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Metallothionein Isoform 3 Expression in Human Skin, Related Cancers, and Human Skin Derived Cell Cultures

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Metallothionein isoform 3 expression in human skin, related cancers and human skin derived cell cultures

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HIGHLIGHTS

- Skin is a target site of As^{+3} toxicity.
- Metallothionein-3 (MT3) is highly expressed in human skin.
- Skin-derived malignancies also expressed MT3.
- Suggested that MT3 is a major sulfhydryl source for dermal As^{+3} binding.

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ABSTRACT

Human skin is a well known target site of inorganic arsenic with effects ranging from hyperkeratosis to dermal malignancies. The current study characterizes the expression of a protein known to bind inorganic, As^{3+}, metallothionein 3 (MT-3). Expression of this protein was assessed immunohistochemically with a specific MT-3 antibody on human formalin-fixed, paraffin-embedded biopsy specimens in normal skin, squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and melanoma. Assessment in normal skin using nine normal specimens showed moderate to intense MT-3 staining in epidermal karatinocytes with staining extending into the basal cells and moderate to intense staining in melanocytes of nevi. MT-3 immunoexpression was shown to be moderate to intense in 12 of 13 of SCC, low to moderate in 8 of 10 BCC, and moderate to intense in 12 melanoma samples. MT-3 expression in cell culture models (normal human epidermal keratinocytes, normal human melanocytes, and HaCaT cells) showed only trace expression of MT-3, while exposures to the histone deacytalase inhibitor, MS-275, partially restored expression levels. These results indicate that the epidermis of human skin and resulting malignancies express high level of MT-3 and potentially impact on the known association of arsenic exposure and the development of skin disorders and related cancers.

1. Introduction

Inorganic arsenic is a potent human carcinogen, and skin is known to be one of the most susceptible human organs affected by chronic environmental exposure to this metalloid (Bolt, 2012). Chronic arsenic exposure causes a dry skin phenotype characterized by melanosis, hyperplasia and hyperkeratosis (Komissarova and Rossman, 2010). The most common arsenic-induced skin cancers include Bowen's disease (BD, SCC *in situ*), squamous cell carcinoma (SCC), and basal cell carcinoma (BCC) (Yeh et al., 1968). There is less evidence for a potential contribution of arsenic exposure to the development of melanoma. However, there is emerging evidence for such an association, especially for melanomas that might arise from co-exposure to ultraviolet radiation (Cooper et al., 2014; Pearce et al., 2012; Dennis et al., 2010). Cell culture models have seen frequent use to investigate the mechanisms involved in arsenic-induced toxicity and cancer development due to the lack of valid animal models. These studies have lead to several theories to explain the carcinogenic effects of arsenic exposure and include the generation of reactive oxygen species (ROS), oxidative DNA damage, genomic instability, aneuploidy, gene amplification, inhibition of

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DNA repair, and epigenetic dysregulation (Ren et al., 2011; Straif et al., 2009; Lee et al., 2012).

This laboratory is interested in how the metallothionein (MT) gene family might participate in the above processes that are associated with arsenic-induced carcinogenesis. A role for this family of proteins might be expected since all MT family members can bind and sequester 6 atoms of As^{+3} and can also serve as an antioxidant (Vasak and Meloni, 2011; Irvine et al., 2013; Garla et al., 2013). In humans, there are four MT isoforms, designated MT-1 through MT-4. The MT-1 and MT-2 isoforms have been the subject of extensive study over the last 50 years and the subject of numerous reviews (see Vasak and Meloni, 2011). The MT-1 and MT-2 isoforms are inducible in almost all tissues by a variety of stress conditions and compounds including glucocorticoids, cytokines, ROS, and metal ions. In contrast, the identification of the MT-3 and MT-4 isoforms is relatively recent (1990s) and both isoforms are largely unresponsive to the above inducers and their expression believed to be confined to far fewer tissue types. The four MT isoforms share a high degree of sequence homology and a specific antibody cannot be produced that can separately identify the MT-1, 2 and 4 isoforms. The MT-3 isoform is unique in that it possesses 7 additional amino acids that are not present in any other member of the MT gene family, a 6 amino acid C-terminal sequence and a Thr in the N-terminal region (Palmiter et al., 1992; Tsuji et al., 1992; Uchida et al., 1991). An MT-3 specific antibody can be generated against the C-terminal sequence (Garrett et al., 1999). Functionally, MT-3 has also been shown to possess several activities not shared by the other MT isoforms. These include a neuronal cell growth inhibitory activity (Uchida et al., 1991), the participation in the regulation of EMT in human proximal tubule cells (Kim et al., 2002; Kim et al., 2002), and the ability to influence the choice between apoptotic and necrotic cell death in proximal tubule cells exposed to cadmium (Somji et al., 2004). This non-duplication of function occurs despite a 63–69% homology in amino acid sequence among MT-3 and the other human MT isoforms (Sewell et al., 1995). These unique features of MT-3, along with its ability to bind and sequester As^{+3}, motivated the present study designed to examine the expression of MT-3 in human skin and related skin cancers. A related question was to determine if human cell culture models used to study As^{+3} effects on skin faithfully recapitulate the *in situ* expression of MT-3.

2. Materials and methods

2.1. Specimens of human skin

Specimens of normal human skin and associated cancers were obtained from archival paraffin blocks 10 years post diagnosis and scheduled for disposal as medical waste. These archival specimens contained no patient identifiers and are in the exempt category for human research. Tissues within these paraffin blocks were routinely fixed in 10% neutral buffered formalin for 16–18 h. The tissues were transferred to 70% ethanol and dehydrated in 100% ethanol. Dehydrated tissues were cleared in xylene, infiltrated, and embedded in paraffin. Tissue sections were cut at 3–5 μm for use in routine histology and immunohistochemical protocols.

2.2. Immunostaining for MT-3 in human skin and associated cancers

Serial sections were cut at 3–5 μm for use in immunohistochemical protocols. Staining was performed by a Leica Bond–Max automatic immunostainer. Major reagents for this procedure were contained in the Bond Polymer Refine Detection kit (Leica, DS9800). Paraffin sections were processed in the machine from deparaffinization to counterstaining by hematoxyline according to the manufacturer's recommended program settings with the following modifications. Briefly, the major steps in the protocol include deparaffinization, antigen retrieval for 20 min in Bond Epitope Retrieval Solution 1 (Leica, Catalog No AR9961), peroxide block for 5 min, incubation with rabbit anti-MT-3 antibody (1:200) for 25 min at room temperature, incubation with Post Primary for 10 min (source of the anti-rabbit IgG antibody), incubation with Polymer (source of the anti-rabbit Poly-HRP antibody) for 10 min, visualization with DAB (diaminobenzidine substrate for color development) for 10 min, counterstaining with hematoxylin for 5 min. Slides were rinsed in distilled water, dehydrated in graded ethanol, cleared in xylene, and coverslipped.The presence and degree of MT-3 immunoreactivity in the specimens was judged by two pathologists. The scale used was 0 to +3 with 0 indicating no staining, +1 staining of mild intensity, +2 staining of moderate intensity, and +3 staining of strong intensity.

2.3. Cell culture

The HaCaT cell line was obtained from Cell Line Services (Eppelheim, Germany). HaCaT were initially isolated from normal skin of a 62 year old Caucasian male donor and spontaneously immortalized through p53 mutation; they are nontumorigenic *in vivo* (Boukamp et al., 1988). The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 4.5 g/L glucose, 2 mM L-glutamine, 10% v/v fetal bovine serum (FBS), 0.25 μg/mL fungizone, 100 U/mL penicillin and 100 μg/mL streptomycin. HaCaT cells were given fresh medium every 72 h and subcultured at a ratio of 1:5. Normal human epidermal keratinocyte (NHEK) primary cells were obtained from Lonza (Walkersville, MD). NHEK were isolated from a 68 year old Caucasian male donor. The cells were maintained in KBM-Gold (Lonza, Walkersville, MD) supplemented with KGM-Gold™–BulletKit™ (Lonza, # 00192060). NHEK were seeded at a density of 3500 cells/cm^2 and given fresh media the day after seeding and then every 48 h until reaching 70–80% confluency. The human epidermal melanocyte primary cells isolated from a light pigmented donor were obtained from Gibco (HEMa-LP) (Carlsbad, CA), and are referred to as normal human melanocytes (NHM). NHM cells were maintained in Medium 254 supplemented with PMA-free Human Melanocyte Growth Supplement-2 (HMGS-2, Gibco, # S-016–5) 0.25 μg/mL fungizone, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were seeded at a density of 5000 cells/cm^2 and given fresh media the day after seeding and then every 48 h until reaching 80% confluency.

2.4. Treatment of cultured cells with 5-Aza-2′-deoxycytidine and histone deacetylase inhibitor MS-275

HaCaT, NHEK and Primary Melanocytes were seeded at a 1:10 ratio and the next day they were treated with 1 or 3 μM 5-Aza-2′-deoxycytidine (5-AZC) (Sigma–Aldrich, St. Louis, MO) or 1, 3 or 10 μM MS-275 (ALEXIS Biochemicals, Lausen, Switzerland). The cells were allowed to grow to confluency and then harvested for RNA isolation.

2.5. RNA Isolation and RT–PCR analysis

Total RNA was isolated from the cells according to the protocol supplied with TRI REAGENT (Molecular Research Center, Inc. Cincinnati, OH) as described previously by this laboratory (Somji et al., 2006). Real time RT–PCR was used to measure the expression level of MT-3 mRNA utilizing a previously described MT-3 isoform-specific primer (Somji et al., 2006). For analysis, 1 μg 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 and compared to that of a standard curve of the MT-3 isoform gene cloned into pcDNA3.1/hygro (+) and linearized with *Fsp* I. Cycling parameters consisted of denaturation at 95 °C for 30 s and annealing at 65 °C for 45 s which gave optimal amplification efficiency of each standard. The level of MT-3 expression was normalized to that of β-actin assessed by the same assay with the primer sequences being sense, CGACAACGGCTCCGGCATGT, and antisense, TGCCGTGCTCGATGGGGTACT, with the cycling parameters of annealing/extension at 62 °C for 45 s and denaturation at 95 °C for 15 s.

3. Results

3.1. MT-3 expression in human skin and related cancers

The expression of MT-3 was determined in normal human skin, *in situ* squamous cell carcinoma, squamous cell carcinoma, basal cell carcinoma, nevus, dysplastic nevus, *in situ* melanoma, superficial spreading melanoma, and deeply invasive melanoma (Table 1). Nine independent specimens of human skin were evaluated for the expression of MT-3. All nine specimens displayed immunoreactivity for MT-3 in the epidermis. For each specimen, the immunoreactivity for MT-3 was uniform throughout the epidermis and included staining of the basal keratinocytes (Fig 1A). Six of the specimens exhibited moderate to strong staining for MT-3 while the other 3 displayed mild to strong intensity of staining (Table 1). All squamous cell carcinomas (SCC) exhibited staining for MT-3 (Table 1), five strongly (Fig 1C), six moderately (Fig 1E), and one mild to moderate, whereas many of the basal cell carcinomas (BCC) exhibited low staining (Table 1) with two being totally devoid of MT-3 expression (Fig 2F), three weakly, and the rest (five samples) were either mild or mild to moderate (Fig 1D) in MT-3 staining. The staining of MT-3 was determined on 9 specimens of nevus. Staining for MT-3 was found for all 9 specimens, exhibited moderate to strong intensity, and was present in over 80% of the cells comprising the nevus (Table 1,Fig 2A). Staining of MT-3 was also performed on 1 case of dysplastic nevus and 3 cases of *in situ* melanoma. The staining was similar to that found in the nevus with all specimens displaying moderate to strong staining in over 80% of the cells (Table 1, Fig 2B). Staining of MT-3 was also performed on 4 cases of superficial spreading melanoma and 5 cases of deeply invasive melanoma. Again, the staining was similar to that found for *in situ* melanoma with moderate to strong staining of MT-3 in over 80% of the melanoma cells (Table 1, Fig 2C, D, E).

3.2. MT-3 expression in primary normal human keratinocytes (NHEK) and immortalized human HaCat keratinocytes

Proliferating and confluent cultures of NHEK and HaCaT cells were assessed for their expression of MT-3 mRNA and protein. Real time PCR demonstrated that both resting and dividing NHEK and HaCaT cells had only background levels of MT-3 mRNA expression (Fig 3A, B). Both sets of cells were also shown to express only background levels of MT-3 protein (data not shown). Both the NHEK and HaCaT cells were treated with MS-275, a histone deacetylase inhibitor, and 5-aza-2′-deoxycytidine, a DNA methylation inhibitor, to determine if MT-3 expression might be silenced by a mechanism involving histone modification or DNA methylation. The results demonstrated that treatment with MS-275 was effective in restoring MT-3 mRNA expression in both the NHEK and HaCaT cells (Fig 3A,B). While MS-275 treatment was effective in both cell lines, MS-275 increased MT-3 mRNA levels in NHEK cells 10 to 20 fold greater than those of the HaCaT cell line. Treatment of

Table 1
Immunostaining of MT-3 in normal skin, SCC[a], BCC[b], Nevi, and Melanoma.

Sample #	Diagnosis	Intensity[c]	Percentage[d]	Comments[e]
T06–02611	Normal skin	2–3+		
T06–04747	Normal skin	2+		
T06–08104	Normal skin	2–3+		
T06–12296	Normal skin	2–3+		
T06–13822	Normal skin	2–3+		
T06–15093	Normal skin	1–2+		
T06–15738	Normal skin	2–3+		
T06–16832	Normal skin	1–2+		
T06–17515	Normal skin	1–2+		
T06–05920-2	SCC	3+	90	Normal skin 2+
T06–08560-1	SCC	2–3+	80	Normal skin 2+
T06–10237-1	SCC	1+	60	Normal skin 1–2+
T06–17492-3	SCC	2+	80	Normal skin 2+
T06–18288	SCC	2–3+	90	No normal skin
T06–19042-2	SCC	2+	80	No normal skin
T07–02859-3	SCC	2–3+	70	Normal skin 2+
T07–04441-1	SCC	2+	60	Normal skin 2–3+
T07–05223-3	SCC	2+	80	Normal skin 1+
T07–07073-2	SCC	1–2+	30	No normal skin
T08–11243	SCC	2+	60	Normal skin 2+
T09–09643-1	SCC	2–3+	80	Normal skin 2+
T06–13947-1	SCC, in situ	2+	80	No normal skin
T06–00002	BCC	0–1+	30	Normal skin 1–2+
T06–03724-2	BCC	1+	60	No skin
T06–06686-8	BCC	0–1+	80	Normal skin 1–2+
T06–06889	BCC	1–2+	80	Normal skin 2+
T06–07160-2	BCC	0–1+	40	Normal skin 2+
T06–08133-2	BCC	NEG	0	Normal skin 1–2+
T06–09327-1	BCC	1+	60	Normal skin 1–2+
T06–10671-1	BCC	1+	40	Normal skin 1–2+
T07–00753-2	BCC	NEG	0	No normal skin
T07–07197-3	BCC	1–2+	60	Normal skin 1–2+
T06–05434	Nevus	2–3+	80	
T06–13829	Nevus	2–3+	80	
T06–15352	Nevus	2–3+	80	
T06–16045	Nevus	2–3+	80	
T06–16174	Nevus	2–3+	80	
T06–16832	Nevus	2–3+	80	
T06–16983	Nevus	2+	80	
T06–18076	Nevus	2+	80	
T06–18753	Nevus	2–3+	80	
T06–17364	Nysplastic nevus	2–3+	80	
T06–02621	in situ melanoma	2–3+	80	
T06–09160	in situ melanoma	2–3+	80	
T06–11933	in situ melanoma	2–3+	80	
T06–08497	Nuperficial spreading melanoma	2–3+	80	
T06–11336	Superficial spreading melanoma	2–3+	80	
T06–11854	Superficial spreading melanoma	2–3+	80	
T06–17070	Superficial spreading melanoma	2–3+	80	
T06–02282	Deeply invasive melanoma	2–3+	80	
T06–03697	Deeply invasive melanoma	2–3+	80	
T06–05435	Deeply invasive melanoma	2–3+	80	
T06–07039	Deeply invasive melanoma	2–3+	80	
T06–16580	Deeply invasive melanoma	2+	80	

a: Squamous Cell Carcinoma, b: Basal Cell Carcinoma,c: Intensity, scored by a pathologist see Materials and methods, d: Percentage of the microscopic field that exhibits staining, e: description of the staining within the normal skin component of the sample.

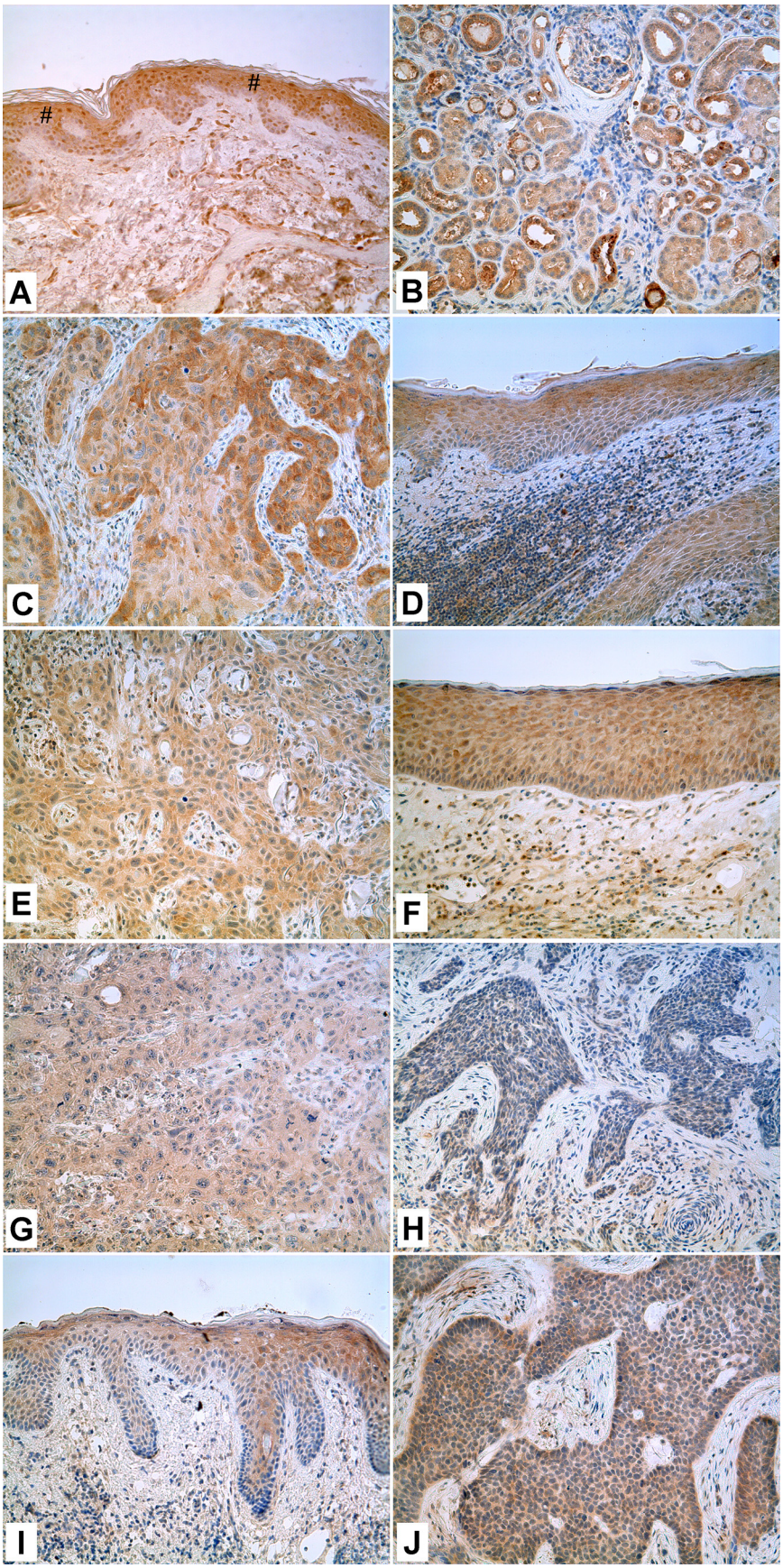

Fig. 1. Immunostaining of MT-3 in Skin Pigment Lesions. A: normal skin shows mild to moderate staining of MT in epidermis (#). In some cases the staining of MT-3 in epidermis can be moderate to strong (not shown); B: normal kidney as positive control; C: squamous cell carcinoma (SCC) (T06–5920) with strong staining of MT-3 (3 +); D: normal skin from the same slide in "C", showing moderate positivity of MT-3 (2 +); E: SCC (T07–4411) with mild staining of MT-3 (2 +); F: normal skin from the same slide as

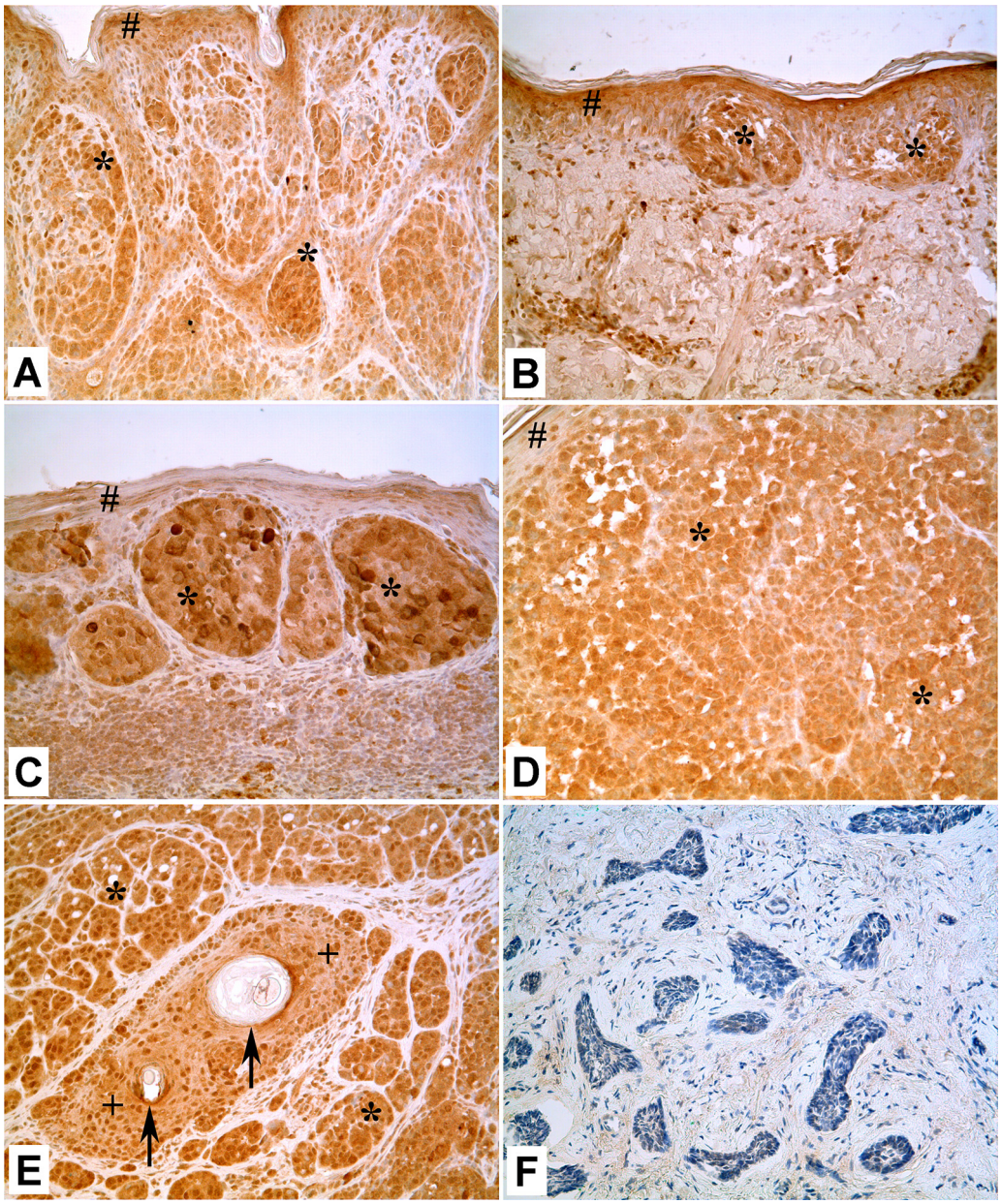

Fig. 2. Immunostaining of MT-3 in Nevi and Melanoma Cells. A: in this intradermal nevus, the neval cells, which located in the dermis, show moderate to strong expression of MT-3 (*). The epidermis at the top of the image is moderately positive for MT-3 (#); B: in this case of in situ melanoma, two nests of melanoma cells in the epidermis are moderately to strongly positive for MT-3 (*). The epidermis is moderately positive for MT-3 (#); C: in this superficial spreading melanoma, some melanoma nests, located in the superficial dermis, show strong staining of MT-3. The overlying epidermis is mildly positive for MT-3 (#); D: in this deeply invasive melanoma, the tumor cells are strongly positive for MT-3 (*). In the top-left corner is a small section of epidermis (#), which is mildly positive for MT-3 (#); E: another case of deeply invasive melanoma. In the center of this image is a hair follicle (+) with two keratin pearls (arrows) in it. The hair follicle shows moderate staining of MT-3. Around the follicle are nests of invasive melanoma cell (*), which show strong staining of MT-3. F: BCC (T06–8133) with negative staining of MT-3.

the NHEK and HaCaT cells with 5-aza-2′-deoxycytidine, resulted in a small, but statistically insignificant increase in MT-3 mRNA expression for both cell types (Fig 3A, B).

3.3. MT-3 expression in normal human melanocytes (NHM)

Normal human melanocytes (from a light pigmented donor) were assessed in proliferating and confluent cultures for expression of MT-3 mRNA and protein. Both cell cultures showed only background levels of mRNA as demonstrated with real-time PCR (Fig 4) and only background levels of protein (data not shown). In order to test for epigenetic regulation, cultures were treated with the histone deacetylase inhibitor, MS-275 and the DNA methylation inhibitor, 5-aza-2′-doxycytidine. The results demonstrated that treatment with MS-275 was effective in restoring MT-3 mRNA expression in the NHM cells (Fig 4). Treatment of the NHM with 5-Aza-2′-deoxycytidine, resulted in only a slight but statistically significant increase in MT-3 mRNA expression (Fig 4).

shown in “E”, showing moderate staining of MT-3 (2 +); G: SCC (T06–10237) with mild staining of MT-3 (1 +); H: basal cell carcinoma (BCC) (T06–00002) with focal, very weak staining of MT-3 (0–1 +); I: Normal skin from the same slide as shown in “H”, showing mild to moderate staining of MT-3 (1–2 +); J: BCC (T06–7889) with mild-moderate staining of MT-3 (1–2 +). All images taken at 200X magnification.

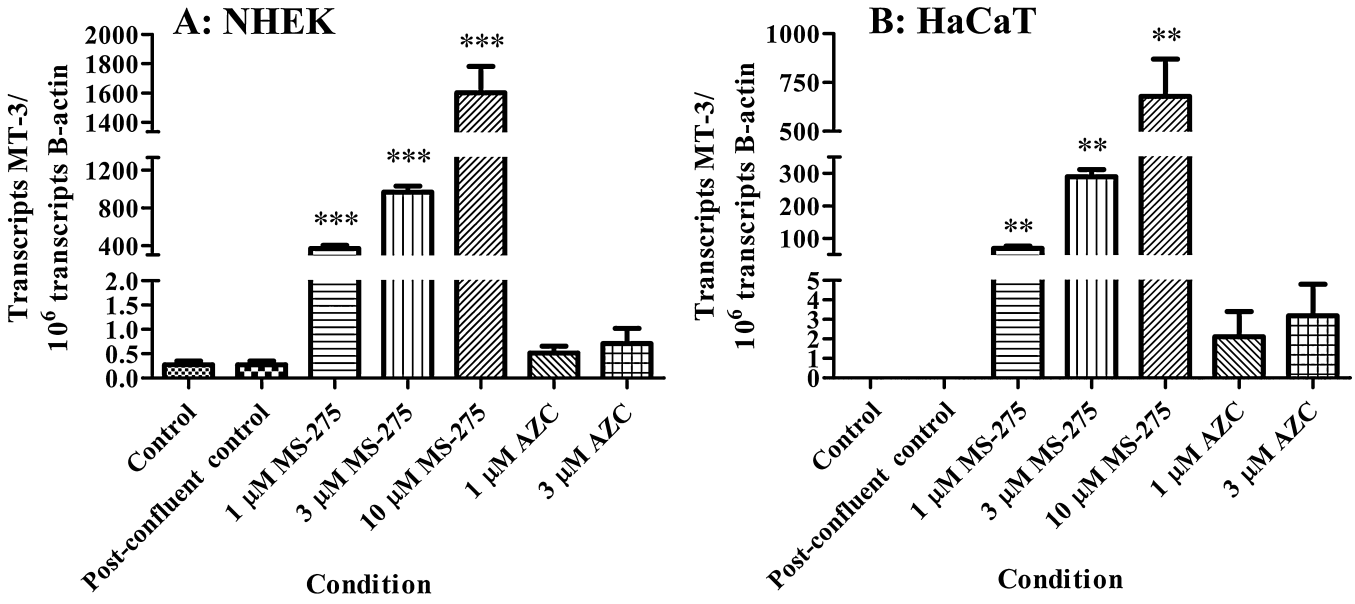

Fig. 3. Expression of MT-3 in primary normal human keratinocytes (NHEK) and Immortalized Human HaCaT Keratinocytes. HaCaT and NHEK cells were exposed to 1, 3, or 10 μM of the histone deacetylase inhibitor MS-275 for up to 72 h, or to 1 or 3 μM 5-azacytidine for 24 h. The post-confluent control was held at confluency for at least 72 h. Messenger RNA levels for MT-3 were assessed using real-time PCR and gene specific primers on RNA isolated from cells in culture. Expression levels are expressed as transcripts per 1000,000 transcripts of β-actin which was also assessed using real-time PCR. Significant differences from control groups are designated as $^{**}p < 0.005$, $^{***}p < 0.0005$ as determined by student's unpaired *t*-test.

4. Discussion

The present study establishes that MT-3 is expressed in human skin. The immunoreactivity for MT-3 was present in all viable keratinocytes comprising the epidermis. The finding that MT-3 was present in the epidermal keratinocytes has a potential impact on the known association of arsenic exposure and the development of skin disorders and related cancers. All members of the MT gene family (MT-1, -2, -3 and -4) are known to bind heavy metals, including As^{+3} (Vasak and Meloni, 2011; Irvine et al., 2013; Garla et al., 2013). Previous studies employing a monoclonal antibody against the E-9 epitope of the MT-1, -2, and -4 isoforms demonstrated that these 3 isoforms are poorly expressed in human skin and with expression restricted to the basal keratinocytes (Van den Oord and Delay, 1994; Karasawa et al., 1991; Zamirska et al., 2012). The high sequence homology of these 3 isoforms prevents the generation of specific antibodies to the individual isoforms. In contrast, the present study shows that a large majority of keratinocytes in the epidermis of normal human skin are moderately to strongly immunoreactivity for MT-3. These findings were consistent for 9 independent samples of human skin. The antibody used for the localization of MT-3 is specific since it was generated against the unique C-terminal amino acid sequence that is present only in this MT isoform (Garrett et al., 1999). The fact that human keratinocytes contain substantial levels of MT-3, and that MT-3 can bind As^{+3}, suggests a possible role for MT-3 in the selective accumulation and sequestering of As^{+3} in skin. One hypothesis to explain why skin is highly responsive to arsenic exposure and cancer development is that skin localizes and store As^{+3} due its high keratin content and the corresponding favorable interaction with sulfhydryl groups (Kitchen, 2001; Lindgren et al., 1982). The current finding suggests that MT-3 might play an additive, or possibly larger, role in the ability of skin to sequester and store As^{+3} in individuals chronically exposed to this metalloid.

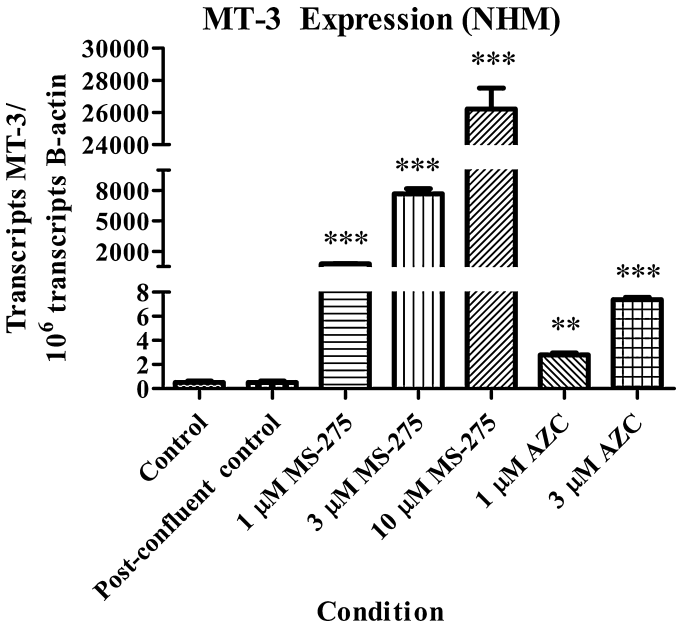

Fig. 4. Expression of MT-3 in normal human melanocytes (NHM). HEMa-LP cells were exposed to 1, 3, or 1 μM of the histone deacetylase inhibitor MS-275 for up to 72 h, or to 1 or 3 μM 5-azacytidine for 24 h. The post-confluent control was held at confluency for at least 72 h. Messenger RNA levels for MT-3 were assessed using real-time PCR and gene specific primers on RNA isolated from cells in culture. Expression levels are expressed as transcripts per 1000,000 transcripts of β-actin which was also assessed using real-time PCR. Significant differences from control groups are designated as $^{**}p < 0.001$, $^{***}p < 0.0001$ as determined by student's unpaired *t*-test.

Evidence to support the concept that MT expression in a normal target tissue can elicit chronic effects can be found in the nephropathy associated with chronic exposure to cadmium. The MT-1 and MT-2 isoforms are predominantly expressed in the proximal tubules of the human kidney, and this expression is initiated during the early development of the kidney (Mididoddi

et al., 1996). The MT-3 isoform is also expressed in the proximal tubules and other tubular elements of the human kidney (Garrett et al., 1999). The cortex of the human kidney has been shown to accumulate cadmium, as a function of age, in humans without occupational exposure (Satarug et al., 2002, 2010). Accumulation is assumed to occur through cadmium's interaction with MT and accumulation has been shown to reach a plateau at approximately 50 years of age. Despite the MT's being looked upon as having a protective role against heavy metal toxicity in general, and the proximal tubule in particular (Liu et al., 1995, 1998, 2000; Masters et al., 1994), the fact remains that the kidney and the proximal tubule is the cell type critically affected by chronic exposure to cadmium (Andrews, 2000; Bernard et al., 1976; Bosco et al., 1986; Gonich et al., 1980). It has been shown in human population studies that even low exposure to cadmium alters renal tubule function (Akesson et al., 2005). Thus, there is evidence in the kidney that pre-existing expression of MT in the renal tubules both protects the kidney from cadmium exposure, but this expression might also render the organ susceptible to the chronic effects of the metal. There is little evidence, either for or against, that would support a similar role for MT-3 expression in human skin as regards the chronic effects of exposure to arsenic.

The present study demonstrates that MT-3 is prominently expressed in the majority of cells comprising the nevus, dysplastic nevus, *in situ* melanoma, superficial melanoma, and deeply invasive melanoma. Although the sample set was relatively small, there was no indication that expression was variable within or between disease categories. A consequence of this pattern of constant MT-3 expression is that the melanocytes, in all stages of progression, are able to continue to bind and accumulate As^{+3} in an environment where exposure to As^{+3} is at elevated levels. Unfortunately, there is very little information in the literature on conditions or mechanisms *in vivo* that would influence the release of As^{+3} from MT-3 inside a cell or tissue. One could speculate that if ultraviolet radiation influenced the release of As^{+3} from MT-3, it might impact on emerging research which suggests a linkage between the development of melanoma and co-exposure to As^{+3} and ultraviolet radiation (Cooper et al., 2014). The expression of MT-1 and -2 has been examined in patients with melanoma. It was shown that a gain of expression of MT-1 and -2 is an adverse prognostic and survival factor for patients with this cancer (Weinlick et al., 2003, 2006). In contrast to MT-3, MT-1 and -2 is not expressed in the nevus and is gained later during the development of the cancer.

The present study also shows that MT-3 is expressed in the normal human epidermal keratinocytes that would give rise to these cancers. The examination of these cancers show that all SCC exhibit robust expression of MT-3, and that the majority of BCC express MT-3 although a significant proportion express mild levels and some BCC failed to immunostain for this protein.

The results of the present study also show that cell cultures of NHEK, HaCaT immortalized human keratinocytes, and normal human melanocytes do not express MT-3 as would be unexpected from their *in situ* patterns of MT-3 expression. This observation shows that these cell lines are lacking a protein that can both bind and sequester As^{+3} as well as serving as an antioxidant. The MT-3 protein has also been shown to have growth inhibitory activity outside the neural system (Gurel et al., 2003), be involved in necrotic and apoptotic cell death (Somji et al., 2004, 2006) and in the epithelial to mesenchymal transition (Kim et al., 2002; Bathula et al., 2014). Exactly how this might impact on studies using these cell lines to elucidate the mechanism/s of As^{+3} toxicity and carcinogenicity is unknown, but may need to be considered in the interpretation of past and future studies. The loss of MT-3 expression in cell cultures derived from tissues where MT-3 is expressed may be a result of the cell culture environment. This is suggested by studies on MT-3 expression in bladder cancer and breast cancer cell lines. This laboratory has shown that the epithelial cells of the human bladder and breast do not express MT-3, but that the majority of patient specimens of breast and bladder cancers do express MT-3 (Sens et al., 2000, 2001; Zhou et al., 2006; Somji et al., 2010). In studies examining MT-3 expression in As^{+3} and Cd^{+2} transformed bladder cancer cell lines and in MCF-7, T-47D, Hs 578 t, MDA-MB-231 breast cancer cell lines it was demonstrated that none of the cell lines expressed MT-3 (Zhou et al., 2006). However, when these cell lines were transplanted into immune compromised mice, all the resulting tumors showed prominent expression of MT-3. It has also been shown that the expression of MT-3 mRNA could be induced under cell culture conditions in the MT-3 non-expressing cell lines following treatment with MS-273, a histone deacetylase inhibitor (Somji et al., 2010, 2011). These results suggest that MT-3 is silenced under cell culture conditions by a mechanism involving histone acetylation.

Previous to the submission of this manuscript, no studies of MT-3 expression in human skin or derived cancers existed in the literature; however, recently a study was published during the review process that documents the expression of MT-3 in human skin, both in normal as well as BCC and SCC (Pula et al., 2014). The findings of this study are in overall agreement with the above findings with the exception that they have found higher levels of MT-3 in SSC whereas the current study did not. Nevus, melanoma and cultured cell models were not assessed in this study.

Contributions

AS, YZ, XDZ, MAS: Performed the immunohistochemical studies on human skin and derived cancers.

AS, SHG, SS: Performed the studies on MT-3 expression in NHEK, HaCaT, and Human Melanocytes.

DAS: Designed the study, organized group meetings, provided core facility support, and wrote the manuscript with assistance (SHG) and graduate student (AS).

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2014.09.028.

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