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shRNA Silencing of AS3MT Expression Minimizes Arsenic Methylation Capacity of HepG2 Cells

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Abstract

Several methyltransferases have been shown to catalyze the oxidative methylation of inorganic arsenic (iAs) in mammalian species. However, the relative contributions of these enzymes to the overall capacity of cells to methylate iAs have not been characterized. Arsenic (+3 oxidation state) methyltransferase (AS3MT) that is expressed in rat and human hepatocytes catalyzes the conversion of iAs, yielding methylated metabolites that contain arsenic in +3 or +5 oxidation states. This study used short hairpin RNA (shRNA) to knock down AS3MT expression in human hepatocellular carcinoma (HepG2) cells. In a stable clonal HepG2/A cell line, AS3MT mRNA and protein levels were reduced by 83 and 88%, respectively. In comparison, the capacity to methylate iAs decreased only by 70%. These data suggest that AS3MT is the major enzyme in this pathway, although an AS3MT-independent process may contribute to iAs methylation in human hepatic cells.

Introduction

The metabolism of inorganic arsenic (iAs)¹ in humans and in other mammalian species is a complex process that yields various reactive and toxic intermediates and products. Reactions involved in this process are enzymatically catalyzed (1). Enzymes have been identified that catalyze the reduction of As^V-containing species (2–6) and the *S*-adenosylmethionine-dependent methylation of As^{III}-containing species (7,8). However, the significance of these enzymes for in vivo metabolism of iAs and the linkage between steps in the metabolic pathway for iAs remains unclear. Identification of a novel arsenic (+3 oxidation state) methyltransferase (AS3MT) in rat liver (9) has allowed the study of iAs methylation at the molecular level. Results of studies with recombinant rat AS3MT (rAS3MT) (10,11) showed that this enzyme catalyzes the entire sequence of reactions that converts trivalent iAs, arsenite (iAs^{III}), to As^{III}- or As^V-containing methylarsenic (MAs), dimethylarsenic (DMAs), and trimethylarsenic (TMAs) species that have been identified as intermediates or products in in vivo methylation of iAs in rats (12,13). The presence of a synthetic reductant [e.g., tris-(2-carboxylethyl)phosphine] or of one of the cellular reducing systems, thioredoxin/thioredoxin reductase/NADPH, lipolic

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¹Abbreviations: shRNA, short hairpin RNA; AS3MT, arsenic (+3 oxidation state) methyltransferase; HepG2, human hepatocellular carcinoma cells; rAS3MT, rat arsenic (+3 oxidation state) methyltransferase; hAS3MT, human arsenic (+3 oxidation state) methyltransferase; iAs, inorganic arsenic; MAs, methylated arsenicals; DMAs, dimethylated arsenicals; TMAs, trimethylated arsenicals; RNAi, RNA interference; siRNA, small interfering RNA; MOPS, 3-(N-morpholino)propanesulfonic acid; PVDF, poly(vinylidene difluoride); RISC, RNA-induced silencing complex; ORF, open reading frame.

acid/thioredoxin reductase/NADPH, or glutaredoxin/glutathione/glutathione reductase/NADPH, is required for the activity of recombinant rAS3MT (11). Orthologues of rAS3MT have been identified in genomes of other mammalian species, including human (14). An orthologue of *AS3MT* (*hAS3MT*) [human arsenic (+3 oxidation state) methyltransferase] is expressed in cultured primary human hepatocytes (15) that methylate iAs^{III} to MAs and DMAs. MAs and DMAs, but not TMAs, have also been found as products of iAs methylation by recombinant hAS3MT (16). The methylation patterns for iAs by human hepatocytes and by recombinant hAS3MT are consistent with the results of epidemiological studies that found MAs^{III}, MAs^V, DMAs^{III}, and DMAs^V, but not TMAs, in the urine of individuals chronically exposed to iAs in drinking water (17–21). Notably, recent studies have linked hASMT polymorphisms to specific methylation patterns for iAs in primary cultures of human hepatocytes (15) and to specific urinary profiles of iAs metabolites found in residents of arsenosis endemic areas of Mexico (22). Studies examining the characteristics of iAs methylation by recombinant genetic variants of rAS3MT and hAS3MT are underway in several laboratories, providing new data on the associations between AS3MT genotypes and specific metabolic phenotypes (14,23).

Despite growing evidence that links the hAS3MT-catalyzed reactions to the metabolism of iAs in humans, the relative contribution of this enzyme to the overall capacity to methylate iAs has not been determined (24). In this study, we have silenced *hAS3MT* expression using RNA interference (RNAi) in human hepatocellular carcinoma (HepG2) cells that constitutively express *hAS3MT*. Short hairpin RNA (shRNA) vectors were used to deliver small interfering RNA (siRNA) oligonucleotides complementary to *hAS3MT* transcript into HepG2 cells. Quantitative analysis of hAS3MT mRNA and protein levels and of the pattern of iAs metabolism in clonal HepG2/A cells that stably express hAS3MT-specific siRNA oligonucleotides show that reduced *hAS3MT* expression is associated with a significant reduction in the capacity to methylate iAs. This finding suggests that hAS3MT is the key enzyme in the pathway for methylation of iAs in human hepatic cells.

Experimental Procedures

Caution

iAs has been classified as a human carcinogen (25) and should be handled accordingly.

Arsenicals

iAs^{III}, sodium salt (>98% pure), was purchased from Sigma (St. Louis, MO). Radiolabeled carrier-free [⁷³As]iAs^{III} was prepared by reduction of [⁷³As]iAs^V (Oak Ridge National Laboratory, Oak Ridge, TN) with metabisulfite–thiosulfate reagent (26). The yield of [⁷³As] iAs^{III} in this reaction as determined by TLC (27) typically exceeded 95%.

Cells

HepG2 cells were obtained from the Tissue Culture Facility of the Lineberger Comprehensive Cancer Center of the University of North Carolina at Chapel Hill. Cells were maintained in minimum essential medium (MEM, Gibco BRL, Grand Island, NY) with 2 mM glutamine, 1 mM sodium-pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 µg/mL) (all from Gibco) at 37 °C in a humidified incubator in a 5% CO₂ atmosphere.

Design and Cloning of shRNA Cassettes

The BLOCK-iT RNAi Designer algorithm (Invitrogen, Carlsbad, CA) was used to analyze hAS3MT mRNA (GenBank accession number, NM_020682) and to identify three target

sequences for siRNA. Three shRNA constructs (*shRNA/A*, *shRNA/B*, and *shRNA/C*) that contained the corresponding target sense and antisense sequences and the loop sequence (Figure 1) were designed using the Small Interfering RNA Hairpin Oligonucleotide Sequence Designer (Clontech Laboratories, Mountain View, CA). This design ensured that *shRNA* constructs delivered into cells were efficiently transcribed and the transcripts folded properly to form hairpin-like structures that were recognized by cellular enzymes. Selected shRNA oligonucleotides were synthesized by Operon Biotechnologies (Huntsville, VA), purified by PAGE, and cloned into pSIREN-RetroQ vectors, following the siRNA Hairpin Oligonucleotide Sequence Designer protocol. Complete pSIREN-RetroQ-*shRNA/A*, */B*, and */C* expression vectors were sequenced to verify insert orientation and nucleotide sequence.

Expression of shRNA Oligonucleotides in HepG2 Cells

The pSIREN-RetroQ-*shRNA/A*, */B*, and */C* expression vectors and an empty pSIREN-RetroQ vector were used for transfection of the packaging AmphoPack-293 cells (Clontech). Here, AmphoPack-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% FBS, penicillin (50 units/mL), and streptomycin (50 µg/mL). Cells were transfected using GeneJuice transfection reagent (Novagen, Darmstadt, Germany) and selected in DMEM medium containing puromycin (1 µg/mL, Sigma). Stable AmphoPack-293 cell lines producing retroviral particles containing pSIREN-RetroQ expression vectors were established. Medium containing retroviral particles and polybrene (4 µg/mL, Sigma) was used to infect HepG2 cells. Clonal HepG2 cell lines were then selected in MEM in the presence of puromycin (2 µg/mL). Stable clonal HepG2 cell lines expressing the empty pSIREN-RetroQ vector or vectors encoding for the *shRNA/A*, */B*, and */C* were established.

Analysis of hAS3MT mRNA

hAS3MT mRNA levels in parental and clonal HepG2 cell lines were analyzed by touch-down PCR and quantified by real-time PCR as previously described (15,27). Briefly, 1 µg of DNase-treated and purified total RNA was reverse transcribed. The following primers were used for the touch-down PCR: hAS3MT/sense, 5'-CGTCTATACGAGCCTTGAA-3'; hAS3MT/antisense, 5'-TTAGCAGCTTTTCTTTGTGC-3'. These primers amplify a 616 bp segment of hAS3MT cDNA (nucleotide no. 555–1170) in which all three sequences targeted by shRNA constructs are located. β -Actin mRNA was analyzed in parallel with hAS3MT mRNA, using the following PCR primers: sense, 5'-TCATGAAGTGTGACGTGGACATCCGC-3'; antisense, 5'-CTAGAAGCATTTGCGGTGGACGATG-3'. These primers amplify a 284 bp segment of β -actin cDNA. Quantitative PCR analysis was carried out with 100 ng of total RNA, using a computerized LightCycler Instrument (Roche Applied Science, Mannheim, Germany). The set of hAS3MT-specific primers designed for touch-down PCR was also used for quantitative PCR. The PCR product was detected using LightCycler FastStart DNA Master^{Plus} SYBR Green I (Roche Applied Science). A hAS3MT expression (pRSET/hAS3MT) vector was used as a positive control and to generate a calibration curve for quantification of hAS3MT mRNA (10,15,27).

Immunoblot Analysis

The hAS3MT protein levels in parental and clonal HepG2 cells were analyzed by immunoblot using a rabbit polyclonal anti-hAS3MT antibody generously provided by Professor R. M. Weinshilboum (Mayo Clinic College of Medicine, Rochester, MN). Cell lysates were prepared in RIPA buffer as previously described (27). Aliquots of 30 µg of total protein were separated by NuPAGE in a 10% Bis-Tris gel and 3-(N-morpholino)-propanesulfonic acid (MOPS) SDS running buffer (Invitrogen) and electroblotted to polyvinylidene difluoride (PVDF) membrane. After it was blocked with 5% nonfat milk for 1 h at room temperature, the membrane was probed with a rabbit anti-hAS3MT antibody (1:2000) for 1 h at room temperature. β -Actin was

analyzed in parallel with hAS3MT as a loading control, using a mouse monoclonal antibody (Abcam, Cambridge, MA). Antibody-antigen complexes were visualized by enhanced chemiluminescence detection (Amersham, Biosciences, Pittsburgh, PA) after incubation with the corresponding HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The chemiluminescence signal was analyzed by a FluorChem Imaging System (Alpha Innotech Corp., San Leandro, CA), using AlphaE-aseFCTM software (Alpha Innotech). The β -actin signal was used to normalize hAS3MT protein contents in parental and clonal HepG2 cell lines.

Analysis of iAs Metabolism

Metabolism of iAs was examined in parental and clonal HepG2 cells incubated with carrier-free [^{73}As]iAs^{III}. Radiolabeled metabolites in cells and culture media were analyzed by TLC (28). Radioactivity associated with iAs, MAs, and DMAs was quantified with a computerized Fuji FLA-5000 imaging system (Fujifilm, Stamford, CT).

Statistical Analysis

Results of quantitative PCR analysis were statistically evaluated using GraphPad Instat software package (GraphPad Software Inc., San Diego, CA). One-way ANOVA followed by Tukey's multiple comparison test evaluated differences between experimental groups. Differences at the $p < 0.05$ level were considered statistically significant.

Results and Discussion

Techniques involving the posttranscriptional silencing by RNAi have widely been used for analyses of gene functions in mammalian cells. Initially, transfection was used for the delivery of siRNA oligonucleotides complementary to target gene sequences. As compared with this approach, a relatively new methodology using shRNA offers a significant advantage in silencing longevity and delivery options. Retrovirus-delivered shRNA permits efficient delivery and immediate selection of stable knockdown cell lines. Integrated shRNA is transcribed in cells from a DNA template as a single-stranded RNA molecule. Complementary regions spaced by a small loop (Figure 1) cause the transcript to fold in a "short hairpin" conformation that resembles cellular microRNA. This molecule is a substrate for the endogenous enzyme Dicer (29). The siRNA released by shRNA digestion enters into a nuclease complex to form an RNA-induced silencing complex (RISC). RISC targets a complementary endogenous gene transcript, cleaving mRNA or suppressing translation (30,31). Notably, an shRNA construct has recently been used by Dhankher and associates (32) to silence expression of arsenate reductase (ACR2) in *Arabidopsis thaliana*. ACR2 silencing resulted in a significant decrease in ACR2 protein level, loss of the capacity to reduce arsenate, and hyperaccumulation of arsenic in the shoots of the plant.

In this study, we tested three sequences in the open reading frame (ORF) of *hAS3MT* gene as potential targets for siRNA. Blast analysis using the BLOCK-iT RNAi Designer database (Invitrogen) confirmed that each selected sequence is unique and highly specific for *hAS3MT*. Selected sequences are located 885–905, 919–939, and 973–993 bp in the ORF of *hAS3MT*. shRNA constructs were designed containing siRNA oligonucleotides that target each selected sequence (*shRNA/A*, *shRNA/B*, and *shRNA/C*). A retroviral expression pSIREN-RetroQ vector delivered shRNA constructs to HepG2 cells and allowed selection of stable genetically modified HepG2 cells. Those clonal cell lines expressing *shRNA/A*, */B*, and */C* were designated as HepG2/A, HepG2/B, and HepG2/C, respectively. The clonal cell line expressing empty pSIREN-RetroQ vector was designated as HepG2/Empty.

Cell morphology, proliferation rates, hAS3MT expression, and capacity for iAs methylation were compared in parental and clonal HepG2 cells. No significant differences in cell morphology or in proliferation rates were found between parental and clonal cell lines during 12 weeks in culture (i.e., about 24 passages). Analysis of hAS3MT expression by conventional (touch-down) RT-PCR found that HepG2/A cells expressed less hAS3MT mRNA than did parental and other clonal cell lines (Figure 2a). Quantitative analysis by real-time PCR confirmed that HepG2/A cells contained only 17% of hAS3MT mRNA found in parental HepG2 cells (Figure 2b). Significantly less hAS3MT mRNA was detected in HepG2/B but not in HepG2/C or HepG2/Empty cells, indicating that the capacity to suppress hAS3MT expression varied markedly among the three shRNA constructs. Consistent with PCR data, immunoblot analysis showed that HepG2/A and HepG2/B cells contained significantly less hAS3MT protein than did HepG2, HepG2/Empty, or HepG2/C cells (Figure 3). The normalized levels of hAS3MT protein in HepG2/A and HepG2/B cells were less than 12 and 30% of that found in parental HepG2 cells, respectively. A statistically significant linear correlation ($R = 0.997$, $p < 0.0001$) between the hAS3MT mRNA level and the normalized hAS3MT protein level was found for the five HepG2 cell lines examined (data not shown). To examine how the suppression of hAS3MT expression affected capacity to methylate iAs, parental HepG2 and clonal cell lines were incubated for 72 h in the presence of carrier-free [^{73}As]iAs^{III} and radiolabeled metabolites were analyzed in cell lysates and culture medium. Parental HepG2 cells methylated about 63% of iAs; MAs and DMAs that were found in both cell lysates and culture medium accounted for 10 and 53% of total ^{73}As , respectively (Figure 4). A similar portion of iAs (58%) was methylated by HepG2/Empty and HepG2/C cells. For both cell lines, MAs and DMAs accounted for about 8 and 50% of total ^{73}As , respectively. In contrast, HepG2/B cells methylated less than 44% and HepG2/A cells methylated less than 25% of iAs. MAs accounted for 13% of total ^{73}As in both cell lines; DMAs represented 30 and 11% of total ^{73}As in HepG2/B and HepG2/A, respectively. Thus, given the percentage of methylated iAs, silencing of hAS3MT expression in HepG2/A resulted in about a 60% decrease of the methylation capacity. However, on the basis of the number of methyl groups transferred to iAs (one for MAs and two for DMAs), the capacity of HepG2/A cells to methylate iAs represents only 30% of the original capacity found in HepG2 cells.

HepG2 cells have frequently been used to study metabolism of nutrients and xenobiotics in human liver (33,34). The liver is also thought to play a key role in the metabolism of iAs. Notably, HepG2 cells are relatively efficient methylators of iAs. The rates and patterns of iAs methylation by HepG2 cells (35) resemble those described in cultured primary human hepatocytes (15). In addition, like primary human hepatocytes, HepG2 cells express hAS3MT. Thus, HepG2 cells are an appropriate cell culture model in which the enzymatic processes underlying metabolism of iAs in human liver can be examined. Because nonproliferating primary hepatocytes are not suitable for RNAi-mediated gene silencing and for selection of stable clonal cell lines, we chose HepG2 cells to examine the significance of hAS3MT expression in iAs methylation in human liver. Comparisons of hAS3MT mRNA and protein levels and the methylation patterns for iAs in parental HepG2 and clonal cells show that the capacity of these cells to methylate iAs was strongly dependent on hAS3MT expression. The quantitative association between hAS3MT silencing by RNAi and reduced capacity of HepG2 cells to methylate iAs suggest that hAS3MT may be the predominant enzyme in the pathway for iAs methylation in human liver. However, the disproportional decrease in hAS3MT mRNA (by 83%), hAS3MT protein (by 88%), and methylation capacity (by 70%) in HepG2/A cells indicates that a minor hAS3MT-independent mechanism may contribute to the hepatic methylation of iAs.

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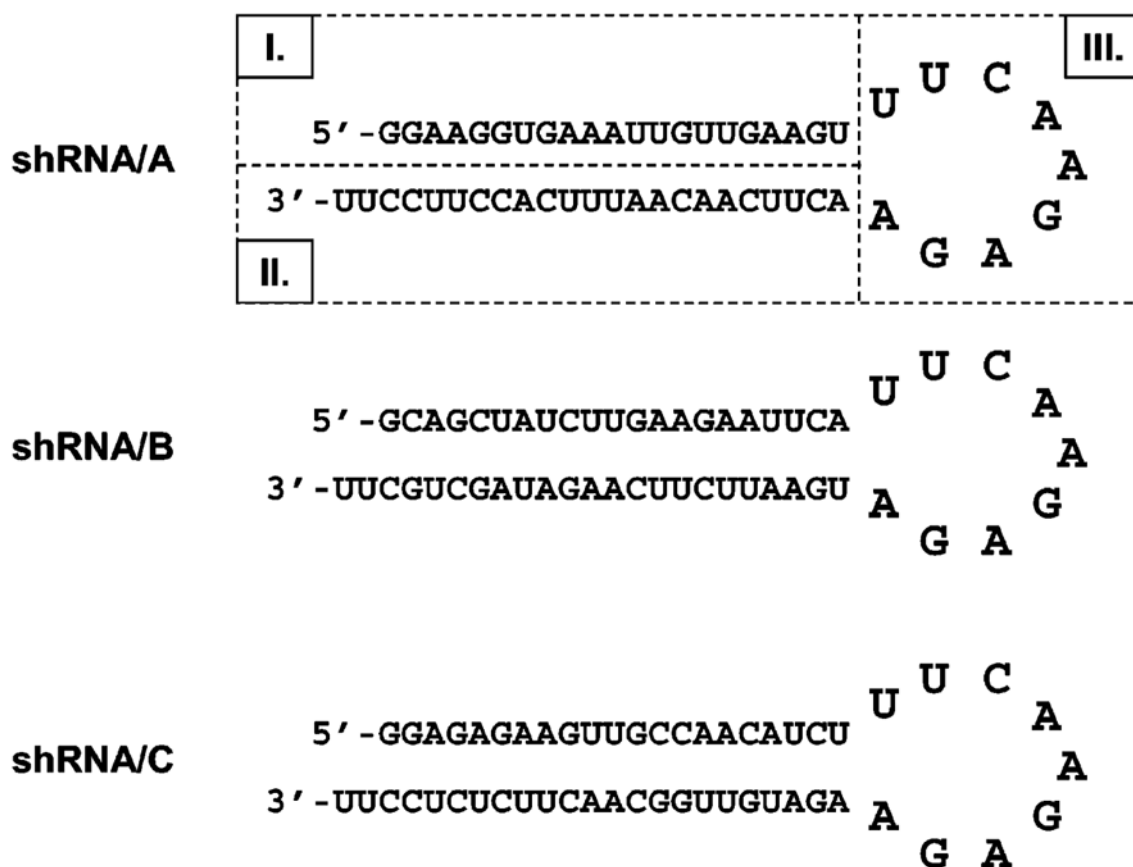


Figure 1. Sequences and predicted folding for three shRNA oligonucleotides designed to silence hAS3MT expression in HepG2 cells: I, target sense sequence; II, target antisense sequence (siRNA); and III, loop sequence.

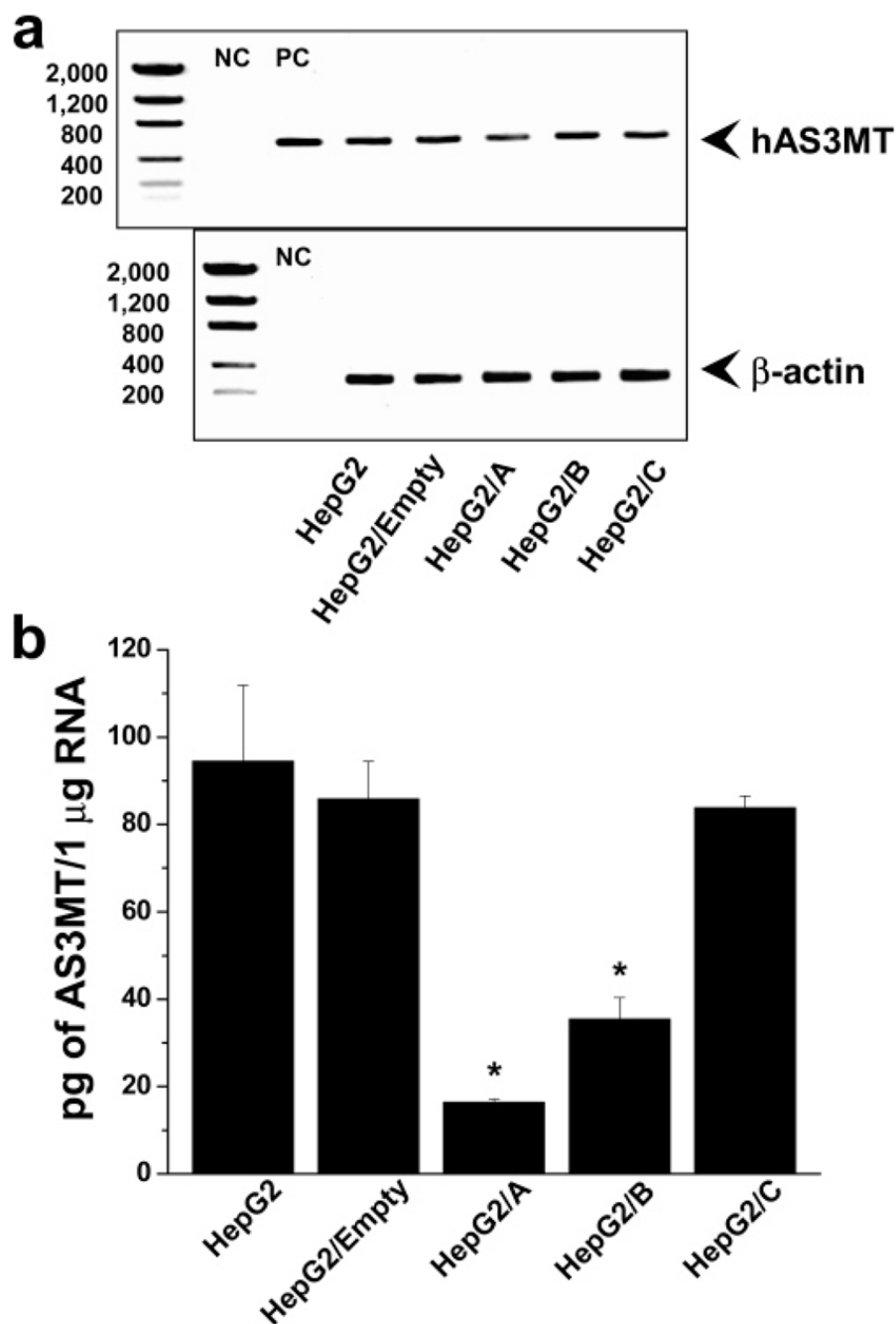


Figure 2. hAS3MT mRNA levels in parental and clonal HepG2 cells. (a) PCR products generated by touch-down PCR from hAS3MT and β -actin cDNA. Negative control (NC) represents a PCR reaction, in which cDNA was replaced with water. Positive control (PC) represents a PCR reaction in which cDNA was replaced with pRSET/hAS3MT expression vector. (b) hAS3MT mRNA levels quantified by real-time PCR. Mean and SD values for three independent measurements are shown. *The mean value is significantly different ($p < 0.001$) from that in parental HepG2 cells.

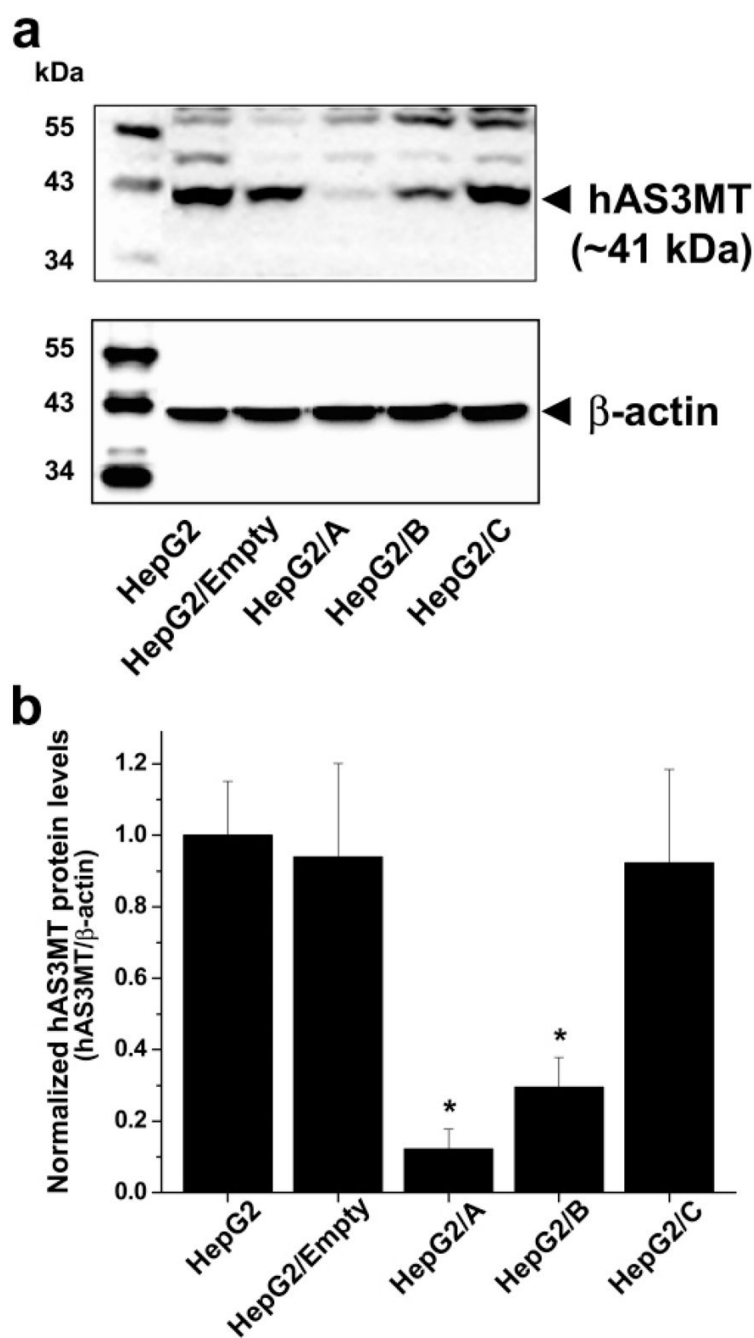


Figure 3. hAS3MT protein levels in parental and clonal HepG2 cells. (a) Immunoblot images of hAS3MT and β -actin. A representative image from one of three independent cultures is shown. (b) hAS3MT protein levels normalized for β -actin contents. Mean and SD values for the three independent cultures are shown. *The mean value is significantly different ($p < 0.05$) from that in parental HepG2 cells.

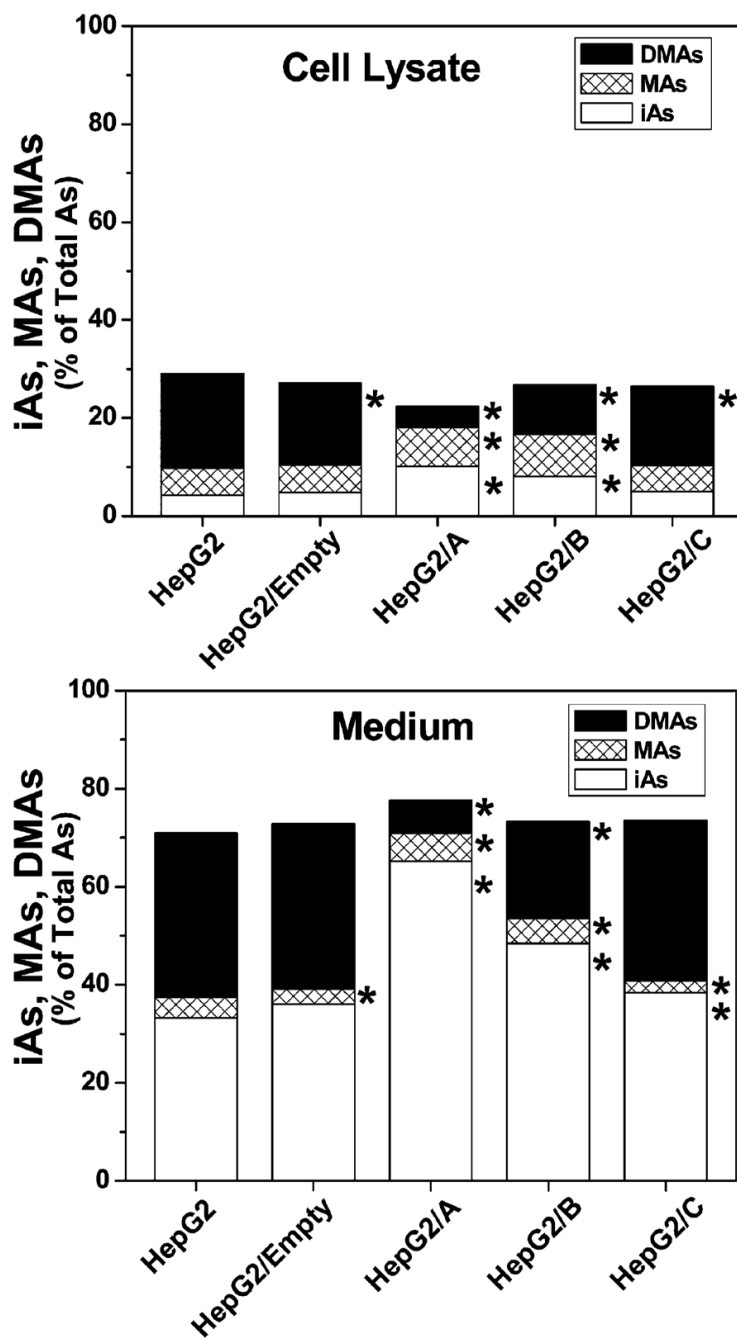


Figure 4. Methylation of $i\text{As}^{\text{III}}$ by parental and clonal HepG2 cells: Radiolabeled metabolites in cell lysates and media after a 72 h exposure to carrier-free ^{73}As . Amounts of iAs, MAs, and DMAs are expressed as a percentage of total ^{73}As . The mean value for three culture wells is shown for each metabolite. Although not shown, SD values did not exceed 10% of the corresponding mean value. *The mean value for a metabolite is significantly different ($p < 0.05$) from the corresponding value in parental HepG2 cells.