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Sodium Arsenite Delays the Differentiation of C2C12 Mouse Myoblast Cells and Alters Methylation Patterns on the Transcription Factor Myogenin

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Abstract

Epidemiological studies have correlated arsenic exposure with cancer, skin diseases, and adverse developmental outcomes such as spontaneous abortions, neonatal mortality, low birth weight, and delays in the use of musculature. The current study used C2C12 mouse myoblast cells to examine whether low concentrations of arsenic could alter their differentiation into myotubes, indicating that arsenic can act as a developmental toxicant. Myoblast cells were exposed to 20 nM sodium arsenite, allowed to differentiate into myotubes, and expression of the muscle-specific transcription factor myogenin, along with the expression of tropomyosin, suppressor of cytokine signaling 3 (Socs3), prostaglandin I₂ synthesis (Ptgis), and myocyte enhancer 2 (Mef2) were investigated using QPCR and immunofluorescence. Exposing C2C12 cells to 20nM sodium arsenite delayed the differentiation process, as evidenced by a significant reduction in the number of multinucleated myotubes, a decrease in myogenin mRNA expression, and a decrease in the total number of nuclei expressing myogenin protein. The expression of mRNA involved in myotube formation, such as Ptgis and Mef2 mRNA, was also significantly reduced by 1.6-fold and 4-fold during differentiation. This was confirmed by immunofluorescence for Mef2, which showed a 2.6-fold reduction in nuclear translocation. Changes in methylation patterns in the promoter region of myogenin (−473 to +90) were examined by methylation-specific PCR and bisulfite genomic sequencing. Hypermethylated CpGs were found at −236bp and −126bp, whereas hypomethylated CpGs were found at −207bp in arsenic exposed cells. This study indicates that 20 nM sodium arsenite can alter myoblast differentiation by reducing the expression of the transcription factors myogenin and Mef2c, which is likely due to changes in promoter methylation patterns. The delay in muscle differentiation may lead to developmental abnormalities.

Keywords

arsenic; C2C12; myogenin; methylation; Mef2c; Ptgis; muscle; myocyte

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Conflict of interest statement

None

Introduction

Arsenic is present in drinking water systems around the world as both a natural element and as a contaminant due to normal geological processes and human disturbances (Mandal and Suzuki, 2002). Epidemiological studies indicate that long-term exposure to arsenic can result in adult-onset diseases, such as cancer, cardiovascular disease, diabetes, and skin lesions (Guha Mazumder, 2008; Mink *et al.*, 2009; Plataniias, 2009; Schuhmacher-Wolz *et al.*, 2009; States *et al.*, 2009). Although most studies have focused on outcomes such as cancer, epidemiological evidence indicates that arsenic can also act as a developmental toxicant (Hopenhayn-Rich *et al.*, 2000; Milton *et al.*, 2005; Rahman *et al.*, 2007; Cherry *et al.*, 2008; Rahman *et al.*, 2009). Arsenic has been shown to cross the placental barrier in humans and rodents (Concha *et al.*, 1998; Xie *et al.*, 2007; Jin *et al.*, 2010), and studies in rodent models have shown that dams exposed to arsenic in their drinking water had an increase in aborted fetuses, while their offspring had low birth weight and changes in locomotor activity (Ahmad *et al.*, 2001; Waalkes *et al.*, 2003; Waalkes *et al.*, 2004; Jin *et al.*, 2005; He *et al.*, 2007).

Moreover, *in utero* arsenic exposure also alters muscle function and muscle-related protein expression in the offspring. For example, arsenic exposure *in utero* reduced the ability of smooth muscle to relax and resulted in changes in the vascular matrix by decreasing the amounts of collagen and elastin (Srivastava *et al.*, 2007; Hays *et al.*, 2008). In fish species, such as killifish, parental arsenic exposure increased tropomyosin and myosin light chain 2 expression in the offspring (Gonzalez *et al.*, 2006). Zebrafish exposed to arsenic during embryogenesis showed altered heart development, and their somite and neuromuscular patterning correlated to altered cell proliferation and genomic DNA methylation (Li *et al.*, 2009). Alterations in muscle regulatory factors during zebrafish somitogenesis resulted in impaired development of fast skeletal muscle (Tilton and Tanguay, 2008). Additionally, arsenic exposure to frogs prior to metamorphosis resulted in reductions in swimming ability (Chen *et al.*, 2009).

Skeletal muscle development occurs in several defined steps. Mesoderm-derived precursor cells become committed to the myogenic lineage and develop into myoblasts, which subsequently fuse to form myotubes. Later, myotubes mature into multinucleated, highly specialized muscle fibers that show cross striation (reviewed in Brand-Saberi, 2005; Darabi *et al.*, 2008). During muscle differentiation, myogenin is the key transcription factor that initiates the conversion of a myoblast to a myotube (Buckingham *et al.*, 2003). Recently, it has been reported that myogenin regulates several genes during muscle differentiation. For example, both *in vitro* and *in vivo* studies in mice indicate that conditional knockout of myogenin represses muscle stem cell differentiation along with reducing the expression of genes that are involved in myogenesis, including prostaglandin I₂ synthesis (Ptgis), suppressor of cytokine signaling 3 (Socs3), and myocyte enhancer 2 (Mef2) (Meadows *et al.*, 2008).

To date, it has been shown that methylation status of the myogenin promoter plays an important role in the activation of myogenin. For example, in undifferentiated C2C12 myoblast cells, the myogenin promoter is maintained in a completely methylated state, which results in low myogenin expression. However, during differentiation, the promoter region undergoes demethylation within 48 hours, and subsequently recruits transcription factors, such as MyoD, to initiate transcription (Fusoa *et al.*, 2001; Lucarelli *et al.*, 2001; Sartorelli and Caretti, 2005). Interestingly, when S-adenosylmethionine (SAM) is administered to C2C12 cells, it can repress expression of myogenin by delaying the demethylation of the 5'-flanking region on the myogenin gene (Fusoa *et al.*, 2001; Lucarelli *et al.*, 2001).

In many cells, arsenic undergoes enzymatic mono- and dimethylation, which is mediated by arsenic methyltransferase (As3MT) using SAM as a methyl group donor (Liu *et al.*, 2007; Vahter *et al.*, 2007; Tsai *et al.*, 2009). However, SAM is also the universal methyl donor for the majority of methyltransferases that modify DNA, RNA, histones, and other proteins that dictate replication, transcription and translation fidelity, mismatch repair, chromatin modeling, epigenetic modifications, and imprinting (Loenen, 2006). Since the SAM methyltransferase pathway for biotransformation of arsenic overlaps with the DNA methylation pathway, one could hypothesize that the arsenic-induced adverse outcomes may be due to an imbalanced SAM pool within the organism. To this end, Zhao *et al.* have shown that arsenic-induced global DNA hypomethylation occurred in the presence of reduced levels of SAM in mice, resulting in aberrant gene expression (Zhao *et al.*, 1997). Individuals chronically exposed to arsenic in their drinking water had hypermethylation on the promoters of p53 and p16 (Chanda *et al.*, 2006), whereas whole genome hypermethylation in persons exposed to arsenic in drinking water has also been reported (Majumdar *et al.*, 2009). Collectively, studies indicate that arsenic exposure results in both DNA hypomethylation and hypermethylation, leading to aberrant gene expression (Benbrahim-Tallaa and Waalkes, 2008; Salnikow and Zhitkovich, 2008).

The objectives of the present study were to: 1) determine whether a low concentration of sodium arsenite delayed the differentiation of C2C12 cells from myoblasts to myotubes by repressing the transcription factor myogenin; 2) examine whether the reduction in myogenin would impact the expression of myogenic proteins involved in contraction and myotube formation; and 3) determine whether DNA methylation patterns on the myogenin promoter were altered.

Methods

Arsenic exposure and quantification of multinucleated myocytes

The mouse myoblast C2C12 cell line (ATCC, Manassas, VA) was seeded at 860 cells/well in 6 well plates, and cultured with or without 20 nM sodium arsenite in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (growth media) at 37°C in a humidified incubator containing 5% CO₂. To differentiate the cells, the medium was replaced with DMEM supplemented with 2% horse serum, 1% L-glutamine, and 1% penicillin/streptomycin (differentiation media) (Kubo, 1991) with or without 20nM arsenic.

Myotube formation was examined on differentiation days 0, 1, 2, 3, and 4 (n=6 per group per day) by fixing the cells in methanol and incubating them in Giemsa stain. Cells were considered to be multinucleated if they contained 3 or more nuclei (Duan and Gallagher, 2009). A multinucleation index was calculated by dividing the number cells containing ≥3 nuclei by the total number of cells.

QPCR

C2C12 cells were plated at 5000 cells/100 mm dish in either growth medium or in growth medium containing 20 nM sodium arsenite, and on the fourth day, differentiation medium alone or differentiation medium containing 20 nM sodium arsenite was added. Cells were harvested on days 0 or 4 (n=4 per group per day) and RNA was extracted using TRIreagent (Sigma-Aldrich, St. Louis, MO). RNA (2 µg) was reverse transcribed into cDNA, and the expression of myogenin, myosin light chain 2, tropomyosin, prostaglandin I₂ synthase (Ptgis), suppressor of cytokine signaling 3 (Socs3), and myocyte enhancer factor 2 (Mef2c) was quantified by QPCR using SybrGreen (SABiosciences, Frederick, MD) and the appropriate primers (myogenin-F: 5'-ccaaccaggagatcatttg-3', myogenin-R: 5'-

acgatggacgtaaggagtg-3'; Ptgis-F: 5'-tgccagcttcttaccaggatgaa-3', Ptgis-R: 5'-taagagtgtgggtccaggagaaca-3'; tropomyosin-F: 5'-tgaaagtcattgaaagccgagccc-3', tropomyosin-R: 5'-agcaatgtgcttgccctcttcag-3'; Mef2c-F: 5'-aggatcaccggaacgaattccact-3', Mef2c-R: 5'-gcatcgcttgactgaaggactt-3'; Socs3-F: 5'-agcagatggagggttctgctttgt-3'; Socs3-R: 5'-attggctgtgttggtccttg-3'; Gapdh-F: 5'-tgcgactcaacagcaactc-3'; Gapdh-R: 5'-atgtaggccatgaggtccac-3'). Samples were run in triplicate and the entire experiment was repeated at least twice. The cycle threshold values (Ct) were converted into number of molecules per 100 ng cDNA using known concentrations of the specific gene product, and then normalized using GAPDH.

Cellular expression and localization of myogenin, tropomyosin, and Mef2c

C2C12 cells were seeded in Lab-tek II eight well coverglass slides (Nunc) at a concentration of 100 cells/well. Cells were cultured with or without 20 nM sodium arsenite as described above and examined for specific protein expression on differentiation days 0, 1, 2, 3, and 4 (n=8 chambers per protein per group per day). Cells were blocked in 1% bovine serum albumin, 0.1% Triton-X100 in PBS, and incubated with the appropriate primary antibody for 1 hour (myogenin: 1:100 dilution, Imgenex; tropomyosin: 1:50 dilution, Santa Cruz Biotechnologies; Mef2: 1:50 dilution, Santa Cruz). The secondary antibody (1 µg/mL) conjugated to Alexa Flour 488 (Invitrogen, Carlsbad, CA) was incubated with the cells, which were counterstained with DAPI (Invitrogen). Cells were examined by conventional immunofluorescence on a Ti Eclipse Inverted Microscope (Nikon, Melville, NY). Myogenin and Mef2c expression was quantified by determining the percentage of nuclei expressing each protein. Tropomyosin expression was quantified by determining the mean pixel intensities in individual cells by examining 4 fields per slide.

Bisulfite genomic sequencing of the myogenin promoter

Bisulfite genomic sequencing PCR was performed using specific primer sets to amplify nucleotides -473 to +90 relative to the transcription start site (TSS) of myogenin (CpG#1-7-F: 5'-ttttgattatggaggagaga-3', CpG#1-7-R: 5'-ccccaccctcaaacctac-3'; CpG#8-11-F: 5'-ggaaggggaattatgttaattt-3', CpG#8-11-R: 5'-aaataatttccccatcataaa-3'). C2C12 cells were seeded at 1.5×10^5 /150 mm tissue culture plate and were cultured with or without 20 nM sodium arsenite as described above. Genomic DNA was extracted on differentiation day 2 using Qiagen's Genomic DNA extraction kit (Valencia, CA; n=3 plates per group). Sodium bisulfite treatment of the DNA was performed using the EpiTect Kit (Qiagen). The BS-PCR reaction was performed for 40 cycles and the products were cloned into the pCR2.1 vector (Invitrogen) and transformed into Top10 chemically competent *E. coli* (Invitrogen). Twenty to thirty randomly selected clones from the control and arsenic-exposed group were sequenced with the M13 reverse primer to determine whether each one of the 12 CpG sites was methylated or not. A chi-square test was used to determine statistical significance between control and arsenic-exposed cells.

Results

Arsenic delays C2C12 muscle cell differentiation

Initial cell viability assays indicated that the LC₅₀ of sodium arsenite in C2C12 cells was 5 µM (data not shown), so we used a 250-fold lower concentration to determine whether arsenic caused a delay in C2C12 cell differentiation. Cells were cultured either with or without 20 nM sodium arsenite in growth media for 3 days and then in differentiation medium for up to 4 days. Both control and arsenic-exposed cells appeared predominantly as myoblasts during culture in growth medium (Fig. 1A–B). After the switch to differentiation medium, myotube formation was reduced in the arsenic exposed cells compared to the controls (Fig. 1C–D). Quantifying the number of multinucleated cells as an indicator of

myotube formation indicated that the number of multinucleated cells was significantly reduced between 1.5- and 2.9-fold in the cells exposed to arsenic (Fig. 1E).

Myogenin transcript and nuclear localization is reduced during arsenic exposure

Since arsenic appeared to delay C2C12 cell differentiation and multinucleation, the expression of myogenin, the transcription factor that initiates the differentiation of a myoblast to a myotube, was examined. When C2C12 cells were cultured in growth medium, there was very low expression of myogenin mRNA in both control and arsenic-exposed cells. However, by day 2 of differentiation, myogenin transcript was significantly reduced 7-fold in the arsenic-exposed cells, and on day 4, myogenin RNA levels were still reduced by 4.1-fold in the arsenic-exposed cells compared to control cells (Fig. 2H). The reduced levels of RNA also correspond with alterations in the percentage of nuclei expressing myogenin. In growth medium, myogenin is localized in the cytoplasm in both control and arsenic-treated cells (Fig. 2A and D). After 2 days of differentiation, myogenin in the control cells shifts to the nuclei (Fig. 2B and E), while the number of nuclei expressing myogenin was significantly reduced in the arsenic-exposed C2C12 cells by 1.6-fold (Fig. 2G). The reduced expression of myogenin is likely causing the delay in differentiation in arsenic-exposed cells.

Altered Cellular Expression and Localization of Muscle Proteins

Since myogenin expression was reduced after arsenic exposure, we next determined whether this would lead to alterations in myogenic proteins. The expression of tropomyosin mRNA, a part of the contractile apparatus, was unchanged after arsenic exposure (Fig. 3A). Its protein expression, as examined by immunofluorescence, was also not altered (data not shown). Similarly, Socs3, a protein that has been reported to induce the differentiation of myoblasts (Spangenburg, 2005), was also not changed after exposure to arsenic (Fig. 3C). However, the RNA expression of Mef2c, a transcription factor that is expressed during late myogenesis (McDermott *et al.*, 1993) that appears to control myofiber subtype (Wu *et al.*, 2000; Potthoff *et al.*, 2007b), was reduced by 4-fold after arsenic exposure (Fig. 3B). Additionally, Ptgis, which is thought to be involved in myoblast fusion (Prisk and Huard, 2003; Bondesen *et al.*, 2007), was also significantly reduced by 1.6-fold in cells treated with 20 nM arsenic (Fig. 3D). Immunofluorescence corroborated the QPCR results for Mef2c, showing a reduction in nuclear translocation by 2- to 2.7-fold in the arsenic treated cells (Fig. 4).

Changes in Myogenin Promoter Methylation

To investigate potential mechanisms responsible for the changes in myogenin expression, we examined histone modifications and DNA methylation patterns on the myogenin promoter, as well as the expression of DNMT1, DNMT3a, and DNMT3b protein and cellular concentrations of SAM. There were no changes in methyltransferase or SAM levels between control and arsenic-exposed cells (data not shown). In addition, chromatin immunoprecipitation (ChIP) assays performed to investigate histone modification at H3K9-Me2/Me3 on the myogenin promoter from -481bp to -28bp showed no difference between the control and arsenic exposed cells (data not shown). However, there were changes in DNA methylation patterns. Bisulfite sequencing PCR was performed to amplify the nucleotides -473 to +90 relative to the transcription start site (TSS) of myogenin, which contains a total of 12 CpG sites (Fig. 5A). Chi-square analysis indicated that hypermethylated CpGs were found at CpG No. 3 and 8 (-236 and -126, respectively), whereas hypomethylated CpGs were found at CpG No. 7 (-207) in arsenic exposed cells (Fig. 5B and 5C). This increase in methylated CpG sites on the myogenin promoter may be, in part, responsible for the reduction in myogenin mRNA and nuclear localization in arsenic-exposed C2C12 cells.

Discussion

These results illustrate that 20 nM sodium arsenite can alter myoblast differentiation by reducing the expression of the transcription factors myogenin and Mef2c, which is likely due to changes in promoter methylation patterns. To our knowledge, this is the first study that has examined the effects of low nanomolar arsenic concentrations on the development of skeletal muscle cells. Previous studies have used much higher arsenic concentrations, ranging from 0.1 – 267 μ M, when examining cardiac, skeletal, and smooth muscle development *in vivo* or *in vitro* (He *et al.*, 2007; Lantz *et al.*, 2008; Li *et al.*, 2009; Yen *et al.*, 2010). For example, when zebrafish embryos were exposed to 2 mM arsenic, the embryos had malformations of the musculature (Li *et al.*, 2009). *In utero* and postnatal exposure of 100 ppb arsenic in mice increased the amount of smooth muscle mass and the amount of actin in the lung (Lantz *et al.*, 2008). Additionally, when pregnant mice ingested drinking water containing 20 ppm or higher sodium arsenite, there was a decrease in their fecundity, and if offspring were produced, their placenta experienced defective formation of blood vessels (He *et al.*, 2007). All these studies provide evidence that arsenic can act as a developmental toxicant.

During skeletal muscle formation, myogenin is the transcription factor that regulates the differentiation of a myoblast to a myotubes. Myogenin knockout mice die shortly after birth due to malformation of skeletal muscles and lack of diaphragm formation (Hasty *et al.*, 1993). In the present study, myogenin mRNA was reduced in the arsenic-treated cells during days 2, 3 and 4 of differentiation. A recently published study also saw reductions in myogenin after exposure of C2C12 cells to arsenic (Yen *et al.*, 2010), although the arsenic levels used in that study were an order of magnitude higher than in ours. In our research, the reduction in myogenin coincided with a delay in differentiation and a reduction in the total number of multinucleated myotubes, which indicates that nanomolar arsenic levels are having a physiological effect on cellular differentiation.

Additionally, our results indicate that Mef2c gene expression and nuclear translocation was also reduced an average of 2.6-fold in arsenic exposed cells. Mef2c is a transcription factor that is essential for muscle differentiation and is activated by myogenin (Dodou and Xu, 2003). The promoter for myogenin contains a Mef2 binding site, which provides a way for maintaining myogenin expression in muscle cells (Edmondson *et al.*, 1992; Molkenin and Olson, 1996). Thus, the 2 sets of transcription factors work cooperatively to regulate skeletal muscle genes. Mef2c deletion in the skeletal muscle of mice results in lethality before birth (Lin *et al.*, 1997) and disorganized myofibers, which are due to disorganized sarcomeres and the loss of M-line encoding proteins such as myomesin (Potthoff *et al.*, 2007a). Like myogenin, Mef2 appears to play a role in myotube and myofiber formation. To our knowledge, this is the first time that arsenic has been shown to alter Mef2 levels. However, whether the reduction in Mef2c is an indirect effect due to the loss of myogenin or whether arsenic can directly alter Mef2c expression is unclear.

It has been shown that the methylation status of the myogenin promoter correlates with myogenin expression and with muscle differentiation (Lucarelli *et al.*, 2001; Palacios *et al.*, 2010). To this end, we examined the DNA methylation patterns in the myogenin promoter region. Although there is a weak CpG island in myogenin promoter, there is a relatively high density of CpGs from -473 bp to +90 bp. The results indicate that there are two hypermethylated CpGs at -236 and -126 in the arsenic exposed C2C12 cells. Interestingly, a hypomethylated CpG at -207 was also detected in the arsenic treatments. Since the methylated CpGs may directly interfere with gene activation by preventing the binding of potential transcription factors or preventing the modification of histone tails, identification of potential arsenic-mediated transcription factors that could interact with the

hypomethylated CpG at -207, as well as hypermethylated CpG at -236 and -126, is currently under study. Recently, demethylation of CpGs in the myogenin promoter from -192bp to +58bp has been reported in during differentiation of C2C12 cells (Palacios *et al.*, 2010). Our results show a hypermethylated CpG (CpG #8, -126bp) in arsenic-exposed C2C12 cells. Moreover, an enhancer binding site (-255bp to -241bp) was predicted in the myogenin promoter, which is in the same area where we identified a hypermethylated CpG (CpG #3, -236bp). Therefore, our findings suggest that these two hypermethylated CpGs may reduce the transcriptional activation of myogenin, or block enhancers binding to the myogenin promoter during arsenic exposure.

Collectively, our results indicate that 20 nM sodium arsenite results in delayed differentiation in C2C12 mouse myocyte cells. The delayed muscle differentiation was caused by reductions in myogenin mRNA expression and reduced appearance of myogenin protein in the nuclei. Repressed myogenin expression may be due to abnormal DNA methylation in the myogenin promoter, as well as the reduced expression of Mef2c, although additional studies are needed to validate this correlation. Ultimately, a delay in muscle cell differentiation could lead to improper organization of myofibers, which can have serious physiological consequences on the organism at arsenic concentrations that are below the current drinking water standard.

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Fig. 1. Arsenic exposure delays C2C12 cell differentiation

After 3 days, myoblast cells grown in growth medium with or without 20 nM arsenic (A and B) were switched to differentiation medium for 4 days (C and D). Myotube formation is indicated by the arrows. Photographs are representative examples from 4 plates/day/group. The percentage of myotubes (cells that had 3 or more nuclei) were counted from 4 random areas per plate (n=4 plates/day/group) (E) and statistical differences (*) in the percentage of myotubes formed was determined by Student's t-test ($p < 0.05$).

Fig. 2. Arsenic alters myogenin nuclear localization and mRNA expression

Control cells on day 0, 2, and 4 of differentiation (A–C) and 20 nM arsenic treated cells on day 0, 2, and 4 of differentiation (D–F) were used to examine myogenin localization by immunofluorescence. White arrows indicate nuclei that are expressing myogenin. Pictures are representative examples from 4 wells/day/group. The percentage of nuclei expressing myogenin (G) was determined by counting the number of nuclei expressing myogenin per total cells in each photo taken ($n \geq 5$). Myogenin RNA expression was determined by QPCR (H). Values were normalized against Gapdh as a housekeeping gene, with each sample run in triplicate ($n=4$ plates/day/group). Statistical differences (*) were determined by Student's t-test ($p < 0.05$).

Fig. 3. Changes in the mRNA expression of tropomyosin, Mef2C, Socs3, and Ptgis
Tropomyosin (A), Mef2C (B), Socs3 (C), and Ptgis (D) RNA expression was determined by QPCR. The data are expressed in number of molecules/100 ng cDNA $\pm$ standard deviation, with each sample run in triplicate ($n=3-5$ plates/day/group). Values were normalized against Gapdh as a housekeeping gene. Statistical differences (*) were determined by Student's t-test ($p < 0.05$).

Fig. 4. Immunofluorescence of Mef2C

Mef2C protein expression was examined on day 0, 2, and 4 in control (A–C) and 20 nM arsenic treated cells (D–F). Pictures are representative examples from 4 wells/time point/group. Differences in expression between control and treated cells (G) were determined by measuring the intensities of the nuclei in each photo taken ($n \geq 5$). Nuclear intensities were normalized to background values and statistical differences (*) were determined by Student's t-test ($p < 0.05$).

Fig. 5. Arsenic alters the methylation pattern on the myogenin promoter

A portion of the myogenin promoter (-473 to +90) was examined for CpG sites and potential transcription factor binding sites by MOTIF Search (<http://motif.genome.jp/>). Bolded nucleotides indicate a CpG site and the number denotes its order. Underlines signify putative transcription factor binding sites, and the transcriptional start site is indicated with an arrow (A). Bisulfite sequencing was performed to determine the methylation status at each of the CpG sites in clones from control and 20 nM arsenic exposed cells. Open circles denote nonmethylated sites and closed circles denote methylated sites (B). The percentage of methylated Cs was calculated for each CpG site (C), and statistical differences (*) were determined using a Chi-Square test ($p < 0.05$).