Overabundance of Putative Cancer Stem Cells in Human Skin Keratinocyte Cells Malignantly Transformed by Arsenic

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Arsenic is a human skin carcinogen. Cancer is probably a disease driven by stem cells (SCs), and SCs are likely a key target during arsenic oncogenesis. *In utero* arsenic exposure predisposes mice to skin cancers that overproduce cancer SCs (CSCs) and have distorted CSC signaling and population dynamics. Therefore, we hypothesized CSC accumulation may occur during arsenic-induced malignant transformation *in vitro* of human skin keratinocytes. Thus, the HaCaT cell line, malignantly transformed by arsenite (100nM, 30 weeks; termed As-TM cells) in prior work, was further studied for the quantity and nature of SCs after this transformation. SCs were isolated from passage-matched control and As-TM cells by a magnetic bead system that enriches for CD34-positive cells. There were 2.5 times more SCs isolated from As-TM cells than control. Holoclone production from As-TM putative CSCs was 2.5-fold higher by 1 week and 3.5-fold higher by 2 weeks than control SCs. Potential malignant phenotype was assessed in isolated SC/CSCs. Transcript level of SC/CSC markers were elevated in both isolated As-TM CSCs and control SCs compared with parental cells, but compared with control SCs, As-TM putative CSCs had elevated *CD34*, *K5*, *K14*, *K15*, and *K19* transcripts and dramatically stronger staining for p63, Rac1, K5, Notch1, and K19. As-TM putative CSCs also showed markedly elevated MMP-9 secretion and colony formation, indicators of cancer phenotype, even compared with total population of As-TM cells. Thus, malignant phenotype is particularly pronounced in CSCs after arsenic-induced transformation of human skin cells and occurs concurrently with a potential overproduction of these cells.

Key Words: arsenic; skin cancer; cancer stem cell.

Inorganic arsenic is an environmental drinking water contaminant and affects millions of people (IARC, 2004). The skin is a major target of arsenic, and the metalloid is considered a human skin carcinogen (IARC, 2004; Straif *et al.*, 2009). Chronic exposure to inorganic arsenic in humans causes various skin cancers, including squamous cell carcinoma (SCC) (IARC, 2004). Although inorganic arsenic does not

seem sufficient alone to cause skin cancer in animals, it definitely acts together as a cocarcinogen, stimulating skin cancer induced by UV irradiation (Rossman *et al.*, 2001, 2002) or by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Germolec *et al.*, 1997, 1998) in mouse skin. SCCs resulting from combined arsenic and UV treatments are also more aggressive than those produced by UV alone (Rossman *et al.*, 2001, 2002). Additional work has shown *in utero* arsenic exposure predisposes mice to subsequent formation of chemically induced SCCs in Tg.AC mice and that these carcinomas are similarly more highly aggressive than usual (Waalkes *et al.*, 2008). Interestingly, premalignant lesions of the skin induced by arsenic in humans are considered more clinically aggressive than is typical for such lesions (Schwartz, 1996). Furthermore, *in vitro* inorganic arsenic causes malignant transformation of human skin keratinocytes that produce SCCs upon inoculation into mice (Pi *et al.*, 2008).

Human skin epidermis can be viewed as an initial line of external defense to protect against environmental injury (Mimeault and Batra, 2006), regulated by a finely tuned network of interactions at the cell-to-cell and cell-to-matrix levels. The regenerative capacity of skin epidermis is maintained by different stem cell (SC) subpopulations located in specialized niches (Fuchs, 2008). In this regard, epidermal SCs are likely key target cells in skin carcinogenesis (Kangsamaksin *et al.*, 2007). The idea of cancer SCs (CSCs) that arise from normal SCs was initially proposed 150 years ago (see Sell, 2004) but has yet to be completely established. SCs and CSCs share several important properties including the self-renewal capacity, conditional immortality, and high proliferative potential (Wicha *et al.*, 2006), characteristics normally allowing pluridirectional replenishment of mature cells via SC differentiation. However, in oncogenesis, the deranged parallel functions of CSCs contribute to heterogeneous aberrant cancer cell production and overgrowth (Kangsamaksin *et al.*, 2007; Pardal *et al.*, 2003). CSCs

normally form a very small portion of the tumor. Nonetheless, they are critical in oncogenesis because they can essentially regenerate tumors through the processes of self-renewal and differentiation into heterogeneous tumor cell types (Pardal *et al.*, 2003).

Accumulating evidence shows that SCs may be key targets during inorganic arsenic oncogenesis. Inorganic arsenic can slow epidermal SC exit into differentiation pathways, thereby distorting population dynamics in human skin models *in vitro* (Patterson and Rice, 2007; Patterson *et al.*, 2005). In doing so, arsenic may increase the proportion of SCs and create more target cells for carcinogen attack (Patterson and Rice, 2007; Patterson *et al.*, 2005). Furthermore, *in vitro* chronic arsenic exposure can directly induce malignant transformation of human SCs (Tokar *et al.*, 2010a) providing clear evidence that SCs can be targeted. In mice, *in utero* arsenic exposure predisposes offspring to skin SCC formation in adulthood in association with the production of an overabundance of tumor CSCs and distortion of CSC signaling and population dynamics, while creating significantly more aggressive cancers (Waalkes *et al.*, 2008). Additionally, CSCs are overproduced in a human heterogeneous prostate cell line as it is malignant transformed by inorganic arsenic (Tokar *et al.*, 2010b), which is consistent with skin SCC facilitated by *in utero* arsenic exposure, which shows a distinctive CSC overabundance (Waalkes *et al.*, 2008). The prostate is also a suspected target of arsenic carcinogenesis in humans (Straif *et al.*, 2009). Thus, defining the role of CSCs is critical to understanding the process involved in arsenic-induced oncogenesis.

Therefore, in this study, SCs/CSCs were isolated from a human skin keratinocyte line (HaCaT cells) using a magnetic bead isolation system that enriches CD34-positive cells. The HaCaT cell line is normally nontumorigenic and was malignant transformed by chronic inorganic arsenic exposure (100nM, 30 weeks) in prior work (Pi *et al.*, 2008). These arsenic-exposed cells (termed arsenic transformed malignant; As-TM) produced aggressive SCC in a mouse xenograft study (Pi *et al.*, 2008). CD34 is a robust surface marker for both normal skin SCs and CSCs (Malanchi *et al.*, 2008; Trempus *et al.*, 2003, 2007). After isolation, quantity was estimated, and malignant characteristics were assessed in CD34-positive putative SC/CSCs derived from control cells and As-TM cells.

MATERIALS AND METHODS

Chemicals and antibodies. Sodium arsenite (NaAsO_2) was obtained from Sigma Chemical Co. (St Louis, MO). Type IV collagen was obtained from Trevigen (Gaithersburg, MD). Arsenite was dissolved in sterile distilled water to make stock solutions. The primers for real-time PCR analysis were synthesized by Sigma-Genosys (The Woodlands, TX). Primers include *CD34*, *K1*, *K5*, *K10*, *K13*, *K14*, *K15*, *K18*, *K19*, *p63*, and *Notch1*. The antibodies against CD34, K1, and K5 were from Abcam Inc. (Cambridge, MA), antibodies against K13, K19, Rac1, Notch1, and p63 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the antibody against β -actin was from Sigma Chemical Co.

Cell culture and treatments. The HaCaT skin keratinocyte cell line was originally derived from normal human adult skin and was spontaneously immortalized through p53 mutation (Boukamp *et al.*, 1988). HaCaT cells are nontumorigenic *in vivo*, are initially hypodiploid, and maintain full epidermal differentiation capacity (Boukamp *et al.*, 1988). As-TM cells, derived from HaCaT cells, are malignant transformed by chronic exposure to a low nontoxic level of arsenic (100nM) in a prior study and form SCCs upon inoculation into nude mice (Pi *et al.*, 2008). As-TM cells and passage-matched control HaCaT cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mixture (all from Gibco, Rockville, MD). Cultures were maintained at 37°C and in a humidified 5% CO_2 atmosphere and passaged subconfluent once a week. The culture medium was refreshed every 3 days. For the As-TM line, cells were maintained continuously in a medium containing 100nM of arsenic.

In some instances, a HaCaT cell line, malignant transformed by ultraviolet (UV) irradiation that will also form SCC upon inoculation into nude mice (He *et al.*, 2006), was used with similar culture conditions. It was used for assessment of CD34-positive SC quantity and formation of holoclones (see below). Since malignant transformation occurred at a different time point for these UV transformed cells, data are expressed as percent control for comparative purposes.

SCs selection and holoclone formation. HaCaT SCs and As-TM putative CSCs were selected from passage-matched control HaCaT cells and As-TM cells, respectively, using Dynal CD34 Progenitor Cell Selection System (Invitrogen, Carlsbad, CA). CD34 is a recognized surface marker for both human normal skin SCs and CSCs (Malanchi *et al.*, 2008; Trempus *et al.*, 2003, 2007). CD34 can also be expressed in a variety of nonskin SCs. Cells were trypsinized with 0.25% trypsin (Invitrogen), washed by PBS buffer, and transferred into 50 ml tubes. After centrifuging for 5 min at 2000 rpm, the supernatant was removed, and the cell pellet was suspended in PBS buffer. Cells were passed through a 40 μm cell strainer (BD Biosciences) to obtain a single cell suspension. Cell number was then counted and volume was measured. Dynabeads were resuspended, and the desired volume ($100 \mu\text{l}/4 \times 10^7$ cells) was transferred to a 15 ml tube. Cells were incubated with Dynabeads at 4°C for 30 min with gentle tilting and rotation. After washing with cold PBS three times in a magnet (DynaMag-15, Invitrogen), bead-bound cells were resuspended in PBS buffer. The bead-bound cells were incubated at room temperature with DETACHaBEAD for 45 min with gentle tilting and rotation. Supernatant containing released cells was transferred to a fresh tube, and the beads were washed three times in PBS buffer. The cells were washed in 10 ml PBS buffer and centrifuged for 10 min at $600 \times g$ to remove excess DETACHaBEAD. The isolated cells were counted and cultured in type IV collagen-coated ($2 \mu\text{g}/\text{cm}^2$) tissue culture dishes.

A holoclone is a colony of cells enriched in SCs/CSCs derived from a single cell and is thought to be indicative of the self-renewal capability typical of SCs or CSCs (Li *et al.*, 2008). Holoclones are large and round colonies with a smooth perimeter mostly containing small cells. For holoclone formation, the same number of isolated CD34-positive putative SCs/CSCs was plated in collagen-coated dishes (5000/dish), and formation was assessed after 1 and 2 weeks. Holoclones were used because they are typically formed by, and contain, stem-like cells (Li *et al.*, 2008). They are distinguished from merclones and paraclones by morphology (Tokar *et al.*, 2010b). Merclones and paraclones were not included in the present assessment because of their more differentiated phenotype (Li *et al.*, 2008). To further distinguish holoclones, a single holoclone was repeatedly subcultured from As-TM CD34-positive cells and found able to produce more holoclones.

Real time PCR analysis. Total RNA was isolated with TRIzol (Invitrogen) and purified with RNeasy Mini kit (Qiagen, Valencia, CA) from cells subcultured for 1 week from holoclones (from CD34-positive putative SCs/CSCs) or cultured normally (i.e., parental lines, control HaCaT, and As-TM). The quality of RNA was determined by the 260/280 ratios (1.7–1.8). RNA was reverse transcribed with MuLV reverse transcriptase and oligo-d(T) primers. The primers for selected genes were designed using Primer Express Software

(Applied Biosystems, Foster City, CA). The Absolute SYBR Green ROX Mix (ThermoFisher Scientific, Rochester, NY) was used for real-time PCR analysis. Relative differences in gene expression between groups were expressed using cycle time (Ct) values. These Ct values were first normalized with that of *GADPH* in the same sample and then expressed as percentage with control set to 100%. Real-time fluorescence detection was carried out using a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

Immunofluorescence. Cells subcultured for 1 week from holoclones (from CD34-positive putative SCs/CSCs) or cultured normally (parental lines) were plated on Lab-Tek chambered cover glass chamber slides (Nunc, Rochester, NY). Cells were washed twice with PBS and fixed for 2 min in acetone:methanol (1:1). The cells were incubated for 1 h with 3% bovine serum albumin (BSA) in PBS to block nonspecific antibody binding followed by incubation for 1 h with primary antibodies against K1 and K5, p63, K13, Rac1, K19, and Notch1, each diluted 1:100 in 3% BSA in PBS. The cells were washed three times in PBS and then incubated in dark for 1 h at 37°C with Alexa Fluor 488 goat anti-rabbit IgG (H + L) and Alexa Fluor 568 goat anti-mouse IgG (H + L) secondary antibodies (Invitrogen) diluted 1:200 in PBS buffer containing 3% BSA. The cells were washed three times with PBS and incubated in DAPI (4',6-diamidino-2-phenylindole) solution (1:1000 in PBS, Invitrogen) for 5 min in the dark. After washing with PBS twice, cells were imaged by fluorescent microscopy.

Zymographic analysis of metalloproteinase-9 (MMP-9) activity. Cells at 70–80% confluence were washed three times with PBS, and the medium was changed to serum-free DMEM. After 48 h, the conditioned medium was collected on ice for zymographic analysis of metalloproteinase-9 (MMP-9). MMP-9, a member of matrix-degrading enzyme family, plays a crucial role in tumor invasion (Bernhard *et al.*, 1994). An elevated expression level of MMP-9 is strongly correlated with malignant phenotype in SCC (Bernhard *et al.*, 1994) and is characteristic of malignant transformation of arsenic-induced cells (Pi *et al.*, 2008). MMP-9 activity was detected as described previously (Sun *et al.*, 2009). After staining with SimplyBlue Safestain (Invitrogen), the bands were quantified with Image J software.

Soft agar cloning assay. Cells were subcultured for 1 week from holoclones (from CD34-positive putative SCs/CSCs) and passed through a 40 μ m cell strainer (BD Biosciences, Bedford, MA) to get a single cell suspension. Plates were prepared by adding 2 ml agar medium (0.8 ml of 1.25% agar and 1.2 ml of DMEM with 10% FBS and 10% Dulbecco's Phosphate-Buffered Saline) and allowed to harden at room temperature. The cells (12,500 cells/35 mm plate) were suspended in 1 ml DMEM with 10% FBS and 0.33% agar and plated. These plates were maintained at 37°C for 21 days. On the final day of the assay, 1 ml of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT, Sigma-Aldrich, St Louis, MO) was added to each plate and incubated at 37°C for 24 h. Colonies were then counted using an automated colony counter.

Statistical analysis. Data are expressed as mean $\pm$ SEM of 3–6 determinations. For statistical comparisons, a Student's *t*-test or an ANOVA followed by a Tukey-Kramer multiple comparisons test were employed as appropriate. The level of significance was set at $p < 0.05$ in all cases.

RESULTS

More Putative CSCs Are Isolated from As-TM Cells Than SCs from Control

Putative SCs/CSCs were enriched from control cells and As-TM cells by assessing CD34-positive cells using a magnetic bead separation system (see "Materials and Methods"). The portion of CD34-positive SCs isolated from control cells was 0.13% of the total cells (Fig. 1A). In marked contrast, the

FIG. 1. CD34-positive SCs/CSCs isolation and holoclone formation and depiction. (A) Isolation of CD34-positive putative CSCs from As-TM cells compared with SCs from control cells. (B) Holoclone production from control-derived SCs and in As-TM-derived putative CSCs over 2 weeks. (C) Morphological imaging of typical holoclones from control-derived SC and As-TM-derived putative CSC (bar = 20 microns). An asterisk (*) indicates a significant increase ($p < 0.05$) compared with control.

portion CD34-positive putative CSCs isolated was 0.30% of the total As-TM cell pool (Fig. 1A), 2.3-fold higher than the number isolated from total control cells.

CD34-positive SCs were also enriched from HaCaT cells malignantly transformed by UV irradiation (He *et al.*, 2006). Unlike As-TM cells, enrichment of UV transformed cells resulted in an amount of CD34-positive SCs (113% of control $\pm$ 22.7%, mean $\pm$ SEM, $n = 6$) that was statistically insignificant from control cell enrichment (100% $\pm$ 1.1%; $n = 6$).

Enhanced Holoclone Production from CSCs Isolated from As-TM cells

A holoclone is a group of cells enriched in SCs/CSCs, which is derived from a single cell and is indicative of the self-renewal capability typical for SCs or CSCs. Holoclones are large and round growing colonies, have a smooth perimeter, and mostly contain small cells. The same number of CD34-positive putative SCs/CSCs isolated from control and As-TM cells was plated in collagen-coated dishes, and holoclone formation was assessed after 1 and 2 weeks. Holoclones were generated by both control-derived SCs and As-TM-derived putative CSCs (Fig. 1B). However, by 2 weeks of subculture, the number of holoclones produced by As-TM-derived putative CSCs was 3.5-fold higher than from control-derived SCs. In addition, holoclones from As-TM-derived putative CSCs generally appeared to grow faster and typically appeared larger

than those generated by the SCs derived from control cells (Fig. 1C). This is indicative of rapid and poorly controlled proliferation, suggesting distorted self-renewal. Thus, arsenic-induced malignant transformation apparently increases putative CSCs in this human skin keratinocyte cell line.

In contrast, the UV-transformed HaCaT cells (He *et al.*, 2006), when similarly cultured formed no more holoclones than control over a 2-week period. For example, holoclone formation rate using CD34-positive cells from UV-transformed HaCaT cells ($81\% \pm 22\%$, $n = 5$) was not significantly different from the rate of holoclone formation for that of CD34-positive cells from control cells ($100\% \pm 16\%$; $n = 5$).

Expression of SC/CSC Markers

Characteristics of SC/CSCs isolated from control and As-TM cells were assessed and compared with their heterogeneous parental lines. CD34 is a cell surface marker for both SCs and CSCs in skin (Trempey *et al.*, 2003). Transcript level of *CD34*

and other SC/CSC self-renewal-related markers (*K5*, *K14*, *K15*, and *K19*) were elevated in both putative CSCs derived from As-TM cells and SCs derived from control cells when compared with their parental cells (Fig. 2). However, the transcript levels of these markers were consistently much more highly expressed in As-TM-derived putative CSCs than in control-derived SCs. Furthermore, the expression of the keratinocyte differentiation marker *K18*, although decreased in both SC groups compared with parental cells (Fig. 2), showed lower expression in As-TM-derived putative CSCs than control-derived SCs. Western blot confirmed these increases in CD34 and K5 (data not shown). Additionally, it was quite evident that Notch1 and K19 were coexpressed specifically in As-TM putative CSCs and showed increased protein levels compared with control-derived SCs (Fig. 3). Furthermore, p63 and Notch1 were coexpressed and overexpressed in As-TM putative CSCs (Supplementary fig. 1). Likewise, Rac1, a gene critical to skin SC self-renewal, was

FIG. 2. Expression of SC/CSC markers. Expression of selected skin SC/CSC markers at the transcript level (see “Materials and Methods”) in CD34-positive control SCs or As-TM putative CSCs. An asterisk (*) indicates a significant difference ($p < 0.05$) compared with cell-matched total control. A cross (†) indicates a significant difference ($p < 0.05$) compared with control-derived SCs. A triangle (▲) indicates a significant difference ($p < 0.05$) compared with As-TM cells. Multiple comparisons († and ▲) are shown only for As-TM putative CSCs. (A) *CD34*, (B) *K5*, (C) *K14*, (D) *K15*, (E) *K19*, (F) *K18*.

FIG. 3. Immunofluorescence images of SC/CSC markers Notch1 and K19 expression. Expression of various skin SC/CSC markers at the protein level in CD34-positive control SCs or As-TM putative CSCs by fluorescence microscopic imaging (bar = 20 microns). DAPI is a blue stain showing cells, and the colocalization of Notch1 and K19 is shown in the far right column (yellow).

overexpressed, and K5 was highly expressed in As-TM putative CSCs (Supplementary fig. 2). Thus, the putative CSCs derived from As-TM cells overexpress various genes that are markers typical for skin CSCs at the transcript and protein levels (Gerdes and Yuspa, 2005; Kanitakis *et al.*, 1999; Liu *et al.*, 2003; Lyle *et al.*, 1998; Malanchi *et al.*, 2008; Trempus *et al.*, 2003).

Enhanced Malignant Phenotype in As-TM-Derived CSCs

Studies were performed to assess further if arsenic had indeed produced a subset of cells with a high malignant phenotype (i.e., CSCs). Increased secreted MMP-9 activity is an excellent marker for arsenic-induced malignant transformation (Pi *et al.*, 2008; Tokar *et al.*, 2010a). Total population As-TM cells showed elevated secreted MMP-9 activity compared with total control cells (Fig. 4A). Furthermore, As-TM-derived CSCs showed even more elevated MMP-9 activity than total

population As-TM cells. A greatly increased *MMP-9* transcript level in As-TM-derived CSCs was consistent with this increased MMP-9 activity (data not shown). Colony formation in soft agar is another marker for malignant transformation. Again, both total population As-TM cells and As-TM-derived CSCs showed significant increases in colony formation compared with total control cells and control-derived SCs. In addition, the number of colonies formed from As-TM-derived CSCs was approximately 1.6-fold higher than total population As-TM cells (Fig. 4B). *PTEN* acts as a tumor suppressor that modulates key cell signaling pathways, has critical roles in SC differentiation, and is often inactivated in malignancies (Di Cristofano and Pandolfi, 2000). We find it is also often inactivated with arsenic transformation (Tokar *et al.*, 2010a). The expression of *PTEN* in As-TM-derived CSCs was markedly reduced when compared with total control or control-derived SCs (Fig. 4C). Thus, the subset of As-TM-derived CSCs

FIG. 4. Assessment of malignant phenotype in isolated SCs/CSCs. Secreted activity of MMP-9, colony formation, and *PTEN* expression as indicators of cancer phenotype in CD34-positive As-TM putative CSCs. (A) Secreted MMP-9 activity. (B) Colony formation in soft agar. (C) Transcript level of *PTEN*. An asterisk (*) indicates a significant increase ($p < 0.05$) compared with cell-matched total control. A cross (†) indicates a significant increase ($p < 0.05$) compared with control-derived SCs. A triangle (▲) indicates a significant increase ($p < 0.05$) compared with As-TM cells. Multiple comparisons († and ▲) are shown only for As-TM putative CSCs.

displays levels of various major markers typical for a malignant phenotype that are consistent with characterization as CSCs.

Expression of *K13*

K13 is a biomarker for dermal cancer progression (He *et al.*, 2006). The expression at the transcript level of *K13* was higher in both total As-TM cells and As-TM-derived CSCs when compared with respective control (Fig. 5A). The expression was also much higher in As-TM-derived CSCs compared with total population As-TM cells (Fig. 5A). Similar increases were seen at the protein level by Western blot analysis (data not shown) and fluorescent immunostaining micrographic analysis (Fig. 5B).

Expression of *K1/10*

Increased *K1* and *K10* expression is seen in hyperkeratosis induced by chronic arsenic exposure (Yu *et al.*, 2001), in arsenic-induced transformed HaCaT cells (Sun *et al.*, 2009), and in primary and explant SCC (Commandeur *et al.*, 2009). Both genes were highly expressed in As-TM cells and As-TM-derived putative CSCs compared with control cells (Fig. 6A). The transcript levels of *K1* and *K10* were increased up to 5-fold in As-TM-derived CSCs compared with total population As-TM cells (Fig. 6A). The increase in *K1* was also confirmed at the protein level by Western blot analysis (data not shown) and fluorescent immunostaining micrographic analysis (Fig. 6B).

DISCUSSION

Inorganic arsenic is a human skin carcinogen and causes various skin cancers, including SCC (IARC, 2004; Straif *et al.*, 2009). Evidence *in vivo* in mice shows inorganic arsenic acts as a cocarcinogen in skin, and it induces much more aggressive skin SCCs than those typically produced by skin carcinogens

alone (Rossman *et al.*, 2001, 2002; Waalkes *et al.*, 2008). Furthermore, *in vitro* inorganic arsenic malignantly transforms human skin keratinocytes, which produce aggressive SCCs upon inoculation into mice (Pi *et al.*, 2008). Accumulating evidence indicates that SCs are a probable key target in arsenic carcinogenesis (Tokar *et al.*, 2010a,b; Waalkes *et al.*, 2008). Inorganic arsenic likely increases the population of SCs in human skin models *in vitro* (Patterson and Rice, 2007; Patterson *et al.*, 2005), arsenic can directly induce malignant transformation of SCs *in vitro* (Tokar *et al.*, 2010a), and it also causes an overproduction of CSCs *in vitro* and *in vivo* associated with acquired malignant phenotype or cancer production (Tokar *et al.*, 2010b; Waalkes *et al.*, 2008). Indeed, in the present study, when CD34-positive cells were isolated from a human skin keratinocyte line that had been malignantly transformed by chronic exposure to inorganic arsenic (Pi *et al.*, 2008), proportionally more putative CSCs were isolated from As-TM cells than normal SCs were isolated from control cells. This indicates that a likely accumulation of CSCs in this skin model system occurred with arsenic-induced acquired malignant phenotype. This CSC overaccumulation does not occur after transformation with all chemical or physical carcinogens (Tokar *et al.*, 2010b, 2011; present study). Quantitation of CSCs in isogenic prostate cell transformants showed a CSC overabundance was unique to arsenic-induced malignant phenotype but did not occur after similar transformation by either cadmium or N-methyl-N-nitrosourea (Tokar *et al.*, 2010b). Similarly, in the present study, UV-transformed HaCaT cells, although clearly of a malignant phenotype (He *et al.*, 2006), showed a similar level of CD34-positive cells (likely SCs/CSCs) and holoclone formation rate when compared with control HaCaT cells. Furthermore, in a study where mice were exposed to inorganic arsenic from gestation through adulthood, resulting arsenic-induced lung and liver carcinoma showed a marked overabundance of CSCs compared with spontaneous tumors or N-nitrosoethylurea-induced lung

FIG. 5. Expression of the dermal cancer progression marker *K13* in CD34-positive control SCs or As-TM putative CSCs. (A) Transcript levels of *K13*. (B) Fluorescence microscopic imaging of *K13* protein (bar = 20 microns). An asterisk (*) indicates a significant increase ($p < 0.05$) compared with cell-matched total control. A cross (†) indicates a significant increase ($p < 0.05$) compared with control-derived SCs. A triangle (▲) indicates a significant increase ($p < 0.05$) compared with As-TM cells. Multiple comparisons († and ▲) are shown only for As-TM putative CSCs.

adenocarcinoma (Tokar *et al.*, 2011). Further study will be required to see if this predilection for a higher rate of production of CSCs is true for other target tissues of arsenic carcinogenesis. This is not to say that other carcinogenic agents do not convert normal cells to CSCs as part of the carcinogenic process, as, for instance, with cadmium when it produces CSCs during malignant transformation of human breast epithelia cells

FIG. 6. Expression of *K1* and *K10* in CD34-positive control SCs or As-TM putative CSCs. (A) Transcript levels of *K1* and *K10*. (B) Fluorescence microscopic imaging of *K1* protein (bar = 20 microns). An asterisk (*) indicates a significant increase ($p < 0.05$) compared with cell-matched total control. A cross (†) indicates a significant increase ($p < 0.05$) compared with control-derived SCs. A triangle (▲) indicates a significant increase ($p < 0.05$) compared with As-TM cells. Multiple comparisons († and ▲) are shown only for As-TM putative CSCs.

(Benbrahim-Tallaa *et al.*, 2009). However, with arsenic the portion of CSCs relative to total cells appears to consistently increase with malignant transformation or tumor initiation (Tokar *et al.*, 2010b, 2011; Waalkes *et al.*, 2008; present study).

Colony-forming human epidermal cells are heterogeneous in their capacity for sustained growth, and clonal types of epidermal keratinocytes include those that produce holoclones (Barrandon and Green, 1987). Holoclones are tightly packed small cells with high reproductive capability, and very few of which will undergo terminal differentiation (Barrandon and Green, 1987). Recent work reveals that keratinocyte holoclones contain self-renewing SCs and the ability to form a holoclone is an intrinsic property of the adult SCs of the hair follicle (Claudinet *et al.*, 2005). Increased holoclone formation from As-TM cell-derived putative CSCs compared with control

cell-derived SCs likely indicates an overproduction via distorted self-renewal of CSCs in these arsenic-transformed skin keratinocytes. Thus, it appears, based also on the ability to form holoclones, arsenic-induced malignant transformation increases the portion of putative CSCs to total cells in this skin cell line. This again is consistent with a variety of evidence both *in vitro* and *in vivo* indicating arsenic produces an overabundance of CSCs during acquisition of malignant phenotype (Tokar *et al.*, 2010a,b, 2011; Waalkes *et al.*, 2008).

SCs and CSCs share a variety of fundamental properties, including self-renewal capacity, although typically dysregulated in CSCs (Pardal *et al.*, 2003). Nonetheless, many genes that are expressed in normal skin SCs are also expressed in CSCs, including *CD34*, *K5*, *K14*, *K15*, *K19*, *p63*, *Notch1*, and *Rac1* (Benitah *et al.*, 2005; Gerdes and Yuspa, 2005; Kanitakis *et al.*, 1999; Liu *et al.*, 2003; Lyle *et al.*, 1998; Pellegrini *et al.*, 2001; Trempus *et al.*, 2003; Waalkes *et al.*, 2008). For example, in skin, *CD34* is specifically expressed in keratinocyte SCs (Trempus *et al.*, 2003), and *CD34* expression is key to skin carcinogenesis (Malanchi *et al.*, 2008; Trempus *et al.*, 2007). *K5* and *K14* are markers of undifferentiated skin SCs or CSCs (Gerdes and Yuspa, 2005; Lyle *et al.*, 1998), whereas *K15* and *K19* are specific skin keratinocyte SC/CSC markers (Kanitakis *et al.*, 1999; Liu *et al.*, 2003), and *K18* is a marker of differentiated cells. Essential for SC self-renewal, *p63* also regulates the proliferative potential of epidermal SCs in skin (Pellegrini *et al.*, 2001). *Rac1* is a key gene in skin SC population dynamics, which stimulates keratinocyte SC self-renewal while suppressing transient-amplifying cells production (Benitah *et al.*, 2005). *Rac1* is highly overexpressed in the SCCs seen after prenatal arsenic exposure when followed by phorbol ester treatment in adulthood (Waalkes *et al.*, 2008). The expression of *Rac1* appears to be key to the maintenance of a malignant phenotype in SCC cells *in vitro* (Kwei *et al.*, 2006). Thus, distortion of SC self-renewal is common during the process of CSC formation (Kanitakis *et al.*, 1999) and appears to occur with arsenic-induced CSC formation. Thus changes of common SC/CSC markers (*p63*) and keratinocyte SC markers (*CD34*, *K5*, *K14*, *K15*, *K19*) occurred in As-TM-derived putative CSCs compared with control-derived SCs along with alterations suggesting that arsenic caused aberrant self-renewal (*Rac1*) and differentiation (*K18*) as the cells acquired a malignant CSC phenotype. This again indicates that arsenic exposure may target SCs and facilitates CSC accumulation during malignant transformation, consistent with prior work in other target cell line models (Tokar *et al.*, 2010a,b).

The role of *Notch1* in skin cancer is complex, and ablation of *Notch1* expression will enhance skin carcinogenesis in some cases, as with *Kras* driven tumor formation in mice (Mazur *et al.*, 2010). This would make the *Notch1* activation seen in the present study appear contrary to an acquired malignant phenotype. However, recent findings indicate *Notch1* can have dual actions, acting either as an oncogene or as a tumor

suppressor gene in skin, depending on the tumor type and the activation of other signaling pathways (Panelos and Massi, 2009). Indeed, it appears that *Notch1* activation is linked to non-UV-associated SCCs (Panelos and Massi, 2009). In this regard, SCCs have been associated with prenatal arsenic exposure in mice (Waalkes *et al.*, 2008), and arsenic transformed HaCaT cells produce SCC when inoculated into mice. SCC is a skin tumor type associated with arsenic exposure in humans and occurs frequently in non-UV-exposed areas (IARC, 2004).

It is thought that tumors arise and grow as a result of the formation of CSCs, which may constitute only a minority of the cells within a malignancy but nevertheless are critical for its propagation (Pardal *et al.*, 2003). As-TM cells produce SCC after inoculation into mice (Pi *et al.*, 2008). The current data suggest that CSCs derived from As-TM cells not only have SC properties but also have acquired malignant characteristics that are amplified from the total population of As-TM cells. Elevated expression of *MMP-9* is strongly correlated with malignant phenotype in SCC (Bernhard *et al.*, 1994) and more specifically, hypersecretion of *MMP-9* is often seen in cells malignantly transformed with arsenic (Pi *et al.*, 2008; Tokar *et al.*, 2010a). Anchorage independence is a hallmark of transformed cells but is also a consistent property of SCs (Dontu *et al.*, 2003). In the present study, As-TM-derived CSCs showed a marked increase in soft agar colony formation and secreted *MMP-9* activity compared with the total population of As-TM or control cells, fortifying oncogenic potential of As-TM CSCs. *PTEN* is a tumor suppressor gene frequently lost in human cancers (Stambolic *et al.*, 1998). *PTEN* expression is suppressed by UV exposure in HaCaT cells (He *et al.*, 2006) and is often inactivated with arsenic transformation (Tokar *et al.*, 2010a). Poor *PTEN* expression can increase SC-like cell number and causes cancer initiation in some cases (Signoretti and Loda, 2007). In this work, the expression of *PTEN* in As-TM-derived CSCs was markedly reduced consistent with expansion of SC numbers. Overexpression of *K13* similarly is a good marker for skin tumor progression (Warren *et al.*, 1993). The expression of *K13* increases in the HaCaT cells malignantly transformed by UV or arsenic exposure (He *et al.*, 2006; Pi *et al.*, 2008). Again, *K13* overexpression in *CD34*-positive holoclone-derived cells derived from As-TM cells is consistent with characterization as potential CSCs.

Expression of *K1/10* is often considered typical for terminal differentiation in the skin (Prado *et al.*, 2011). However, in our previous studies, *K1/10* are clearly overexpressed during arsenic transformation in HaCaT cells (Sun *et al.*, 2009), and arsenic is known to cause hyperkeratosis *in vivo*. These same arsenic-transformed cells produced aggressive SCC in mouse xenograft study (Pi *et al.*, 2008). In this regard, both primary SCC and *in vitro* explant SCC models overexpress *K1/10*, even though they represent aggressive tumors (Commandeur *et al.*, 2009). Thus, the activation of these “differentiation” keratins with

arsenic seems a consistent phenomenon but defining their exact role in the carcinogenic process initiated by the metalloid in the skin will require additional research.

In conclusion, SCs appear to be key targets in arsenic carcinogenesis (Tokar *et al.*, 2010a,b; present study). The current work shows *in vitro* arsenic-induced oncogenic phenotype is particularly pronounced in SCs during malignant transformation, and this occurs concurrently with a potential overproduction of CSCs, consistent with the prior data *in vivo* (Tokar *et al.*, 2011; Waalkes *et al.*, 2008) and *in vitro* (Tokar *et al.*, 2010a,b).

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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