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Enrichment of genes associated with squamous differentiation in cancer initiating cells isolated from urothelial cells transformed by the environmental toxicant arsenite

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Abstract

Arsenic is an environmental toxicant with long-term exposure associated with the development of urothelial carcinomas. Our lab has developed an *in-vitro* model of urothelial carcinoma by exposing the immortal, but non-tumorigenic bladder cell line, the UROtsa, to arsenite (As^{3+}). These transformed cells form tumors in immune-compromised mice, which resemble urothelial carcinomas with components of the tumor exhibiting squamous differentiation. The goal of the present study was to determine the differences in global gene expression patterns between the As^{3+} -transformed UROtsa cells and the urospheres (spheroids containing putative cancer initiating cells) isolated from these cell lines and to determine if the genes involved in the development of squamous differentiation were enriched in the urospheres. The results obtained in this study show an enrichment of genes such as KRT1, KRT5, KRT6A, KRT6B, KRT6C, KRT14 and KRT16 associated with squamous differentiation, a characteristic feature seen in aggressive basal subtypes of urothelial cell carcinoma (UCC) in the urospheres isolated from As^{3+} -transformed UROtsa cells. In addition, there is increased expression of several of the small proline-rich proteins (SPRR) in the urospheres and overexpression of these genes occur in UCC's displaying squamous differentiation. In conclusion, the cancer initiating cells present in the urospheres are enriched with genes associated with squamous differentiation.

Keywords

Urothelial cell carcinoma; arsenite; squamous differentiation; stem cells; UROtsa

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Conflict of interests

The authors declare that they have no competing interests.

1. Introduction

Environmental exposure to As^{3+} has a strong association with urothelial cell carcinomas (UCC) and the incidence correlates to the same endemic areas of the world where populations have been identified with arsenic-induced skin cancer (Cantor et al., 2007; Smith et al., 1998; Chiou et al., 1995; Steinmaus et al., 2000). These carcinomas are the most common type of bladder cancer being the second most diagnosed cancer of the genitourinary tract, behind prostate cancer (Ferlay et al., 2015; Andreassen et al., 2016). Worldwide, UCC is the 5th most common cancer in men and the 12th most common cancer in women. Approximately 20% of UCCs are characterized as more aggressive, muscle-invasive tumors, which arise independent of the papillary pathway and have a poor outcome with an overall 5-year survival rate of only 5% (van der Horst et al., 2012). Gene expression profiling is currently being used to predict if bladder cancers will progress resulting in poor patient outcome. The profiling is based on previous studies that have successfully utilized gene expression profiling in breast cancer, resulting in the identification of molecular subtypes (basal/triple negative, HER2^+ , luminal A, and luminal B) that behave clinically as distinct disease entities (Rouzier et al., 2005). Several investigative teams have defined the gene expression signatures of UCC and proposed several intrinsic subtypes of the disease (Sjödahl et al., 2013; Choi et al., 2014; Patschan et al., 2015). One study analyzed 237 tumors and defined three molecular subtypes of UCC as urobasal (Uro), genomically unstable (GU), and squamous-cell-carcinoma like (SCCL). The study showed the three subtypes did not correspond directly to pathological state and grade, and differed in fundamental biological aspects and survival patterns (Sjödahl et al., 2013; Lindgren et al., 2012). A second study examined 167 primary stage T1 tumors and demonstrated the rapidly progressing T1 tumors were of subtype GU or SCCL, possessing either a high progression risk score or an elevated CD3^+ cell count (Patschan et al., 2015). Ultimately, the subtypes were expanded to six categories due to the complexity of muscle-invasive UCCs (Aine et al., 2015; Sjödahl et al., 2017; Cancer Genome Atlas Research Network, 2014). A third study focused only on samples from muscle-invasive UCCs and defined two distinct cell subtypes, basal and luminal (Choi et al., 2014). The basal muscle-invasive UCCs showed a disease more aggressive at presentation and significantly enriched with squamous features. Damrauer and coworkers (2014) have found similar results for muscle-invasive UCCs with segregation into basal and luminal subtypes. A general finding in the above studies is the association of more basal subtypes with aggressive disease, including the enrichment of genes and histological features associated with squamous differentiation.

The finding that squamous differentiation is associated with more aggressive UCCs is of interest, since studies from our laboratory have shown the immortal human urothelial cell line (UROtsa), when transformed with As^{3+} or cadmium (Cd^{2+}), gives rise to tumors exhibiting prominent focal areas of squamous differentiation (Rossi et al., 2001; Sens et al., 2004; Somji et al., 2011; Cao et al., 2010). Thus exposure to these metals can give rise to aggressive forms of bladder cancer. In addition, the presence of squamous differentiation in the UROtsa-derived tumors also suggests they exhibit the basal gene signature determined for muscle-invasive UCCs. The possible relationship between squamous differentiation and basal gene expression was confirmed in a study by Hoggarth and coworkers (2018) where

tumors produced by the As³⁺ and Cd²⁺-transformed UROtsa cells were assessed for the expression of the basal and luminal markers as defined by Choi and coworkers (2014) for muscle-invasive bladder cancers. The study showed the tumors to have a gene expression pattern similar to the pattern seen in muscle-invasive UCCs. The study also determined the expression of nine basal and 16 luminal markers as defined by Choi and coworkers (2014) in the transformed cell lines and in urospheres (spheroids) isolated from the cell lines. Previous studies from this laboratory have shown the urospheres generated from the As³⁺ and Cd²⁺-transformed cell lines to form tumors in immune-compromised mice with a histology indistinguishable from the originating cell lines, including focal areas of squamous differentiation (Slusser-Nore et al., 2016; Sandquist et al. 2016). The results from the Hoggarth et al., 2018 study showed the cell lines to display a gene signature with identity to the basal subtype, and this signature was further enriched in urospheres isolated from the cell lines (Hoggarth et al., 2018). The observation also provides evidence, but not definitive proof, based on the gene signature that urospheres represent the cell population that is originally malignantly transformed by exposure to As³⁺ or Cd²⁺.

Cancer stem cells (CSCs) or cancer-initiating cells (CICs) can be isolated from different tumors since they can grow as spheroids (Ishiguro et al., 2017). These tumor-derived spheroids are a unique *in-vitro* system enriched with CSCs or cells with stem-cell related characteristics. The spheroids are maintained in culture as floating spheres using serum-free media and cell culture vessels that do not promote cell attachment. Spheroids isolated from cultured tumor cell lines are believed to represent a minority population of CSCs within the culture that are capable of establishing a tumor in an appropriate animal model. The relationship of this minority population of CICs to the remaining of the cells in the culture is not known. The present study was designed to ask several questions regarding urospheres isolated from the As³⁺-transformed UROtsa cell lines shown to form tumors in immune compromised mice (Slusser-Nore et al., 2016; Sandquist et al., 2016). The As³⁺-transformed isolate was chosen for this study since exposure to As³⁺ is linked to the development UCC. The goals of this study were to determine the difference in gene expression patterns between the As³⁺-transformed cell line and the urospheres isolated from the cell line and determine if the genes involved in squamous differentiation that were noted in our As³⁺-transformed cells and other pathways involved in the development of UCCs were enriched in the urospheres. The final goal of this study was to determine if the urospheres maintained their gene expression profile when cultured under conditions similar to those used for the parental cell lines.

2. Materials and Methods

2.1. Cell culture

The UROtsa parent cells and the As³⁺-transformed isolate were cultured in 75 cm² tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% v/v fetal bovine serum as described previously (Sens et al., 2004). The cells were sub-cultured at a 1:4 ratio using trypsin-EDTA and the cultures were fed fresh growth medium every three days. The UROtsa cell line has been authenticated using Short tandem repeat analysis (Slusser-Nore et al., 2016). The As³⁺-transformed isolate used in the current study has been

previously characterized for its ability to form colonies in soft agar, form tumors when injected subcutaneously in immune-compromised mice and form spheroids when grown in ultra-low attachment flasks (Sens et al., 2004; Somji et al., 2011; Cao et al., 2010; Slusser-Nore et al., 2016; and Sandquist et al., 2016).

Urospheres (spheroids) were generated by seeding the UROtsa parent and the As³⁺-transformed cell line at a density of 10⁵ cells in T-25 cm² Ultra-low attachment flasks (Corning Inc., Corning NY). The growth medium consisted of a 1:1 mixture of DMEM and Hams's F-12 growth medium supplemented with selenium (5 ng/mL), insulin (5 µg/mL), transferrin (5 µg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL). The urospheres were harvested after 8 days by centrifugation for RNA and protein isolation.

For passaging of the urospheres, they were placed in either serum-containing or serum free media in flasks that allowed them to attach until they reached confluency (passage 1, P1). At confluency they were passaged 7 more times and at P1, P4 and P8, and cells were harvested for RNA and protein isolation.

2.2. RNA sample isolation and microarray analysis of global gene expression

Total RNA was purified from triplicate cultures of the As³⁺-transformed cell line and urospheres isolated from the cell line using the RNeasy Mini kit (Qiagen, Valencia, CA). Purity and concentration of RNA samples were determined from OD_{260/280} readings using a dual beam UV spectrophotometer and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) as per the manufacturers instructions. Microarray analysis was performed by Genome Explorations. Total RNA (200 ng) was converted to cDNA and amplified by the random primed RT-IVT-RT method, fragmented and labeled with biotin using GeneChip® WT PLUS Reagent Kit according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Fragmented biotinylated cDNA was hybridized for 20 hr at 45°C to Clariom™ D, Human Arrays (Applied Biosystems™). The arrays were washed and stained with phycoerythrin-conjugated streptavidin (Life Technologies, Carlsbad, CA) in a Fluidics Station 450 (Affymetrix) according to manufacturer's recommended procedures. Fluorescence intensities were determined using a GCS 3000 7G high-resolution confocal laser scanner, and analyzed using programs resident in the AGCC, Expression Console (Affymetrix), & Transcriptome Analysis Console (TAC) 4.0 (Affymetrix). RMA Quality Control outputs from Expression Console & TAC 4.0 were used to identify potential outlier arrays; outlier evaluation was also performed by Principal Components Analysis in GeneMaths XT (Applied Maths, Austin TX).

2.3. Microarray data analysis

Expression data was analyzed following background correction, probe set signal summarization and normalization by SST[EMS1]-RMA, (Irizarry et al., 2006). Gene-level transcript clusters exhibiting differential expression were identified in TAC 4.0 by using linear regression and hierarchical clustering analysis—hierarchical clustering analysis performed in TAC 4.0 was subsequently confirmed and carried out in GeneMaths XT.

Significantly differentially expressed transcript clusters were identified based on absolute signal fold-change ≥ 2.0 and false-discovery rate (FDR) adjusted *p*-value ≤ 0.05 (when performing pairwise comparisons). When performing linear regression analysis, the FDR was controlled at 5% using the Benjamini-Hochberg step-up procedure.

Sample and Gene-level unsupervised hierarchical clustering was performed in TAC 4.0 & GeneMaths XT 2.0 (Applied Maths, Belgium), following row mean centering of log₂ transformed RMA signal values; sample and gene-based clustering was performed by the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) method using Pearson correlation as the similarity metric.

Gene annotations, gene ontology information and biochemical pathway information were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), NetAffx (www.affymetrix.com), the Gene Ontology (GO) Consortium (<http://amigo.geneontology.org>), the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg), WebGestalt (<http://bioinfo.vanderbilt.edu/webgestalt>), and the Broad Institute's Gene Set Enrichment Analysis (GSEA) desktop application v2.2.3 (<http://software.broadinstitute.org/gsea/index.jsp>).

To investigate group-specific pathway enrichment, lists of differentially expressed genes, or DEGs (and fold-change values), were submitted to WebGestalt and pathway enrichment against GO, KEGG, Wikipathways, and Pathway Commons databases was determined. When submitting DEGs to WebGestalt, categories/pathways involving ≤ 2 genes were discarded and categories/pathways were only considered significantly enriched if FDR-adjusted hypergeometric test *p*-value ≤ 0.05 . In addition to individual lists of DEGs, GSEA of the entire dataset (against KEGG, GO, Reactome, and Biocarta MSigDB gene-sets) was performed per default parameters with the exception that transcript clusters were collapsed to gene-symbols based on median probe expression and not maximum probe expression. After GSEA, the FDR was controlled at 5% and gene-sets were considered significantly enriched if *q*-value ≤ 0.05 & normalized enrichment score ≥ 1.0 .

2.4. Real-time PCR analysis

The expression of various genes was assessed with reverse transcription quantitative polymerase chain reaction (RT-qPCR) using commercially available primers. The list of the genes, along with the source of the primers and catalogue numbers are listed in Table 1, supplemental materials. Total RNA was purified as described above and 0.1 μ g was subjected to cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories), in a total volume of 20 μ L. Real-time RT-qPCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) with 2 μ L cDNA and 0.2 μ M primers in a total volume of 20 μ L in an CFX96 real-time detection system (Bio-Rad Laboratories). Amplification was monitored by SYBR Green fluorescence. Cycling parameters consisted of a 30 s hot-start followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30s, which gave optimal amplification efficiency. The resulting levels were normalized to the change in β -actin expression.

2.5. Western blot analysis

Western blot analysis was performed as described previously (Hoggarth et al., 2018). The cell line and urospheres were harvested in ice cold radio-immunoprecipitation assay lysis buffer supplemented with PMSF, protease inhibitor cocktail and sodium orthovanadate (Santa Cruz Biotechnology). Protein lysates were quantified by Pierce BCA protein assay kit (Thermo-Scientific Pierce) and diluted to equal concentrations. Prior to loading, samples were reduced and denatured. The protein extracts were resolved by SDS PAGE and transferred to a 0.2 μ m PVDF membrane using semi-dry transfer. The blots were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% [w/v] non-fat milk for 90 min at room temperature. The membranes were probed overnight at 4 °C with the primary antibody diluted in 5% [w/v] bovine serum albumin. All antibodies were purchased from commercial suppliers and were validated against known positive and negative expressing cell lines by Western analysis prior to use in experimental protocols. The source of the antibody along with their catalogue numbers have been published previously (Hoggarth et al., 2018). After washing 3 times in TBS-T, the membranes were incubated with the anti-mouse or anti-rabbit secondary antibody (1:2000) for 90 min at room temperature. The blots were visualized using the Clarity™ Western ECL Blotting Substrates (Bio-Rad Laboratories).

2.6. Statistical analysis

Statistical analysis consisted of one-way ANOVA with Dunnet's multiple comparisons test using GraphPad PRISM 7 software. All experiments were done in triplicates and unless otherwise noted, all graphs are plotted as the mean $\pm$ SEM of triplicate determinations.

3. Results

3.1 Differences in gene expression between urospheres (CICs) and the UROtsa As³⁺-transformed cell line

Triplicate total RNA samples from the As³⁺-transformed cell line and urospheres isolated from this cell line were subjected to gene expression analysis using the Clariom™ D human array. From the 135,750 genes in the probe set, 4,415 genes passed the filter criteria (3.3%) and of these 4,415 genes, 2,150 genes were up-regulated (48.7%) and 2,265 genes were down-regulated (51.3%) in the urospheres when compared to the As³⁺-transformed parent (Fig. 1A and B). Filtering transcript clusters by group revealed that of the 4,415 differentially expressed transcript clusters, 459 interrogated coding genes, 571 interrogated non-coding genes, 108 interrogated pseudogenes, 58 interrogated small RNAs, 44 interrogated precursor mRNA, 16 interrogated rRNA, and 1 interrogated tRNA. Moreover, 3,010 of the differentially expressed transcript clusters interrogated multiple/complex genes while 148 were unassigned/interrogated putative or predicted genes. An examination of the dataset for up-regulated genes identified nine genes that were up-regulated 1,000 to 9,000 fold, 28 that were up-regulated 100 to 999 fold, and 178 that were up-regulated 10 to 99 fold in the urospheres compared to the parent As³⁺-transformed cell line. The remaining genes were up-regulated 2.0 to 9.9 fold compared to the parent cell line. A similar examination of the down-regulated genes identified only one gene that was down-regulated over 1,000 fold (1549.9), one down-regulated between 100 and 999 fold (249.0), and 89 that were down-

regulated 10 to 99 fold in the urospheres compared to the parent As³⁺-transformed cell line. The remaining genes were down-regulated 2.0 to 9.9 fold compared to the parent cell line. The up-regulated and down-regulated genes included only identified genes and no pseudogenes, non-coding genes, or RNA's were included in the assessment (Table 2, Supplemental Materials).

3.2 Expression of urosphere genes identified as basal and luminal markers for muscle invasive urothelial carcinoma

The research by Choi and coworkers (2014) identified a panel of genes that defined a basal subtype of muscle invasive bladder cancer (MIBC). The gene signature consisting of CDH3, CD44, KRT1, KRT5, KRT6A, KRT6B, KRT6C, KRT14, and KRT16 along with KRT17 and P63 was used by this laboratory to show the As³⁺ and Cd²⁺-transformed UROtsa cells display a gene signature pattern similar to the basal subtype of MIBC (Hoggarth et al., 2018). In the present study, microarray analysis was performed on the As³⁺-transformed cell line (As#4) grown in serum-containing media and the urospheres isolated from this cell line. The results demonstrate that KRT1 was induced 1,500 fold, KRT6A 10.4 fold, KRT6B 11.4 fold, KRT6C 20.3 fold, KRT14 54 fold, KRT16 258 fold and KRT17 7.0 fold in urospheres compared to the parent transformed cell line. There was no difference in expression of CDH3, CD44, KRT5 or P63 between the urospheres and the parent cell line (Table 2, Supplemental Materials). The microarray data was validated on triplicate RNA samples isolated from the urospheres and the As³⁺-transformed parental cells grown in serum as well as serum-free media using real-time qPCR. The results showed the expression of the above genes identified by the microarray as up regulated in the urospheres also increased when assessed individually using real-time qPCR (Fig. 2) and Western analysis (Fig.3). The KRT6 antibody does not distinguish between the isoforms and recognizes proteins made by the KRT6A, KRT6B and KRT6C genes. The expression of CDH3, CD44, and P63 genes were down-regulated in urospheres compared to the As³⁺-transformed parent cells, but at levels below or near the cut-off values of the microarray analysis. Thus, our data suggests the As³⁺-transformed cell line and the urospheres isolated from this cell line have a gene signature similar to the basal subtype of MIBC.

The panel of genes used to identify the luminal subtype of MIBC in patients were CD24, CYP2J2, ERBB2, ERBB3, FABP4, FGFR3, FOXA1, GATA3, GPX2, KRT7, KRT8, KRT18, KRT19, KRT20, PPARG, and XBPI (Choi et. al, 2014). Of these genes CD24 (induced 9.5 fold), ERBB2 (induced 2.2 fold), ERBB3 (induced 10.3 fold), FGFR3 (induced 3.1 fold) and KRT8 (repressed 2.3 fold) were differentially expressed in the microarray analysis (Table 2, Supplemental Materials). Validation of these individual genes on RNA isolated from urospheres and the As³⁺-transformed parent cells demonstrated the CYP2J2, FABP4, FOXA1, GATA3, KRT7, KRT20 and PPARG genes to be at the lower limits of detection of the real-time qPCR reaction (0.0005 to 0.000002 transcript per β -actin). The remaining genes of the luminal signature were expressed in the As³⁺-transformed cell line as well as the urospheres (Fig. 4). Western analysis showed the differences in protein levels to be similar to the differences seen in mRNA levels for the genes for KRT8, KRT18 and KRT19, whereas for CD24, the protein levels were either lower in the urosphere or were approximately the same as the cell line in the urospheres (Fig. 5).

3.3 Differential expression of genes involved in keratinization

The microarray results comparing the As³⁺-transformed cell line to urospheres isolated from the cell line were analyzed for the differential expression of genes that might be involved in squamous differentiation. This study was undertaken since, as detailed in the introduction, squamous differentiation of UCCs is associated with a poor patient prognosis. An analysis of the microarray using the GSEA enrichment method and the Reactome pathway for enrichment analysis demonstrated keratinization and formation of the cornified envelope to be the most significant positively related categories. Since the gene lists are identical between these two positive related categories, results are shown for the keratinization category (Table 1). It is noteworthy that genes induced in the urospheres that associated with the basal subtype of MIBC noted above were also present as a subset of the genes found in the keratinization dataset (Table 1, Fig 2). The normal tissue distribution of genes identified as being induced over 100 fold in the microarray, as well as those identified as a gene signature for basal MIBCs, were examined using the NCBI database (<https://www.ncbi.nlm.nih.gov/gene/>). The database provides RNA-seq results of tissue samples from 95 human individuals representing 27 different tissues. Microarray analysis showed that 35 genes were induced greater than 100 fold in the urospheres (Table 1). These genes were also expressed in the skin, esophagus and to a lesser extent in the human bladder (Table 1). In addition, the normal tissue distribution of nine other genes that were included in the basal gene signature were also analyzed (Table 2). For two of the genes, there was no data available on NCBI. From a total of 42 genes, 92.85% (39/42) were expressed in the skin, 88.1% (37/42) were expressed in the esophagus and 35.7% in urinary bladder (15/42). When compared to the 27 normal tissues samples, skin had expression of 16 (38.1%) of the genes, esophagus for 19 (45.2%) of the genes, and urinary bladder for two (4.8%) of the genes. Only five (11.95%) genes were highly expressed in other tissues. Twenty (47.6%) of the total of 42 genes showed restricted tissue expression limited to skin, esophagus or urinary bladder (Tables 1 and 2).

3.4 Stability of the urosphere gene signature

Microarray analysis was performed to determine if the gene signature pattern of the urospheres would be maintained if they were cultured under conditions used for the parental UROtsa As³⁺-transformed cell line. Triplicate samples of the urospheres were grown in regular cell culture flasks that promote attachment in serum-containing growth media that was also used for the growth of As³⁺-transformed parental cells. Total RNA samples were prepared when the inoculated urospheres reached confluency (passage 1, P1) and at passage 4 (P4) and 8 (P8). Gene expression analysis was determined using the Clariom™ D human array. This experiment was contiguous with the comparison of the urospheres with the As³⁺-transformed parent described above. The results of this analysis demonstrate the return of the gene expression signature of the urospheres to that of the As³⁺-transformed parental cell line when placed under similar cell culture conditions (Fig. 6, Table 2, Supplemental Materials). In addition, upon confluency, P1, 71.4% of the 4,415 differentially expressed genes in the urospheres return to those expressed by the As³⁺-transformed parent cell line. This increases to 90.6% at P4 and 95.8% at P8. The results are similar when the array is analyzed for up regulated and down regulated genes with a 61.4% (P1), 91.2% (P4), and 95.4% (P8) reduction in up regulated genes and a 79.7% (P1), 83.4% (P4), and 95.6%

reduction in down regulated genes. Analysis of the various RNA categories of the induced and repressed probes (i.e. multiple complex, coding, noncoding, small RNA, ribosomal RNA, and precursor micro RNA) at 1, 4 and 8 passages showed an increase in the number of genes in the multiple complex category. However, non-coding genes as well as genes for small RNA, ribosomal RNA, and precursor micro RNA decreased (Supplemental Figs 1, 2 and 3) when compared to the As³⁺-transformed parent. Analysis of the gene sets of the induced and repressed genes from the cells at P1, P4 and P8 did not identify any obvious pattern to the reductions in expressed genes or their functions (Table 2, Supplemental Materials). Real-time qPCR analysis was also performed on RNA samples for the basal and luminal genes that define MIBC and the results showed the reversal of expression of these genes to the levels seen in parental As³⁺-transformed cells (data not shown).

It was also determined if the urospheres could retain their gene signature if they were cultured in serum-free media in adherent culture vessels or their signature would reverse to the parental As³⁺-transformed cells as seen when they were cultured in serum-containing media. For this purpose, the urospheres were cultured in serum-free growth media in cell culture vessels that promoted attachment and RNA was isolated at P1, P4 and P8. The genes analyzed were the same basal and luminal markers of MIBC that were used to validate the microarray. The results of this analysis showed that the expression pattern was similar to that seen when urospheres were cultured in serum-containing media (data not shown). Thus, our data shows that culturing the urospheres in serum-containing growth media or serum-free growth media and culture vessels that promote attachment results in the urospheres returning to a gene signature similar to the parental As³⁺-transformed UROtsa cells.

3.5 Role of epigenetic regulation on urosphere gene expression

In order to determine if epigenetic regulation played a role in maintaining the gene expression profile, the urospheres were cultured in the presence of the demethylating agent, 5-Aza-2'-deoxycytidine (AZC) or the histone deacetylase inhibitor, N-(2-Aminophenyl)-4-[(pyridine-3ylmethoxycarbonyl) aminomethyl]benzamide (MS-275). The urospheres were cultured in flasks that permit cell attachment and the growth media containing serum was supplemented with 1.0, 3.0, or 5.0 μ M MS-275 or 0.5, 1.0, or 3.0 μ M AZC. Upon reaching confluency, the cells were passaged and total RNA was isolated from cells at P1, P4 and P8. Eleven (11) genes were chosen from the microarray that varied in their level of expression when the urospheres were compared to the As³⁺-transformed parental cells (Table 2, Supplemental Materials). These genes were: DSG1 (3,864 fold); SPRR2E (1,918 fold); SPRR4 (1345 fold); SPRR2D (776 fold); CYP2C18 (245 fold); SBSN (178 fold); CEACAM6 (109 fold); GJB2 (96 fold); MAF (69 fold); CX3CL1 (–15 fold); and MMP13 (–57 fold). The results of this analysis showed the reversion of the gene expression profile of the urospheres back to the As³⁺-transformed parental cells and AZC or MS-275 has no effect on the expression of the above genes. The results are shown for 3.0 μ M AZC and 5.0 μ M MS-275 for the cell line, urospheres, P1 and P8. (Table 3).

4. Discussion

This laboratory has shown that tumors generated from the subcutaneous injection of UROtsa cells transformed by either Cd^{2+} or As^{3+} produce tumors with prominent focal areas of squamous differentiation (Sens et al., 2004; Somji et al., 2011; Cao et al., 2010). These findings are consistent among the six independent isolates of As^{3+} -transformed cells and seven isolates of Cd^{2+} -transformed cells, although the extent of squamous differentiation of tumors does vary among the isolates as does the formation of keratin pearls. Despite the development of tumors that display squamous differentiation, neither the parent UROtsa cells nor the As^{3+} or Cd^{2+} -transformed cell lines show any evidence of squamous differentiation when grown in cell culture using serum-containing growth medium (Rossi et al., 2001; Sens et al., 2004; Somji et al., 2011; Cao et al., 2010). These differences could be explained by the concept that only a small number of cells exist within a tumor or a cancer cell line that have self-renewal properties, the ability to generate cellular tumor heterogeneity via differentiation and they are ultimately responsible for tumor growth and viability (Garg, 2015; Ishiguro et al., 2017). A popular method to isolate these cells is by culturing them in non-adherent plastic surfaces using serum-free growth media where the cells proliferate as free-floating multicellular spheroids. Two isolates of the As^{3+} -transformed UROtsa cells formed urospheres and when inoculated subcutaneously in immune-compromised mice, produced tumors with a histology indistinguishable from those generated by the parent cell line (Sandquist et al., 2016). One of these isolates (As#4) was used in the present study to determine the difference in gene expression patterns between the urospheres and parent cell line and see if this explains the difference in squamous differentiation seen between the cultured As^{3+} -transformed cell lines and their derived tumors. The results showed 4,415 transcript clusters and 3,469 identifiable genes differentially expressed between the As^{3+} -transformed cell line and urospheres isolated from the cell line.

Microarray analysis of the urospheres demonstrated enrichment of genes involved in squamous differentiation. The GSEA enrichment method and the Reactome pathway both showed genes involved in keratinization and formation of the cornified envelop to be the most highly enriched set of genes. In addition, an analysis of 35 genes induced over 100 fold and 9 genes that aligned with the basal subtype of MIBC demonstrated that many of these genes were expressed in normal skin and esophagus, but not the urinary bladder. These findings are consistent with the esophagus and skin being composed of squamous epithelium and the normal bladder composed of the transitional epithelium. According to the NCBI, 24 of these 44 genes are expressed in the skin or esophagus, and not the normal urinary bladder and 20 of them have a role in structure, function, or development of the stratified squamous epithelium (A2ML1, CERS3, DSG1, SPRR3, SPRR2E, SPRR4, SPRR2D, SPRR1B, SPRR2A, SPRR2B, SULT2B1, KRT1, KRT10, KRT16, KRTDAP, SCEL, ZFP750, KRT14, KRT6A, KRT6B, and KRT6C). It is not unique to find genes expressed in UCC and not in the normal bladder. The squamous cell carcinoma-like (SCCL) subtype of UCC express high levels of the basal keratins KRT6A, KRT6B, KRT6C, KRT14 and KRT16, which are not normally expressed in the urothelium and the expression of these genes is an indicator of poor prognosis (Sjödahl et al., 2012). These keratins have long been associated with

squamous differentiation in UCC (Dyrskjöt et al., 2003; Chu et al., 2001; Harnden and Southgate, 1997; Tungekar et al., 1988). The large number of small proline-rich family (SPRR) genes in the urospheres, many of which are not present in the normal urinary bladder, are also involved in the formation of the cornified cell envelope in stratified squamous epithelia (Cabral et al., 2001a,b). Several of these genes are over-expressed in UCCs displaying squamous differentiation (Lindgren et al., 2012). The current findings also extend a previous study performed using all six of the As³⁺-transformed isolates and seven of the Cd²⁺-transformed isolates. The studies showed the tumors generated from these cell lines to align with the basal subtype of human MIBC (Choi et al., 2014; Hoggarth et al., 2018). The analysis compared the cell lines, urospheres and tumors of all 13 cell lines with the gene signatures for the basal and luminal subtypes of MIBC identified by Choi and coworkers (2014). The present study clearly defines the relationship of the urospheres with genes involved in squamous differentiation, a feature of the basal subtype of MIBC. Together, the above findings reinforce that genes involved in squamous differentiation are enriched in urospheres compared to the originating As³⁺-transformed cell line, potentially explaining why the originating culture shows no overt signs of squamous differentiation.

A previous study explored the relationship between UCCs and squamous cell carcinomas (SCCa) of the bladder and showed UCCs to express very few unique dysregulated genes and share the majority of its dysregulated genes relative to normal urothelium with SSCa (Hansel et al. 2013). In contrast, SCCa, while expressing many genes common with UCCs, express many more dysregulated genes common in SCCa arising at other sites. The authors advance two possibilities to explain the close relationship between UCCs and SSCa. One, that MIBC may represent a default pathway of UCC development, with clonal changes resulting in SCCa development and overgrowth of a pre-existing UCC. The hypothesis is supported in part by the finding of mixed morphology UCCs with well-documented areas of squamous differentiation and/or glandular differentiation. A second possibility is that an early UCC stem cell exists, either prior to invasion or early in the course of invasion, which gives rise to distinct morphological entities along discrete morphological linkages that are pure subtypes. In either case, the studies by Handel and coworkers (2013) suggest a close evolution between UCCs and SCCa, with gene expression changes in UCCs primarily reflecting morphological correlates of the SCCa arising from different sites. A previous study from our laboratory has shown the immortal, but non-tumorigenic UROtsa cell line and urospheres isolated from this cell line to express genes associated with the basal subtype of MIBC (Hoggarth et al., 2018). There was an increase in expression of five genes (KRT1, KRT6B, KRT6C, KRT14 and KRT16) in the urospheres compared to the parental cell line, suggesting the urospheres generated from the non-transformed UROtsa cells possess a subpopulation of cells enriched for markers associated with squamous differentiation. Furthermore, it suggests that this minority population of cells are those that undergo transformation with both As³⁺ and Cd²⁺ that ultimately produce tumors with squamous differentiation when transplanted into immune compromise mice. The selective transformation would have had to occur in 13 separate instances since six isolates of As³⁺ and seven isolates of Cd²⁺-transformed cells were independently isolated from the UROtsa non-transformed cell line when they were exposed long-term to As³⁺ or Cd²⁺. As stated above, all 13 transformed cell lines produce tumors with squamous differentiation (Sens et

al., 2004; Somji et al., 2011; Cao et al., 2010). Compared to the transformed cell lines, the UROtsa parent cells produce very few urospheres (unpublished data). The author's at present have no data to suggest or speculate on why this minority cell population is the one susceptible to malignant transformation, but the model does lend itself to such a future examination.

The fate of the urosphere gene signature was determined when the urospheres were placed back into cell culture under conditions used to grow the originating cell line. The data shows the gene signature of the urospheres reverts to the originating As³⁺-transformed cell line. The return is rapid with over 70% of the differentially expressed genes no longer expressed upon reaching initial confluency compared to the originating cell line. Seven more serial passages at a 1:3 subculture ratio show 95% of the genes expressed in the urosphere are no longer differentially expressed by the cells, thus indicating the influence of the culture environment on the gene expression pattern of the urospheres. In addition, epigenetic modifications do not influence the changes in gene expression when the urospheres are placed back into serum-containing growth media. There is a lack of literature to determine if the above results are what one would expect when the urospheres are reintroduced back into the growth media used for the originating As³⁺-transformed cell line. It is not known if this occurs in all cancer cell lines, but there are some practical consequences to the findings. From our findings, it cannot be assumed that the gene signature of the urospheres are maintained when they are placed back into serum-containing growth media and adherent culture flasks. This impacts studies that propose to examine chronic response of urospheres over a long time period. Despite the limitations for the long-term culture of urospheres, the present findings does allow for future studies looking at the relationship between the urospheres and the majority of the cells present in the originating culture.

From this study, we can conclude that the UROtsa cell line transformed with the environmental carcinogen As³⁺ gives rise to carcinomas that have gene signature patterns similar to MIBC. In addition, the As³⁺-transformed cell line contains a population of CICs enriched with genes for squamous differentiation and keratinization. The presence of squamous differentiation in urothelial cancers is significant since these cancers are aggressive, with increase resistance to chemotherapeutic agents and have the potential to metastasize. Thus, exposure of humans to arsenic may result in the development of aggressive urothelial cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

As³⁺	Arsenite
AZC	5-Aza-2'-deoxycytidine
Cd²⁺	Cadmium
CSCs	Cancer stem cells
CICs	Cancer-initiating cells
DEGs	Differentially expressed genes
DMEM	Dulbecco's modified Eagle's medium
FDR	False-discovery rate
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GU	Genomically unstable
KRT	Keratin
MIBC	Muscle invasive bladder cancer
MS-275	N-(2-Aminophenyl)-4-[(pyridine-3ylmethoxycarbonyl)aminomethyl]benzamide
RPKM	Reads Per Kilobase of transcript, per Million mapped reads
SPRR	Small proline-rich protein
SCCL	Squamous-cell-carcinoma like
SCCa	Squamous cell carcinomas
TAC	Transcriptome Analysis Console
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing 0.1% Tween-20
UPGMA	Unweighted Pair Group Method using Arithmetic Averages
UCC	Urothelial cell carcinoma
Uro	Urobasal

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Highlights

- UROtsa cells transformed with arsenite show areas of squamous differentiation.
- Urospheres are enriched in squamous differentiation and keratinization genes.
- There is lack of stability in the urosphere gene expression pattern.
- Epigenetic regulation does not play a role in maintaining urosphere gene expression.

Fig.1.

Hierarchical cluster analysis and classification of differentially expressed genes in urospheres and arsenite-transformed UROtsa parent cells. (A). Hierarchical cluster diagram of urospheres and arsenite-transformed UROtsa parent cells using all differentially expressed transcript clusters. Significantly differentially expressed transcript clusters are defined as those with FDR ≤ 0.05 and fold change ≥ 2.0 . (B). Classification of differentially expressed genes in urospheres versus arsenite-transformed UROtsa parent cells. The total number of transcript clusters is given in parentheses. The cluster class is as indicated with multiple complex defined as a transcript cluster having more than one class type.

Fig.2.

Gene expression analysis of basal markers in urospheres and As³⁺-transformed UROtsa cells. Real-time PCR was performed on urospheres and As³⁺-transformed UROtsa cells cultured in serum and serum-free media. (A) Expression of CDH3. (B) Expression of CD44. (C) Expression of KRT1. (D) Expression of KRT5. (E) Expression of KRT6A. (F) Expression of KRT6B. (G) Expression of KRT6C. (H) Expression of KRT14. (I) Expression of KRT16. (J) Expression of KRT17. (K) Expression of P63. Analysis was done in triplicates and is plotted as the mean $\pm$ SE. The data is plotted on a log base 2 scale per

transcript of β -actin times 1000. * indicates significantly different at $p < 0.05$ from parent As³⁺-transformed UROtsa cells.

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Fig. 3.

Western blot analysis of basal genes in urospheres and As^{3+} -transformed UROtsa cells. (A-J). Western blots for CDH3, CD44, KRT1, KRT5, KRT6, KRT14, KRT16, KRT17, P63 and β -actin respectively. Integrated optical density (IOD) of each band was normalized to that of β -actin. * indicates significantly different at $p < 0.05$ from parent As^{3+} -transformed UROtsa cells.

Fig. 4.

Gene expression analysis of luminal markers in urospheres and As^{3+} -transformed UROtsa cells. Real-time PCR was performed on urospheres and As^{3+} -transformed UROtsa cells cultured in serum and serum-free media. (A) Expression of CD24. (B) Expression of CYP2J2. (C) Expression of ERBB2. (D) Expression of ERBB3. (E) Expression of FABP4. (F) Expression of FGFR3. (G) Expression of FOXA1. (H) Expression of GATA3. (I) Expression of GPX2. (J) Expression of KRT7. (K) Expression of KRT8. (L) Expression of KRT18. (M) Expression of KRT19. (N) Expression of KRT20. (O) Expression of PPARG. (P) Expression of XBP1. Analysis was done in triplicates and is plotted as the mean $\pm$ SE. The data is plotted on a log base 2 scale per transcript of β -actin times 1000. * indicates significantly different at $p < 0.05$ from parent As^{3+} -transformed UROtsa cells.

Fig. 5.

Western blot analysis of luminal genes in urospheres and As³⁺-transformed UROtsa cells. (A-E). Western blots for CD24, KRT8, KRT18, KRT19 and β-actin respectively. Integrated optical density (IOD) of each band was normalized to that of β-actin. * indicates significantly different at $p < 0.05$ from parent As³⁺-transformed UROtsa cells.

Fig. 6. Analysis of differentially expressed genes. The total number of differentially expressed genes between the As^{3+} -transformed UROtsa cells (control) were compared to the urospheres as well as the urospheres after they were cultured in serum-containing media as adherent cultures for 1, 4 and 8 passages.

Table 1.

Differential Expression of genes involved in keratinization in CIC's and tissue samples

Gene	Fold Induced in CIC [^]	Skin (Mean RPKM) *** (n=3)	Esophagus (Mean RPKM) *** (n=3)	Bladder (Mean RPKM) *** (n=2)	Expression in other tissues
KRT10	9473	7171.9±2165.1	-	-	None
SPRR3	4130	-	6973.3±1231.8 *	-	None
DSG1	3863	314.6±124.9 *	12.5±2.4	-	None
SPRR1A	2834	-	-	594.4(1) *	None
SPRR2E	1918	204.4±67.0	706.9±391.2 *	-	None
KRT1	1500	6502.3±1932.4 *	-	-	None
SPRR4	1345	6.2±0.4 *	-	-	1 other, low
KRTDAP	1191	815.2±111.5 *	-	-	None
A2ML1	1105	25.2±12.6	189.9±111.8 *	-	None
SPRR1B	761	47.3±28.2	746.1±185.7 *	-	None
SPRR2D	750	30.8±15.5	644.6±172.6 *	-	None
SPRR2A	620	16.1±8.7	1789.3±509.2 *	-	None
SPINK5	556	127.7±54.9	472.7±149.1 *	-	None
ZNF185	538	20.8±6.9	112.2±25.5 *	3.5±0.8	Low in all others (24)
GJB6	444	8.3±3.0	49.2±10.4 *	3.9±3.0	3 others low
FETUB	440	0.6±0.1	2.5±1.1	-	Liver high *
PGLYRP3 **	307	-	-	-	None
KRT16	268	209.9±40.3 *	198.8±74.6	-	None
KLK10	249	8.9±1.5	28.7±2.7 *	-	3 others low
CYP2C18	245	1.9±0.9	12.2±1.8	-	High in 4 and low in 4
SPRR2B	237	17.2±9.9	318.5±91.0 *	-	None
IVL	202	46.6±15.7	127.7±11.5 *	2.1±0.5	None
SULT2B1	198	46.5±11.7	55.8±3.0 *	-	8 others low
SBSN	178	753.6±198.0 *	339.2±73.6	-	None
CERS3	174	15.5±5.3 *	13.5±0.8	-	Testis low
GRHL3	151	14.0±2.2	31.7±9.3 *	10.2±5.1	2 others low
ZNFP750 **	144	-	-	4.66	(Mouse)
CLIC3	139	10.5±6.3	23.8±7.1 *	0.5±0.1	Ubiquitous but low in others
LYPD3	136	226.0±44.6	319.1±51.0 *	12.3±3.9	3 others very low
ABCA12	117	10.7±3.8 *	0.3±0.1	-	3 others intermediate

Gene	Fold Induced in CIC [^]	Skin (Mean RPKM) *** (n=3)	Esophagus (Mean RPKM) *** (n=3)	Bladder (Mean RPKM) *** (n=2)	Expression in other tissues
KLK12	108	0.2±0.1	4.7±4.0 *	-	3 others very low
ALDH3B2	107	44.3±8.4 *	44.2±14.6	5.7±3.5	3 others low
BNIP1	106	64.5±9.2 *	30.1±4.8	1.1±0.4	6 others low
SCEL	106	141.4±55.0	207.5±45.0 *	-	Lung low
MUC15	101	13.7±5.7	7.1±1.4		High in thyroid * 5 others low

- : No expression

* : Highest Expression

** : No data on NCBI

*** : RPKM, Reads Per Kilobase of transcript, per Million mapped reads

± : Standard error of the mean (SEM)

[^] : Fold induced in the CIC isolated from the As³⁺-transformed cell line compared to the parental transformed cell line.

Table 2.

Differential Expression of basal genes in CIC's and tissue samples

Gene	Fold Induced in CIC [^]	Skin (Mean RPKM) (n=3)	Esophagus (Mean RPKM) (n=3)	Bladder (Mean RPKM) (n=2)	Other Tissues
KRT17	69	53.6±19.1	32.0±5.7	147.9±33.2 *	2 others
KRT14	54	3550.7±780.1 *	156.9±35.4	“	None
KRT6C	20.3	66.0±15.0	948.6±460.3 *	-	None
KRT6B	11.4	58.2±13.9	785.7±354.6 *	-	None
KRT6A	10.37		2985.3±1450.0 *	-	None
KRT5	-	2611.0±550.8 *	1766.7±156.2	38.2±0.1	1 other
CDH3	-	13.2±0.8 *	5.1±0.9	3.0±0.9	24 others low
CD44	“	96.7±23.1 *	37.1±7.3	29.6±0.2	24 others varied
TP63	-	45.0±18.4 *	18.6±2.8	9.6±6.9	4 very low

- : No expression

* Highest Expression

± : Standard error of the mean (SEM)

[^] : Fold induced in the CIC isolated from the As³⁺-transformed cell line compared to the parental transformed cell line.

Table 3.

Effect of epigenetic regulators on the expression of listed genes following urosphere generation and subsequent passages 1 (P1) and 8 (P8).

Gene	Cell line	Urosphere	Control P1	AZC 1µM P1	MS-275 1 µM P1	Contr ol P8	AZC 1 µM P8	MS-275 1 µM P8
DSG1	1.00E+00+/-3.12E-02	2.75E+02 ^a +/-1.60E+01	1.76E+02 ^a +/-3.07E+00	2.43E+02 ^a +/-1.58E+01	2.15E+01 ^a +/-1.54E+00	9.31E-01+/-6.04E-02	1.94E+00+/-1.88E-01	2.88E-01 +/- 4.36E-02
SPRR2E	1.00E+00+/-5.28E-02	3.66E+01 ^b +/-1.53E+00	1.05E+02 ^a +/-4.76E+00	1.67E+02 ^a +/-1.35E+01	1.62E+02 ^a +/-2.28E+01	7.15E-01+/-7.89E-02	1.24E+00+/-8.72E-02	2.39E-01 +/- 1.40E-02
SPRR4	1.00E+00+/-8.21E-02	5.42E+01 ^a +/-3.93E+00	5.89E+01 ^a +/-5.19E+00	1.21E+02 ^a +/-1.42E+01	2.96E+01 ^a +/-3.86E+00	1.42E+00+/-5.02E-02	1.34E+00+/-9.65E-02	1.12E+ 00 +/- 5.43E-02
SPRR2D	1.00E+00+/-5.58E-02	1.56E+01 ^a +/-3.71E-01	5.22E+01 ^a +/-1.63E+00	1.09E+02 ^a +/-6.45E+00	5.85E+01 ^a +/-2.13E+00	7.92E-01+/-6.35E-01	1.40E+00+/-1.39E-01	1.78E-01 +/- 1.11E-02
SBSN	1.00E+00+/-4.77E-02	9.57E+01 ^a +/-3.33E+00	9.66E+01 ^a +/-1.94E+00	1.36E+02 ^a +/-5.21E+00	7.39E+01 ^a +/-3.34E+00	5.17E+00+/-5.10E-01	7.20E+00+/-7.72E-01	2.81E+ 00 +/- 2.93E-01
CEACAM6	1.01E+00+/-1.61E-01	9.75E+00 ^d +/-5.43E-01	1.59E+02 ^a +/-3.96E+00	2.41E+02 ^a +/-5.85E+00	1.89E+02 ^a +/-1.17E+01	1.08E+00+/-1.53E-01	2.10E+00 ^d +/-5.55E-02	2.94E-01 +/- 2.71E-02
GJB2	1.00E+00+/-6.66E-02	8.42E+00 ^a +/-7.46E-01	8.55E+00 ^a +/-6.03E-01	9.23E+00 ^a +/-2.68E-01	6.55E+00 ^a +/-2.53E-01	1.57E+00+/-9.82E-02	1.94E+00 ^d +/-4.10E-01	2.20E+ 00 ^b +/- 6.89E-02
CX3CL1	1.00E+00+/-3.47E-02	1.26E-01 ^a +/-6.71E-03	6.21E-01 ^a +/-3.65E-02	3.92E-01 ^a +/-4.72E-03	5.72E-01 ^a +/-2.73E-02	1.07E+00+/-3.34E-02	1.08E+00+/-5.68E-02	8.93E-01 +/- 2.79E-02
MMP13	1.00E+00+/-4.56E-02	1.14E-02 ^a +/-6.44E-04	8.28E-01 ^a +/-3.17E-02	7.19E-01 ^a +/-3.83E-02	8.28E-01 ^a +/-2.34E-02	4.74E-01 ^a +/-3.05E-02	4.98E-01 ^a +/-3.04E-02	4.97E-01 ^a +/- 2.00E-02
MAF	1.00E+00+/-3.12E-02	5.82E+00 ^a +/-4.86E-01	1.61E+00 ^d +/-5.84E-02	1.74E+00 ^C +/-1.01E-01	1.54E+00 ^d +/-4.84E-02	2.79E+00 ^a +/-1.75E-01	3.25E+00 ^a +/-1.94E-01	1.95E+00 ^b +/-5.94E-02
CYP2C18	1.00E+00+/-1.14E-01	5.67E+01 ^a +/-5.61E+00	5.96E+01 ^a +/-5.45E+00	5.92E+01 ^a +/-3.16E+00	2.92E+01 ^a +/-4.44E+00	1.43E+00+/-1.51E-02	1.34E+00+/-8.49E-02	1.09E+00+/-6.05E-02

Gene expression was measured by RT-qPCR and analyzed for differential expression by the Livak Method [$2^{-(Cq)}$] using the cell line (prior to urosphere formation) as the calibrator sample and β -actin as the reference gene. Each determination was done in triplicate. Shown is the fold change in gene expression compared to the cell line $\pm$ standard deviation. The data was analyzed for statistical significance using a one-way ANOVA with Dunnet's multiple comparisons test. Levels of significance are represented by the following symbols:

^a indicates $p < 0.0001$;

^b indicates $p < 0.001$;

^c indicates $p < 0.01$;

^d indicates $p < 0.05$

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