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Expression of selected pathway-marker genes in human urothelial cells exposed chronically to a non-cytotoxic concentration of monomethylarsonous acid

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ABSTRACT

Bladder cancer has been associated with chronic arsenic exposure. Monomethylarsonous acid [MMA(III)] is a metabolite of inorganic arsenic and has been shown to transform an immortalized urothelial cell line (UROtsa) at concentrations 20-fold less than arsenite. MMA(III) was used as a model arsenical to examine the mechanisms of arsenical-induced transformation of urothelium. A previous microarray analysis revealed only minor changes in gene expression at 1 and 2 months of chronic exposure to MMA(III), contrasting with substantial changes observed at 3 months of exposure. To address the lack of information between 2 and 3 months of exposure (the critical period of transformation), the expression of select pathway marker genes was measured by PCR array analysis on a weekly basis. Cell proliferation rate, anchorage-independent growth, and tumorigenicity in SCID mice were also assessed to determine the early, persistent phenotypic changes and their association with the changes in expression of these selected marker genes. A very similar pattern of alterations in these genes was observed when compared to the microarray results, and suggested that early perturbations in cell signaling cascades, immunological pathways, cytokine expression, and MAPK pathway are particularly important in driving malignant transformation. These results showed a strong association between the acquired phenotypic changes that occurred as early as 1–2 months of chronic MMA(III) exposure, and the observed gene expression pattern that is indicative of the earliest stages in carcinogenesis.

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1. Introduction

Arsenic is a ubiquitous environmental metalloid. Chronic exposure to low concentrations of inorganic arsenic increases risks for the development of skin, lung, and bladder cancers [1,2]. The exact biological events by which inorganic arsenic causes cancer have not been fully elucidated. However, oxidative stress, increased cell proliferation, inhibited DNA repair, genotoxicity, and altered cellular signals have all been suggested as critical events in arsenic-induced carcinogenesis [3–5].

Most human exposure to arsenic is via ingestion of inorganic arsenate [As(V)] and arsenite [As(III)]. Biotransformation of these inorganic arsenic species in humans

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produces methylated metabolites, that are found in the urine of arsenic-exposed individuals. As the urine accumulates in the bladder, the bladder urothelium is clearly exposed to the inorganic arsenic species as well as the methylated metabolites [6–8]. Additionally, *in vitro* studies of urothelial cells (UROtsa) demonstrated that these cells accumulated a greater amount of arsenic species when compared to human primary hepatocytes [9].

Both the trivalent arsenic metabolites, monomethylarsonous acid [MMA(III)] and dimethylarsinous acid [DMA(III)], have been shown to be more cytotoxic than both the methylated pentavalent forms and inorganic arsenic [10–12]. In particular, MMA(III) has been shown to be a potent generator of reactive oxygen species (ROS), and appears to be more clastogenic than inorganic arsenic [13–15]. Cytotoxicity assays of inorganic arsenic and MMA(III) have been performed in UROtsa, where MMA(III) had a 20-fold more toxic response than As(III), supporting earlier work that methylated trivalent arsenic species were highly toxic to immortalized rat (MYP3) and human (1T1) urothelial cells [16,17]. The IC_{50} value for MMA(III) was determined to be 5 μM in UROtsa cells, with no significant effects observed on cell morphology and mitochondrial activity during 24 h of exposure [16]. Thus 0.05 μM (50 nM) has been used in this current study and several others [18–21] as a non-cytotoxic concentration for long-term exposure studies.

Inorganic arsenic and its methylated metabolites have been shown to modify the expression of genes, and cause distinct profiles of cellular responses in urothelial cells and keratinocytes [22–25]. MMA(III) appeared to exhibit a greater carcinogenic potential, more pro-inflammatory signals, and altered expression of growth factors in human keratinocytes compared to inorganic arsenic exposure. Likewise, fundamental similarities and differences were found between As(III)-, MMA(III)-, and DMA(III)-induced transcriptional perturbations in urothelial cells (SV-HUC-1), and that some of these changes were attributed to epigenetic alterations [24]. Even with these bodies of work, the understanding of the relationship between chronic MMA(III) exposure and the development of bladder cancer is still limited.

UROtsa cells chronically exposed to 50 nM MMA(III) for a period of 3 months underwent phenotypic changes consistent with malignant transformation, i.e., increased rate of proliferation, anchorage-independent growth, and formation of tumors in immuno-compromised mice [21]. These acquired phenotypic changes were sustained even after withdrawal of MMA(III). Additionally, chronic exposure to MMA(III) had substantially impacted the global expression of genes at 3 months of exposure with roughly 8.4% of genes significantly changed from 30,000 genes assessed [26]. Surprisingly, significantly altered genes in the first and second months of exposure amounted to less than 1% of the total genes assessed. The transition from a normal to a malignant phenotype occurred between the second and third months of exposure. The early changes described by the gene array analysis implicated the involvement of perturbed immune and inflammatory pathways and related signaling. Similar observations of altered pro-inflammatory genes have been observed previously using the same model [18,19].

Since the gene array study in UROtsa cells was only performed at monthly intervals, resolving the window of transformation between 2 and 3 months of exposure into shorter intervals should define the specific changes that induce malignant transformation with chronic MMA(III) exposure. Therefore, the goal of the present study was to dissect the gene expression changes at weekly intervals in UROtsa cells chronically exposed to a non-cytotoxic concentration of MMA(III) between 2 and 4 months of exposure to resolve the changes contributing to malignant transformation. The study used a customized PCR array to profile the expression of selected genes associated with several important pathways, i.e., apoptosis, immune/inflammatory response, and proliferation as wells as correlate the phenotypic changes that occur within that timeframe. Concomitantly, cell growth kinetics, anchorage-independent growth, and tumorigenicity in SCID mice were also assessed to determine the earliest and persistent phenotypic changes and their association with the changes in expression of selected genes.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic solution (containing penicillin, streptomycin, and amphotericin B), and 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) were acquired from Gibco Invitrogen/Molecular Probes Corporation (Carlsbad, CA). Diiodomethylarsine [MMA(III) iodide, CH_3AsI_2] was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) using the technique of Millar et al. [27]. Dissolution of diiodomethylarsine in water yields monomethylarsonous acid [MMA(III)] and the concentration of MMA(III) was verified using high performance liquid chromatography-inductively coupled mass spectrometry (HPLC ICP-MS) in the analytical core of our NIEHS Superfund Research Program. MMA(III) solutions in distilled, de-ionized water were stable for approximately 4 months at 4 °C with no degradation observed when monitored using HPLC ICP-MS [28].

2.2. Preparation of MMA(III) dosing solution

Preparation of dosing solution and procedures were derived from Bredfeldt et al. [16]. Pure MMA(III) was stored in ampules at 4 °C. Fresh stock solutions of 25 mM MMA(III) were made and diluted to a final concentration of 5 μM prior to dosing (1:100 dilution) to obtain a final concentration of 50 nM MMA(III) in the culture media. The level of MMA(III) used in this study (50 nM) represents a relevant physiological concentration as this specific metabolite has been detected in the urine of humans with MMA(III) levels at or near 50 nM when exposed to environmentally relevant concentrations of inorganic arsenic in their drinking water [29,30]. All dosing solutions were sterile filtered with a 0.2 μM Acrodisc (Sigma–Aldrich) and stored in sealed,

sterile tubes at 4 °C that were opened only for dosing in a sterile cell culture hood.

2.3. Cell culture

UROtsa cells [31] were a generous gift from Drs. Donald and Maryann Sens (University of North Dakota, ND). Cell culture conditions were derived from those previously described by Rossi et al. [32] and Bredfeldt et al. [33]. UROtsa cells (beginning at passage 26) were cultured in a growth media of DMEM containing 5% (v/v) FBS and 1% (v/v) antibiotic–antimycotic. Cultured cells were incubated at 37 °C and 5% CO_2. Confluent cells were removed from plates with trypsin–EDTA (0.25%) and subcultured at a ratio of 1:3. All cell lines used in this study tested negative for the presence of mycoplasma contamination. Arsenical-treated cultures of UROtsa were continuously grown in media supplemented with 50 nM MMA(III) and refreshed every 2 days.

2.4. Control cell lines

The control cell lines consisted of untreated passage-matched UROtsa cells and a treated positive control cell line derived from [21]. Treated positive control cell line began with malignant URO-MSC36 and exposed for an additional month to 50 nM MMA(III).

2.5. Quantitative reverse transcription PCR

Total RNA was isolated from all cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), and DNase digestion performed with DNase I Set (Qiagen). RNA quantification was performed on NanoDrop 2000 Spectrophotmeter (Thermo Scientific). Gene expression was measured using quantitative real-time reverse transcription (QRT)-PCR on a custom RT^2 Profiler™ 96-well PCR Array (Qiagen) according to manufacturer's protocols. The selected panel of genes used in this study is shown in Table 1A. Briefly, total RNA from controls and treated UROta cells was reverse transcribed to complementary DNA (cDNA) using the RT^2 First Strand Kit (Qiagen). cDNA synthesis reaction product was then combined with RT^2 SYBR Green Mastermix containing HotStart DNA *Taq* Polymerase. Reaction mixture was then distributed into custom 96-well PCR arrays (http://www.sabiosciences.com/PCRArrayPlate.php).
PCR cycling was performed on Applied Biosystems StepOnePlus™ Real-Time PCR System with cycling conditions recommended for RT^2 SYBR Green PCR quantification. Post-run threshold cycle (CT) values were calculated using dedicated ABI software, normalized to GAPDH and RPLP0, and then transferred to SABiosciences PCR Array Data Analysis Template in Excel. These files were then uploaded to the SABioscience website (http://sabiosciences.com/pcr/arrayanalysis.php) for web-based data analysis and generated plots.

2.6. Selection of genes

Selection for the majority of the genes evaluated in this study was based on established changes from previous arsenic *in vitro* studies, changes observed in the gene array study [26], and some PCR confirmations. JUN and FOS have been shown to respond to arsenic challenge and oxidative stress [34]. The over-expression of JUN has been implicated in anchorage-independent growth [35]. Escudero-Lourdes et al. [19,36] also demonstrated perturbations in the expression of a number of pro-inflammatory cytokines. Those included in the study were IL6 and IL8. In addition, other inflammatory/immune mediators such as CXCL10/11, CCRL1, S100A8, IFIT2, and TRIM21 were also included. Acute exposure to 50 nM MMA(III) was found to induce PTGS2 (COX2) expression [18]. Similar responses have been observed with endothelial cells and keratinocytes challenged with As(III) [37,38]. Both PTGS1 and PTGS2 were selected. The previous gene array study demonstrated a consistent down-regulation of STAT1 and STAT2 at 1, 2, and 3 months of exposure to MMA(III). STATs respond to cytokines and growth factors and mediate complex immune responses [57]. Arsenic has been implicated in the suppression of the JAK-STAT pathway based on *in vitro* immunotoxicity studies on HepG2 cells exposed to arsenic [39], and animal models of the immune system [40]. As such, these genes were monitored during the exposure period in this study, in addition to the STAT1-regulated IRF1, a regulator of interferon activation, apoptosis and tumor suppression. Apoptotic-related genes, PDCDLG2, CFLAR, GPC3, FAS, and XAF1 were also included to gauge changes in this particular pathway since apoptosis was an important gene ontology term to appear in SPIA analysis of the gene array study within the first 2 months of exposure. As oxidative stress has been implicated in the pathogenesis from arsenic exposure, genes responsive to oxidative stress were also included in the study. Wnek et al. [20] previously reported the production of ROS and DNA damage in UROtsa chronically exposed to MMA(III), with significant changes occurring at 3 months of exposure. The genes selected to monitor oxidative stress were SOD1, OXSR1, MT1X, DUOX2, GPX8, DUSP1, and CRYAB. The pathway categorization of all of these genes, based on Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/), is given in Table 1B.

2.7. Cell growth kinetics

Growth curves for UROtsa cells chronically exposed to 50 nM MMA(III) from 4 to 20 weeks were obtained by MTT/PMS assay [16]. Alterations in mitochondrial activity were used as an indicator of cell viability/growth. The methylthiazoletetrazolium (MTT, 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide) assay measures mitochondrial activity [41]. Cells were seeded in six-well plates at a density of 32,500 cells per well ($n = 4$). Cells were removed from six-well plates with trypsin–EDTA (0.25%) and counted by the trypan blue exclusion method. Average growth curves were generated based on changes in cell number after 24, 48, 72, and 96 h for each of the n values. Cell doubling times were calculated using the formula $N/N_0 = e^{kt}$, where N is the cell number at time t, N_0 is the initial cell number (time 0), and k is a constant. The k value was calculated for each time point between 24 and 96 h and an average value used to calculate the time t when

Table 1A
Selected genes for custom RT^2 Profiler™ quantitative PCR array. Refseq# is the NCBI reference sequence database number (http://www.ncbi.nlm.nih.gov/refseq/). GAPDH and RPLP0 are included in this list since they served as control housekeeping genes for PCR raw data analysis. HGDC is a proprietary probe included in the custom arrays for assessing genomic DNA contamination. The genes categorized by pathway are given at the end of the table.

Gene symbol	Refseq#	Official full name
JUN	NM_002228	Jun proto-oncogene
PDGFRA	NM_006206	Platelet-derived growth factor receptor, alpha polypeptide
PDCD1LG2	NM_025239	Programmed cell death 1 ligand 2
CFLAR	NM_003879	CASP8 and FADD-like apoptosis regulator
GPC3	NM_004484	Glypican 3
FAS	NM_000043	Fas (TNF receptor superfamily, member 6)
XAF1	NM_199139	XIAP associated factor 1
STAT1	NM_007315	Signal transducer and activator of transcription 1, 91 kDa
STAT2	NM_005419	Signal transducer and activator of transcription 2, 113 kDa
IRF1	NM_002198	Interferon regulatory factor 1
CCRL1	NM_016557	Chemokine (C–C motif) receptor-like 1
SOD1	NM_000454	Superoxide dismutase 1, soluble
OXSR1	NM_005109	Oxidative-stress responsive 1
MT1X	NM_005952	Metallothionein 1X
DUOX2	NM_014080	Dual oxidase 2
GPX8	NM_001008397	Glutathione peroxidase 8 (putative)
DUSP1	NM_004417	Dual specificity phosphatase 1
CRYAB	NM_001885	Crystallin, alpha B
DCLRE1C	NM_022487	DNA cross-link repair 1C
FOS	NM_005252	FBJ murine osteosarcoma viral oncogene homolog
IL6	NM_000600	Interleukin 6 (interferon, beta 2)
IL8	NM_000584	Interleukin 8
PTGS1	NM_000962	Prostaglandin-endoperoxide synthase 1 (or COX1)
PTGS2	NM_000963	Prostaglandin-endoperoxide synthase 2 (or COX2)
S100A8	NM_002964	S100 calcium binding protein A8
CXCL10	NM_001565	Chemokine (C–X–C motif) ligand 10
CXCL11	NM_005409	Chemokine (C–X–C motif) ligand 11
IFIT2	NM_001547	Interferon-induced protein with tetratricopeptide repeats 2
TRIM21	NM_003141	Tripartite motif containing 21
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase
RPLP0	NM_001002	Ribosomal protein, large, P0
HGDC	SA_00105	Human genomic DNA contamination

$N/N_0 = 2$, similar to methods described by Butterworth et al. [42].

2.8. Colony formation in soft agar

UROtsa cells chronically exposed to 50 nM MMA(III) from 4 to 20 weeks were assessed for anchorage-independent growth by colony formation in soft agar similar to the methods described by Bredfeldt et al. [16]. Cells were removed from culture flask with 1× trypsin–EDTA (0.25%) and suspended in DMEM containing 5% (v/v) FBS and 1% antibiotic–antimycotic supplemented with 0.3% agar. The agar enriched with cells was overlaid onto 0.6% agar medium in a 24-well plate with a density of 1×10^4 cells per well. After 14 days of incubation, colonies were manually counted using an Olympus CK2 microscope (Olympus America, Inc., Melville, NY). Data represent colonies counted from 5 fields chosen at random within each well ($n = 3$) for a total of 15 fields.

2.9. SCID mouse colony

A SCID mouse colony was developed at the University of Arizona using original SCID obtained from Taconic (Germantown, NY). The mice were housed in microisolator cages (Allentown Caging Equipment Company, Allentown, NJ) and maintained under specific pathogen-free conditions. The mice were fed NIH-31 irradiated pellets

Table 1B
Selected genes listed by pathways. The genes categorized by pathway are based on Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) listings.

Oxidative stress genes	MAPK signaling	Immune system	Inflammatory response	Apoptosis
SOD1	DUSP1	JUN	FOS	PDCD1LG2
OXSR1	JUN	STAT1	IL6	CFLAR
MT1X	PDGFRA	STAT2	IL8	GPC3
DUOX2	FOS	IRF1	PTGS1	FAS
GPX8		CCRL1	PTGS2	XAF1
DUSP1		FOS	S100A8	
CRYAB		TRIM21	CXCL11	
			CXCL11	
			IFIT2	

(Tekland Primier, Madison, WI). Sentinel mice were screened monthly for mycoplasma, mouse hepatitis virus, pinworms, and Sendai virus via ELISA. Male mice 6–8 weeks of age were bled (200 μl) by retro-orbital puncture in order to screen for the presence of mouse immunoglobulin (Ig) using ELISA. Only mice with Ig levels ≤20 μg/ml were used for the xenografts experiments.

2.10. Tumorigenicity in SCID mouse xenografts

To assess malignant transformation, UROtsa cells chronically exposed to 50 nM MMA(III) from 4 to 20 weeks were injected subcutaneously (1×10^7 cells per condition) into the lower right flank of the mouse in a total volume of 100 μl of sterile saline using a 27-gauge needle (Becton Dickinson, Franklin Lakes, NJ). Four mice were used per condition. At 60 days from the time of injection, tumor volumes were measured. The tumor volumes (mm^3) were estimated in accordance with the ellipsoid volume formula, $4/3 \times \pi \times a^2 \times b$, where a is the length of minor axis and b is the length of the major axis [68]. Mice were euthanized by carbon dioxide inhalation until listless and without respiration, followed by cervical dislocation or exsanguination. Tumors were harvested from each animal and portions of the tumors were placed in 10% neutral buffered formalin for 24 h then transferred to 70% ethanol. The tissues were taken to Cellular Imaging Facility Core at the University Health Sciences Center. To evaluate histology, tumor samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E), and analyzed via light microscopy by Dr. David G. Besselsen (Veterinary Pathologist, University Animal Care, University of Arizona). All procedures were performed in accordance with approved protocols of the University of Arizona Institutional Animal Care and Use Committee.

3. Results

Gene expression of pathway markers in UROtsa cells exposed to 50 nM MMA(III) from 4 to 20 months indicate perturbations in immune, inflammatory, MAPK, and apoptotic pathways

PCR assessment of selected genes showed gradual changes early in the exposure period with substantial alterations in the expression of some genes detected between 12 and 16 weeks of exposure to 50 nM MMA(III). Measured cumulative expression fold change shows a gradual increase in the absolute magnitude of change as time progresses with exposure (Fig. 1). From 15 to 20 weeks of exposure there is a substantial increase in the cumulative fold change in gene expression. The proportion of down-regulated genes primarily dominates throughout the profile. Table A.1 lists the direction and magnitude changes of genes for the time points evaluated in this study.

The most perturbed pathway was the inflammatory response (Fig. 2). Gradual changes in the expression of these genes between 4 and 12 weeks of exposure give way to erratic changes between 13 and 20 weeks of exposure. Genes, such CXCL10 and CXCL11, involved in chemotactic responses, exhibited abnormal expression later in the exposure period. CXCL10 expression declined at 13 weeks

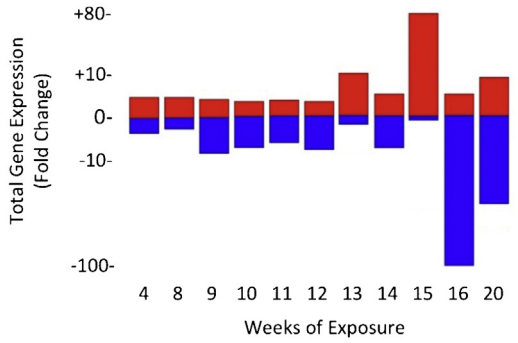

Fig. 1. Total gene expression change of 29 selected genes from PCR array study in UROtsa exposed chronically to 50 nM MMA(III) from 4 to 20 weeks. Total expression change for given time interval with red indicating summation of fold changes for genes up-regulated and blue indicating summation of fold changes for genes down-regulated.

of exposure and then peaked at 15 weeks, while CXCL11 expression peaked at 15 weeks of exposure. Arsenic has been shown to alter the expression of pro-inflammatory genes PTGS1 and PTGS2 [18,58], however, the expression changes in PTSG1 and PTGS2 exhibited in this study were distinctly different in that there was a down-regulation in PTGS1 expression at around 16 weeks. Both FOS and PTGS2 expression were down-regulated at 9 and 10 weeks of exposure, with FOS expression continuing in this down-regulated state at 11 and 12 weeks of exposure. Although not discernable in Fig. 2, the earliest changes detected were the up-regulation of PTGS2 and FOS with at least two-fold change in expression (Table A.1).

Genes associated with the immune system pathway exhibited no major changes until between 14 and 20 weeks of exposure (Fig. 3). Arsenic is known to impair immune function, and such changes were characterized in the previous gene array study [26]. Although direct effects of arsenic exposure on STAT2 have not been well established, STAT2 expression within this study peaked at 15 weeks of exposure, and then declined at 16 weeks. A similar pattern of change was observed for JUN expression. CCRL1, a gene that encodes for C–C type chemokine receptors, has been reported to be involved in cancer invasion and metastasis, and may serve as a prognostic marker of cancer progression [67]. CCRL1 expression was elevated later in the exposure period, reaching a peak at 20 weeks.

With the exception of a sustained down-regulation of FOS from 9 to 12 weeks of exposure, the expression of genes associated with the MAPK pathway was not characterized by major changes until later in the exposure period (Fig. 4). Gradual increase in the expression of PDGFRA, a potent mitogen, with a peak at 15 weeks of exposure was paralleled by a gradual increase in JUN expression and slight elevation in DUSP1 expression. Although not discernable in Fig. 4, the earliest change detected was the up-regulation of PDGFRA, with at least two-fold change in expression (Table A.1).

Subtle changes occurred in the expression of genes involved the apoptotic pathway (Fig. 5). More intense changes in the expression of some of these genes occurred much later in the exposure period. The expression of CFLAR,

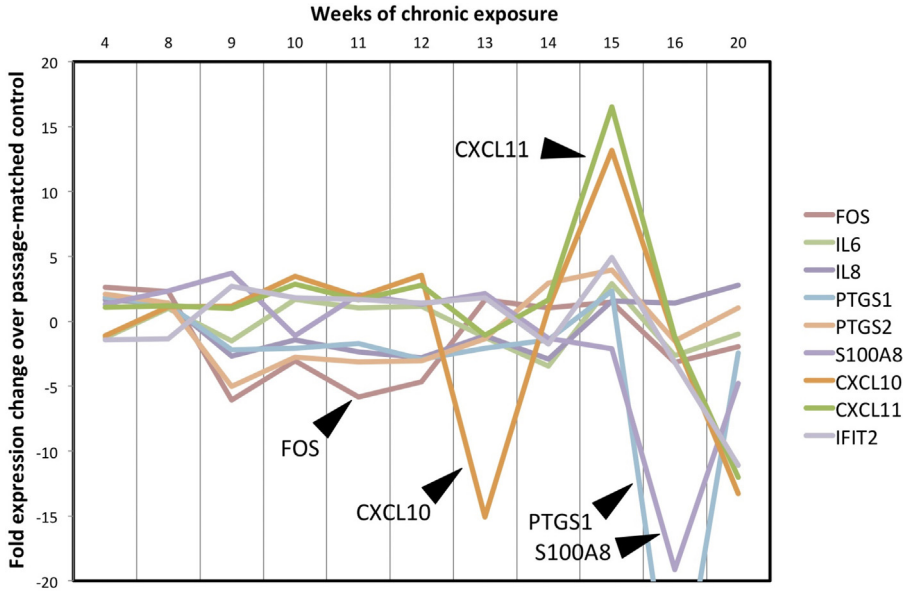

Fig. 2. Plot of the chronological expression of genes associated with the inflammatory response in UROtsa cells chronically exposed to 50 nM MMA(III). Plot depicts the level of expression of FOS, IL6, IL8, PTGS1, PTGS2, S100A8, CXCL10, CXCL11, and IFIT2 relative to control for UROtsa cells exposed chronically to 50 nM MMA(III) from 4 to 20 weeks.

an inhibitor FAS-induced apoptosis, declined at around 16 weeks of exposure. XAF1 is a known mediator and inhibitor of apoptosis was down-regulated by 20 weeks of exposure. GPC3 has been implicated in the liver cancer, control of growth factors and apoptosis [61]. Previous gene array study demonstrated an elevation in the expression of GPC3 with 12 weeks of chronic MMA(III) exposure [26]; however, within this study, GPC3 expression did not start to increase until 16 weeks of exposure.

Surprisingly, genes involved with oxidative stress did not exhibit any substantial change throughout the exposure period (Fig. 6). Genes such as MT1X and SOD1, which

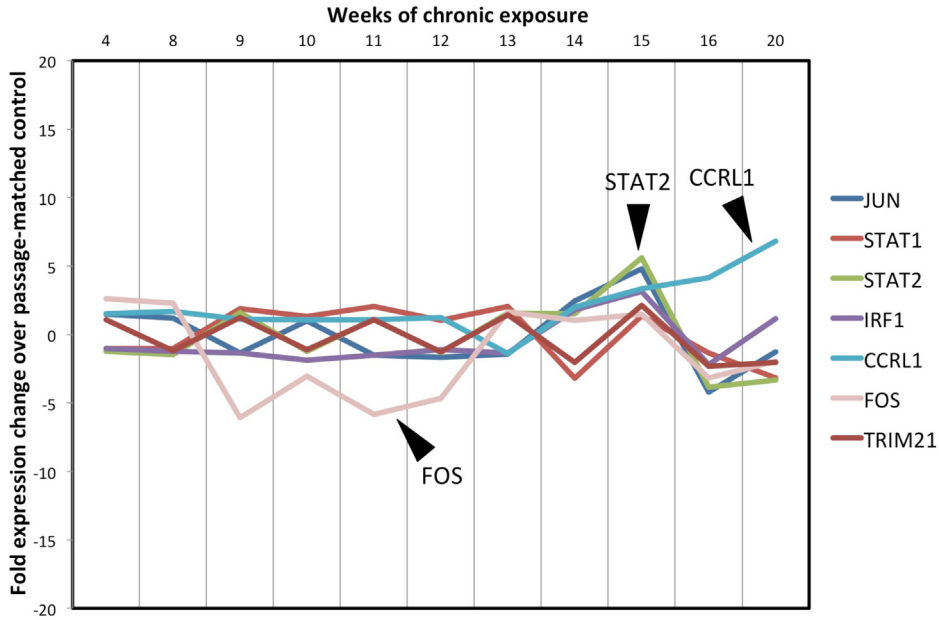

Fig. 3. Plot of the chronological expression of genes associated with the immune system pathway in UROtsa cells chronically exposed to 50 nM MMA(III). Plot depicts the level of expression of JUN, STAT1, STAT2, IRF1, CCRL1 FOS, and TRIM21 relative to control for UROtsa cells exposed chronically to 50 nM MMA(III) from 4 to 20 weeks.

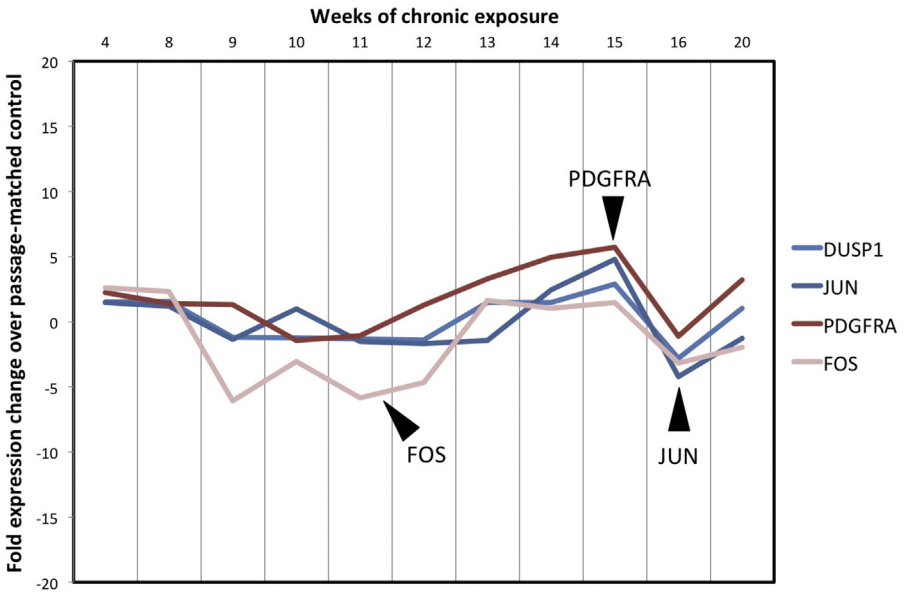

Fig. 4. Plot of the chronological expression of genes associated with MAPK signaling pathway in UROtsa cells chronically exposed to 50 nM MMA(III). Plot depicts the level of expression of DUSP1, JUN, PDGFRA, and FOS relative to control for UROtsa cells exposed chronically to 50 nM MMA(III) from 4 to 20 weeks.

have previously been observed altered during short-term exposure to arsenic, were unaltered [69,70].

A little over a half of the genes surveyed within the PCR array study were in agreement with the change and direction of expression measured in the gene array study [26]. Of particular interest was the comparative up-regulation observed in PDGFRA, S100A8, and SOD1; and suppression in STATs, DUOX2, DUSP1, PTGS1, and PTGS2 at 12 weeks of exposure to 50 nM MMA(III).

3.1. MMA(III) induces hyperproliferation in UROtsa cells

To determine the transition from a normal to a malignant phenotype in UROtsa cells following chronic exposure to 50 nM MMA(III), cellular growth rates were assessed in UROtsa cells chronically exposed to 50 nM MMA(III) from 4 to 20 weeks (Fig. 7). With increasing time of exposure to MMA(III), cells displayed an increase in proliferation rate. Statistical significance was found at 16 weeks

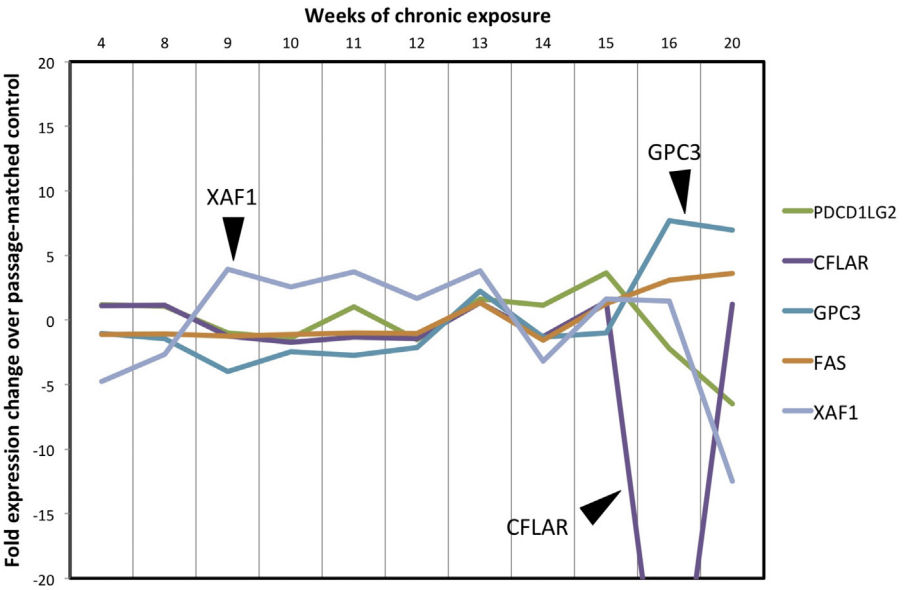

Fig. 5. Plot of the chronological expression of genes associated with apoptosis in UROtsa cells chronically exposed to 50 nM MMA(III). Plot depicts the level of expression of PDCD1LG2, CFLAR, GPC3, FAS, and XAF1 relative to control for UROtsa cells exposed chronically to 50 nM MMA(III) from 4 to 20 weeks.

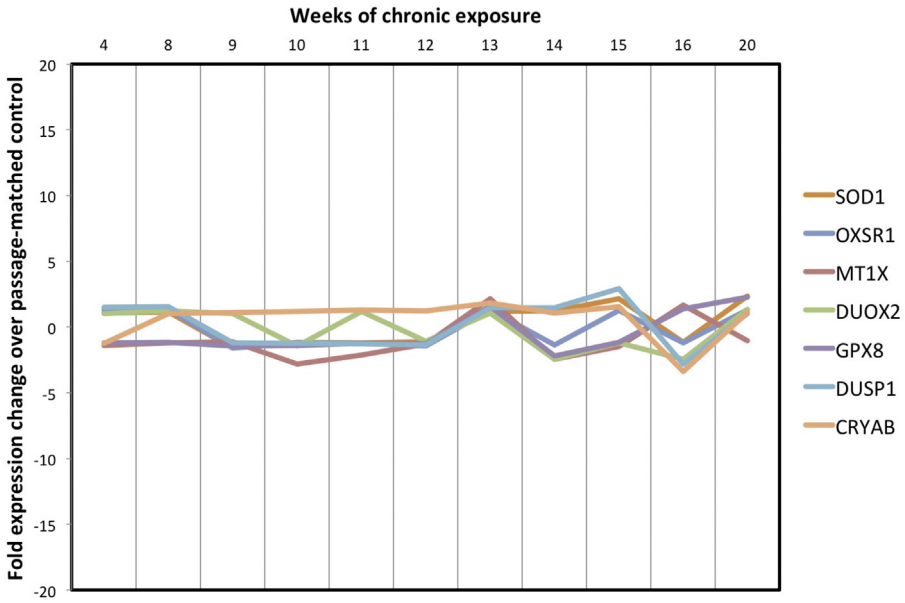

Fig. 6. Plot of the chronological expression of genes associated with oxidative stress in UROtsa cells chronically exposed to 50 nM MMA(III). Plot depicts the level of expression of SOD1, OXSR1, MT1X, DUOX2, GPX8, GPX8, DUSP1, and CRYAB relative to control for UROtsa cells exposed chronically to 50 nM MMA(III) from 4 to 20 weeks.

and 20 weeks when compared to UROtsa untreated control.

3.2. MMA(III) induces anchorage-independent growth of UROtsa

Anchorage-independent growth is a property displayed by many tumorigenic cell lines. Measurement of colony forming potential allows for the assessment of anchorage-independent growth. To determine if the increases in cell proliferation rates were associated with anchorage-independent growth, UROtsa cells chronically exposed to 50 nM MMA(III) from 4 to 20 weeks were tested for the ability to form colonies in soft agar (Fig. 8). Colony formation was detected in UROtsa after 4 weeks of exposure to 50 nM MMA(III). Colony formation continued to increase in a time-dependent manner from 8 to 16 weeks but leveled off at 20 weeks.

3.3. MMA(III) enhanced tumorigenicity in UROtsa cells following 4 weeks of exposure

Because significant colony formation was demonstrated in nearly all conditions compared to untreated control, tumorigenicity of select conditions was then examined. UROtsa cells exposed to 50 nM MMA(III) for 4 weeks, 8 weeks, 16 weeks, and 20 weeks had significantly elevated tumor burdens compared to control at 60 days from the time of injection (Fig. 9). The presence of tumors at 4 and 8 weeks of exposure was a surprising new finding,

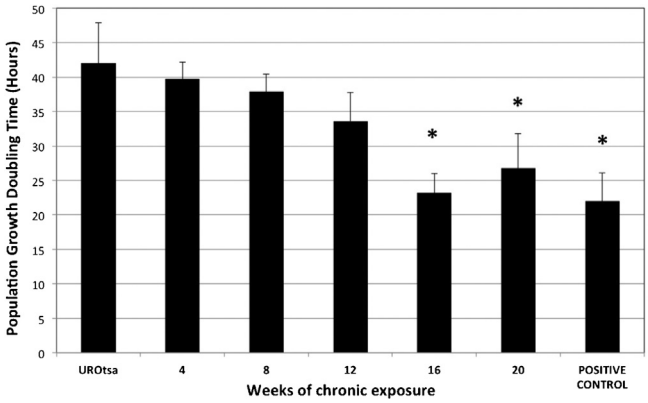

Fig. 7. Cell population doubling times for UROtsa exposed chronically to 50 nM MMA(III) from 4 to 20 weeks. Declining trend in doubling times was apparent, but significance was not reached until 16 weeks of exposure when compared to UROtsa untreated control (UROtsa). (*) Marks a statistically significant difference ($p < 0.02$) for the treated cells compared to passage-matched untreated UROtsa following ANOVA detected difference and Bonferroni's correction. Error bars represent the standard error of the mean.

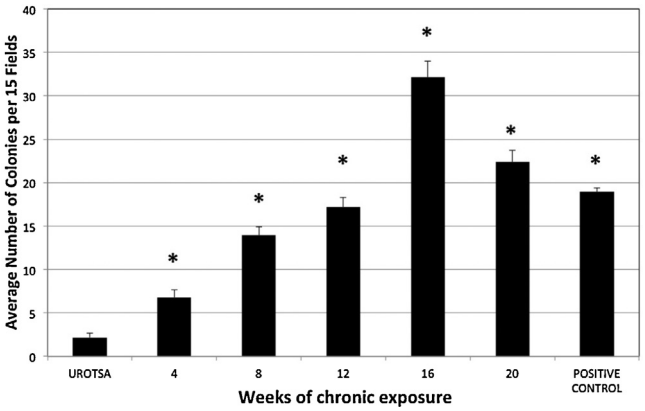

Fig. 8. Anchorage-independent growth measured for UROtsa exposed chronically to 50 nM MMA(III) from 4 to 20 weeks. Graphs depict colony formation measured after 2 weeks following inoculation of cells into soft agar. Within each well 5 fields were randomly selected and colonies counted in three independent experiments. (*) Marks a statistically significant difference ($p < 0.02$) in treated cells compared to untreated UROtsa following ANOVA detected difference and Bonferroni's correction. Error bars represent the standard error of the mean.

since a previous study did not show tumors burdens until 12 weeks of exposure [21]. Although our previous study demonstrated significant tumor formation following 12 weeks of exposure, in this study the tumor burden at 12 weeks of exposure was not statistically different from the control. The tumor morphology was indicative of squamous cell differentiation and neoplasia/dysplasia (Figs. A.1 and A.2).

4. Discussion

It has been previously shown that chronic exposure of UROtsa to a trivalent monomethylated arsenical, MMA(III), leads to malignant transformation [16,21]. Gene array analysis at each month of exposure revealed gradual changes in the first and second months followed by substantial alterations in the expression of genes at 3 months that corresponded to phenotypic changes in cellular proliferation and growth [26].

Within this more detailed time-course study, low-level MMA(III) was shown to perturb the expression of a small group genes in the first 4 weeks, those associated with the inflammatory response, MAPK signaling, and the immune system. However, the variable direction in the regulation of these genes did not clearly establish a stable alteration associated with direct MMA(III) insult. Hence, the attempt to resolve the exposure period into weekly intervals did not yield any additional information in regards early gene changes leading to malignant transformation, as the changes were too subtle. However, the substantial changes with inflammatory genes later in the exposure period would suggest malignant transformation is likely consequential of and coupled with these changes. The results herein also suggest that the effects from exposure to low-level MMA(III) are not strongly linked with

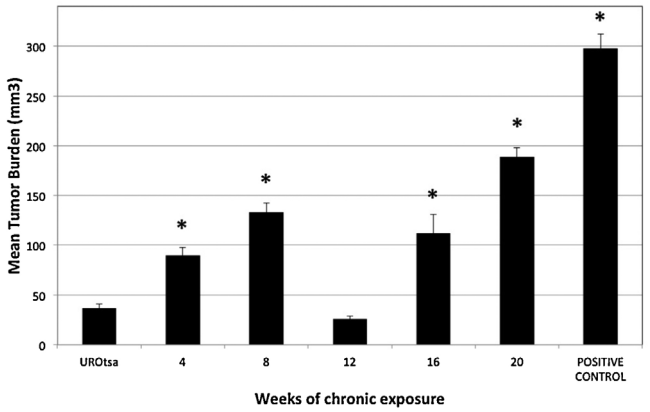

Fig. 9. Tumorigenicity measured for UROtsa exposed chronically from 4 to 20 weeks in SCID mice. Cells were injected into SCID mice and allowed to grow for 60 days to assess tumorigenicity. Tumor volumes were measured twice weekly, but data represent only measurements made at 60 days post-injection. Mean tumor burden represents the average tumor volume measured in 4 individual mice per condition. Significant changes in tumor volume were identified with ANOVA followed by Bonferroni's correction. (*) Marks statistically significant changes compared to UROtsa untreated ($p \leq 0.005$). Error bars indicate the standard error of the mean for each condition.

elevation in ROS, but more likely an outcome due to the perturbation in cell signaling cascades that occur further along in the exposure period. Genes associated with the immune, inflammatory, MAPK signaling, and apoptosis pathway appear to be more perturbed than genes involved with the oxidative stress response. These results provide some unique insight on arsenic-induced carcinogenesis, and demonstrate that even low-level arsenic exposure must be carefully evaluated, since such considerations on arsenic dose and risk thresholds for carcinogenesis are still being debated [65].

Arsenic has been shown to induce the expression of a number of ROS-responsive genes such as SOD-1, heme oxygenase 1 (HMOX-1), NADPH oxidoreductase, glutathione, heat-shock proteins, and multidrug-resistant transporters [22,43–46]. These observations have been suggested as one of the hallmarks underlining arsenic-induced carcinogenesis [15,63]. In considering the conditions established, all these studies have applied concentrations of arsenicals anywhere from 1 to 25 μM for periods of a few hours to a few weeks, and have utilized animal models and other *in vitro* systems. However, the oxidative stress genes assessed in the present study did not change appreciably throughout the exposure period for the low-level, non-cytotoxic concentration of the arsenical. A similar finding has been reported, where oxidative stress-responsive genes did not appear to be significantly altered after 12 weeks of low-level exposure with various trivalent arsenicals in urothelial cells [24]. Such findings suggested that ROS might be playing a more critical role much earlier in the exposure period, or since the concentration is so low that oxidative stress is not as important as the effects from chronic perturbation in cellular signals.

Perturbations in immunological and inflammatory pathways have long been considered a component in the development of cancer [60,64]. It has been suggested that immunological impairment in arsenic-exposed populations is a possible mechanism allowing transformed cells to survive [59]. Likewise, transformed cells with perturbed immunological gene expression likely hinder the appropriate response from the immune system. Within this study, marked perturbations of immunological genes from MMA(III) exposure occurred in the latter part of the exposure period. Additionally, the severe perturbations in the expression of inflammatory genes are reminiscent of the altered inflammatory responses seen in other studies on arsenic exposure ([37,66]; Wu et al., 2008). The inflammatory cytokine, IL-8, has been reported to increase the expression of PTGS2 (elevated at 4 weeks of exposure in this study) and most likely promotes cell survival and growth [18,47]. In the weeks that followed, inflammatory genes associated with cancer were elevated. At later time points (between 12 and 16 weeks of exposure), the malignant alterations were characterized by the abnormal expression of STAT1, in addition to FOS, IL-8, and PTGS2. PTGS2 has been shown to be over-expressed in aggressive colon cancers [48]. However, arsenic-induced down-regulation of PTGS2 has only been reported with the use of arsenic trioxide in the treatment of acute myeloid leukemia, HL-60, through the inhibition of NF-kB activity [49].

The effects of arsenic exposure on the MAPK signaling system have been well documented. Increased cellular proliferation has been observed with chronic arsenic exposure [16,50–52]. In this study, significant phenotypic changes were not observed until 16 weeks of exposure. However, it is clear that a steady increase in the proliferation rate was observed and by 3 months of exposure a 20% change was evident when compared to untreated control. Wnek et al. [21] first reported a significant and irreversible change in the proliferation rate of UROtsa cells at 3 months of exposure to MMA(III). In the previous gene array study perturbations were identified in the MAPK signaling pathway contributing to enhanced proliferation [26]. In the present study, the proliferation rate assay did not show a significant change in the doubling time at 4 or 8 weeks of exposure. Consequently, DUSP1, a negative regulator of the MAPK pathway, was up-regulated early in the exposure period, likely attributing to FOS, JUN, and PDGFRA down-regulation between 8 and 12 weeks of exposure. Since PDGFRs are distinct mitogens that activate downstream effectors such as PI3K, Erk, JNK, and STAT [62], any uncontrolled expression of PDGFRs would promote cell migration, proliferation, and survival. Up-regulation of PDGFRA beginning in latter part of the exposure period reflects one element of enhanced proliferation due to arsenic-induced carcinogenesis. Coupled to this was the perturbation in the expression of apoptotic genes. Although the expression fluctuates throughout, near the end of the exposure period there were major changes in the expression of XAF1, PDCD1L2, and CFLAR, which could potentially be modifying the survival aspects of the cells.

The growth of colonies in soft agar is a commonly used method to characterize malignant transformation [16,52,53]. UROtsa cells exposed for 4 weeks to MMA(III) resulted in the development of colonies in soft agar. According to a previous study, this period of exposure was not sufficient to induce colony formation [21]. As anchorage-dependent growth is a tightly regulated system where cell growth is coupled to signals from growth factor receptors and integrin [54], it is not clear from the present study what patterns of gene changes are contributing to the acquisition of this phenotype. However, perturbations in the expression of IL6 were observed at several points in the exposure period, and IL6 has been implicated in anchorage-independent growth with colorectal carcinoma cell lines [55]. Another method for determining malignant transformation is to examine the tumorigenicity of cells when injected into immuno-compromised mice (SCID) [56]. The development of tumors at 4 weeks of exposure is a new finding, and similar to the study on anchorage-independent growth, not previously seen with this model. These studies demonstrate that as early as 4 weeks of exposure to low-level physiologically-relevant levels of MMA(III), urothelial cells acquire phenotypic changes that are characteristic of a malignant transformation.

In summary, the changes observed in the expression of specific genes covering a broad range of biological processes from mitogenesis to apoptosis provide a simplified picture of alterations that accompany chronic exposure of the bladder urothelium to a trivalent arsenical. The variability of contributing factors in arsenical-induced

transformation of UROtsa attests to the very nature of arsenic-induced carcinogenesis: the path and progression toward the development of cancer is diverse and arsenic is affecting multiple pathways.

Transparency document

The Transparency document associated with this article can be found in the online version.

Appendix A.

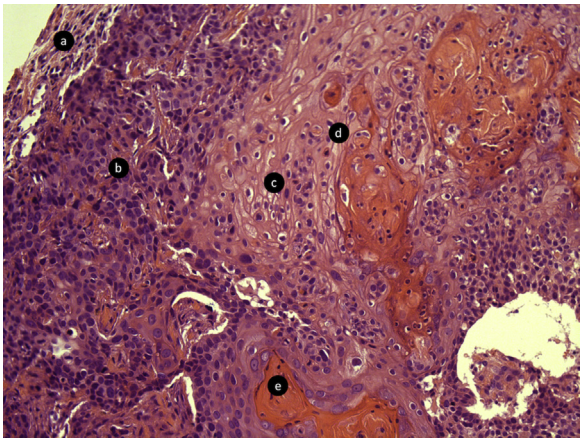

Fig. A.1. H&E stain of control untreated UROtsa cells in SCID mice at 60 days post-injection. Legend: (a) edge/fat tissue; (b) disorganized transitional epithelium; (c) vacuolated stratified squamous epithelial cells; (d) keratin; (e) stratified squamous cells (atypical urothelial morphology).

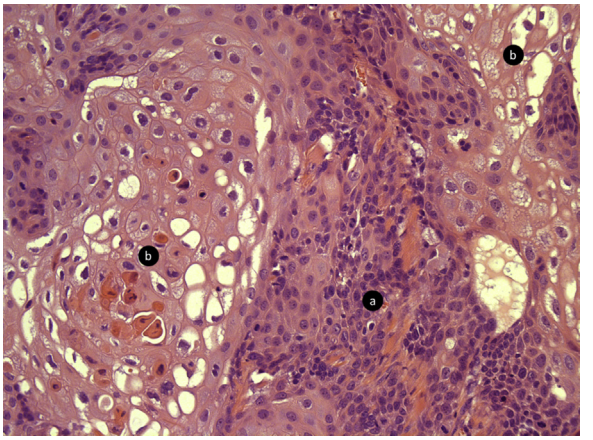

Fig. A.2. H&E stain of malignantly transformed UROtsa cells (positive control) in SCID mice at 60 days post-injection. Legend: (a) mitotic cells; (b) vacuolated cells. Overall morphology is indicative of squamous cell differentiation and neoplasia/dysplasia.

Table A.1
Magnitudes of change in the expression of genes in UROtsa cells from 1 month to 6 months of chronic exposure to 50 nM MMA(III).

	4 weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks	13 weeks	14 weeks	15 weeks	16 weeks	20 weeks	24 weeks
JUN	1.47	1.19	-1.34	1.01	-1.53	-1.66	-1.43	2.45	4.78	-4.21	-1.25	-17.81
PDGFRA	2.24	1.39	1.32	-1.44	-1.06	1.28	3.28	4.96	5.73	-1.11	3.22	-5.34
PDCD1LG2	1.19	1.08	-1.02	-1.41	1.03	-1.53	1.61	1.15	3.67	-2.20	-6.49	-12.66
CFLAR	1.12	1.15	-1.25	-1.75	-1.34	-1.46	1.33	-1.27	1.42	-35.77	1.24	-1.65
GPC3	-1.05	-1.44	-4.00	-2.44	-2.73	-2.12	2.25	-1.33	-1.01	7.68	6.97	-2.96
FAS	-1.10	-1.09	-1.26	-1.11	-1.00	-1.05	1.33	-1.57	1.26	3.08	3.59	-2.03
XAF1	-4.76	-2.68	3.95	2.57	3.72	1.69	3.80	-3.18	1.61	1.49	-12.49	-3.69
STAT1	-1.04	-1.03	1.89	1.32	2.04	1.02	2.04	-3.19	1.34	-1.35	-3.16	-2.40
STAT2	-1.24	-1.45	1.60	-1.24	1.12	-1.30	1.58	1.53	5.62	-3.84	-3.32	-6.93
IRF1	-1.04	-1.24	-1.35	-1.89	-1.51	-1.12	-1.35	1.82	3.15	-2.20	1.17	-17.38
CCRL1	1.51	1.70	1.12	1.08	1.07	1.24	-1.39	2.00	3.35	4.16	6.83	-3.40
SOD1	1.07	1.14	-1.60	-1.14	-1.22	-1.14	1.18	1.21	2.16	-1.15	2.35	1.65
OXSR1	1.32	1.36	-1.60	-1.21	-1.26	-1.44	1.29	-1.38	1.27	-1.21	1.30	1.25
MT1X	-1.41	-1.19	-1.11	-2.82	-2.13	-1.30	2.14	-2.45	-1.50	1.66	-1.03	-19.55
DUOX2	1.04	1.22	1.04	-1.44	1.17	-1.10	1.07	-2.47	-1.20	-2.44	1.36	-7.49
GPX8	-1.20	-1.18	-1.46	-1.42	-1.26	-1.29	1.60	-2.20	-1.17	1.39	2.29	-1.36
DUSP1	1.53	1.56	-1.21	-1.22	-1.30	-1.38	1.48	1.48	2.91	-2.83	1.03	-2.72
CRYAB	-1.25	1.02	1.11	1.18	1.32	1.21	1.82	1.06	1.54	-3.40	1.10	-2.93
DCLRE1C	1.14	1.26	-1.19	-1.16	-1.14	-1.22	1.41	1.52	3.16	-1.43	1.37	-2.48
FOS	2.61	2.31	-6.07	-3.05	-5.85	-4.65	1.64	1.02	1.47	-3.19	-1.95	-12.28
IL6	-1.27	1.01	-1.50	1.69	1.05	1.17	-1.24	-3.46	2.89	-2.66	-1.00	-2.24
IL8	1.63	1.42	-2.70	-1.45	-2.35	-2.81	-1.11	-2.91	1.56	1.39	2.76	-4.10
PTGS1	1.86	1.27	-2.21	-2.06	-1.71	-2.91	-2.07	-1.44	2.34	-31.59	-2.46	-6.68
PTGS2	2.09	1.40	-5.02	-2.77	-3.14	-3.03	-1.36	2.92	3.95	-1.53	1.04	-9.83
S100A8	1.34	2.34	3.71	-1.12	2.06	1.30	2.12	-1.32	-2.10	-19.15	-4.81	-10.47
CXCL10	-1.10	1.12	1.17	3.47	1.90	3.56	-15.08	1.00	13.18	-1.37	-13.26	-3.56
CXCL11	1.09	1.19	1.01	2.85	1.66	2.76	-1.07	1.63	16.50	-1.19	-12.02	-7.59
IFIT2	-1.41	-1.36	2.68	1.81	1.68	1.42	1.81	-1.77	4.91	-3.18	-11.09	-6.48
TRIM21	1.09	-1.18	1.24	-1.09	1.08	-1.26	1.45	-2.04	2.11	-2.32	-2.03	-66.17

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