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Elevated connexin 43 expression in arsenite-and cadmium-transformed human bladder cancer cells, tumor transplants and selected high grade human bladder cancers

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Abstract

Connexin 43 has been shown to play a role in cell migration and invasion; however, its role in bladder cancer is not well defined. Previous studies from our laboratory have shown that the environmental pollutants arsenite and cadmium can cause malignant transformation of the immortalized urothelial cell line UROtsa. These transformed cells can form tumors in immune-compromised mice. The goal of the present study was to determine if connexin 43 is expressed in the normal human bladder, the arsenite and cadmium-transformed UROtsa cells as well as human urothelial cancer. The results obtained showed that connexin 43 is not expressed in the epithelial cells of the human bladder but is expressed in immortalized cultures of human urothelial cells and the expression is variable in the arsenite and cadmium-transformed urothelial cell lines derived from these immortalized cells. Tumor heterotransplants generated from the transformed cells expressed connexin 43 and the expression was localized to areas of squamous differentiation. Immuno-histochemical analysis of human bladder cancers also showed that the expression of connexin 43 was localized to areas of the tumor that showed early features of squamous differentiation. Treatment of UROtsa cells with various concentrations of arsenite or cadmium did not significantly alter the expression level of connexin 43. In conclusion, our results show that the expression of connexin 43 is localized to the areas of the tumor that show squamous differentiation, which may be an indicator of poor prognosis. This suggests that connexin 43 has the potential to be developed as a biomarker for bladder cancer that may have the ability to invade and metastasize.

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Keywords

Connexin 43; UROtsa; Arsenic; Cadmium; Urothelial cancer; Squamous differentiation

1. Introduction

Our laboratory has developed an *in-vitro* model of human urothelial cancer by exposing the UROtsa cell line, a cell line retaining characteristics of human urothelium to cadmium (Cd^{+2}) and arsenite (As^{+3}) (Rossi et al., 2001; Sens et al., 2004). The immortalized UROtsa cell line is a non-tumorigenic line that demonstrates characteristic features of the transitional urothelium of the bladder when cultured on a serum-free growth medium (Rossi et al., 2001). The initial study (Sens et al., 2004) performed by our laboratory was expanded and we subsequently isolated and characterized 5 additional As^{+3} -transformed cell lines and 6 additional Cd^{+2} -transformed cell lines (Cao et al., 2010; Somji et al., 2010; Somji et al., 2011). These malignantly transformed cell lines displayed morphologies that were consistent with human urothelial cancer and had phenotypic differences indicative of tumor heterogeneity. These transformed cell lines formed tumors when they were injected subcutaneously into immune-compromised mice and these tumors had histological features similar to those of the human urothelial carcinomas. There were areas within the tumors that displayed moderate to prominent squamous differentiation (Cao et al., 2010; Somji et al., 2010; Somji et al., 2011). This is an important feature since urothelial cancer is the most prominent type of bladder cancer in western countries and accounts for over 90-95% of all cases and is 5th in overall occurrence (Bischoff and Clark, 2009). The remaining cancers contain aberrant differentiation which can occur in approximately 10-60% of the patients (Lopez-Beltram et al., 2007, Martin et al., 1989). The presence of squamous differentiation in areas of urothelial cancers that occur in the western countries is not that common, however, when they are present within transitional carcinomas, they may indicate poor prognosis for the patient. Focal squamous differentiation has been shown to be an unfavorable prognostic feature for patients undergoing radical cystectomy (Frazier et al., 1993) or radiation therapy (Martin et al., 1989; Akdas and Turkeri, 1990), and it is associated with a poor response to systemic chemotherapy (Logothetis et al., 1989). Recently it has been shown that the presence of squamous differentiation and/or glandular differentiation in urothelial cancers is associated with poorer cancer specific survival and overall survival when compared to individuals with a pure form of urothelial carcinoma (Lee et al., 2014). Thus it seems that squamous differentiation may play a role in determining an individual's clinical outcome and risk stratification as well as selection and response to various therapies (Gellert et al., 2015), however it may not be an independent predictor of worse prognosis in patients with urinary tract cancers.

One of the goals of this laboratory is to employ the UROtsa cell culture and tumor transplant model to identify putative biomarkers associated with the development and/or progression of arsenic - or Cd^{+2} - induced human bladder cancer. To identify candidate biomarker gene signatures, RNA from the parent UROtsa cell line and the As^{+3} - and Cd^{+2} -transformed counterparts were subjected to a microarray analysis using the Affymetrix Human Genome U133 Plus 2.0 gene array (Garrett et al., 2014). The results of this analysis suggested that the

over expression of the connexin 43 (Cx43) gene might be, if expression was validated, a candidate biomarker of As⁺³- induced transformed urothelial cells. Cx43 is one of the 21 member family of human connexin proteins which have been the subject of several recent reviews (Su and Lau, 2014; Defamie et al., 2014; Zhou and Jiang, 2014). An examination of the literature has shown that the study of Cx43 is limited in human bladder and bladder cancer in general, and there are no studies regarding its role in heavy metal-induced human bladder cancer. In one study, Cx43 was found by northern analysis to be expressed in urothelial cells cultured from the human bladder, however its expression was variable in a series of human bladder cancer cell lines, with some lines showing an increase in expression while other had reduced expression (Grossman et al., 1994). A second study using explant cultures from ureters showed that treatment with nitrosamine reduced the Cx43 protein to undetectable levels (Lyng et al., 1996). Recently it was shown that high expression of Cx43 in bladder cancer was associated with poor patient prognosis (Poyet et al., 2015).

The goals of the present study were several-fold. The first was to validate the expression of Cx43 mRNA and protein in parent UROtsa cells and the As⁺³- and Cd⁺²- transformed cells and tumor transplants. The second goal was to confirm the expression of Cx43 protein in formalin-fixed, paraffin-embedded specimens of normal urothelium using immuno-histochemistry. The last goal was to determine if Cx43 is expressed in archival specimens of human urothelial cancer.

2. Materials and methods

2.1. Cell culture

The parental UROtsa cell line was grown in Dulbecco's modified Eagle's medium containing 5% v/v fetal calf serum in a 37°C, 5% CO₂: 95% air atmosphere (Rossi et al., 2001). The isolation of the As⁺³- and Cd⁺²-transformed UROtsa cells has been described previously (Sens et al., 2004; Somji et al., 2011). These transformed cells were also cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 5% v/v fetal calf serum in a 37°C, 5% CO₂: 95% air atmosphere. Confluent flasks were sub-cultured using trypsin-EDTA and the cells were fed fresh growth medium every 3 days. The J82, RT4 and TCCSUP human bladder cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were grown in DMEM supplemented with 5% (v/v) fetal calf serum. The cells were fed every 3 days and were passaged at confluency. For short term exposure to As⁺³ and Cd⁺², the UROtsa parental cells were exposed to 1, 2 or 4 µM sodium arsenite (NaAsO₂) or to 0.5, 1 or 2 µM cadmium chloride (CdCl₂) for 48 h. CdCl₂ was purchased from Sigma-aldrich, Cat No. 202908 (purity 99.99%), whereas NaAsO₂ was purchased from Fluka, Cat No. 71287 (purity 99%). Both the solutions were prepared in water prior to dilution in cell culture medium.

2.2. Expression of Cx43 mRNA and protein in the UROtsa derived cell lines and transplanted tumors

The preparation of total RNA and protein from cultured cells and tissues have been described previously (Sens et al., 2004; Somji et al., 2010). The RNA and protein extracts from the mouse tumor transplants were prepared from pre-existing specimens obtained from

previous studies done by this laboratory (Sens et al., 2004; Somji et al., 2010). This study adhered to all recommendations dictated in the Guide for the Care and Use of Laboratory Animals of the NIH. The specific protocol was approved by the University of North Dakota Animal Care Committee (IACUC #1110-2C). The expression of Cx43 mRNA and aldehyde dehydrogenase 1 (ALDH1) mRNA was determined using real time RT-PCR and Cx43 and ALDH1 specific primers were obtained from Qiagen (Valencia, CA). For PCR analysis, 1 µg of purified RNA was subjected to complementary DNA (cDNA) synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 µL. Real-time PCR was performed utilizing the SYBR Green kit (Bio-Rad Laboratories) with 2 µL of cDNA, 0.2 µM primers in a total volume of 20 µL in an iCycler iQ real-time detection system (Bio-Rad Laboratories). Amplification was monitored by SYBR Green fluorescence. The level of Cx43 mRNA and ALDH1 mRNA was determined relative to that of UROtsa cells using serial dilutions of this sample as the standard curve. The resulting relative levels were then normalized to the fold change in β -actin expression assessed by the same assay using the primers, sense: CGACAACGGCTCCGGCATGT and antisense: TGCCGTGCTCGATGGGGTACT, giving a product size of 194 base pairs.

The expression of Cx43 protein was determined by Western blotting using protocols that have been previously published (Sens et al., 2004; Somji et al., 2010). Twenty micrograms of total cellular protein was separated on a 12.5% SDS-polyacrylamide gel and transferred to a hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Following transfer, the membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% (wt/vol) nonfat dry milk for 1 h at room temperature. After blocking, the membranes were probed with the Cx43 primary antibody (Cell Signaling Technology, Beverly, MA, Cat# 3512) at a dilution of 1:1000 in blocking buffer overnight at 4°C. After washing 3 times with TBS-T, the membranes were incubated with the anti-rabbit secondary antibody (1:2000) in blocking buffer for 1 h. The blots were visualized using the Phototope-HRP (horseradish peroxidase) Western blot detection system (Cell Signaling Technology).

2.3. Immunohistochemical analysis of Cx43 expression

Archival patient specimens of normal human bladder and urothelial cancer possessing no patient identifiers were fixed in 10% neutral buffered formalin for 16-18 hours. Since these samples were 10 years post diagnosis and contained no patient identifiers they are in the exempt category for use and this was approved by the University of North Dakota Internal Review Board. The tissue samples were dehydrated in 100% ethanol following which they were cleared in xylene, infiltrated, and embedded in paraffin. Tissue sections were cut at 3-5 µm for use in immunohistochemical protocols. Antigen retrieval was performed by immersing the sections in preheated citrate buffer pH 6.0 and the samples were heated in a steamer for 20 minutes. The sections were allowed to cool to room temperature and immersed into Tris buffered saline with Tween 20 (Dako, Carpinteria, CA) for 5 minutes. The Cx43 antibody was used at a dilution of 1:100. Liquid diaminobenzidine was used for visualization. The slides were rinsed in distilled water following which they were dehydrated in graded ethanol and cleared in xylene. The protocol for the immunohistochemical analysis

of the keratin 6 protein in archival patient specimens has been described previously (Somji et al., 2008).

2.4. Immunolocalization and functional coupling of Cx43 in As⁺³- and Cd⁺²-transformed and parent UROtsa cells

For immunolocalization, the cells were grown in 24 well plates containing 12 mm glass coverslips at 37° C, 5% CO₂. Cells at confluent density were then fixed and stained using previously described procedures (Cao et al., 2010; Somji et al., 2010). Briefly, cells were fixed in 3.7% buffered, methanol-free formaldehyde (Polysciences, Inc, Warrington, PA) for 15-20 min at room temperature. Coverslips were then quenched of free aldehyde with 0.1 M ammonium Chloride (NH₄Cl) for 15 min, followed by permeabilization with 0.1% Igepal for 10 min at room temperature. Cells were stained for Cx43 by incubation for 45 min at 37° C with a 1:75 dilution of Cx43 antibody in phosphate buffered saline (PBS). The Cx43 primary antibody was detected by incubating cells with 2.0 mg/ml of Alexa-Fluor 488 goat anti-rabbit antibody (Invitrogen, Carlsbad, CA) for 45 minutes at 37° C followed by staining for F-actin using a 1:100 dilution of Phalloidin-Alexa-Fluor 568 (Invitrogen). Coverslips were mounted in ProLong Gold anti-fade reagent with DAPI (Invitrogen) for nuclear counter staining. Controls consisted of coverslips treated with PBS instead of primary antibody. All controls stained appropriately and had virtually no specific staining when photographed under the same settings that were used for experimental cells.

A Gap Junction Intercellular Communication (GJIC) assay was used to determine if the cells were functionally coupled via connexons by qualitatively assessing the spread of Lucifer Yellow dye in a region wounded by a cut with a razor blade (Rhett et al., 2011; Zemljic et al., 2014; Li et al., 2014). Briefly, UROtsa cells were grown to confluence on 12mm glass coverslips and rinsed twice with 37° C DMEM-F12 (phenol-red free) medium. Coverslips were then rinsed 3 times with ice cold PBS (without Ca⁺² or Mg⁺² salts). PBS containing 1.33 mM Lucifer Yellow (Invitrogen) and 0.25 mg/ml 10 kD Dextran Alexa Fluor 594 (Invitrogen) was then placed on the coverslips and the monolayer was then cut 2-3 times using a razor blade followed by ice cold incubation for 3 minutes. Coverslips were then rinsed 3 times with ice cold PBS and incubated in DMEM-F12 (phenol-red free) medium for 5 min on ice. Coverslips were rinsed three times with ice cold PBS and then fixed with 3.7% formaldehyde as described above. Free aldehyde groups were quenched with 0.1 M NH₄Cl in PBS for 15 min and cells were mounted in ProLong Diamond anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Cell lines were tested in quadruplicate. Stained cells were observed using a Leica TCS SPE, DM5500 laser scanning confocal microscope with LAS AF software (Leica Microsystems, Buffalo Grove, IL). Images were obtained by capturing z-slices at the indicated optimal depth per objective lens.

2.5. Preparation of Cancer-Initiating-Cells from As⁺³-and Cd⁺²-transformed UROtsa cells

Cancer-Initiating-Cells (CICs) were isolated from one of the As⁺³- transformed UROtsa cell lines (As #1) and one of the Cd⁺²- transformed UROtsa cell lines (Cd #1). The cells were seeded at a density of 1×10⁵ cells in T-25 cm² Ultra-low attachment flasks (Corning Inc., Corning NY). The growth medium consisted of 1:1 mixture of Dulbecco's modified Eagles' medium 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 cells were allowed to form spheres for 8 days following which the CICs were harvested by centrifugation for RNA isolation and injection into immune compromised mice. The protocol for the transplantation of the transformed UROtsa cell lines into immune compromised mice has been described previously (Sens et al., 2004). A similar protocol was used for the transplantation of spheroids. Briefly, 10³ spheroids were injected into five nude (NCR-nu/nu) mice subcutaneously in the dorsal thoracic midline. All mice were sacrificed by 10 weeks post-injection or when clinical conditions dictated euthanasia. The maximum tumor size was 2.5 cm.

2.6. Statistics

Statistical analysis was performed using ANOVA with Tukey *post-hoc* testing performed by Graphpad PRISM 4.

3. Results

3.1. Validation of elevated Cx43 mRNA expression in As⁺³- and Cd⁺²-transformed UROtsa cells

The initial goal of the present study was to validate the microarray study that suggested Cx43 might be elevated in As⁺³- and Cd⁺²-transformed UROtsa cells. For this determination, total RNA was isolated from confluent cultures of parental UROtsa cells, the 6 independent isolates of As⁺³-transformed UROtsa cell lines, and the 7 independent isolates of the Cd⁺²-transformed UROtsa cell lines and analyzed for Cx43 expression using real-time PCR. An analysis for the expression of the Cx43 protein by Western blotting was also performed on protein isolated from the parental UROtsa cells and the independent isolates of the As⁺³- and Cd⁺²-transformed UROtsa cells. For the non-transformed parental UROtsa cells, this analysis showed that the parent UROtsa cells expressed both Cx43 mRNA and protein (Fig. 1). The expression of Cx43 mRNA was significantly increased in all of the As⁺³-transformed UROtsa cell lines when compared to that of the parental UROtsa cells (Fig. 1A), however the fold increase varied between the isolates with As#2 showing the smallest induction with a 2-fold increase in expression. The corresponding analysis of the Cx43 protein followed that of Cx43 mRNA, with Cx43 protein being significantly increased in 5 of the 6 independent isolates of the As⁺³-transformed cells (Fig. 1B and C). The As⁺³-transformed cell line (As #6) that showed only an approximate 2-fold increase in Cx43 mRNA did express Cx43 protein, but the level was not significantly increased over that noted in parental UROtsa cells (Fig. 1B and C). An identical analysis of Cx43 expression in the Cd⁺²-transformed UROtsa cells showed that only 2 of the 7 Cd⁺²-transformed cell lines had significant elevated expression of Cx43, and only one of these isolates (Cd #1) exceed a 2-fold elevation (Fig. 1D). The corresponding analysis of the Cx43 protein followed that of Cx43 mRNA with Cx43 protein being significantly increased in only 3 of the 7 independent isolates of the Cd⁺²-transformed cells (Fig. 1E and F).

3.2. Immuno-fluorescence localization of Cx43 expression in parental UROtsa Cells and the As⁺³- and Cd⁺²-transformed cell lines

The intracellular localization of Cx43 was determined using laser scanning confocal microscopy for the parental UROtsa cells and one isolate each of the As⁺³- and Cd⁺²-transformed cell lines that showed elevated expression of Cx43 (Fig. 2). Cells were stained for Cx43 followed by F-actin staining and nuclear counterstaining to show the intracellular localization of the connexin. Results showed that Cx43 was mostly located to the plasma membrane in intensely stained gap junctional plaques with lower levels of Cx43 visible as small, weak intensity stained punctate structures located intracellularly, consistent with endoplasmic reticulum and Golgi staining. There was a clear and substantial increase in the expression levels of Cx43 in the transformed cell lines As#3 and Cd#1 compared to the parental UROtsa cells (Fig. 2A-F). Although the localization pattern of both small and larger stained connexons at the plasma membrane appeared to be the same between all three cell lines, the number of gap junctional complexes at the plasma membrane was greatly increased in the transformed cell lines compared to the parent cell line. These results are consistent with results from Western blot analysis showing high levels of Cx43 protein in the As#3 and Cd#1 transformed cell lines in comparison to the parental UROtsa cell line. GJIC was determined by Lucifer Yellow movement beyond the wounded cells stained by 10 kD Dextran Red and showed that the gap junctional channels were functional in the parental UROtsa cell line as well as the As⁺³- and Cd⁺²-transformed cell lines (Fig. 2 G-I). Although this method is generally qualitative, the parental cells did appear to have less Lucifer Yellow transfer compared to their As⁺³- and Cd⁺²-transformed counterparts. Overall, the results showed that there were functional gap junctional complexes in all of the cell lines.

3.3. Expression of Cx43 mRNA and protein in tumor transplants generated from As⁺³- and Cd⁺²-transformed UROtsa cells

Total mRNA and protein were analyzed for the expression of Cx43 from subcutaneous tumor transplants derived from As⁺³- and Cd⁺²-transformed UROtsa cells inoculated into immune compromised mice. The expression of Cx43 mRNA was significantly elevated in all 6 tumor transplants derived from the As⁺³-transformed cell lines when compared to the level present in the parental UROtsa cells or when compared to the corresponding As⁺³-transformed cell line grown under cell culture conditions (Fig. 3A). While the transplants from all 6 tumors were elevated for Cx43 mRNA, there was no evidence of a direct relationship between the level of Cx43 mRNA in the cell line under culture conditions and the level of Cx43mRNA in the corresponding tumor transplant (Fig. 3A vs 1A). The corresponding analysis of the Cx43 protein showed that 5 of the 6 As⁺³-transformed tumor transplants had significantly elevated levels of Cx43 protein, with one isolate (As #2) showing a decrease in expression (Fig. 3B and C). Similar to that found for the Cx43 mRNA expression in the tumor transplants, there was no evidence for a direct relationship between Cx43 mRNA and protein expression with a given isolate's tumor or with the expression levels of the originating cell line (Fig. 1A, B and C vs 3A, B and C).

The expression of Cx43 mRNA and protein was significantly elevated in the tumor transplants originating from all 7 independent Cd⁺²-transformed cell lines (Fig. 3D, E and F). This is in contrast to the originating Cd⁺²-transformed cell lines where only two isolates

demonstrated an increase in the expression of Cx43 mRNA and protein (Fig. 1D, E and F). There was also no evidence of a relationship between Cx43 mRNA expression and that of the Cx43 protein for any of the tumor transplants derived from the Cd⁺²-transformed cells (Fig. 3D, E and F).

3.4. Immunohistochemical localization of Cx43 protein in tumor transplants generated from As⁺³- and Cd⁺²-transformed UROtsa cells

The localization of Cx43 was determined on the tumor transplants generated from the As⁺³- and Cd⁺²-transformed cell lines using immuno-histochemistry on formalin-fixed, paraffin embedded tumor specimens. All tumor transplants generated from the 6 As⁺³- and 7 Cd⁺²-transformed cell lines stained positive for Cx43 and yielded similar patterns of Cx43 staining. The staining of Cx43 in tumors derived from 3 As⁺³- (As #1, 4 and 5) and 3 Cd⁺²-derived cell lines (Cd #1, 4 and 7) are shown for reference (Fig. 4 A-F). The staining of Cx43 in each of the respective transplants was focal with the strongest staining localized to the well-differentiated cells in the center of the tumor nests. Very weak or no Cx43 staining was present in the less differentiated cells located at the periphery of the tumors. The only deviation from this pattern was one tumor transplant (As #4, Fig. 4C), where the less differentiated cells were also Cx43 positive. The well-differentiated areas that stained for Cx43 are areas of the tumors having characteristics consistent with squamous differentiation. For all the tumors, the most intense Cx43 staining was localized to the cell membrane of the cells with no evidence of nuclear-associated staining or appreciable cytoplasmic staining.

3.5. Effect of short-term As⁺³ and Cd⁺² exposures on Cx43 expression in parental UROtsa cells

Since the validation protocol showed that UROtsa cells express low levels of Cx43, the effect of a short-term exposure to As⁺³ and Cd⁺² on the expression level of Cx43 was determined. For this purpose, confluent cultures of parental UROtsa cells were exposed to 1.0, 2.0 or 4.0 μ M As⁺³ for 48 h or to 0.5, 1.0 and 2.0 μ M Cd⁺² for 48 h at which time total RNA and protein were prepared from the cells. The concentrations and exposure times were chosen from a previous study and only caused a loss of cell viability at the highest concentration after 48 h of exposure (Soh et al., 2012). The results of this analysis showed that exposure of the parental UROtsa cells to 1.0, 2.0 or 4.0 μ M As⁺³ for 48 h elicited a step-wise reduction in the expression of Cx43 mRNA (Fig. 5A). The Cx43 protein was reduced compared to control following exposure for 48 h to either 1.0, 2.0 or 4.0 μ M As⁺³ (Fig. 5B and C). UROtsa cells were also exposed to 1.0 μ M As⁺³ for 12, 24 and 48 h. This dose was chosen since this was the lowest dose (Fig. 5A) that caused a decrease in expression of Cx43 mRNA. The results of this analysis showed that the level of Cx43 mRNA decreased at 24 and 48 h of exposure (Fig. 5D). The corresponding analysis of the Cx43 protein showed an initial rise in Cx43 expression at 12 and 24 h of exposure, followed by a return to control levels at 48 h of exposure (Fig. 5E and F). Exposure of the UROtsa parental cells to 0.5, 1.0 or 2.0 μ M Cd⁺² for 48 h resulted in a decrease in Cx43 mRNA levels at the 2.0 μ M Cd⁺² concentration (Fig. 6A). The expression of the Cx43 protein was not altered for the parental UROtsa cells exposed to 0.5, 1.0 and 2.0 μ M Cd⁺² for 48 h (Fig. 6B and C). Exposure of the parental UROtsa cells to 2.0 Cd⁺² for 12, 24 or 48 h did not change the expression level of Cx43 at 2 or 24 h, however by 48 h the level of mRNA decreased significantly (Fig. 6D).

The level of the Cx43 protein showed an initial increase in levels at 12 and 24 h following which the levels returned to control at 48 h (Fig. 6E and F).

3.6. Expression of Cx43 mRNA in cancer-initiating-cells Isolated from an As⁺³-and Cd⁺²-transformed cell lines

CICs were prepared from one As⁺³-transformed cell line (As #1) and one Cd⁺²-transformed cell line (Cd #1). Total RNA was isolated from the CICs for the determination of ALDH1 and Cx43 mRNA levels. The CICs from both the As⁺³- and Cd⁺²-transformed cell lines showed an elevated expression of aldehyde dehydrogenase 1 (ALDH1), a stem cell marker (Fig. 7A) and Cx43 (Fig. 7B) when compared to parental UROtsa cells and the cell used for the isolation of the CICs.

3.7. Expression of Cx43 in bladder cancer cell lines and human bladder cancers

The expression of Cx43 was also determined in three commonly utilized human bladder cancer cell lines. The results showed that each of these commonly utilized bladder cancer cell lines expressed both Cx43 mRNA and protein (Fig. 8A and B). For reference, the levels of expression are shown opposite that of the parental UROtsa cells and one As⁺³-transformed cell line (As #3). The expression of Cx43 protein was determined on 3 archival formalin fixed, paraffin-embedded samples of normal human urothelium. The results showed staining for the Cx43 protein in the smooth muscle cells of the underlying muscular layer of the bladder, but there was no evidence of staining for the Cx43 protein in the urothelial cells of the urothelium. All the three specimens yielded identical results with a representative example shown by illustration (Fig. 9A). The expression of Cx43 was also determined in 7 randomly chosen specimens of non-invasive high grade urothelial cancer and invasive high-grade urothelial cancer. The results showed that there was no staining for the Cx43 protein in any of the specimens of non-invasive high grade urothelial cancer (Fig. 9B for a representative staining profile). Cx43 expression was negative for 6 of the 7 samples of invasive, high grade urothelial cancers, with one specimen showing a few scattered cells that stained positive for the Cx43 protein, but such cell profiles were very rare (Fig. 9C for a representative positive profile and Fig. 9D for a negative profile). The expression of Cx43 was also examined in three non-invasive and three invasive high grade urothelial cancers that exhibited evidence of squamous differentiation in a study that determined keratin 6a immunoreactivity in urothelial cancer (Cao et al., 2010; Somji et al., 2010; Somji et al., 2008). This analysis showed that both non-invasive and invasive urothelial cancers that were keratin 6a positive and showed evidence of early squamous differentiation (Fig. 9E and G), stained positive for the expression of Cx43 protein (Fig. 9F and H for illustration).

4. Discussion

One of the goals of the present study was to determine if Cx43 is expressed in the normal urothelial cells of the human bladder. The results of the present study demonstrated using immuno-histochemical staining that Cx43 was below the level of detection in the urothelial cells of the human bladder, but was expressed in the underlying smooth muscle cells. The finding that Cx43 is expressed in the underlying smooth muscle cells is in agreement with

previous reports in the literature for both human and animal models (Neuhaus et al., 2002; Haefliger et al., 2002; Neuhaus et al., 2002). There does not appear to be any other studies in the literature regarding Cx43 expression in the *in situ* bladder urothelium. Additional studies confirming the absence of expression of Cx43 in the human bladder urothelium by analyzing Cx43 mRNA expression by RT-PCR and/or protein expression by western blotting is compromised by the difficulty in separating the urothelium from stroma with no cross contamination. This laboratory's attempts at such an analysis were inconclusive due to inconsistent expression patterns between triplicates of independent tissue preparations. Thus, the present results suggest that Cx43 is not expressed in the normal bladder urothelium under conditions where Cx43 expression is found in bladder smooth muscle cells.

The finding that the parental UROtsa cells express a low level of Cx43 mRNA and protein suggests that these cultures do not recapitulate the absence of Cx43 expression found *in situ* for normal human bladder urothelium. In addition, the studies documenting a low level of expression of Cx43 in the parental UROtsa cells were very convincing. The expression of Cx43 in the parental UROtsa cells was confirmed by real-time PCR, western blotting and immunofluorescent localization which confirmed that Cx43 was present in gap junctional complexes at the plasma membrane. The gap junctions present on the UROtsa cells were also determined to be capable of intercellular communication as cells were observed to transfer Lucifer Yellow but not 10 kD Dextran in a GJIC wound based assay. Taken together, these observations suggest that parental UROtsa cell line acquires a low expression of Cx43 when compared to *in situ* human urothelium. There were two other reports in the literature that suggest Cx43 is expressed in cultures of human urothelium. The first of these was a report showing that cultures derived from human bladder urothelium expressed elevated levels of Cx43 compared to several commonly studied human bladder cancer cell lines (Grossman et al., 1994). However, the primary cultures of bladder urothelium were grown using minimal essential medium which the authors' note causes terminal differentiation of the cells into stratified keratinocytes rather than transitional urothelium. Another study using explant cultures from ureters showed that treatment with nitrosamine reduced Cx43 protein to undetectable levels; however, the purity of the explant cultures being free of smooth muscle cells was not addressed by the authors (Lyng et al., 1996). The expression of Cx43 was also determined in UROtsa parent cells that were exposed to various concentrations of As^{+3} and Cd^{+2} . The results demonstrated that short term exposure to As^{+3} or Cd^{+2} does not increase the expression of Cx43 indicating that the actual transformation event may be required to increase the expression of Cx43. One explanation for the low level of Cx43 expression found in the parental UROtsa cells is that the UROtsa cell line is immortal and Cx43 expression may reflect an early alteration in cell growth regulation associated with progression to a fully transformed and tumorigenic cell line.

Another goal of the present study was to validate the results of a microarray study that suggested Cx43 might be induced in As^{+3} - and Cd^{+2} -transformed UROtsa cells. The results of this study demonstrated that Cx43 expression was increased in 5 of the 6 As^{+3} -transformed cell lines based on both mRNA and protein expression. For the Cd^{+2} -transformed cell lines, Cx43 was increased in 2 of the 7 transformed cell lines at the level of mRNA expression and in 3 of the 7 cell lines based on Cx43 protein expression. The finding that the level of Cx43 mRNA expression and Cx43 protein expression did not always

correlate with one another in the individual cell lines, or when comparing expression between and among cell lines, is likely due to the fact that Cx43 protein is known to have a short half-life with multiple post-transcriptional mechanisms controlling protein degradation (Su and Lau, 2014). Generally, protein levels tend to correlate to the corresponding mRNA levels in most studies, however sometimes the global expression of mRNA levels compared to the corresponding protein levels tend to vary considerably as reviewed by Liu et al., 2016. This is due to the multitude of regulation steps in translation that contribute to the level of a particular protein. There is also a considerable amount of heterogeneity in global mRNA expression among the UROtsa transformants with each transformant differing in at least 10% global gene expression. This results in over a thousand genes being differentially expressed from one transformant to the other, as detailed in our recent publication (Garrett et al., 2014). This difference is to be expected among tumor cells and is reflective of the genomic instability commonly seen in tumor cells. With such high proportion of the transcriptome exhibiting such a difference, it is likely that several genes and their resultant proteins involved in the synthesis and turnover of a particular protein would not exhibit consistent levels throughout the UROtsa transformant series, leading to deviations in the ratios of protein to mRNA for each protein. While it is difficult to speculate on the molecular details of why a particular transformant lacks or has excessive levels of a particular protein opposite the corresponding mRNA, occasional differences in correlation among a large series of transformants may occur. The finding that was unexpected was that the tumor transplants derived from all 6 of the As⁺³-transformed cell lines and all 7 of the Cd⁺²-transformed cell lines had highly elevated expression levels of Cx43 mRNA and protein. An immuno-histochemical examination of Cx43 expression demonstrated that Cx43 expression was localized to the urothelial cancer cells showing evidence of squamous differentiation. This finding shows that elevated Cx43 expression is associated with a histological element of urothelial cancers that has been associated in patients having a poor prognosis (Frazier et al., 1993; Martin et al., 1989; Akdas and Turkeri, 1990; Logothetis et al., 1989). Overall, the finding of elevated Cx43 expression in the As⁺³- and Cd⁺²-derived tumor transplants did validate the microarray findings. It was also shown that three commonly utilized human urothelial cancer cell lines expressed Cx43.

The expression of Cx43 was also examined in CIC's produced by the As⁺³- and Cd⁺²-transformed cells in an effort to explain why tumor from the transplanted cells in immune compromised mice gained a high level of expression of Cx43; especially from cell cultures showing only modest or no increase in Cx43 expression. It is generally accepted that CIC's which form spheroids and are isolated from transformed cell cultures under conditions not permissible for cell attachment represent the very small percentage of cells that have the properties of tumor initiation. That these spheroids from the As⁺³- and Cd⁺²- transformed cell lines are capable of tumor initiation and being classified as CICs was confirmed in a recent publication from this laboratory (Slusser et al., 2015). In this study it was shown that spheroids isolated from the As⁺³- and Cd⁺²- transformed cell lines were capable of establishing tumors in immune compromised mice. In addition, it was also shown that these CIC-initiated tumors recapitulated the tumor histology produced by transplantation of the original cell lines into immune comprised mice. These CICs in the present study were shown to have a high level of expression of Cx43 mRNA and ALDH1 (a stem cell marker),

providing strong evidence that the small sub-population of tumor-initiating cells present in the original As⁺³- and Cd⁺²-transformed cell cultures had a high level of Cx43 expression compared to the overall cell population comprising the entire culture. The finding that the Cx43 expressing cell spheroids (CICs) were those responsible for initiating tumor formation in immune compromised mice provides an explanation for the elevated expression of Cx43 in the tumors produced from the transformed cell lines.

The final goal of the study was to determine if there was preliminary evidence to suggest that the findings of Cx43 expression in the As⁺³-and Cd⁺²-transformed cell lines could translate to human urothelial cancer. There was no expression of Cx43 in seven randomly selected archival cases of non-invasive high grade urothelial cancer and in only one of seven cases of invasive high grade urothelial cancer. In this one case, there were only a few rare profiles of individual cells that were positive for Cx43. This finding suggests that Cx43 expression in human urothelial cancer is a rare event. However, a past examination of keratin 6 expression in tumors derived from the As⁺³-and Cd⁺²-transformed UROtsa cell lines showed that keratin 6 expression was localized to areas of squamous differentiation (Cao et al., 2010; Somji et al., 2011; Somji et al., 2008). When this study was expanded to an examination of archival specimens of human urothelial cancer, it was shown that keratin 6 expression was a sensitive marker to identify the few cases in the archive of human urothelial cancer that exhibited early squamous differentiation. Six of the specimens that stained positive for keratin 6 in this past study were examined in the present study for the expression of Cx43 and all showed positive focal staining for Cx43 protein. These findings suggest that Cx43 might also be a marker to determine early squamous differentiation in human urothelial cancer.

A recent review details the evidence that the expression of Cx43 can play a pivotal role in cell migration and invasion (Defamie et al., 2014). While none of these studies were specific to human urothelial cancer, they did show in other cellular systems that the effect of Cx43 seems to rely on the consequences of its functions that are carried out simultaneously or at different times during the acquisition of the invasive process and its maintenance. One aspect is that this accumulation of functions may be favored by the possible intracellular action of Cx43 with various cytosolic proteins involved in the regulation of migration and secretion of proteases. A second aspect, and one interesting for urothelial cancer, is the capacity of Cx43 to mediate transmembrane communication with the microenvironment or heterocellular gap junctional intercellular communication with stromal cells. Of acute interest is the possibility that a urothelial cancer cell that elaborates Cx43 is able to establish gap junctional communication with a stromal smooth muscle cell through the formation of Cx43 between the two cell types. The gap junctional intercellular communication by a urothelial cancer cell with that of a smooth muscle cell of the stroma could promote invasion and metastasis of the urothelial cancer cell. This might provide a mechanism to explain the observation that squamous differentiation is associated with a poor patient prognosis in patients with urothelial cancer.

Conclusions

Our study shows that Cx43 is not expressed in the epithelial cells of the human bladder but is expressed in immortalized cultures of human urothelial cells and the expression is variable in As⁺³- and Cd⁺²- transformed urothelial cell lines derived from these immortalized cells. However, all the tumor heterotransplants generated from the transformed cells expressed Cx43 and the expression was localized to areas of squamous differentiation. Immunohistochemical analysis of human bladder cancers also showed that the expression of Cx43 was localized to areas of the tumor that showed early features of squamous differentiation which is an indicator of poor prognosis.

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Fig. 1. Expression of Connexin 43 mRNA and protein in As³⁺- and Cd²⁺-transformed UROtsa cell lines

(A and D). Real-time PCR analysis of connexin 43 expression in As³⁺- and Cd²⁺-transformed cell lines respectively. Gene expression is denoted by the relative fold change in expression compared to the UROtsa parent, normalized to the change in β-actin. (B and C). Western blot analysis of connexin 43 protein in As³⁺-transformed UROtsa cell lines. (E and F). Western blot analysis of connexin 43 protein in Cd²⁺-transformed UROtsa cell lines. The integrated optical density (IOD) of each band was normalized to the IOD of β-actin. The values obtained for each of the isolate was normalized to the UROtsa parent (control). **

denotes significant difference from UROtsa parent cells ($p < 0.05$). *** denotes significant difference from UROtsa parent cells ($p < 0.001$).

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Fig.2. Immunofluorescent analysis of Cx43 localization in UROtsa cell lines

In panels A-F, cells were stained with Cx43 (green) and F-actin was detected with phalloidin (red) while nuclei were stained with DAPI (blue). Panels A-C show images from a single 0.35 μm capture where Cx43 localization relative to F-actin and nuclei can be discerned. Panels D-F show total Cx43 staining in a wider view of that same field. In panels G-I, cells were stained with Lucifer Yellow (green) and 10 kD Dextran (red) using a GJIC assay. Representative images are shown for the UROtsa parent cell line (A, D, G), UROtsa As#3 cell line (B, E, H), and UROtsa Cd#1 cell line (C, F, I). The white bar shown in C = 10 μm represents the magnification for the top image panel; magnification bar in F = 20 μm for the middle image panel; and likewise the bar in I = 50 μm represents the magnification for the bottom row.

Fig. 3. Expression of connexin 33 mRNA and protein in subcutaneous tumor heterotransplants (A and D). Real-time PCR analysis of connexin 33 expression in As⁺ and Cd⁺- tumor heterotransplants respectively. Gene expression is denoted by the relative fold change in expression compared to the UROtsa parent, normalized to the change in β -actin. (B and C). Western blot analysis of connexin 33 protein in As⁺-tumor transplants. (E and F). Western blot analysis of connexin 33 protein in Cd⁺-tumor heterotransplants. The IOD of each band was normalized to the IOD of β -actin. The values obtained for each of the isolate was normalized to the UROtsa parent (control). ** denotes significant difference from UROtsa

parent cells ($p < 0.05$). *** denotes significant difference from UROtsa parent cells ($p < 0.001$).

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Fig. 4. Immunohistochemical analysis of Cx43 in As³⁺ and Cd²⁺ tumor heterotransplants
Immunohistochemical localization of Cx43 in As#1 (A), As#4 (C) and As#5 (E) tumor heterotransplants. Figures 4 (B), (D) and (F) show the staining and localization of Cx43 in Cd#1, Cd#4 and Cd#7 transplants. All micrographs are at $\times 100$ magnification. The red-brown color indicates Cx43 protein expression. Staining is seen predominantly in boundaries between cells and in focal regions within each field.

Fig. 5. Expression of Cx43 in UROtsa parent cells after exposure to As^{+3}

(A-C) UROtsa parent cells were exposed to 1, 2 or 4 μM As^{+3} for 48 h and the expression of Cx43 was determined. (A). Real-time PCR analysis of Cx43 expression. Gene expression is denoted by the relative fold change in expression compared to the UROtsa parent, normalized to the change in β -actin. (B and C). Western blot analysis of Cx43 protein. The IOD of each band was normalized to the IOD of β -actin. The values obtained for each of the isolate was normalized to the UROtsa parent (control). (D-F). UROtsa parent cells were exposed to 1 μM As^{+3} for 12, 24 and 48 h and the expression of Cx43 was determined. (D). Real time PCR analysis of Cx43 expression. (E and F). Western blot analysis of Cx43 expression. ** denotes significant difference from UROtsa parent cells ($p < 0.05$). *** denotes significant difference from UROtsa parent cells ($p < 0.001$).

Fig. 6. Expression of Cx43 in UROtsa parent cells after exposure to Cd²⁺

(AC) UROtsa parent cells were exposed to 0.5, 1, or 2 μM Cd²⁺ for 48 h and the expression of Cx43 was determined. (A). Real-time PCR analysis of Cx43 expression. Gene expression is denoted by the relative fold change in expression compared to the UROtsa parent, normalized to the change in β -actin. (B and C). Western blot analysis of Cx43 protein. The IOD of each band was normalized to the IOD of β -actin. The values obtained for each of the isolate was normalized to the UROtsa parent (control). (D-F). UROtsa parent cells were exposed to 2 μM Cd²⁺ for 12, 24 and 48 h and the expression of Cx43 was determined. (D). Real-time PCR analysis of Cx43 expression. (E and F). Western blot analysis of Cx43 protein. ** denotes significant difference from UROtsa parent control cells ($p < 0.05$). *** denotes significant difference from the UROtsa parent control cells ($p < 0.001$).

Fig.7. Expression of ALDH1 and Cx43 in putative CIC's isolated from the As⁺³ and Cd⁺²-transformed UROtsa cells

(A and B). Real -time PCR analysis of ALDH1 and Cx43 expression in UROtsa parent cells, As#1 cells , As#1 CIC, Cd#1 cells and Cd #1CIC's respectively. Gene expression is denoted by the relative fold change in expression compared to the UROtsa parent, normalized to the change in β -actin. ** denotes significant difference from UROtsa parent cells ($p<0.05$). *** denotes significant difference from the As⁺³- and Cd⁺²-transformed UROtsa cells ($p<0.001$).

Fig. 8. Expression of Cx43 in human bladder cancer cell lines

(A). Real-time PCR analysis of Cx43 in human bladder cancer cell lines. Gene expression is denoted by the relative fold change in expression compared to the UROtsa parent, normalized to the change in β -actin. *** denotes significant difference from UROtsa parent cells ($p < 0.001$). (B). Western blot analysis of Cx43 protein in human bladder cancer cell lines.

Fig. 9. Immuno-histochemical analysis of Cx43 expression in normal human urothelium and urothelial cancers

(A). Normal Urothelium. (B). Non-invasive high grade urothelial cancer. (C and D). Invasive high grade urothelial cancer. (E and G). Staining for keratin 6 in non-invasive and invasive high grade urothelial cancer with squamous differentiation respectively. (F and H). Staining for Cx43 in non-invasive and invasive high grade urothelial cancer with squamous differentiation respectively. All micrographs are at $\times 100$ magnification.