Published in final edited form as:

Toxicol Appl Pharmacol. 2013 October 1; 272(1): 147–153. doi:10.1016/j.taap.2013.04.035.

Disruption of Canonical TGF β -signaling in Murine Coronary Progenitor Cells by Low Level Arsenic

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Abstract

Exposure to arsenic results in several types of cancers as well as heart disease. A major contributor to ischemic heart pathologies is coronary artery disease, however the influences by environmental arsenic in this disease process are not known. Similarly, the impact of toxicants on blood vessel formation and function during development has not been studied. During embryogenesis, the epicardium undergoes proliferation, migration, and differentiation into several cardiac cell types including smooth muscle cells which contribute to the coronary vessels. The TGF β family of ligands and receptors are essential for developmental cardiac epithelial to mesenchymal transition (EMT) and differentiation into coronary smooth muscle cells. In this *in vitro* study, 18 hour exposure to 1.34 μ Marsenite disrupted developmental EMT programming in murine epicardial cells causing a deficit in cardiac mesenchyme. The expression of EMT genes including TGF β 2, TGF β receptor-3, Snail, and Has-2 are decreased in a dose-dependent manner following exposure to arsenite. TGF β 2 cell signaling is abrogated as detected by decreases in phosphorylated Smad2/3 when cells are exposed to 1.34 μ Marsenite. There is also loss of nuclear accumulation pSmad due to arsenite exposure. These observations coincide with a decrease in vimentin positive mesenchymal cells invading three-dimensional collagen gels. However, arsenite does not block TGF β 2 mediated smooth muscle cell differentiation by epicardial cells. Overall these results show that arsenic exposure blocks developmental EMT gene programming in murine coronary progenitor cells by disrupting TGF β 2 signals and Smad activation, and that smooth muscle cell differentiation is refractory to this arsenic toxicity.

Introduction

The coronary vasculature is formed from an extra cardiac progenitor cell population derived from the epicardium. This progenitor population of cells proliferate, migrate to cover the heart and a subset differentiate into several cardiac cell types including smooth muscle cells which form the coronary vessels of the mature heart. These changes in cell behavior and phenotype require growth factor as well as extracellular matrix influences in the sub epicardial space. The requirement for TGF β family of ligands and receptors for epicardial

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epithelial to mesenchymal transition (EMT) has been well established (Craig et al. 2010, Austin et al. 2008). TGF β 2, but not TGF β 1 or 3, has an exclusive function in cardiac EMT required for valve and septal development (Shull et al. 1992, Sanford et al. 1997, Kaartinen et al. 1995). At 14.5 days of gestation in mice, the type III TGF β receptor (TBRIII) knockout phenotype is lethal as a result of inhibition of the coronary vessel development (Compton et al. 2007). Defects include a hypo-proliferative epicardium and decreased epicardial cell invasion into the myocardium. Epicardial cells isolated from TBRIII null embryos are less invasive in response to TGF β 2 (Sanchez et al. 2011), but still differentiate into smooth muscle lineage *in vitro*. TGF β ligands are classical activators of the Smad family of proteins that are phosphorylated by TGF β receptors (Hoffmann et al. 2005). Receptor Smads, Smad 2 and 3, are phosphorylated and dimerize with Smad4 to translocate to the nucleus and execute transcription of target genes required for cell differentiation; this interaction is required for proper endocardial cushion EMT (Sirard et al. 1998). How the Smad effectors function in epicardial EMT are not clearly delineated, nor is it known whether any naturally occurring substances target the TGF β Smad signaling pathway.

Exposure to arsenic especially at high concentrations is linked to cancers of the bladder, lung, skin and liver (Chen et al. 1985). A unique peripheral vascular disorder, Blackfoot disease, is also prevalent in areas with high levels of arsenic (>300 μ g/L) in drinking water (Smith et al. 1992). Non-cancer ailments from low doses (<ppm) of arsenic are becoming more documented, especially for cardiovascular diseases. Two recent epidemiological studies reveal the impact of exposure to arsenic and heart disease morbidities. The Health Effects of Arsenic Longitudinal Study (HEALS) (Parvez et al. 2010) with over 20,000 participants showed arsenic exposure through the drinking water and confirmed by urine arsenic levels (Chen et al. 2009) is substantially linked to heart disease and cardiovascular morbidities. The second study involves the population in region II area of Chile where the investigators show increased risk for myocardial infarction and death due to both in utero and early childhood consumption of arsenic contaminated drinking water (Yuan et al. 2007). This elevated cardiovascular risk was eliminated once the arsenic was removed from the drinking water. Furthermore, other studies show increased incidence and risk for infant death, spontaneous abortions, and low birth weights from elevated arsenic in drinking water consumed during pregnancy (von Ehrenstein, et al. 2006, Rahman et al. 2007, Rahman et al. 2009). Although there are limitations and challenges inherent to epidemiologic studies, these observations highlight the link between arsenic exposure and the development and progression of cardiovascular diseases. The mechanisms of arsenic's contribution to cardiovascular disorders are not clearly known and there are no data on the impact of arsenic on cellular signaling required for primary blood vessel formation. In order to properly assess how arsenic affects cell signaling, a primary cell source from epicardial tissue is required. This study uses a novel murine epicardial cell model to investigate the effect of exposure to low levels of arsenic (arsenite) on epicardial progenitor cell signaling and differentiation. Specifically, it was tested whether exposure to arsenite disrupts cardiac specific EMT gene programming and TGF β 2 signaling necessary for cardiac mesenchyme production.

MATERIALS AND METHODS

Cell line and culture conditions

Immortalized murine epicardial cells were cultured and maintained as previously reported (Austin et al. 2008, Craig et al. 2010). Briefly, these cells are conditionally immortal at 33°C by the interferon driven expression of temperature sensitive Large T Antigen (TA β). Cells are cultured at 37°C for 24 hours in the absence of interferon to revert cells into a primary state for all experiments.

Reagents

Sodium arsenite[NaAsO₂, As(III)] was provided by the Synthetic Chemistry Core (Southwest Environmental Health Sciences Center, Tucson, AZ) and prepared in distilled deionized sterile water. Recombinant TGFβ2 was purchased from R&D systems (R&D #302-B2). Antibodies to pSmad2/3, Smad2/3, and vimentin were purchased from Santa Cruz Biotechnologies. HRP-conjugated secondary antibodies (Santa Cruz) were used for western blotting and AlexaFluor594 Donkey AntiGoat (Invitrogen) was used for immunofluorescent detection of vimentin and pSmad2/3.

Cell Viability Assays

Cells were grown to 80% confluence and with or without As(III) for 24 and 48 hours in 1% fetal bovine serum and DMEM. Alterations in mitochondrial activity were used as an indicator of cytotoxicity using the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. MTS is bio-reduced by cells into a formazan product that is soluble in tissue culture medium (Promega G5421). The absorbance of the formazan product is measured at 490nm in an M2 Spectramax plate reader (Molecular Devices). Cell viability was graphed on the y-axis corresponding to x axis log[μMAs(III)] and fit to linear regression analysis. The resulting equation of the line: $Y=mX+b$. $IC_{50} = (0.5-b)/m$ to yield log[μM As(III)] IC_{50} . $10^{(\log[\mu MAs(III)])} = \text{calculated } IC_{50} \text{ As(III) in } \mu M$. Samples are in triplicate with values shown $\pm$ S.D. All assays performed in triplicate at each concentration of As(III), and in a minimum of three independent determinations. Statistical significance determined by one-tailed Student's T-test with equal variance.

Gene expression

RNA was isolated and purified using Trizol RNA isolation reagent according to manufacturer's instructions (Invitrogen). cDNA was generated using first strand cDNA synthesis kit (Roche) from 1μg RNA isolated from each experimental condition. Real-Time PCR was performed as previously described using TaqMan Master primer-probe system (Roche). 40S Ribosomal Protein 7 (RPS7) was used as a housekeeping gene. The following oligonucleotides for murine primer sets were used: *RPS7*: (F) AGCACGTGGTCTTCATTGCT, (R) CTGTCAGGGTACGGCTTCTG; *Fibronectin (FN)*: (F) TTTGTTCCTGCACGTGTTTC, (R) GTAGTTGTGGCCGGTGGT; *Has2*: (F) GGCGGAGGACGAGTCTATG, (R) ACACATAGAAACCTCTCACAATGC; *Hmox1*: (F) GTCAAGCACAGGGTGACAGA, (R) ATCACCTGCAGCTCCTCAAA; *SM22a* (F) CCTTCCAGTCCACAAACGAC, GTAGGATGGACCCTTGTGTTGG; *Slug*: (F) GATCTGTGGCAAGGCTTTCT, (R) ATTGCAGTGAGGGCAAGAGA; *Snail*: (F) ACCTGCTCCGGTCTCAGTC, (R) ATTGCAGTGAGGGCAAGAGA; *TBR3*: (F) TCCAAACATGAAGGAGTCCA, (R) GTCCAGGCCCGTGGAAT; *Tgfb1*: (F) TGGAGCAACATGTGGAATC, (R) GTCAGCAGCCGGTTACCA; *Tgfb2*: (F) TGGAGTTCAGACACTCAACACA, (R) AAGCTTCGGGATTTATGGTGT; *VEGF*: (F) CTCGCCTGGGAACTTTTG, (R) CCTCGTCTTCTCACCTCAA.

Western blot analysis

Cellular protein lysates were prepared using Triton X-100 lysis buffer with protease and phosphatase inhibitors as previously reported (Austin et al. 2008). Nuclear fractionation of protein lysates was performed as described (Haspe, et al. 1999). Nuclear and cytosolic fractions were resolved via SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane and subjected to Western blot detection of indicated proteins. Chemiluminescence detection was documented using a ChemiDoc XRS Molecular Imager (BioRad). Densitometry performed twice on Western blot data to calculate average

with standard deviation in representative experiment. Statistical analysis performed using one sided Student's t-test.

Collagen gel invasion assay and immunofluorescence

To recapitulate epicardial cell EMT, mouse epicardial cells were cultured on Rat Tail Type I Collagen gels at 1mg/ml collagen (BD Biosciences, Franklin Lakes, New Jersey). Epicardial cells were dosed with As(III) for 18 hours prior to 4ng/ml TGF β 2 stimulation for 48 hours. Cells were fixed in 4% paraformaldehyde and subjected to immunostaining for vimentin, a marker for cardiac mesenchyme (Perez-Pomares et al. 1998). Collagen gels were mounted on glass slides and Vimentin positive transformed epicardial cells were observed using fluorescence microscopy using a Leica DMLB microscope and documented using a Retiga 200R camera and ImagePro Plus 5.1 software.

pSmad 2/3 immunofluorescence

Epicardial cells were cultured on chamber slides in the presence of 1.34 μ M As(III) for 18 hours prior to a 20 minute stimulation with TGF β 2. Smad 2/3 phosphorylation and nuclear localization was detected and observed via fluorescence microscopy using deconvolution microscopy (Olympus). Nuclei stained with diamidino-2-phenylindole (DAPI, Pierce).

SM22a reporter assay

Epicardial cells isolated from SM22 α -*LacZ* reporter transgenic mice were generated and isolated as described (Compton, et al. 2007) and were used to determine SM22 α promoter activity and epicardial cell differentiation into a smooth muscle cell lineage *in vitro*. SM22 α -*LacZ* epicardial cells were pretreated with arsenic for 18 hours and subsequently stimulated with 4ng/ml TGF β 2 for 24 hours and allowed to transform. Chemiluminescent detection of β -galactosidase activity in cell lysates was performed using the Galacto Light-Plus System (Applied Biosystems) and BioTek2 micro plate luminometer. X-GAL was also detected using the β -galactosidase reporter gene staining kit (Sigma-Aldrich) to further confirm SM22 α promoter activity observed in the luminescence assays.

Results

Characterization of As(III) on epicardial cells

Cell Viability—Since a new cell model was being studied in the context of arsenic toxicity, a dose relationship of cell viability to arsenic concentration was performed. Epicardial cell viability was measured over 24 and 48 hour periods in As(III) concentrations of 1 μ M to 50 μ M (Fig. 1). Low level As(III) (1–10 μ M) had no effect on epicardial cell viability for the first 24 hours, but cells showed high sensitivity at 48 hours. The cytotoxic IC₅₀ value at 24 hours for As(III) is 15.9 μ M, the cytotoxic IC₅₀ value at 48 hour exposure to As(III) is 5.8 μ M. Therefore, concentrations of 1.34 μ M (100 ppb) up to 6.7 μ M of As(III) were examined for the effect on the cardiac EMT pathway.

As(III) Disrupts Cardiac pro-EMT Genes—A specific set of genes are required to drive cardiac EMT (Rosenthal, Harvey 2010). The TGF β family of ligands and receptors, related signaling effectors in the TGF β pathway, and hyaluronan synthase-2 (Has2) and its product hyaluronic acid (HA) are all critical molecules in EMT. TGF β 1, TGF β 2, the type three TGF β receptor (TBR3), the TGF β signaling effector Snail (or Slug 2), and Has2 were selected as a representative defined group of EMT genes. TGF β 3 mRNA was not detected in murine epicardial cells. Hemeoxygenase-1 (Hmox) was used as a positive control for induction by As(III) (Sardana et al. 1981). Epicardial cells were exposed for 18 hours to a small dose range of As(III) (0 – 6.7 μ M) based on the determined IC₅₀ concentrations in

figure 1, and expression of the indicated genes was assessed by real-time PCR (Fig. 2). In figure 2, the TGF β pathway components TGF β 2, TBR111 and Snail are all dramatically attenuated in expression following As(III) exposure. TGF β 1 mRNA levels did not appear to follow this pattern. Snail is substantially down regulated in expression by As(III). This indicates the capacity for TGF β -mediated EMT gene expression programming is disrupted at all doses examined. This is supported by observed down regulation of both TGF β 2 and the type III TGF β receptor. Similarly, the expression of Has2 is also significantly reduced due to As(III) pretreatment. These observations show that transcription of essential cardiac specific EMT genes is disrupted by As(III).

Canonical TGF β Signaling is Blocked by Arsenite—Epicardial cells show robust activation of Smad2 and Smad3 by phosphorylation (pSmad2/3) and nuclear translocation following stimulation with 4ng/mL TGF β 2 for 20 minutes compared to unstimulated control cells (Supplemental Figure 1 and Fig. 3). In contrast, pSmad2/3 is dramatically reduced in epicardial cells exposed to As(III) and then stimulated with TGF β 2 (Fig. 3A). TGF β 2 induces phosphorylation of Smad2/3 as expected (Fig. 3A compare lanes 1 and 2). This phosphorylation is dramatically reduced by 1.34 μ M and 2.34 μ M As(III) pretreatment (compare lanes 3 and 4 with lane 2). As(III) exposure alone also reduces the basal level of pSmad (compare lane 1 with lanes 5 and 6). Densitometry shows a greater than forty percent reduction in detection of pSmad in the arsenic pretreatment samples (Supplemental figure 2A). Nuclear fractionation of protein lysates were prepared for detection of nuclear pSmad2/3. TGF β 2 induced robust phosphorylation and nuclear translocation of pSmad2/3 (Fig. 3A, bottom panels). However, exposure to As(III) attenuates detection of phosphorylated Smad2/3 in the nuclear compartment (Fig. 3A, compare lanes 3 and 4 with lane 2 in bottom panels). This significant reduction in TGF β 2 induced Smad2/3 phosphorylation is observed at both concentrations for As(III) exposure (Supplemental figure 2B). Immunofluorescent detection of pSmad2/3 following exposure to As(III) further shows a dramatic abrogation of Smad2/3 phosphorylation and nuclear translocation (Fig. 3B) relative to TGF β 2 alone (Fig. 3B, white arrows). We detect little to no TGF β 2-stimulated pSmad 2/3 in the nuclear compartment in As(III) pretreated cells. Thus, these immunostaining observations are consistent with immunoblotting data showing arsenic reduces TGF β 2-triggered activation of Smad2/3. Collectively, these data indicate that As(III) has inhibitory effects on epicardial EMT signal transduction.

TGF β 2 Induced Epicardial EMT is Blocked by Arsenite—Vimentin is an intermediate filament whose expression serves as a marker for the mesenchymal cell phenotype (Franke et al. 1982) and was detected by immunostaining in mouse epicardial cells cultured on collagen (Fig. 4). Compared to unstimulated control cells (Fig. 4A), TGF β 2-stimulated epicardial cells execute EMT as vimentin positive cells which adopt an elongated morphology, migrate away from the epithelial sheet, and invade into the three-dimensional collagen matrix (Fig. 4B, white arrows). In contrast, epicardial cells exposed to As(III) and subsequently dosed with TGF β 2 show very little vimentin expression, retain the parental epithelial cell morphology, and do not invade into the collagen matrix (Fig. 4D). This failure to execute TGF β 2 induced EMT coincides with As(III) exposed cells showing a down regulation of key effectors necessary for EMT in figure 2. Collectively these data show that As(III) blocks TGF β 2-mediated epicardial EMT.

Arsenite Does Not Block TGF β 2 Induced Smooth Muscle Differentiation

Smooth muscle 22 α (SM22 α) is a calponin-related protein and a marker for smooth muscle cells (Shanahan et al. 1993). SM22 α -LacZ transgenic epicardial cells were exposed to 1.34 μ M As(III) for 18 hours and subsequently stimulated with 4ng/mL TGF β 2 for 24 hours to induce cellular differentiation. Protein lysates were subject to luminescent detection of β -

galactosidase activity as an indication of SM22 α promoter activity and smooth muscle phenotype. SM22 α promoter activity levels are robustly induced following TGF β 2 stimulation, and surprisingly arsenite pretreatment does not affect this induction (Fig. 5A). To confirm SM22 α promoter activity, cells under identical experimental conditions were subjected to β -galactosidase staining. As expected, TGF β 2 induced robust expression of β -galactosidase as an indicator of active transcription by the SM22 α promoter compared to unstimulated control cells (Fig. 5 B & C). This TGF β 2 triggered SM22 α β -galactosidase induction is not abrogated by As(III) exposure (Fig. 5E). These results indicate that arsenite does not disrupt TGF β 2-induced epicardial smooth muscle differentiation.

Discussion

Arsenic exposure in humans is linked to cardiovascular ailments including hypertension, atherosclerosis, and constriction of the aorta, coronary artery disease, and myocardial infarction (Navas-Acien et al. 2005). In addition, *in utero* exposure to arsenic contributes to spontaneous miscarriages and low birth weights which are usually associated with cardiovascular malformations. A multitude of developmental processes require EMT including formation of the neural tube, the coronary vessels, and heart valves. Previously our laboratory reported an avian heart development model in which As(III) disrupted EMT in endocardial cushions, the tissue which gives rise to heart valves (Lencinas et al. 2010). This current study shows the ability of arsenic to disrupt murine coronary progenitor cell EMT. This suggests conservation of this cardiotoxicity by arsenic as a global-disruptor of developmental cardiac EMT.

This murine epicardial cell line serves as a mammalian model for cardiac EMT (Austin, et al. 2008), therefore whether As(III) attenuates mammalian cardiac mesenchyme production was tested. A cytotoxic IC₅₀ of 15.9 μ M was detected at 24 hours in the coronary progenitor cells following exposure to As(III). These epicardial cells are specialized epithelial cells serving as a protective layer around the heart and as a progenitor source of cells for the coronary vasculature. As such, the sensitivity of these epicardial cells to concentrations of arsenic around 5 μ M is consistent with reports showing similar effects in vascular endothelial cells (Barchowsky et al. 1996). Human urothelial cells (UROtsa) have been used as a model for As(III) induced bladder carcinogenesis. In comparison, the UROtsa cell model has a cytotoxic IC₅₀ of 100 μ M at 24 hours of exposure to As(III) (Rossi et al. 2002, Bredfeldt et al. 2004). Thus, vascular endothelial cells are at minimum 5-times more sensitive to arsenic than UROtsa cells. This highlights a previously underappreciated aspect of arsenical toxicity, in that both progenitor and vascular endothelial cells are more sensitive to arsenic than other tissues. This also underlines the fact that more investigation into arsenic as a developmental toxicant is necessary to fully understand life-long effects of arsenic exposure.

Several genes and their products are known to be essential for cardiac developmental EMT. Has2, TGF β 2, TBR1, and Snail are key molecules needed for EMT. Thus, this panel of cardiac specific pro-EMT molecules was used to gauge the impact of arsenic upon cardiac EMT. A dose-dependent decrease in mRNA transcripts of cardiac EMT specific genes was observed after exposure to arsenic. A decrease in TBR1 and Snail transcripts strongly suggests that As(III) exposure decreases the cellular signaling to TGF β 2 induction of EMT. Although a modest increase in TGF β 1 mRNA is detected with As(III) exposure, the collagen gel invasion data suggests that this effect has no impact on As(III) epicardial EMT blockade. Indeed, the disruption in EMT signals is supported by the detected dramatic reduction in Snail expression following arsenic exposure. Decreased tissue responsiveness to growth factor induction of EMT could constitute a specific mechanism by which As(III) exerts *in utero* toxicities and structural heart defects leading to cardiovascular ailments.

Epicardial cells exposed to arsenic attenuate TGF β 2 induced receptor Smad (Smad2/3) phosphorylation. Furthermore, TGF β signaling via pSmad 2/3 nuclear translocation is abrogated in the presence of As(III) as detected by both Western blot analysis and immunostaining. Thus, As(III) reduces the capacity of epicardial cells to activate pro-EMT pathways in response to TGF β 2 by specifically disrupting signal transduction. This is in part due to the reduced expression of the TGF β type III receptor (TBRIII) as its mRNA expression is decreased in arsenic exposed samples. Arsenic may also reduce endogenous TGF β ligand production or impede receptor kinase activity leading to decreased Smad phosphorylation and nuclear localization. In this regard, TGF β 2-stimulated accumulation of pSmad2/3 in the nuclear compartment is blocked by As(III). Since Smad activation is abrogated by As(III), this suggests arsenic impedes the canonical TGF β signaling pathway.

The collagen gel invasion assay is a widely utilized system for the study of cardiac EMT in multiple species (DeLaughter et al. 2011). This highly relevant model was exploited to evaluate the effect of As(III) on epicardial cell EMT and invasion. Epicardial cells under TGF β 2 induction robustly express vimentin, undergo elongation and cellular invasion into the three dimensional collagen matrix. These observations are indicative of the mesenchymal phenotype which is severely disrupted by concomitant exposure to As(III). The alteration of the normal cardiac EMT process by As(III) could lead to subtle structural malformations that manifest in congenital heart defects or adult acquired congenital heart disease.

TGF β 2 induced epicardial EMT was significantly disrupted in the presence of arsenic, but smooth muscle differentiation is refractory to this toxic effect by arsenic. Previous data shows that TBRIII null epicardial cells do not undergo cell invasion in a collagen matrix in the presence of TGF β 2 (Sanchez et al. 2011). However, TBRIII deficient epicardial cells retain full competency to differentiate into smooth muscle cell lineage. This suggests that epicardial cells do not require the TGF β type III receptor to differentiate into coronary smooth muscle cells *in vitro*, and our observations show that arsenic acts as a chemical silencer of TBRIII gene expression. Since TBRIII is down regulated by As(III), this suggests that other non-Smad or TBRIII-independent mechanisms remain intact in the presence of arsenic preserving the capacity of epicardial cells to differentiate into smooth muscle cells in response to TGF β 2. Furthermore, these observations highlight the importance of non-Smad TGF β 2 signaling pathways in epicardial smooth muscle differentiation, and that full activity of the canonical TGF β pathway is not required to induce the smooth muscle phenotype.

Taken together, our data demonstrate that cardiac EMT specific molecular mechanisms required for development of the coronary vasculature are abrogated in the presence of environmentally relevant concentrations of arsenic. Our observations also support the high sensitivity to arsenic toxicity during developmental processes, related to the formation of the coronary vasculature. Thus, subtle developmental defects in heart vessels could predispose to atherosclerotic lesions or aneurysms which are linked to chronic arsenic ingestion in arsenic endemic regions. These data also define TGF β canonical signaling as a target pathway disrupted by low-dose arsenic exposure. Our findings demonstrate a novel molecular mechanism for the impact by arsenic on the developing heart that can define a developmental basis for adult cardiovascular pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank all members of the Camenisch laboratory for helpful discussions and comments. We acknowledge M. Alabaster for technical insights. We greatly appreciate Dr. A. Jay Gandolfi's review of the manuscript. This work is supported by the following NIH grants: ES04940, ES06694, P30ES006694.

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Highlights

1. Arsenic blocks TGF β 2 induced expression of EMT genes
2. Arsenic blocks TGF β 2 triggered Smad 2/3 phosphorylation and nuclear translocation
3. Arsenic blocks epicardial cell differentiation into cardiac mesenchyme
4. Arsenic does not block TGF β 2 induced smooth muscle cell differentiation

Figure 1. Arsenic exposure impacts viability of epicardial cells

Murine epicardial cells were incubated with the indicated concentrations of As(III) over 24 hours and 48 hours and subjected to MTS cell viability assay. Values are averages $\pm$ S.D. of controls from triplicate samples at each dose representing three independent experiments. 24 hours cytotoxic IC₅₀=15.9 μ M, and 48 hours cytotoxic IC₅₀=5.8 μ M. Asterisks (*P 0.05) and pound sign (#P 0.005) marks the first statistically significant observation for increase in cytotoxicity for 24 hour and 48 hour samples, respectively.

Figure 2. Arsenic decreases expression of key genes required for cardiac EMT

Murine epicardial cells were exposed to the indicated concentrations of As(III) for 18 hours. RNA was isolated from each treatment condition and RT-PCR analysis of cardiac EMT genes was performed. Heme oxygenase (Hmox) was used as a positive control for gene expression induction by arsenic. Clear bars, control; black bars, 1.34 μM; dark grey bars, 5 μM; light grey bars, 6.7 μM. * $p < 0.05$; # $p < 0.005$; + $p < 0.0005$ and & $p < 0.00005$. Statistical significance determined by one-tailed Student's T-test with equal variance. All samples performed in triplicate from a minimum of three independent experiments.

Figure 3. Arsenic blocks TGF β 2 stimulated Smad2/3 phosphorylation and nuclear localization

Epicardial cells were exposed to 1.34 μ M As(III) for 18 hours prior to stimulation with TGF β 2 for 20 minutes. **A.** Smad2/3 phosphorylation in whole cell lysates (top panels) and nuclear localization (bottom panels) was observed via immunoblotting. Actin used for loading control for total cell lysates and detection of LaminA for loading controls for nuclear lysates. **B.** Detection of Smad2/3 phosphorylation by immunostaining; control, top row; 4ng/mlTGF β 2, second row; 1.34 μ M As(III) + 4ng/mlTGF β 2, third row; and 1.34 μ M As(III) alone, last row. Images at 1000 $\times$ magnification, nuclei in blue (DAPI) and pSmad in red.

Figure 4. Arsenic blocks TGF β 2 mediated epicardial cell invasion and mesenchymal transformation

Epicardial cells were cultured on rat type I collagen gels and exposed to 1.34 μ M As(III) for 18 hours, followed by 4ng/ml TGF β 2 stimulation for 48 hours. Epicardial cells were subject to immunostaining for vimentin to visualize epithelial to mesenchymal transition. **A.** Control **B.** 4ng/ml TGF β 2 **C.** 1.34 μ M As(III) **D.** 1.34 μ M As(III) + 4ng/ml TGF β 2. Nuclei in blue (DAPI) and vimentin in red.

Figure 5. Arsenic does not block TGFβ2 mediated smooth muscle differentiation

SM22α-*LacZ* murine epicardial cells were exposed to 1.34μM As(III) for 18 hours and subsequently stimulated with 4ng/mL TGFβ2 for 24 hours. **A.** Chemiluminescence detection of β-galactosidase activity was used to assess SM22α promoter activity. Average values are shown ± S.D. from triplicate samples performed in three independent experiments. **B – E.** X-GAL staining was performed to detect β-galactosidase expression under identical conditions to further assess extent of epicardial smooth muscle differentiation. **B.** Control **C.** 4ng/ml TGFβ2 **D.** 1.34μM As(III) **E.** 1.34μM As(III) + 4ng/ml TGFβ2. X-GAL positive cells are blue. Representative images from four independent experiments are shown at 400× magnification.