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Research Article

Specificity of the Metallothionein-1 Response by Cadmium-Exposed Human Urothelial Cells

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Abstract: Occupational and environmental exposure to cadmium is associated with the development of urothelial cancer. The metallothionein (MT) family of genes encodes proteins that sequester metal ions and modulate physiological processes, including zinc homeostasis. Little is known about the selectivity of expression of the different MT isoforms. Here, we examined the effect of cadmium exposure on MT gene and isoform expression by normal human urothelial (NHU) cell cultures. Baseline and cadmium-induced MT gene expression was characterized by next generation sequencing and RT-PCR; protein expression was assessed by western blotting using isoform-specific antibodies. Expression of the zinc transporter-1 (*SLC30A1*) gene was also assessed. NHU cells displayed transcription of *MT-2A*, but neither *MT-3* nor *MT-4* genes. Most striking was a highly inducer-specific expression of MT-1 genes, with cadmium inducing transcription of *MT-1A*, *MT-1G*, *MT-1H* and *MT-1M*. Whereas *MT-1G* was also induced by zinc and nickel ions and *MT-1H* by iron, both *MT-1*A and *MT-1M* were highly cadmium-specific, which was confirmed for protein using isoform-specific antibodies. Protein but not transcript endured post exposure, probably reflecting sequestration. *SLC30A1* transcription was also affected by cadmium ion exposure, potentially reflecting perturbation of intracellular zinc homeostasis. We conclude that human urothelium displays a highly inductive profile of MT-1 gene expression, with two isoforms identified as highly specific to cadmium providing candidate transcript and long-lived protein biomarkers of cadmium exposure.

Keywords: Metallothionein, urothelium, urothelial cancer, cadmium exposure, zinc transporter

1. Introduction

Occupational and environmental exposure to cadmium has increased as a result of the burning of fossil fuels and widespread use of the heavy metal in anthropological activities, such as battery production, electroplating, smelting and soldering (reviewed [1]). Cadmium ions accumulate in the body in an almost irreversible manner [2], as the metal cannot be metabolized to a less toxic species [3] and has a low excretion rate [4]. This is thought to be due to intracellular sequestration of cadmium ions by metal-binding proteins [5–7]. An association between cadmium exposure and bladder (urothelial) carcinogenesis has been reported, with higher cadmium concentrations demonstrated in the blood [8] and urine [9–11] of patients with bladder cancer. *In vitro* research supports these correlative studies, with malignant transformation of the immortalized RWPE-1, TLR1215, 16HBE and UROtsa cell lines reported after extended chronic cadmium exposure [12–15]. The bladder stores

concentrated urine prior to voiding, meaning that the urothelial lining of the bladder, which functions as one of the tightest epithelial barriers [16], is potentially exposed to excreted xenobiotics [16–18]. It is not known whether exposure to urinary cadmium is limited by the presence of an intact urinary barrier.

The metallothioneins (MTs) are a superfamily of low-molecular weight (~6 kDa), cysteine-rich proteins that are induced by and bind a range of metal ions, including cadmium [19,20]. Through this sequestration of metal ions MTs are considered to play a primary role in metal detoxification [21,22], but also metal (e.g. zinc) homeostasis [23–25] and the scavenging of reactive oxygen species (ROS) [26,27]. MT involvement in the bodily response to cadmium exposure has been well-documented, with numerous studies demonstrating cadmium-induced MT expression both in vitro [28–34] and in vivo [35,36].

Direct binding of MT protein to cadmium ions [37,38] results in a MT-cadmium complex that is highly resistant to degradation [5–7]. In man, four main MT subfamilies exist (MT-1, MT-2/2A, MT-3 and MT-4), with MT-1 consisting of nine isoforms (-A, -B, -E, -F, -G, -H, -L, -M, -X) [39] each encoded on individual genes. It is predicted that the individual MT isoforms have distinct properties including structure [40-41], tissue- and inducer-specific expression [22,34,42–47], induction rate [48], translational efficiency [41] and degradation rate [49,50]. Cadmium is reported to be the most potent inducer of MT expression [51]. This offers the potential that individual MT isoforms may be utilized as specific biomarkers of human exposure to cadmium, although it remains unclear which isoform(s) are responsible for cadmium sequestration. This lack of discrimination is largely due to the high homology between isoforms and the lack of discriminatory reagents, with no validated antibodies able to distinguish MT-1 and MT-2 subfamilies, nor the different MT-1 isoforms [41,52].

MTs are reported to work cooperatively with zinc transporters to regulate cellular zinc homeostasis, potentially by modulating cellular zinc ion concentration [39], although the exact mechanisms are unknown. Thus, a possible consequence of exposure to cadmium ions may be altered cellular zinc homeostasis. Cadmium and zinc possess highly similar properties, and it has been shown that cadmium can substitute for zinc in biological systems [53]. This can disrupt the normal functioning of various biological pathways [54,55], thus indirectly influencing processes involved in carcinogenesis such as cell proliferation and metastasis [56].

Our aims were to investigate MT isoform expression and specificity of induction in human urothelium under baseline and cadmium-exposed conditions, using a well-characterised normal human urothelial (NHU) cell culture system that includes polarized differentiated NHU cell sheets possessing tight barrier function [57,58]. Prior to cadmium exposure, cellular growth assays were performed to assess cytotoxicity. We next exposed NHU cells to a variety of potential MT inducers, including reactive oxygen species (ROS) [59,60], essential metals [44,61–66] and heavy metals [67–70] to define the specificity of response. Previously unpublished MT-1 isoform-specific antibodies were used to discriminate between MT-1 isoform proteins. Lastly, we determined whether transcription of the free zinc efflux regulator, zinc transporter-1 (*SLC30A1*) [71], altered as a consequence of cadmium exposure. These results revealed that MT isoform expression was inducer-specific and that abundance of both *MT-1A* and *MT-1M* transcript and protein was highly cadmium-specific, highlighting their potential as biomarkers of exposure. Cadmium was able to penetrate an intact urothelial barrier and effected transcriptional upregulation of *SLC30A1*, indicating a potential route for cadmium uptake and subsequent substitution in zinc homeostatic mechanisms.

2. Results

2.1 Influence of cadmium on NHU cell culture growth and uptake of cadmium across an intact urothelial barrier

Exposure of non-differentiated NHU cells to cadmium revealed that cell growth was unaffected by concentrations ≤10 μM $CdCl_2$ whilst exposure to 20 μM $CdCl_2$ resulted in distinct cytotoxicity (Fig 1A). Replication in a second independent cell line confirmed that exposure to 10 μM $CdCl_2$ did not

affect NHU cell growth (Fig 1B) and this concentration was therefore selected for further experiments. When differentiated NHU cell cultures (three independent cell lines) were grown on permeable membranes in triplicate and exposed apically to 10 µM $CdCl_2$ for 72 hours, no effect was seen on barrier function (control versus cadmium-exposed transepithelial electrical resistance (TEER) of 3.24 ± 0.48 kΩ.cm² versus 3.17 ± 0.52 kΩ.cm², mean ± SEM; P=0.93; Table S1). The barrier was retained during $CdCl_2$ exposures of at least 7 days, over which time the TEER increased in the cadmium-exposed culture to 1.8 fold over control. Analysis of cell lysates by inductively coupled plasma optical emission spectroscopy (ICP-OES) revealed an intracellular cadmium concentration of 0.94 µM in lysates from cadmium-exposed cultures compared to 0.08 µM for control cultures.

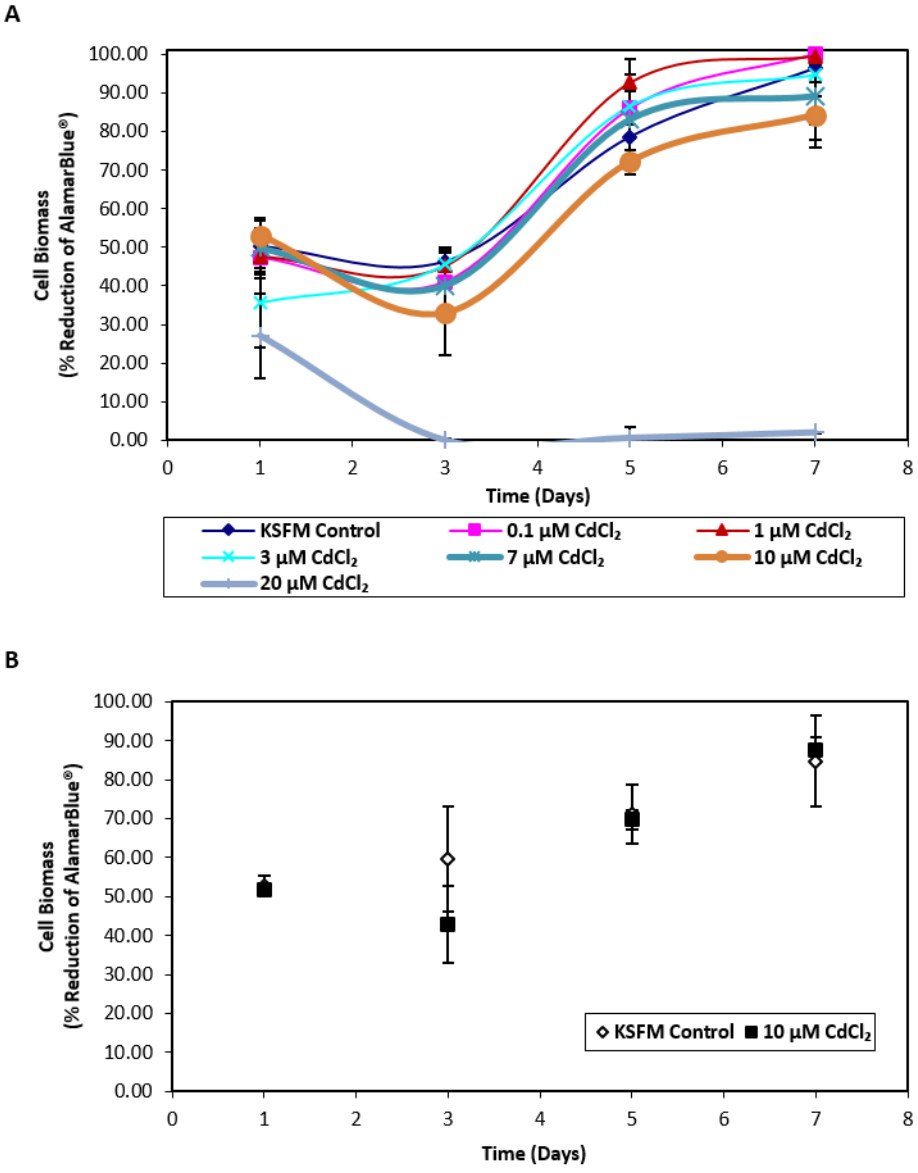

Figure 1. Biomass growth assays for *in vitro* NHU cell cultures exposed to cadmium. AlamarBlue® assays were performed over 7 days on NHU cell cultures seeded at 6 x 10^4 cells/cm². (A) NHU cells were exposed to a range of cadmium concentrations from 0 to 20 µM (n=1 independent cell line). Each data point represents mean percentage reduction in AlamarBlue® ±S.D. from three replicate cultures. (B) NHU cells were exposed to 10 µM $CdCl_2$ for up to 7 days. Data points represent mean percentage reduction in AlamarBlue® ±S.D. from two independent NHU cell lines, each performed in triplicate.

113

114 *2.2 Baseline and cadmium-induced MT transcription in NHU cells*

115 NHU cells maintained in culture in non-differentiated and differentiated states were examined
116 for baseline expression of MT genes. Analysis by mRNA-seq of non-differentiated NHU cells
117 revealed high expression of *MT-1E* and *MT-1X* and low expression of *MT-1A*, *MT-1B*, *MT-1F* and
118 *MT-1G*; there was no detection of *MT-1H* or *MT-1M* transcripts (Fig 2A). *MT-2A* expression was three
119 times greater than all the MT-1 genes combined. No expression was detected for *MT-3* or *MT-4*. In
120 almost all cases where MT gene expression was detected in non-differentiated NHU cells, the
121 expression was reduced in the differentiated state. This was most striking for *MT-2A* ($log_2FC=4.2$;
122 $q=4.08x10^{-3}$) and *MT-1E* ($log_2FC=1.5$; $q=4.0x10^{-4}$), although between-donor variation prohibited
123 statistical significance for many genes with lower expression. The apparent exception was *MT-1X*,
124 where the average expression increased in the differentiated state. However, this was inconsistent
125 between donors, and the differential expression was non-significant. Interestingly, *MT-1L*, which
126 generates a transcript with a premature stop codon [72], was expressed at similar abundance to *MT-*
127 *1E* in the non-differentiated cells, but with a much greater downregulation in the differentiated state
128 ($log_2FC=5.4$; $q=8.4x10^{-4}$). Previous reports of a truncation-rescuing polymorphism [73] was not
129 identified in these donors, so while *MT-1L* is unlikely to form a functional protein, it may play a role
130 in MT-1 transcript regulation. Expression was detected for *SLC30A1* in both non-differentiated and
131 differentiated states (Fig 2A), but there was no significant differentiation-associated change in
132 expression.
133 RT-PCR results supported the NGS data, although the variability in transcript detection in non-
134 differentiated NHU cells indicated a potentially inducible state (Fig 2B). Differentiated NHU cell
135 cultures revealed a more consistent baseline expression of several MT-1 genes, particularly *MT-1X*
136 (Fig 2C).
137 Exposure to cadmium ions caused a massive induction of all eight MT-1 genes (*MT-1A, MT-1B,*
138 *MT-1E, MT-1F, MT-1G, MT-1H, MT-1M, MT-1X*) within 12 hours of initial exposure, as demonstrated
139 by the RT-PCR results, with expression receding over time (Figs 2B and 2C). Whereas expression of
140 *MT-1* transcripts was mostly lost after 48 hours of continuous cadmium exposure in non-
141 differentiated cell cultures (Fig 2B with independent cell line repeats in supplementary Figs S1 and
142 S2), MT-1 subfamily transcript expression was still detectable in differentiated cell cultures after 72
143 hours exposure (Fig 2C; with independent repeats in supplementary Figs S3 and S4).
144 Irrespective of differentiation state and the presence or absence of cadmium, *MT-2A* transcript
145 expression was constitutively high, whilst *MT-3* and *MT-4* transcripts were invariably absent. Based
146 on this, the *MT-2A, MT-3* and *MT-4* genes were not further studied. By contrast, the strong induction
147 of the *MT-1A, -1G, -1H* and *-1M* paralogues, which was consistent following cadmium-exposure in
148 three independent NHU cell lines, was further investigated for specificity. As the RT-PCR results
149 demonstrated a striking on/off transcriptional response of the MT isoforms to cadmium exposure, it
150 was decided to continue with this approach, as quantitative PCR would likely not have added
151 anything to the data.
152

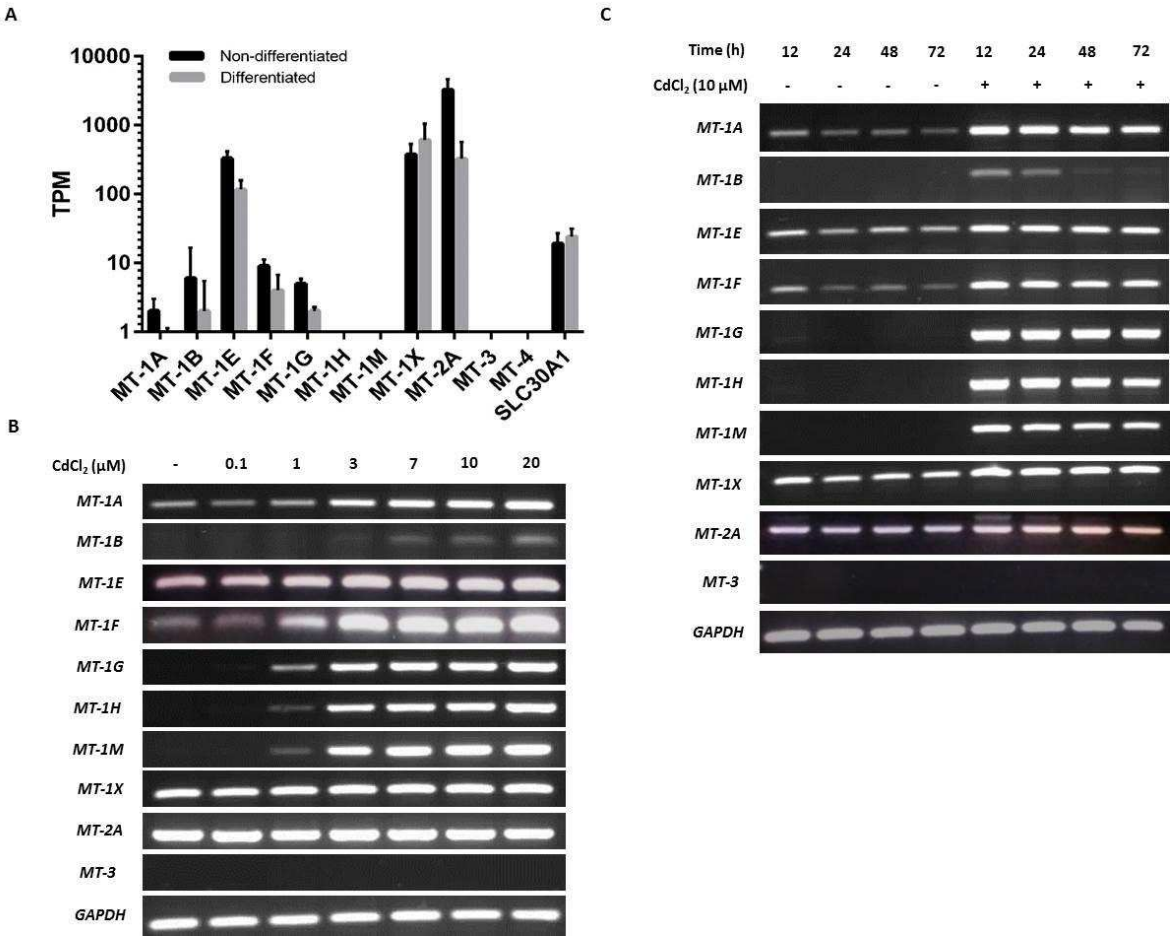

Figure 2. Baseline and cadmium-induced MT transcript expression by NHU cells in vitro. (A) Next generation sequencing data showing baseline MT isoform transcription in non-differentiated and differentiated NHU cells (n=3 independent cell lines; standard deviation is shown). (B-C) MT gene expression in NHU cells assessed by RT-PCR. The total cDNA input was 1 μg and PCR reaction products were removed after 25 cycles; *GAPDH* was included as input control. See Table 1 for primer sequences and product sizes. Note that medium was changed at time T=0 only and there was no renewal of cadmium over the period. The figure shows results representative of n=3 independent NHU cell lines. Additional PCR controls included genomic DNA as a positive control and a no-template (H_2O) negative control; RT-ve samples confirmed absence of genomic contamination.

- In B, the result of exposing non-differentiated NHU cells to different concentrations of cadmium (0-20 μM) for 72 hours on MT gene expression is shown.
- In C, MT gene expression is shown in differentiated NHU cell cultures following exposure to 10 μM $CdCl_2$ for up to 72 hours.

2.3 Specificity of Cadmium-Induced MT Transcription

NHU cell cultures were exposed to a variety of candidate MT inducers identified from the literature.

ROS is reported as by-product of cadmium exposure [59] and we therefore sought to determine the effects of ROS on transcription of MT-1 genes. The ROS-inducing agent sulforaphane ($C_6H_{11}NOS_2$) and ROS-inhibitor ascorbic acid ($C_6H_8O_6$) were titrated against transcription of the ROS-sensitive heme oxygenase-1 (*HMOX1*) gene [74,75] to infer intracellular ROS activity (Figs 3A & 3B, respectively). Induction of expression of the four MT-1 genes in response to cadmium exposure was unaffected by 25 μg/mL ascorbic acid used to inhibit ROS, suggesting that ROS was not responsible

(Fig 3C). This conclusion was supported by the failure of 5 μM sulforaphane to induce MT-1 expression (replicate in supplementary material, Fig S5).

Differential induction of MT-1 paralogues was examined in response to other metal ions, both essential and carcinogenic (replicates in supplementary material, Figs S6 & S7). Both zinc and nickel exposure induced *MT-1G* transcription, whereas *MT-1H* transcript expression was only minimally induced by iron, and nickel had no effect. *MT-1A* transcription was constitutively low under all conditions apart from cadmium exposure, which increased expression, and *MT-1M* transcription was specifically induced by exposure to cadmium alone.

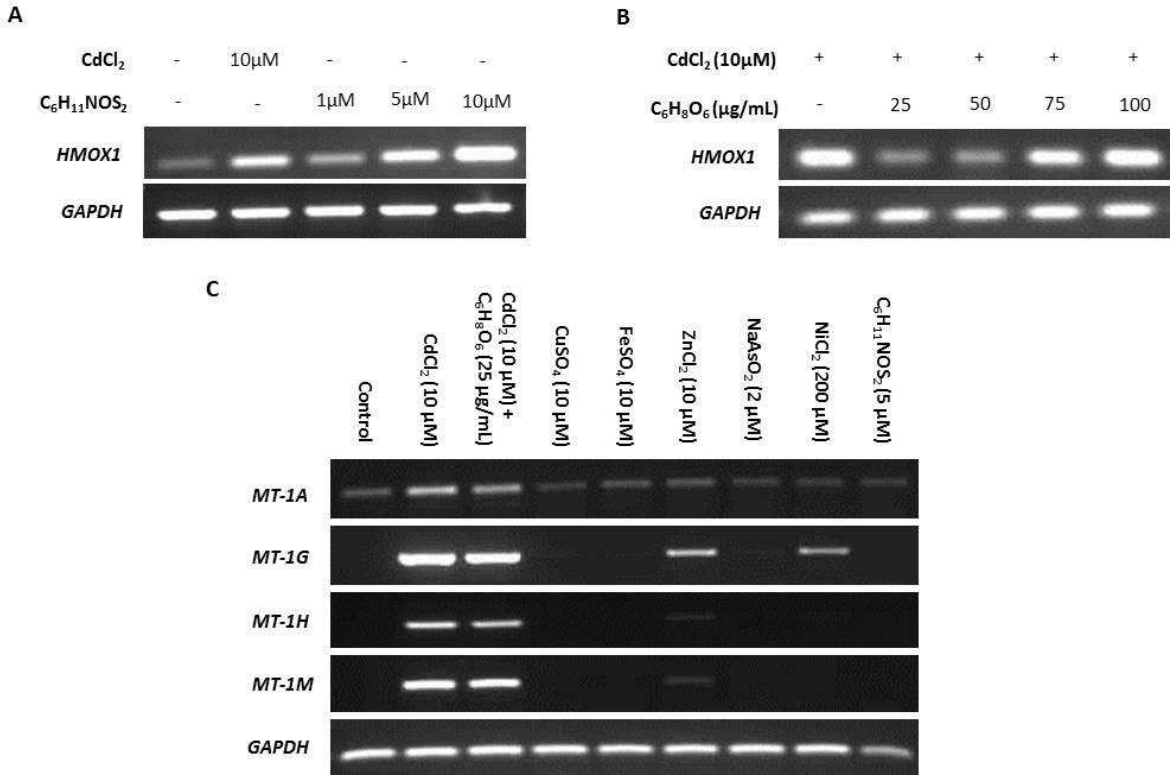

Figure 3. Specificity of MT transcription induction by cadmium. MT gene expression was assessed by RT-PCR in NHU cells exposed to a variety of candidate inducers. The total cDNA input was 1 μg and PCR reaction products were removed after 25 cycles; *GAPDH* was included as input control. Note that medium was changed at time T=0 only and there was no renewal of treatments over the period. Additional PCR controls included genomic DNA as a positive control and a no-template (H_2O) negative control; RT-ve samples confirmed absence of genomic contamination.

- In A, non-differentiated NHU cell cultures were treated with a range of concentrations of sulforaphane ($C_6H_{11}NOS_2$) for 12 hours, and the effect on transcription of the ROS-sensitive gene *HMOX1* assessed in comparison to exposure to 10 μM $CdCl_2$ (n=1 independent cell line). The concentration of $C_6H_{11}NOS_2$ that induced transcription of *HMOX1* to a comparable extent to cadmium was selected.
- In B, non-differentiated NHU cell cultures were treated with a range of concentrations of ascorbic acid ($C_6H_8O_6$) for 12 hours in combination with 10 μM $CdCl_2$ and the effect on *HMOX1* transcription assessed. The concentration of $C_6H_8O_6$ that caused the biggest decrease in *HMOX1* transcription was selected.
- In C, NHU cell cultures (n=2 independent cell lines) were exposed to a range of candidate regulators and transcript expression was assessed for the MT-1 genes shown above to be most sensitive to cadmium induction (from Figures 2B & C). Candidate inducers tested were cadmium (10 μM $CdCl_2$), cadmium combined with ascorbic acid (10 μM $CdCl_2$ + 25 μg/mL $C_6H_8O_6$), copper (10 μM $CuSO_4$), iron (10 μM $FeSO_4$), zinc (10 μM $ZnCl_2$), arsenite (2 μM $NaAsO_2$), nickel (200 μM $NiCl_2$), and sulforaphane (5 μM $C_6H_{11}NOS_2$). Essential metals were applied at equivalent concentrations to cadmium. Arsenite

and nickel were both used at their highest non-cytotoxic concentrations based on initial titration experiments (not shown).

2.4 Immunoblotting with isoform-specific antibodies

To examine if the observed induction of MT-1 gene expression translated to protein, antibodies specific to the MT-1A and MT-1M isoforms were used to perform western blotting. Control non-differentiated NHU cells lacked MT-1A and MT-1M protein expression (Fig 4A). Cadmium exposure caused induction of both MT-1A and MT-1M proteins after 72 hours (replicate in supplementary material, S8). Both proteins were also induced in cadmium-exposed differentiated NHU cells (Fig 4B; replicate in supplementary material, S9).

The specificity of MT-1A and MT-1M protein induction was examined in response to the wider range of candidate inducers. Western blotting revealed that the MT-1M isoform was induced only by cadmium exposure, supporting the RT-PCR results (Fig 4C; replicate in supplementary material, S10). MT-1A protein expression was highly induced by cadmium exposure, although low protein expression was observed under other conditions. As assessed by densitometry, only cadmium was capable of increasing MT-1A protein expression over control, resulting in a ~6-fold increase in MT-1A protein expression (Fig 4D).

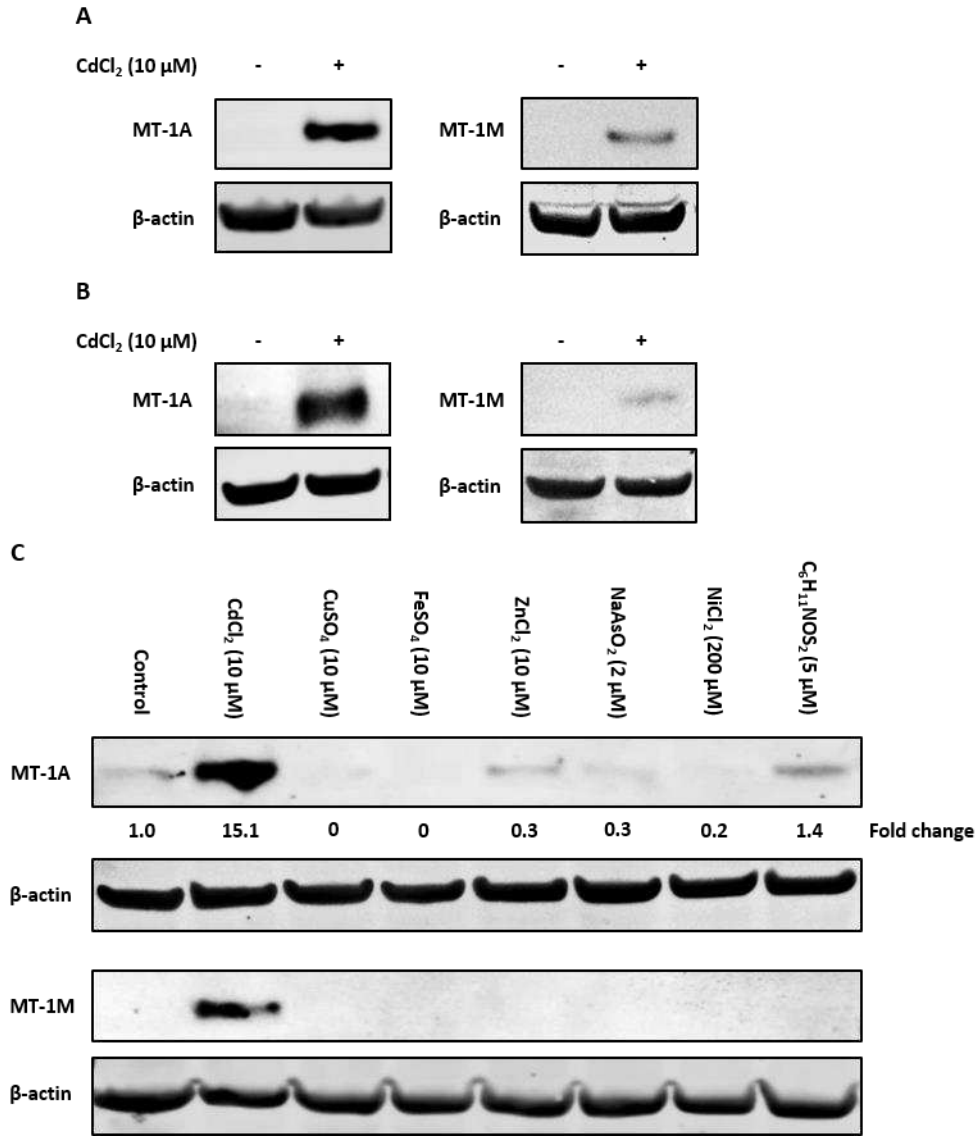

Figure 4. Western blot detection of cadmium-induced MT-1A and MT-1M expression in NHU cells using subtype-specific antibodies. MT-1A and MT-1M protein induction was observed by western blotting of NHU cells exposed to 10 μM $CdCl_2$ for 72 hours. Total protein input was 20 μg per track, with β-actin expression used to verify equal protein loading. Figures show western blots probed with MT-1A and MT-1M subtype-specific antibodies in (A) proliferating and (B) differentiated NHU cell cultures exposed to cadmium for 72 hours (representative blots shown from n=3 independent cell lines). In (B), differentiated barrier formation was confirmed by TEER (see Figure 3B). (C) Western blot showing specificity of MT-1A and MT-1M protein induction (representative of n=2 independent cell lines tested). Proliferating NHU cells were exposed to a range of potential inducers for 72 hours and protein expression assessed. The candidate inducers tested were cadmium (10 μM $CdCl_2$), copper (10 μM $CuSO_4$), iron (10 μM $FeSO_4$), zinc (10 μM $ZnCl_2$), arsenite (2 μM $NaAsO_2$), nickel (200 μM $NiCl_2$), and sulforaphane (5 μM $C_6H_{11}NOS_2$). MT-1A protein expression is reported as fold-change relative to unexposed control cells.

2.5 Upregulation of zinc transporter-1 (SLC30A1) transcription in cadmium-exposed NHU cells

RT-PCR of both non-differentiated and differentiated NHU cells revealed that cadmium exposure resulted in increased *SLC30A1* gene transcription compared to unexposed controls (Figure 5A; replicate in supplementary material, S11). After cessation of exposure, *SLC30A1* transcript expression receded over time, and this decrease was observed to occur most rapidly in non-differentiated (after 24 hours; Figure 3B) compared to differentiated (after 11 days; Figure 5C) cell cultures.

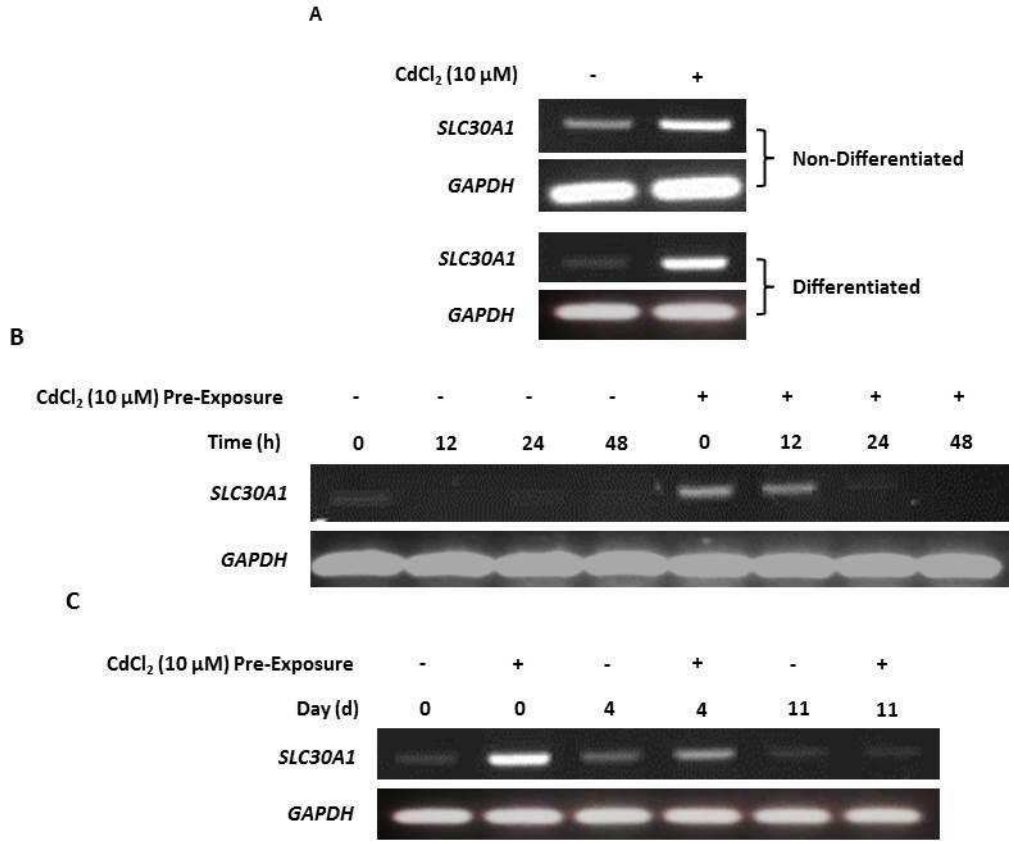

Figure 5. Cadmium induction of zinc transporter-1 (*SLC30A1*) gene transcription. *SLC30A1* gene transcript expression was assessed by RT-PCR, with PCR reaction products removed after 25 cycles. PCR controls included genomic DNA as a positive control and a no-template (H_2O) negative control. Total cDNA input was 1 μg with GAPDH used as an input loading control; RT-ve samples confirmed the absence of genomic contamination. For differentiated NHU cells, differentiation was confirmed by assessment of TEER as >1000 $\Omega.cm^2$ (see supplementary materials, Table 1).

- A: NHU cells in non-differentiated and differentiated states were maintained in control medium or with 10 μM $CdCl_2$ for up to 72 hours. Note that medium was changed at time T=0 and there was no renewal of the cadmium over the period. The figure shows representative results from n=3 (non-differentiated) and n=1 (differentiated) independent NHU cell lines.
- B: RT-PCR of NHU cells in a non-differentiated state maintained in standard medium (control) or with 10 μM $CdCl_2$ for up to 24 hours prior to the experiment ('pre-exposure'), whereupon cadmium was removed from the medium (time point 0) and culture continued for a further 48 hours.
- C: RT-PCR of NHU cells in a differentiated state maintained in standard medium (control) or with 10 μM $CdCl_2$ for up to 3 days prior to the experiment ('pre-exposure'), whereupon cadmium was removed from the medium (time point 0) and culture continued for up to 11 days.

3. Discussion

To the authors' knowledge, this is the first study to investigate if cadmium exposure affects urothelial tight barrier function. Previous studies have examined the effect of cadmium exposure on renal [76] and bronchial [77] epithelia, with both studies reporting a decrease or complete collapse of barrier function. By contrast, the results from this study showed that urothelial barrier function was retained and even tightened. This may reflect the type of epithelium being tested, as urothelial cells are known to form one of the tightest epithelial barriers in the human body and thus may be unique in their resistance to cadmium. Although cadmium did not compromise urothelial barrier function, our results found that apical exposure induced expression of MT-1 genes in underlying NHU cells and using ICP-OES, cadmium was detected in differentiated urothelial cell sheets. Taken together, these results indicate that the urothelium remains intact and may even mount a protective response to cadmium in terms of tight junction tightening, yet the heavy metal is able to penetrate the urothelial barrier.

Also for the first time, we describe specific MT gene expression by normal human urothelium. Our results are consistent with previous reports in other tissues that MT-1 is the inducible subfamily [22,40,78], whereas *MT-2A* is the most widely expressed isoform, accounting for up to 50 % total MT expression in humans [6,79]. In agreement with the consensus that MT-3 is neural-restricted [80], we found no agents that could induce *MT-3* transcription in urothelial cells. As an anomaly, one group has previously suggested a link between heavy metal exposure, MT-3 induction and urothelial carcinogenesis based on high MT-3 expression in malignant bladder cancer [81] and supported by in vitro studies in cadmium-transformed UROtsa cells [82,83]. However, we were not able to replicate those findings in our normal cell system.

Of the *MT-1* genes, transcription of *MT-1F* and *MT-1G* has previously been described as constitutive in human umbilical vein epithelial cells (HUVEC) under baseline conditions [42]. Both these genes exhibited low basal but inducible expression in NHU cells, therefore it cannot be certain whether the expression differences between tissues are constitutive, or due to inducers present in the culture medium. Whereas we found the majority of *MT-1* paralogues to be inducible by cadmium exposure, transcript expression was transient, presumably due to sequestration leaving fewer free cadmium ions to maintain induction. By contrast, expression of MT-1 proteins was more stable and in line with the sequestering nature of the formed MT-cadmium complex. Previously, MT-1 protein isoform detection has been performed using mass-spectrometry [41], but the demonstrated

availability of specific antibodies now opens the door to detection using immunochemical approaches.

Exposure of proliferating NHU cell cultures to a range of divalent metal ions revealed differential and inducer-specific induction of MT-1 isoforms. For example, zinc ions induced strong transcription of *MT-1G*, but not *MT-1A* genes, whereas nickel ions caused *MT-1G* expression exclusively. Inducer-specific expression of MT-1 genes has been noted in other cell types [34,44–47], supporting the hypothesis that individual isoforms have very selective metal ion sequestering functions [5,6,22,39,40,84]. Western blotting revealed that cadmium was the most potent inducer of MT-1A protein expression, whereas MT-1M protein expression was highly specific to cadmium, revealing the potential of both as biomarkers of cadmium exposure. Neither arsenic nor nickel induced MT-1A or MT-1M expression, further demonstrating the specificity of MT-1 genes to differentiate between different non-genotoxic carcinogenic metals [85,86].

Alongside a pathophysiological role in sequestering carcinogenic metals, the MTs are considered to contribute to the normal homeostasis of zinc, which, as a cofactor involved in many key cellular processes [56], is under tight control. MT sequestration and zinc transporter efflux coordinated by a common transcription factor *MTF-1* [87] is thought to regulate the availability of zinc [88–91]. Changes in intracellular zinc concentration have been associated with tumour growth and progression [92]. The displacement of zinc from the proteome by cadmium may affect the intracellular concentration and/or availability of zinc ions and can substitute and destabilise the functional sites of zinc-containing proteins, such as zinc-finger transcription factors, changing the character and/or rendering them non-functional [54,55].

Preliminary investigation into the effect of cadmium exposure on zinc homeostasis in normal urothelial cells revealed an upregulation of the zinc transporter-1 (*SLC30A1*) gene transcript in non-differentiated NHU cells. *SLC30A1* upregulation was also observed in cadmium-exposed differentiated NHU cell sheets possessing a functional barrier, further suggesting that cadmium can penetrate an intact urothelial barrier. Such results agree with a previous study using the human hepatic HepG2 cell line, which demonstrated increased *SLC30A1* protein expression and localization at the cell membrane after acute cadmium exposure [93]. A later study showed cadmium exposure resulted in a 93% increase in the intracellular labile zinc concentration, suggesting a large displacement of zinc ions from the proteome, possibly due to substitution by cadmium [94].

MT expression is often seen as a 'double-edged sword' as on the one hand, it functions to protect the cell, but by the same mechanisms can also facilitate malignant events [6,22,95,96]. MTs may contribute to cell survival by increasing resistance to ROS-induced apoptosis [97] and increasing cellular proliferation [32]. Cadmium exposure can result in inhibition of DNA repair [98], which coupled with increased cellular protection *via* cadmium-induced MT expression, increases the probability of deleterious cells surviving and passing on defects to their progeny [96]. The ability of MT to counteract ROS could also play a role in chemotherapy resistance, and high expression of MTs has been correlated with treatment resistance in bladder cancer [99,100]. Specifically, after radical surgery and adjuvant chemotherapy, 100% patients with high tumour MT expression progressed within nine months, whereas in patients with low MT expression, only 65% had progressed after five years [101].

Our study supports a concordant induction of MT isoforms and *SLC30A1* transcription in response to cadmium exposure. Whereas this does not directly contradict hypotheses that cadmium exposure increases cellular zinc concentration or that cellular zinc homeostasis is maintained through the cooperative regulation of MT and zinc transporters [39], it does proffer a more direct relationship with cadmium responsible for regulating *SLC30A1* expression. This may reveal new insight as to the role of cadmium in (bladder) cancer, where previously reported *SLC30A1* and MT changes may reflect increased concentrations of intracellular cadmium rather than zinc. Our demonstration of differential MT-1 gene paralogue induction by zinc and cadmium should help design future (e.g. knockout) experiments to clarify the respective roles. Further experiments might also directly quantify intracellular zinc in NHU cells after cadmium exposure and determine the consequences of cadmium on zinc homeostasis and dysregulated cadmium-substituted proteins.

355

356 **4. Materials and Methods**

357 *4.1 NHU cell culture and exposure to cadmium and other agents*

358 Normal human urothelial (NHU) cells were maintained *in vitro* as non-immortalised cell lines,
359 as detailed elsewhere [57,102]. For routine culture, NHU cells were grown as adherent monolayers
360 on Primaria™ plasticware (BD Biosciences) in low calcium [0.09 mM] keratinocyte serum-free
361 medium (KSFM) containing bovine pituitary extract and recombinant epidermal growth factor (Life
362 Technologies) supplemented with 30 ng/ml cholera toxin (KSFMc). NHU cell lines were sub-cultured
363 by trypsinisation at just-confluence and used in experiments between passages 3-5.
364 For cadmium exposure, medium was replaced with fresh medium containing 10 μM cadmium
365 chloride ($CdCl_2$). This concentration was selected after preliminary titration for toxicity (Figs 1A &
366 1B). For other treatment agents, concentrations were selected following initial titration and target
367 gene expression assessment (Figs 3A & 3B).
368 NHU cell lines retain the capacity to differentiate to form a functional tight barrier epithelium
369 (as described in [58]). Differentiation was induced by switching NHU cells into medium
370 supplemented with 5% adult bovine serum for 5 days before sub-culture onto semi-permeable
371 ThinCert™ (Greiner Bio-One) membranes with a 0.4 μm pore size. After 24 hours, the exogenous
372 calcium (Ca^{2+}) concentration was increased to 2 mM (near physiological) and cultures were
373 maintained for a further 7-9 days to develop a tight barrier. For cadmium exposure of differentiated
374 NHU cell cultures, medium was removed from the apical chamber after establishment of a barrier
375 >1000 $\Omega.cm^2$ (see below) and replaced with fresh medium supplemented with 10 μM $CdCl_2$, in order
376 to mimic apical exposure. RNA and protein were then harvested from these membranes for further
377 analysis.

378 *4.2 Measurement of transepithelial electrical resistance*

379 The barrier function of differentiated NHU cell sheets was assessed in triplicate cultures by
380 measuring the transepithelial electrical resistance (TEER) using an EVOM™ voltohmmeter (World
381 Precision Instruments), as described [103]. A blank (no cell) membrane measurement was subtracted
382 from each TEER reading.

383 *4.3 MT transcript abundance quantification by next generation sequencing*

384 mRNA-seq data for three donor-matched NHU cultured non-differentiated and differentiated
385 samples were previously generated by our group [104]. Sequencing reads were 'pseudoaligned' to
386 the Ensembl v.91 human transcriptome (GRCh38.p10) using kallisto v0.44.0 [105] and relative gene
387 abundance was calculated as transcripts per million (TPM) following gene-level aggregation with
388 tximport v1.8.0 [106]. Differentiation-associated expression changes in the MT gene family were
389 detected by a differential-expression analysis conducted by sleuth [107] accounting for matched-
390 donor samples. Differentially-expressed genes are reported with their log_2 transformed fold change
391 (log_2FC) and 'q' value (Benjamini-Hochberg correction).

392 *4.4 Reverse transcriptase-polymerase chain reaction (RT-PCR)*

393 RT-PCR was performed to observe actual patterns of MT isoform induction (rather than relative
394 change). RNA was extracted from cells using TRIzol® (ThermoFisher Scientific) and treated with a
395 DNA-free™ kit (Ambion). cDNA synthesis was performed on 1 μg RNA with random hexamers and
396 the SuperScript®II First-Strand Synthesis System (Life Technologies). PCR primers were designed
397 specifically to detect all known splice variants for each MT-1 isoform gene, with GAPDH used as the
398 internal transcript control [108]. Primer sequences and optimised PCR conditions are provided in
399 Table 1. PCR was carried out in a T100 thermal cycler (Bio-Rad) using 25 reaction cycles. Controls
400 consisted of genomic DNA (gDNA as template positive control), water (no template control) and no

401 reverse transcriptase (gDNA negative control). PCR products were separated on 2 % (w/v) agarose
402 gels, stained using SYBR® Safe DNA gel stain (Invitrogen) and visualised on a Gene Genius Gel
403 Imaging System (Syngene).

404

405 **Table 1:** Details of primers used for experiments.

Target Gene	Forward or Reverse	Sequence (5´-3´)	Product Size (bp)
GAPDH	Forward	CAAGGTCATCCATGACAACTTTG	90
GAPDH	Reverse	GGGCCATCCACAGTCTTCTG	90
HMOX1	Forward	CCAGCAACAAAGTGCAAGATTC	102
HMOX1	Reverse	GTGTAAGGACCCATCGGAGAAG	102
MT-1A	Forward	CTCGAAATGGACCCCAACT	219
MT-1A	Reverse	ATATCTTCGAGCAGGGCTGTC	219
MT-1B	Forward	GGAACTCCAGGCTTGTCTTGG	77
MT-1B	Reverse	TTGCAGGAGGTACATTTG	77
MT-1E	Forward	TGCGCCGGCTCCTGCAAGTC	118
MT-1E	Reverse	ATGCCCCTTTGCAGACGCAGC	118
MT-1F	Forward	CCTGCACCTGCGCTGGTTCC	110
MT-1F	Reverse	ACAGCCCTGGGCACACTTGC	110
MT-1G	Forward	CTTCTCGCTTGGGAACTCTA	309
MT-1G	Reverse	AGGGGTCAAGATTGTAGCAAA	309
MT-1H	Forward	CCTCTTCTCTTCTCGCTTGG	317
MT-1H	Reverse	GCAAATGAGTCGGAGTTGTAG	317
MT-1M	Forward	CTAGCAGTCGCTCCATTTATCG	180
MT-1M	Reverse	CAGCTGCAGTTCTCCAACGT	180
MT-1X	Forward	GGACCCAACTGCTCCTGCTC	151
MT-1X	Reverse	TTTGCAGATGCAGCCCTGGGC	151
MT-2A	Forward	CCGACTCTAGCCGCCTCTT	259
MT-2A	Reverse	GTGGAAGTCGCGTTCTTTACA	259
MT-3	Forward	AGTGCGAGGGATGCAAATG	98

MT-3	Reverse	GCCTTTGCACACACAGTCCTT	98
SLC30A1	Forward	GCATCAGTTTATGAGGCTGGTCCT	352
SLC30A1	Reverse	CAGGCTGAATGGTAGTAGCGTGAA	352

406

407

408 *4.5 Western blotting*

409 NHU cell cultures were lysed into electrophoresis sample buffer containing protease inhibitors
410 and sonicated. Twenty µg protein was resolved on 4-12% Bis-Tris NuPage™ polyacrylamide gels
411 (Invitrogen) in 2-(N-morphilino) ethanesulfonic acid (MES) buffer and electro-transferred onto
412 polyvinylfluoride membranes (Millipore). Membranes were blocked with Odyssey® blocking buffer
413 (LI-COR®), incubated with primary antibodies for 16 h at 4°C and bound antibody detected using
414 Alexa Fluor® 680-conjugated anti-mouse secondary antibody (Invitrogen) or an IRDye 800-
415 conjugated anti-rabbit secondary antibody (Tebu-Bio). Antibody binding was visualised using an
416 Odyssey® Sa Infrared Imaging System (LI-COR®). Protein quantification was performed using
417 Odyssey® Image Studio™ software v5.0 (LI-COR®). Details of antibodies are given Tables 2 and 3.
418

419 **Table 2:** Details of primary antibodies used for experiments.

Antigen	Clone	Host	Supplier	Dilution	Molecular Weight (kDa)
Beta-actin	A5441	Mouse	Sigma Aldrich	1:10 000 (WB)	42
MT-1A	B01P	Mouse	Abnova	1:750 (WB)	6
MT-1M	17281-AP	Rabbit	ProteinTech	1 µg/mL (WB)	6

420

421 **Table 3:** Details of secondary antibodies used for experiments.

Antigen	Conjugate	Host	Supplier	Application
Anti-mouse IgG	Alexa 680	Goat	Life Technologies	WB
Anti-rabbit IgG	Alexa 800	Goat	Life Technologies	WB

422

423 **5. Conclusions**

424 MT-1 isoform expression has been characterised in normal human urothelium for the first time,
425 and a unique expression profile described with the use of isoform-specific antibodies. Individual
426 MT-1 genes demonstrated inducer-specific expression and two paralogues with cadmium-specific
427 or -selective induction were identified as candidate biomarkers of cadmium exposure. With the
428 potential for cadmium to interfere and substitute in the homeostatic regulation of zinc, new
429 approaches are proposed for understanding cadmium-induced non-genotoxic carcinogenesis.

430

431 **Supplementary Materials:** Supplementary materials can be found at www.mdpi.com/xxx/s1.

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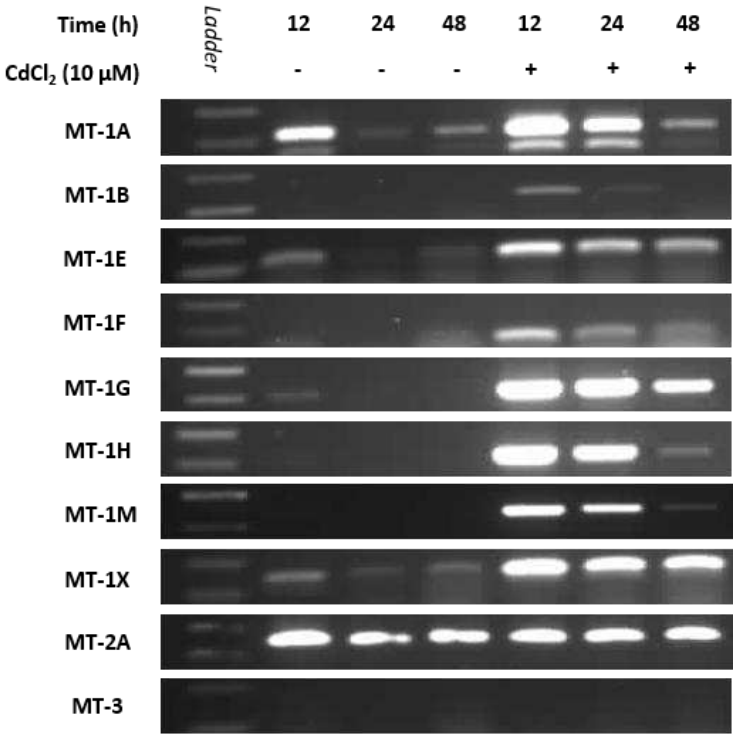
Abbreviations

HMOX1	Heme oxygenase-1
MT	Metallothionein
NHU	Normal human urothelial
SLC30A1	Solute carrier family A member 1
TEER	Trans-epithelial electrical resistance

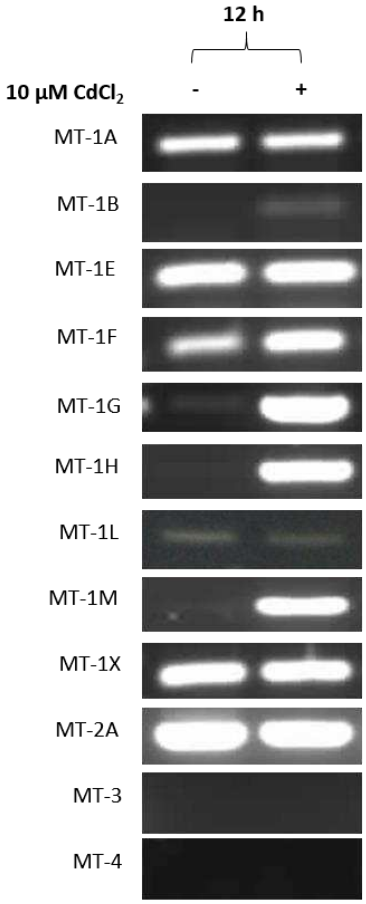
Appendix A

Supplementary Table 1: TEER readings from different NHU cell lines used for experiments. Each reading is an average of three technical replicates. A TEER reading >0.5 kΩ.cm^2 is considered to reflect a functional urothelial barrier.

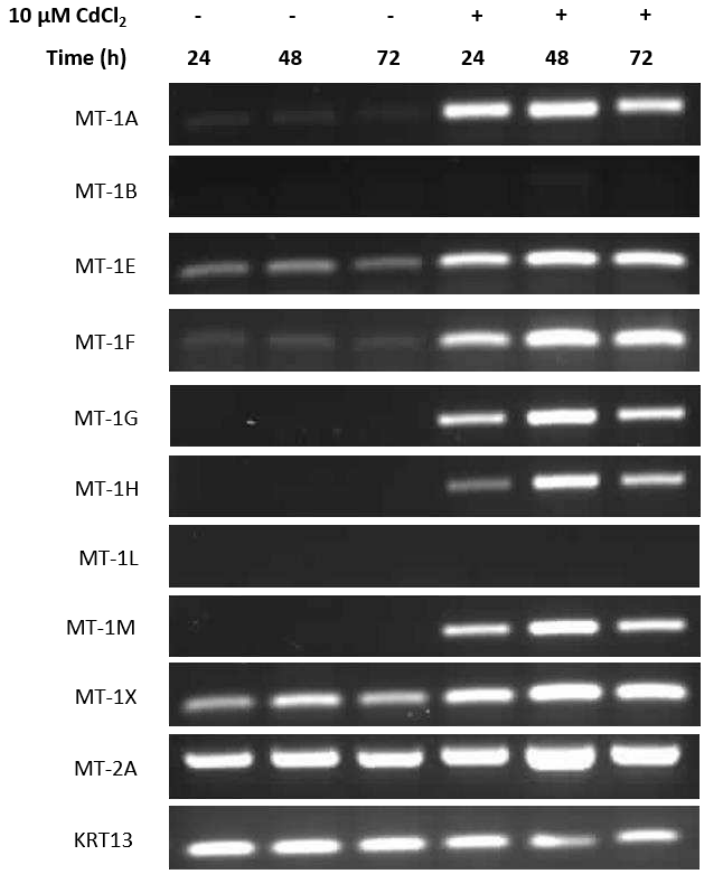
Cell Line	Figure	Time Point	TEER (kΩ.cm^2)	
			Control	Cadmium
Y1456	1B, Suppl.	12h	3.54	4.88
		24h	3.58	3.96
		48h	4.63	2.14
		72h	3.78	4.09
Y1493	Suppl.	24h	2.00	2.65
		48h	3.20	2.99
		72h	2.27	2.30
Y1426	4C, Suppl.	72h	3.33	4.43



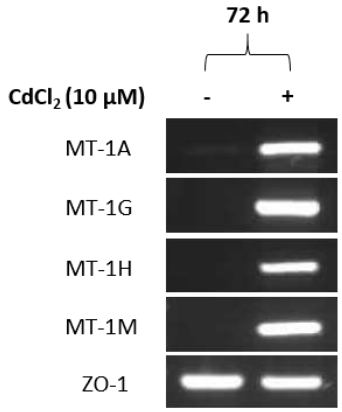
Supplementary Figure 1: RT-PCR showing MT isoform transcription in proliferating NHU cells exposed to cadmium (experimental replicate from Fig 2B). Non-differentiated NHU cells were exposed to 10 µM $CdCl_2$ for up to 48 hours. The total cDNA input was 1 µg and PCR reaction products were removed after 25 cycles.



Supplementary Figure 2: RT-PCR showing MT isoform transcription in proliferating NHU cells exposed to cadmium (experimental replicate from Fig 2B). Non-differentiated NHU cells were exposed to 10 μM $CdCl_2$ for up to 12 hours. The total cDNA input was 1 μg and PCR reaction products were removed after 25 cycles.

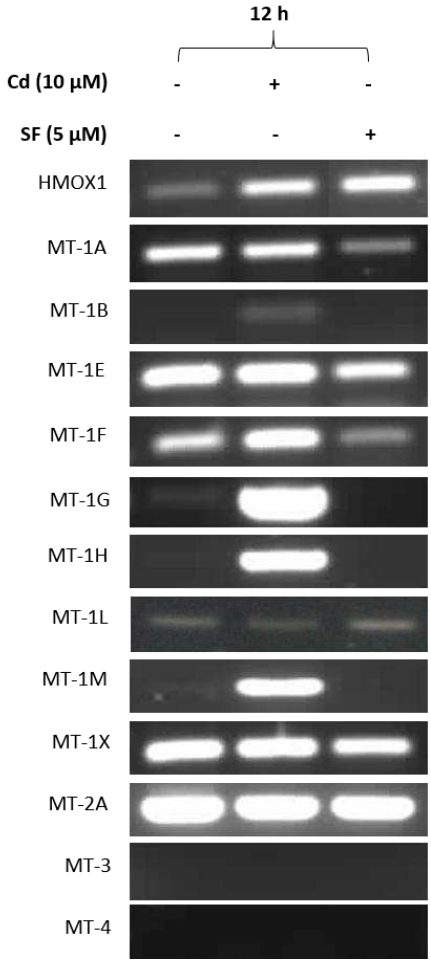


Supplementary Figure 3: RT-PCR showing MT isoform transcription in cadmium-exposed differentiated NHU cell sheets that demonstrated a functional barrier (experimental replicate from Fig 2C). NHU cells were differentiated and exposed to 10 μM $CdCl_2$ for up to 72 hours. Differentiation was confirmed using TEER readings and expression of KRT13. The total cDNA input was 1 μg and PCR reaction products were removed after 25 cycles.

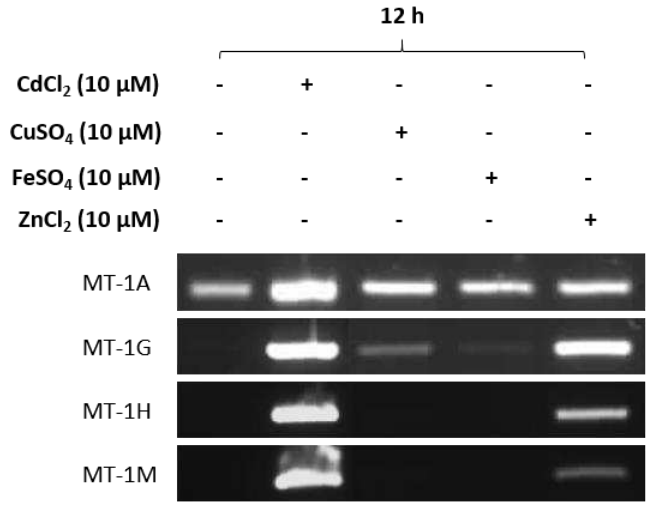


Supplementary Figure 4: RT-PCR showing MT isoform transcription in cadmium-exposed differentiated NHU cell sheets that demonstrated a functional barrier (experimental replicate from Fig 2C). NHU cells were differentiated and exposed to 10 μM $CdCl_2$ for up to 72 hours. Differentiation

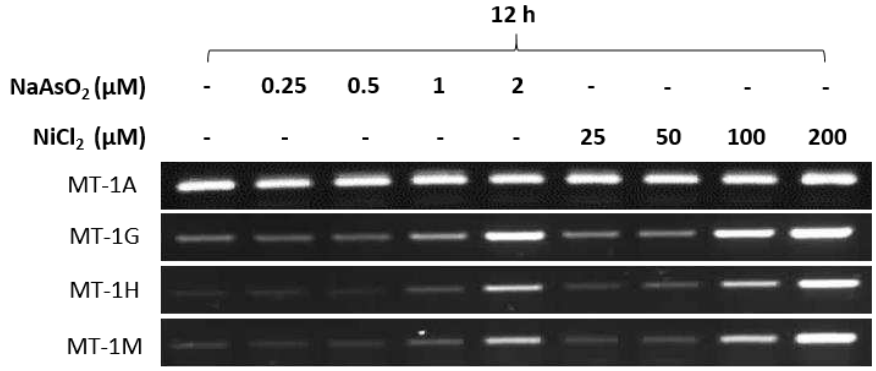
was confirmed using TEER readings and expression of ZO-1. The total cDNA input was 1 μg and PCR reaction products were removed after 25 cycles.



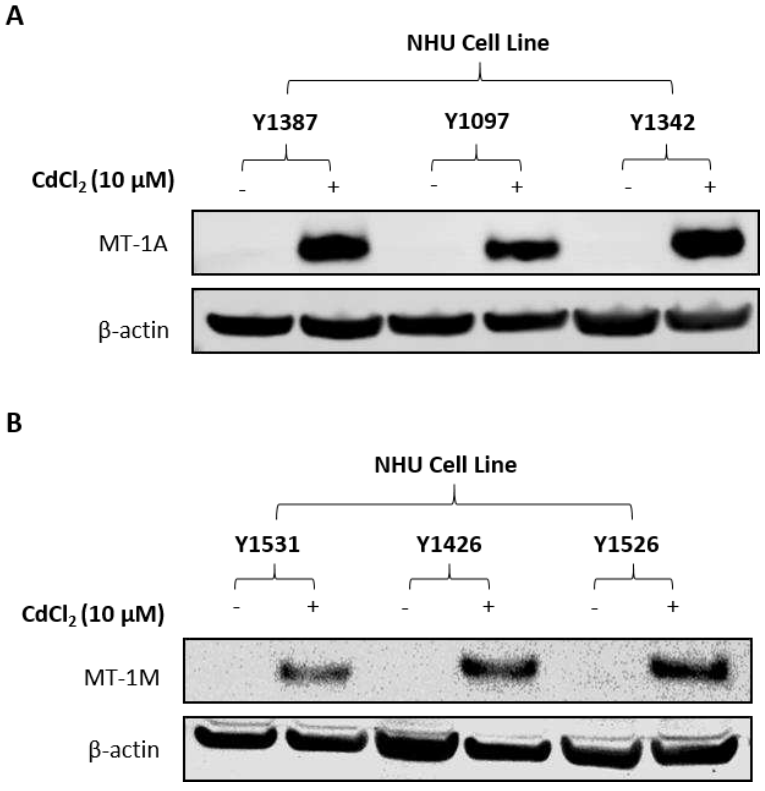
Supplementary Figure 5: RT-PCR showing the effects of ROS on MT isoform transcription in NHU cells (experimental replicate from Fig 3C). The chemical Sulforaphane ($C_6H_{11}NOS_2$) was used to induce ROS, having been titrated to a concentration that mimicked the levels of cadmium-induced ROS. Non-differentiated NHU cells were treated with either 10 μM $CdCl_2$ or 5 μM $C_6H_{11}NOS_2$ for 12 hours. The total cDNA input was 1 μg and PCR reaction products were removed after 25 cycles.



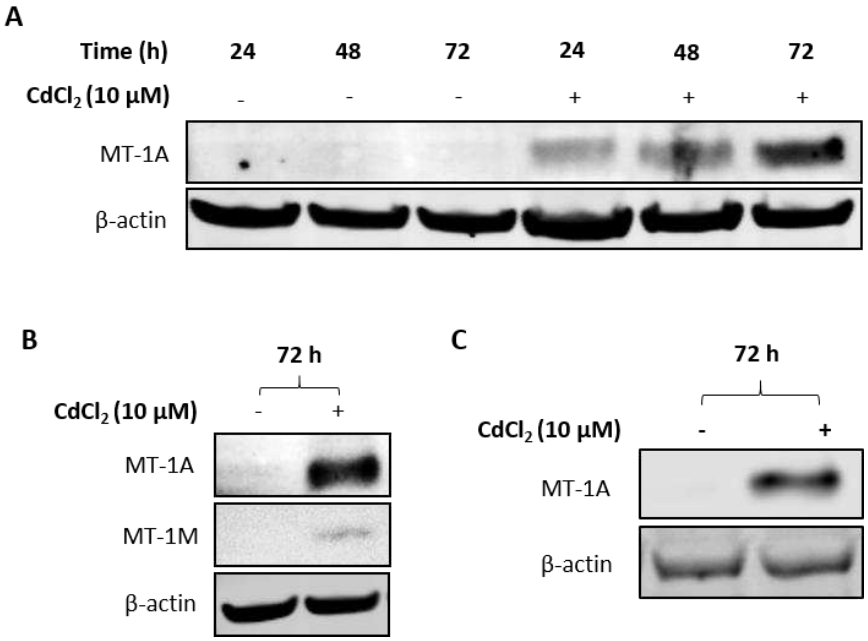
Supplementary Figure 6: RT-PCR showing the effects of essential metal exposure on MT-1 isoform transcription in NHU cells (experimental replicate from Fig 3C). Non-differentiated NHU cells were exposed to either 10 μM $CdCl_2$, 10 μM $CuSO_4$, 10 μM $FeSO_4$, or 10 μM $ZnCl_2$ for 12 hours. The total cDNA input was 1 μg and PCR reaction products were removed after 25 cycles.



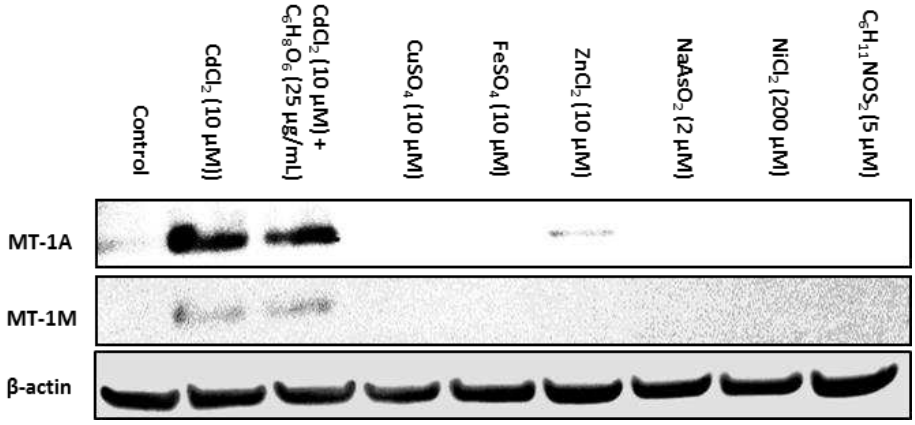
Supplementary Figure 7: RT-PCR showing the effects of exposure to the carcinogenic metals arsenite and nickel on MT-1 isoform transcription in NHU cells (experimental replicate from Fig 3C). Non-differentiated NHU cells were exposed to a range of concentrations of arsenite (0.25 μM – 2 μM; NaAsO2) and nickel (25 μM – 200 μM; $NiCl_2$) for 12 hours. The total cDNA input was 1 μg and PCR reaction products were removed after 25 cycles.



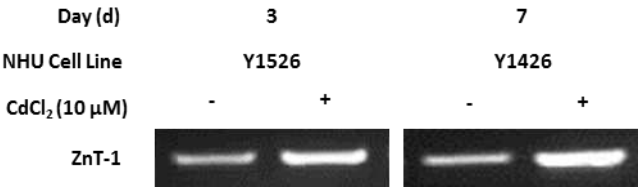
Supplementary Figure 8: Western blots showing MT-1A and MT-1M protein expression in non-differentiated NHU cells exposed to cadmium (experimental replicate from Fig 4A). Non-differentiated NHU cells (n=3) were exposed to 10 μM $CdCl_2$ for 72 hours and protein expression of (A) MT-1A and (B) MT-1M determined using novel, isoform-specific antibodies. β-actin protein expression was used as a loading control.



Supplementary Figure 9: Western blots demonstrating MT-1A and 1M protein expression in differentiated NHU cells with functional barriers that were exposed to cadmium (experimental replicate from Fig 4B). NHU cells were stimulated to differentiate and form a functional barrier, before exposure to 10 µM $CdCl_2$. (A) MT-1A protein expression was assessed at multiple time-points to ensure exposure time was adequate for protein translation in differentiated NHU cells. (B) Western blots showing MT-1A and MT-1M protein expression and (C) MT-1A protein expression in differentiated NHU cells exposed to cadmium for 72 hours. β-actin protein expression was used as a loading control.



Supplementary Figure 10: Western blot showing the specificity of cadmium-induced MT-1A and MT-1M isoform protein expression in NHU cells (experimental replicate from Fig 4C). Non-differentiated NHU cells were exposed to a range of potential inducers for 72 hours. Candidate inducers were cadmium (10 µM $CdCl_2$), copper (10 µM $CuSO_4$), iron (10 µM $FeSO_4$), zinc (10 µM $ZnCl_2$), arsenite (2 µM $NaAsO_2$), nickel (200 µM $NiCl_2$), and sulforaphane (5 µM $C_6H_{11}NOS_2$). Cadmium in combination with ascorbic acid (25 µg/ml $C_6H_8O_6$) was also included, to support the RT-PCR data demonstrating that inhibition of cadmium-induced ROS did not inhibit cadmium-induced MT expression (Fig 3C). β-actin protein expression was used as a loading control.



Supplementary Figure 11: Effect of cadmium exposure on zinc transporter-1 (SLC30A1) transcription (experimental replicate from Fig 5B). RT-PCR of SLC30A1 gene transcription in non-differentiated NHU cells exposed to 10 µM $CdCl_2$ for 3 or 7 days. Note that medium was changed at time T=0 and that for 3 day exposure there was no renewal of the cadmium by medium change over the period. For 7 day exposure, cadmium-containing medium was renewed on day 4. The total cDNA input was 1 µg and PCR reaction products were removed after 25 cycles.

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