C for 5 min. The chamber slides were incubated with 2% skimmed milk in PBS for 90 min at room temperature. Next, the slides were stained with anti-phospho histone H3 (1:500 diluted in 2% skimmed milk in PBS) in a moist chamber at 37 °C for >90 min. After the washing, the slides were treated with TRITC-labeled anti-rabbit IgG, and analyzed basically same method as described previously (Ochi *et al*., 2004b).

Immunoblotting

Total cellular proteins (20 μg per lane) were electrophoretically separated using SDS–PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories Inc., Berkeley, CA, USA). The membrane was blocked with 5% skimmed milk or 5% BSA dissolved in TBS-T (Tris-Buffered Saline Tween20) for >6 h at 4 °C. The membrane was then incubated with first-antibody, anti-histone H3 (13,500), anti-phospho histone H3 (1:5000), anti-phospho p44/42 ERK MAP kinase (1:3,500) and anti-p44/42 ERK MAP kinase (1:3,500) overnight at 4 °C with gentle agitation. The blot was washed and incubated with HRP-conjugated anti-rabbit IgG (1:20,000) or HRP-conjugated anti-goat IgG (1:20,000) for 90 min at room temperature. The blot was washed and detected using the ECL Plus Western Blotting Detection System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and analyzed with the LAS-3000 mini (Fujifilm, Tokyo, Japan).

Semiquantitative RT-PCR Assay and Quantitative Real-time PCR Assay

Total RNA was extracted from HepG2 cells or HeLa cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), and cDNA was synthesized using the SuperScript III First-strand Synthesis system for real-time polymerase chain reaction (RT-PCR; Invitrogen, Carlsbad, CA, USA) with $Oligo(dT)_{12–18}$ primer.

Semiquantitative RT-PCR assay was performed using the DNA Engine PTC-200 (Bio-Rad Laboratories Inc.) using gene specific primers, and optimum PCR conditions, such as annealing temperature and PCR cycle, were determined for each gene before the experiments (Table 1). The PCR products were analyzed using the Archiver Eclipse (Fotodyne, WI, USA) after electrophoresis through 1.2% agarose gel with ethidium bromide, and quantitated by ImageGauge (Fujifilm).

The quantitative real-time PCR assay was performed using the ABI 7500 real time-PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR *Premix Ex Taq* (TAKARA BIO Inc., Shiga, Japan) according to the manufacturer's instructions. The *JUN*, *FOS*, *EGR1*, *P21*, *GADD45A*, *GADD153*, *HSPA1A*, *IL8* and *GAPDH* specific primers for quantitative real-time PCR were designed using Primer Express (Applied Biosystems).

Construction of Histone H3 Mutant (S10A)

The point mutation of histone H3 at serine 10 to alanine was generated using the KOD-Plus-Mutagenesis kit (Toyobo Co.

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Table 1. The primer sequences, annealing temperature and PCR cycle number for semiquantitative RT-PCR

	Gene	Primer		Annealing	PCR
		Forward	Reverse	temperature	cycle
Transcription factor	*JUN*	TGAAGGAGGAGCCTCAGAC	TGGATACCCTTGGCTTTAG	58	32
	FOS	TGCTTTGCAGACCGAGATTG	TGAAGACGAAGGAAGACGTG	62	32
	MYC	CCACCACCAGCAGCGACTCTG	CCAAGACGTTGTGTGTTCGCG	65	30
	EGR1	CGGCAGAAGGACAAGAAAGC	GGAAGTGGGCAGAAAGGATT	65	30
Stress protein	*HMOX1*	TTCTTCACCTTCCCCAAC	GCATAAAGCCCTACAGCAAC	66	32
	HSPA1A	TACTCCTGCGTGGGGGTGTT	CTGGCTGATGTCCTTCTTGTGT	67	29
	HSPA4	CATCTTATCGCCTGCTTTCA	TCTGCTGCTGTTGCTCTTCA	67	29
	HSPA5	CCGTCCTATGTCGCCTTCAC	TTCTTCTCCCCCTCCCTCTT	60	29
	HSPA8	GATGAGAAGCAGAGGGACAA	AGCACCACCAGAGGGAGGAG	67	29
	HSPA9	TGTCACTCCCCTGTCTCTGG	TCCTGTTTCGCTGTCTTTTC	67	29
	HSPB1	GAGTGGTCGCAGTGGTTAGG	GGCAGCAGGGGTGGGCATCC	67	29
	HSPD1	TGGAAAAACACTGAATGATG	AAACCCTGGAGCCTTGACTG	57	30
	HSP90AA1	CGGTGTTGGTTTTTATTCTG	TCCTTCTTTTCTTCTTCCTC	57	30
	HSP90B1	ATGCTTCTGATGCCTTGGAC	ACTTCTTTGACCCGCCTCTC	60	29
	MT2A	ATGGATCCCAACTGCTCCT	AAGTCGCGTTCTTTACATCT	60	29
	IL8	GCAGAGGGTTGTGGAGAAGT	GAGGTAAGATGGTGGCTAAT	62	32
Cell cycle	*P21*	TTCTCCTTTTCCTCTCTCC	TCTACTCCCCCATCATATACC	60	32
	TP53	AGCACATGACGGAGGTTGTGA	TGGTTTCTTCTTTGGCTGGGG	60	32
	GADD45A	GACCGAAAGGATGGATAAGG	CGCAGGATGTTGATGTCGTT	64	31
	GADD45B	AACATGACGCTGGAAGAGCTCG	ACAGATTCTGCTGCTGGGAAGG	68	32
	GADD153	AGGAGGAAGAGGAGGAAGAC	TGGGAAAGGTGGGTAGTGTG	64	31
	AURKB	CAGAGACATAAAGCCAGAAAATC	ACAAAGGAGGAGGTAGAAAACAG	58	32
Oxidative stress	*SOD1*	AAGGCCGTGTGCGTGCTGAA	CAATAGACACATCGGCCACAC	67	30
	SOD2	CACCAGCAGGCAGCTGGCTCC	TCCACCACCGTTAGGGCTGAGG	67	30
	CAT	AACCGCACGCTATGGCTGAC	CCAGTGATGAGCGGGTTACA	60	32
	GPX1	TGGCTTCTTGGACAATTGCG	CCACCAGGAACTTCTCAAAG	64	32
	GPX4	CGATACGCTGAGTGTGGTTTG	CGCTGGATTTTCGGGTCTGC	64	32
	PRDX1	TGTGTTGGGACTGCTGATAG	CAAAGGAAGAAAGGCTGGTC	67	30
	PRDX2	CCCACGCAGCTTTCAGTCAT	GGATTTTGGAGGGGCAGGTC	65	32
	PRDX3	TGAAGATGGCGGCTGCTGTA	ATTGGTTCCTTGCCTTCTAC	62	34
	PRDX4	CGCCAAGGGACGTGTTTCTG	TGGTAATGAAACCGTGAACT	62	34
	PRDX5	AGGAGGCGGAGTGGAAGTGG	GGGGTGGAGGAAGTAATCTG	68	34
	PRDX6	CCTCATCACCGTCGCCATGC	TCACAGCACCAACTTCTCCA	62	32
	TXN	AGACTCCAGCAGCCAAGATG	TGTTTATTGTCACGCAGATG	62	32
loading control	*GAPDH*	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC	60	22

Ltd, Osaka, Japan) and subcloned into the pcDNA3.1 vector (Invitrogen). The entire coding region of histone H3 was sequenced to confirm to the desired mutation and the absence of any additional mutation. The constructed mutant vector was transfected to HeLa cells using LipofectAMINE2000 (Invitrogen), and stably expressed cells were established by cultivation with a G418 (400 $\mu g\ ml^{-1}$; Invitrogen) for 6 weeks. After the selection, the mRNA expression ratio of wild vs mutant histone H3 was analyzed by semiquantitative RT-PCR using the wild histone H3 (F: AAAGCAGACTGCCCGCAAAT; R: GCTAGCTGGATGTCTTTTGG-CATAA) and S10A histone H3 (F: AAAGCAGACTGCCCGCAAAG; R: GCTAGCTGGATGTCTTTTGGCATAA) specific primers.

Statistical Analysis

The statistical significance of the experimental data was evaluated using Student's *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Effects of iAs(III) and DMA(III) on mRNA Expressions

To evaluate whether or not iAs(III) and DMA(III) induced mRNA expression, we selected 34 genes classified as transcription factor, stress related protein, cell-cycle genes and oxidative stress-related genes; these were analyzed using a semiquantitative RT-PCR assay. Based on the previous experiment, HepG2 cells were treated with equitoxic levels of iAs(III) (50 μM) and DMA(III) (1 μM) for 2 and 5 h, respectively. Inorganic arsenite [iAs(III)] induced expression of 10 genes more than 3-fold, and DMA(III) induced eight genes more than 2-fold that with untreated cells (Table 2). Inorganic arsenite [iAs(III)] induced expression of *JUN*, *FOS*, *EGR1*, *IL8*, *GADD45A* and *GADD153* mRNA more than 6-fold as compared with the untreated control. In contrast, DMA(III) induced mRNA expression 4.27-fold at the most, and in most cases less than 3-fold as compared with the untreated control. Both iAs(III) and DMA(III) induced the same genes except *EGR1*, and only iAs(III) induced *EGR1*.

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Table 2. Effects of iAs(III) and DMA(III) on mRNA expression in HepG2 cells

	Gene	Accession number	iAs(III) 50 μM		DMA(III) 1 μM	
			2 h	5 h	2 h	5 h
Transcription factor	*JUN*	NM_002228	4.68	4.71	2.55	2.35
	FOS	NM_005252	8.48	7.30	1.90	1.86
	MYC	NM_002467	1.59	1.99	1.46	1.56
	EGR1	NM_001964	7.54	6.10	1.02	0.89
Stress-related protein	*HMOX1*	NM_002133	3.57	4.55	1.64	2.83
	HSPA1A	NM_005345	3.50	3.36	2.60	3.54
	HSPA4	NM_002154	1.00	1.06	0.94	1.24
	HSPA5	NM_005347	1.33	0.97	0.94	0.96
	HSPA8	NM_006597	1.47	1.16	1.29	1.54
	HSPA9	NM_004134	0.95	1.08	0.95	0.90
	HSPB1	NM_001540	0.99	1.34	1.06	1.13
	HSPD1	NM_002156	1.13	1.23	1.19	1.29
	HSP90AA1	NM_005348	1.11	1.26	1.52	1.68
	HSP90B1	NM_007355	1.18	1.13	0.98	0.98
	MT2A	NM_005953	1.63	1.99	0.80	1.04
	IL8	NM_000584	5.66	11.91	2.97	4.27
Cell cycle	*P21*	NM_000389	0.74	2.16	2.18	3.72
	TP53	NM_000546	1.02	1.01	1.23	1.73
	GADD45A	NM_001924	3.66	6.60	2.07	3.16
	GADD45B	NM_015675	4.47	4.62	2.23	1.53
	GADD153	NM_004083	7.76	11.32	2.15	1.68
	AURKB	NM_004217	1.17	1.25	1.21	1.11
Oxidative stress	*SOD1*	NM_000454	1.26	1.26	1.14	1.22
	SOD2	NM_000636	1.17	1.07	1.22	1.21
	CAT	NM_001752	0.90	1.15	1.02	1.14
	GPX1	NM_000581	1.20	1.05	1.13	0.86
	GPX4	NM_002085	1.01	0.99	1.01	1.03
	PRDX1	NM_002574	1.24	0.84	1.07	1.25
	PRDX2	NM_005809	1.11	1.29	1.02	1.19
	PRDX3	NM_006793	1.10	1.05	1.10	1.17
	PRDX4	NM_006406	1.26	1.05	1.14	1.25
	PRDX5	NM_012094	1.04	0.96	1.01	1.01
	PRDX6	NM_004905	1.46	0.93	1.26	1.12
	TXN	NM_003329	1.14	1.08	1.15	1.22

Values are normalized with GAPDH and represented as relative ratio against untreated control.
The reproducibility of the results was confirmed by at least two separate experiments.

Next, we investigated time-dependent induction of eight genes that were markedly induced by iAs(III)-treatment by means of quantitative RT-PCR assay. Inorganic arsenite [iAs(III)] induced transcription factors (*JUN*, *FOS*, *EGR1*) and *GADD45A* quickly, then decreased until 8 h. In contrast, *GADD153*, *HSPA1A* and *IL8* were increased until 5 h after iAs(III) treatment, and *P21* increased time dependently up to 8 h after the treatment (Fig. 1).

iAs(III)-induced Phosphorylation of Histone H3 Ser^{10} in Interphase Cells and Effects of ERK Pathway Inhibitor (U0126) on Phosphorylation of Histone H3 Ser^{10}

As iAs(III) dramatically induced mRNA expression of some genes, we investigated the localization of phospho-histone H3 Ser^{10} in HepG2 cells, because phosphorylation of histone H3 in interphase cells is thought to induce chromatin relaxation and then gene expression. Phospho-histone H3 Ser^{10} is generally observed in entire nuclei of mitotic cells and not in interphase cells, but iAs(III) induced phosphorylation of histone H3 Ser^{10} at specific loci as a punctate signal in interphase cells (Fig. 2A).

ERKs are known to be the main kinase that phosphorylates histone H3 in interphase cells. Therefore, we evaluated the effects of ERKs pathway inhibitor (U0126) on iAs(III)-dependent phosphorylation of histone H3 Ser^{10} in HepG2 cells. Inorganic arsenite [iAs(III)] induced phosphorylation of histone H3 Ser^{10} and activation of ERKs after 0.5 h treatment (Fig. 2B). In contrast, inhibition of the ERKs pathway by U0126 effectively reduced activation of ERKs and phosphorylation of histone H3 until 2 h after the treatment.

Effects of ERKs Pathway Inhibitor (U0126) on iAs(III)-induced mRNA Expression

As ERKs pathway inhibitor (U0126) effectively reduced the phosphorylation of histone H3 Ser^{10} resulting from iAs(III) treatment,

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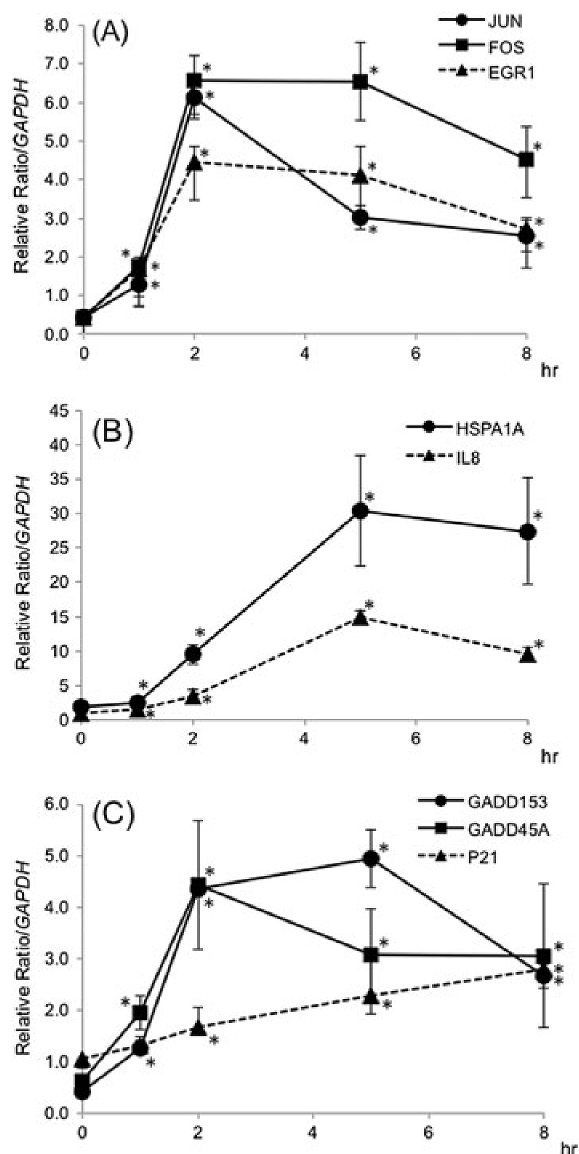

Figure 1. Time-dependent mRNA expression of *JUN*, *FOS*, *EGR1*, *GADD45A*, *GADD153*, *P21*, *HSPA1A* and *IL8* in HepG2 cells exposed to iAs(III). HepG2 cells, seeded at the density of 8×105 cells/6-cm dish and preincubation for 24 h, were treated with 50 μM iAs(III) for 1 h, 2 h, 5 h and 8 h. After incubation, total RNA was extracted and quantitative real-time PCR was performed as described in Materials and methods. (A) Transcription factor, (B) Stress related protein, (C) Cell cycle related gene. The data are given as relative ratio against GAPDH and represented as mean ± SE (n = 3). The reproducibility of the results was confirmed by at least two separate experiments. *: Significantly different form untreated control group ($p < 0.05$).

we next evaluated the effects of U0126 on iAs(III)-dependent gene expression in HepG2 cells. Basal mRNA expression levels [untreated iAs(III)] of *FOS* and *EGR1* were significantly reduced in U0126-treated cells, and iAs(III)-induced expression of *FOS* and *EGR1* were effectively blocked by U0126 treatment (Fig. 3). Although the basal expression level of *IL8* was not changed in U0126-treated cells, iAs(III)-induced expression of *IL8* was significantly reduced by U0126 treatment. Five other genes were not affected by U0126, significantly.

Histone H3 Ser^{10} (S10A) Mutant

To confirm the significance of histone H3 Ser^{10} phosphorylation in iAs(III)-mediated mRNA induction, we constructed a histone H3 nonphosphorylatable mutant by replacing serine 10 of histone H3 with alanine (S10A; Fig. 4A) and transfected it into HeLa cells. The mRNA expression level of wild-type histone H3 vs mutant histone H3 was 4.7:1 in S10A cells (Fig. 4B). The phosphorylation of histone H3 Ser^{10} by iAs(III) was suppressed in S10A cells relative to wild-type cells (Fig. 4 C), and S10A cells were more resistant to iAs(III) toxicity (IC_{50}: wild cells, 28.5 μM; S10A cells, 47.6 μM; Fig. 4D).

Inorganic arsenite [iAs(III)] induced mRNA expression of *JUN*, *FOS*, *EGR1*, *P21*, *GADD45A*, *GADD153*, *HSPA1A* and *IL8* in HeLa cells as well as observed in HepG2 cells (Fig. 5). Basal mRNA expression levels [untreated iAs(III)] of *FOS* and *EGR1* were reduced in S10A cells, and iAs(III)-induced expression of *FOS* and *EGR1* was effectively suppressed in S10A cells as well as in the experiment on ERK pathway inhibition (U0126; Figs 3 and 5). Further, the basal expression level of *IL8* mRNA did not differ between wild-type cells and S10A cells, but induction of *IL8* by iAs(III) was effectively reduced in S10A cells, the same as with U0126 treatment experiment. The mRNA expression levels of five other genes did not differ substantially between wild-type cells and S10A cells.

DISCUSSION

Mitotic phosphorylation of histone H3 Ser^{10} is critical for proper chromosome condensation and segregation under normal physiological conditions. By contrast, interphase phosphorylation of histone H3 Ser^{10} is a somewhat unusual condition that induces chromatin relaxation and gene expression (Prigent and Dimitrov, 2003). We have previously reported that trivalent dimethylarsinous acid [DMA(III)] induced phosphorylation of histone H3 Ser^{10} in mitotic cells and trivalent inorganic arsenite [iAs(III)] induced phosphorylation of histone H3 Ser^{10} in interphase cells (Suzuki *et al.*, 2009). DMA(III) induced mitotic abnormality and then induced mitotic arrest. As histone H3 Ser^{10} is phosphorylated in mitotic cells, DMA(III)-induced phosphorylation of histone H3 Ser^{10} seems to be a result of accumulation of the mitotic cells. In contrast, phosphorylation of histone H3 Ser^{10} in interphase cells by iAs(III) appears to be related to epigenetic regulation of gene expression, because arsenite is known to induce a number of genes (Simeonova *et al.*, 2000; Liu *et al.*, 2001a; Yih *et al.*, 2002). Recently, it was demonstrated that overexpression of histone H3 induced neoplastic cell transformation, and overexpression of a histone H3 mutant that was unable to undergo phosphorylation of Ser^{10} suppressed cell transformation (Choi *et al.*, 2005). In this regard, the significance of arsenic induction of histone H3 phosphorylation is important for elucidating arsenic cytotoxicity and tumorigenesis.

We selected treatment conditions [iAs(III) 50 μM; DMA(III) 1 μM] based on the previous experiment (Suzuki *et al.*, 2009) and a preliminary dose-dependence experiment. The IC_{50} of iAs(III) on HepG2 cells was 70.1 μM and the IC_{50} of DMA(III) was 1.1 μM, and more than 60% of cells survived after 24 h treatment under these conditions. We first selected 34 genes, and tried screening for the iAs(III)-induced genes by means of semiquantitative RT-PCR assay. Inorganic arsenite [iAs(III)] remarkably induced mRNA expression more than DMA(III) when the cells were treated with equitoxic levels of these arsenicals (Table 2). Then we focused on

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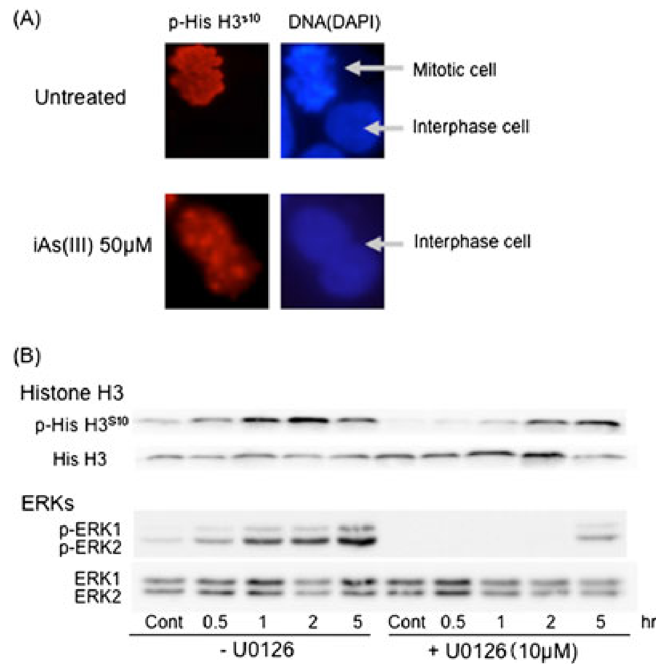

Figure 2. Localization of phospho histone H3 Ser10 and effects of ERKs pathway inhibitor (U0126) on iAs(III)-mediated phosphorylation of histone H3 Ser10 in HepG2 cells. (A) HepG2 cells, seeded at the density of 1-×-104 cells/well in collagen-coated 8-chamber slides and preincubation for 24-h, were placed in fresh medium that contained 50-μM of iAs(III) for 5-h. The cells on the slides were stained for p-histone H3 Ser10 and chromosomal DNA as described in Material and methods. (B) HepG2 cells, seeded at the density of 8-×-105 cells/6-cm dish and preincubation for 24-h, were pre-treated with or without 10-μM of U0126 for 1-h then treated with 50-μM of iAs(III) for specific time. After incubation, cell proteins were extracted and immunoblot analysis was performed as described in Materials and methods. The reproducibility of the results was confirmed by at least two separate experiments.

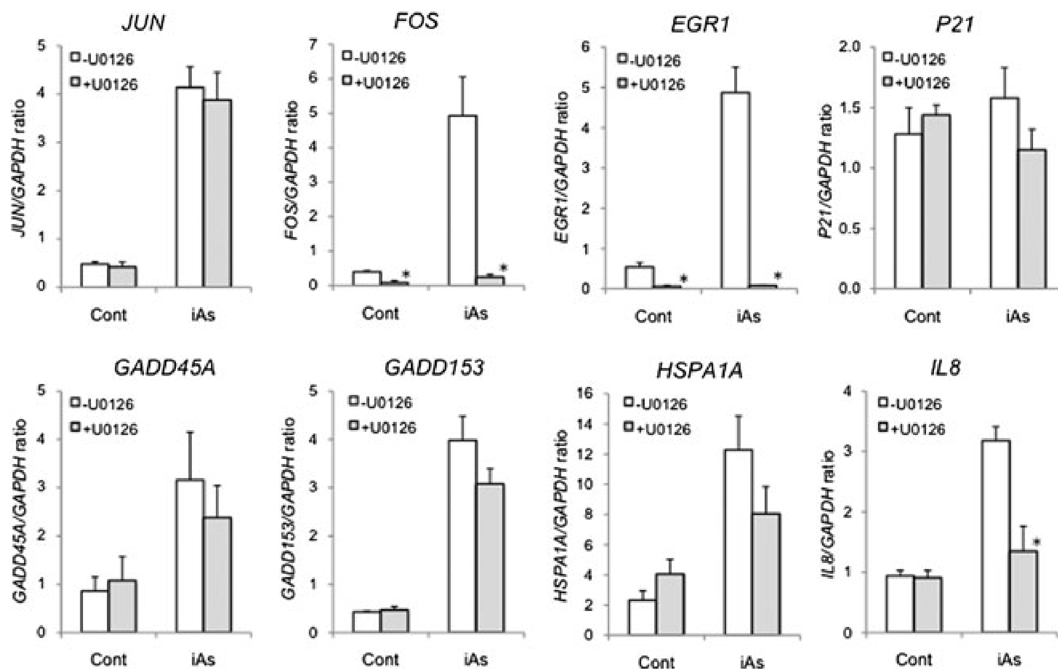

Figure 3. Effects of ERKs pathway inhibitor (U0126) on iAs(III)-mediated mRNA expression of *JUN*, *FOS*, *EGR1*, *GADD45A*, *GADD153*, *P21*, *HSPA1A* and *IL8* in HepG2 cells. HepG2 cells, seeded at the density of 8 × 105 cells/6-cm dish and preincubation for 24 h, were pre-treated with or without 10 μM of U0126 for 1 h then treated with 50 μM of iAs(III) for more 2 h. After incubation, total RNA was extracted and quantitative real-time PCR was performed as described in Materials and methods. The data are given as relative ratio against GAPDH and represented as mean ± SE (n = 3). The reproducibility of the results was confirmed by at least two separate experiments. *: Significantly different form untreated control group ($p < 0.05$).

the effects of iAs(III), in connection with epigenetic regulation of gene expression, because iAs(III) induced phosphorylation of histone H3 Ser^{10} in interphase cells (Fig. 2). Based on the semiquantitaive RT-PCR assay, we selected eight genes, *JUN*, *FOS*, *EGR1*, *IL8*, *HSPA1A*, *GADD45A*, *GADD153* and *P21*, and evaluated them in more detail and more precisely by quantitative RT-PCR

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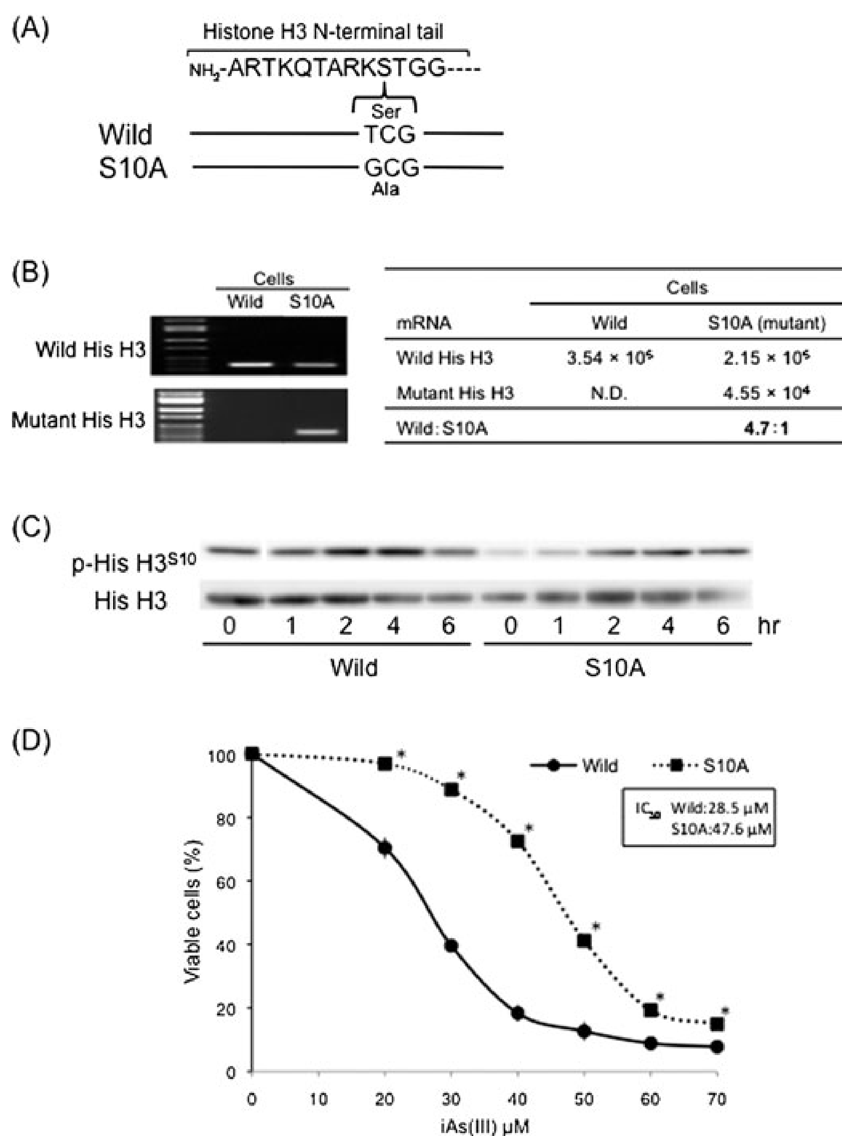

Figure 4. The characteristic of nonphosphorylatable histone H3 mutant cells (S10A). (A) Schematics of histone H3 mutant of serine 10 to alanine (S10A). (B) HeLa cells were transfected with S10A vector and selected with 400 μM G418 for 6 weeks. After the selection, mRNA expression ratio of wild versus mutant histone H3 was analyzed by using the wild histone H3 and S10A H3 specific primers. (C) HeLa cells, seeded at the density of 8 × 105 cells/6-cm dish and preincubation for 24 h, were placed in fresh medium that contained 50 μM of iAs(III) for specific time. After incubation, cell proteins were extracted and phospho histone H3 Ser10 and histone H3 were analyzed by immunoblotting as described in Materials and methods. The reproducibility of the results was confirmed by at least two separate experiments. (D) Wild cells and S10A cells, seeded at the density of 2 × 104 cells/well in 96-well plates and preincubated for 24 h, were placed in fresh medium that contained specified concentration of iAs(III), and then incubated for 24 h. After the incubation, the cytotoxic effects were evaluated by the method of WST-8 as described in Material and methods. The data are given as percent of the untreated control and represented as mean ± SE (n = 4). The reproducibility of the results was confirmed at least by two separate experiments.

assay. When the HepG2 cells were treated with 25 μM of iAs(III), some genes were induced less than 2-fold. In contrast, six genes, but not *GADD45A* and *P21*, were induced more than 10-fold by 100 μM of iAs(III) as compared with control untreated cells (data not shown). mRNA expression of these genes was very sensitive to iAs(III) concentration and treatment time (Fig. 1) as well as cell type (Figs 3 and 5). As sensitivity to iAs(III) was different in HepG2 cells and HeLa cells, further study should be conducted to determine the effects of gene expression on iAs(III) cytotoxicity among the different cell lines.

Phosphorylation of histone H3 is mediated by different kinases that depend on the stage of the cell cycle. Aurora kinase B is known to govern histone H3 phosphorylation during mitosis, and MAP kinases, especially ERKs, are responsible for histone H3 phosphorylation during interphase (Nowak and Corces, 2004). As iAs(III) has been known to activate the ERK pathway (Lau *et al*., 2004; Chowdhury *et al*., 2010), we next evaluated the effects of ERK pathway inhibitor (U0126) on iAs(III)-mediated histone H3 Ser^{10} phosphorylation and gene expression. U0126 reduced phosphorylation of histone H3 Ser^{10} (Fig.2) and effectively blocked *FOS*, *EGR1* and *IL8* induction by iAs(III) (Fig. 3). In addition, expressions of *FOS*, *EGR1* and *IL8* mRNA induced by iAs(III) were effectively reduced in histone H3 nonphosphorylatable mutant (S10A) cells (Fig. 5). These results suggested that iAs

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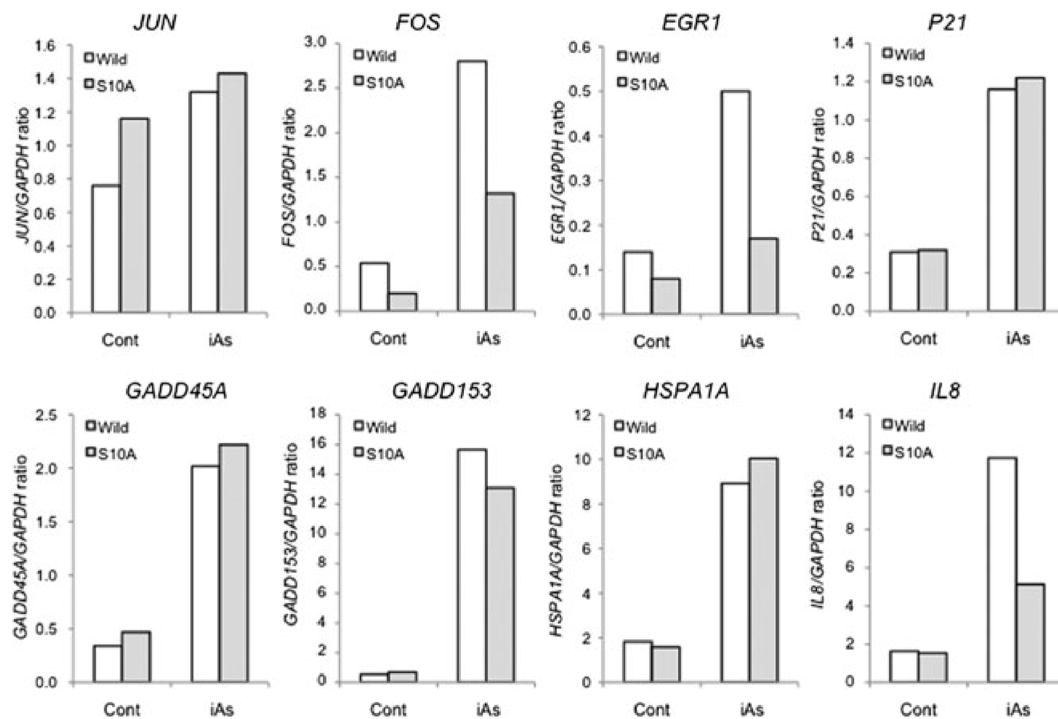

Figure 5. Effects of iAs(III) on mRNA expression of *JUN*, *FOS*, *EGR1*, *GADD45A*, *GADD153*, *P21*, *HSPA1A* and *IL8* in wild and S10A cells. Wild cells and S10A cells, seeded at the density of 8×105 cells/6-cm dish and preincubation for 24 h, were treated with 50 μM of iAs(III) for more 2 h. After incubation, total RNA was extracted and quantitative real-time PCR was performed as described in Materials and methods. The data are given as relative ratio against *GAPDH*. The reproducibility of the results was confirmed by at least two separate experiments.

(III) activated the ERKs pathway and phosphorylated histone H3 Ser^{10}, which might have induced chromatin relaxation, and then induced *FOS*, *EGR1* and *IL8* mRNA expression. These genes are known to be associated with cell growth and thought to promote tumor generation. In contrast, other genes, which were induced by iAs(III) but not affected by U0126 treatment and S10A mutant cells, such as *P21*, *GADD45*, *GADD153* and *HSPA1A*, are known to play a protective role against many types of stresses.

JUN and *FOS* are the main components of activator protein 1 (AP1), which mediates many biological effects of tumor promoters and is an important regulator of cell growth (Shaulian and Karin, 2002). The AP1 pathway contributes to the expression of cyclin D1(Zhang *et al*., 2009) and overexpression of *FOS* increases the proliferation of human hepatocyte by stabilizing nuclear cyclin D1 (Guller *et al*., 2008). Arsenite is known to induce cyclin D1 expression through the AP1 pathway (Hwang *et al*., 2006), and inhibition of cyclin D1 expression by its specific siRNA resulted in impairment of tumor colony formation by arsenite (Ouyang *et al*., 2008; Zhang *et al*., 2009). In this regard, remarkable induction of *FOS* by iAs(III) triggered activation of AP1 pathway and may induce deregulations of the cell cycle.

Early growth response (*EGR*) genes are nuclear transcription factors that are implicated in regulating cell proliferation, as G_0–G_1 transition of the cell cycle in a variety of cell lines upon mitogenic stimulation (Molnar *et al*., 1994). *EGR1* mRNA levels were found to be elevated in human prostate carcinomas in proportion to grade and stage (Eid *et al*., 1998). In addition, inhibition of EGR1 expression by antisense oligonucleotides in prostate cancer cells decreased cell proliferation, whereas stable expression of EGR1 in normal prostate epithelial cells promoted transformation (Baron *et al*., 2003). Functional EGR1 binding sites are found in the promoter domains of a large number of genes involved in cell growth, including oncogenes, growth factors (e.g. transforming growth factor β1 and platelet-derived growth factor A chain; Takimoto *et al*., 1991; Kim *et al*., 1994), and proteins involved in cell cycle control (e.g. thymidine kinase, cyclin D; Molnar *et al*., 1994; Xiao *et al*., 2005). In this regard, remarkable induction of *EGR1* by iAs(III) may disrupt the cell growth control.

In this study, basal mRNA expression levels [untreated iAs(III)] and iAs(III)-mediated induction of *FOS* and *EGR1* were significantly reduced in U0126 treated cells and S10A mutant cells (Figs 3 and 5). In contrast, basal *IL8* mRNA levels were not affected and *IL8* induction by iAs(III) was reduced in U0126-treated cells and S10A mutant cells (Figs 3 and 5). These results suggested that phosphorylation of histone H3 Ser^{10} is essential for *FOS* and *EGR1* expression, and phosphorylation of histone H3 Ser^{10} is responsible for iAs(III)-mediated *IL8* induction. As the *IL8* promoter region contains binding sites for NF-κB, C/EBP and AP1, basal *IL8* expression is not regulated by AP1 and AP1 might regulate iAs(III)-mediated *IL8* expression (Ma *et al*., 2009). In addition, knockdown of *EGR1* inhibited *IL8* production and *IL8*-mediated tumor colony formation (Ma *et al*., 2009). These observations suggest that *IL8* expression is regulated through the *AP1* and/or *EGR1*signaling pathway when the cells are exposed to iAs (III). Interleukin-8 (*IL8*), originally discovered as a chemotactic factor for leukocytes, has been shown to contribute to human cancer progression through its functions as a mitogenic, angiogenic and motogenic factor (Xie, 2001; Waugh and Wilson, 2008). Indeed, *IL8* expression was associated with the progression of prostate cancer (Uehara *et al*., 2005), and localization of *IL8* was changed in correlation with advancing stage of the disease (Murphy *et al*., 2005). In addition, exogenous *IL8* increased the proliferation of cells and

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mitogenic potency (Murphy *et al*., 2005), whereas knockdown of *IL8* reduced tumor colony formation (Ma *et al*., 2009). Although the carcinogenic mechanisms are very complicated and study of animal models is essential, overexpression of *IL8* may be one of the important causes of iAs(III) carcinogenicity.

At interphase, the phosphorylation of histone H3 Ser^{10} affected only a subset of genes, which was thought to be transcriptional activation (Li *et al*., 2003; Prigent and Dimitrov, 2003). In this report, iAs(III) induced phosphorylation of histone H3 Ser^{10} at specific loci as a punctate signal in interphase cells (Fig. 2). Although further studies are required to clarify the mechanisms in detail, phosphorylative loci observed in iAs(III)-treated cells might be within the *FOS* and *EGR1* genes. In the present study, we found epigenetic modification of histone H3 Ser^{10} by iAs(III) through the ERKs pathway, and this modification triggered the induction of transcription factors, such as *FOS* and *EGR1*. These transcription factors further induce downstream effector proteins such as *IL8*, which may play an important role in the iAs(III) cytotoxicity and one of the possible cause of iAs(III) carcinogenicity.

Acknowledgments

This work was partly supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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