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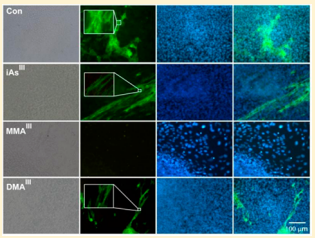
Effect of Arsenic Compounds on the *in Vitro* Differentiation of Mouse Embryonic Stem Cells into Cardiomyocytes

Qian Qian Wang,[†,‡] Yong Fei Lan,[†,‡] Kanwal Rehman,[†,‡] Yu Han Jiang,[§] Yasen Maimaitiyiming,[†,‡] Dan Yan Zhu,[†] and Hua Naranmandura*[,†,‡]

[†]Department of Toxicology, School of Medicine and Public Health, [‡]Department of Toxicology and Pharmacology, College of Pharmaceutical Sciences, [§]Ocean College, Zhejiang University, Hangzhou 310058, China

Supporting Information

ABSTRACT: Arsenic is a known carcinogen; however, there is no information on the toxic effects of inorganic arsenic and its intermediate metabolites, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), during the differentiation of embryonic stem (ES) cells into cardiomyocytes. The objective of this study was to evaluate the effects of arsenic compounds on ES cell differentiation into cardiomyocytes *in vitro* and to predict the associated toxic effects. Although iAs^{III} is known to be toxic, here we found that iAs^{III} and DMA^{III} did not influence ES cellular differentiation, whereas MMA^{III} inhibited ES cell differentiation into cardiomyocytes, suggesting that MMA^{III} has adverse effects on embryonic stem cells.



Various environmental pollutants and heavy metals are known to influence fetal development, resulting in embryolethal effects. Arsenic is a known human carcinogen,[1] and, globally, millions of people are chronically exposed to arsenic by arsenic-contaminated drinking water and food. In particular, the high level of arsenic in groundwater is considered to be a major cause of skin lesions and peripheral vascular diseases as well as cancers of the skin, lungs, and urinary bladder.[2−4] However, until now, little has been known about the mechanisms underlying the teratogenic adverse effects induced by arsenic in humans. Nevertheless, concern is developing over the adverse effects of arsenic on fetal development and birth. In mothers exposed to arsenic via contaminated drinking water, premature birth as well as babies with low birth weight and size has been observed.[5] Embryonic stem (ES) cells, which are derived from the inner mass of the preimplantation embryo, have been known to differentiate into cardiomyocytes *in vitro*.[6] Because ES cells are capable of differentiating into various cell types, these cells have engendered attention as a novel means of investigating cardiovascular therapeutics or toxicants.[7,8] Mouse ES cells differentiate into cardiomyocytes via the formation of embryoid bodies (EBs). Moreover, these ES cells cultured in EBs are able to recapitulate cardiomyocyte development starting from their precursors until reaching the differentiated cells.[6] Nevertheless, the toxic effects of inorganic arsenic and its intermediate metabolites on ES cell differentiation into other cell types still need to be elucidated.

Although the pathways involved in the metabolism of arsenic are still under debate,[9] it is generally believed that iAs^{III} is primarily biotransformed into mono- and dimethylated metabolites enzymatically by AS3MT after being administered to mammals.[10] Usually, biomethylation of iAs^{III} results in the formation of MMA^{III}, DMA^{III}, monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), of which the three trivalent arsenic species are considered to be more toxic than MMA^{V} and DMA^{V}.[11,12] Therefore, the toxic effects of these trivalent arsenic species on different cell types need to be clearly identified. Here, we show the association between exposure to arsenicals and their effect on mouse embryonic cell differentiation into cardiomyocytes.

ES-D3 cells were maintained in an undifferentiated state by culturing them on a monolayer of embryonic fibroblast cell feeders. The mouse ES cells were observed to be in ovoid- and/or nodule-shaped structures, forming colonies with clear boundaries. For determination of cardiomyocyte differentiation, EBs were formed using the hanging drop method, and the continuous proliferation and differentiation of these cells were permitted. After 5 days of induction of differentiation, representative EBs were formed (Figure S1). Later, after 5 days on culture plates, cells were found to grow from EBs, confirming the initiation of EB differentiation.

However, cells treated with MMA^{III} failed to show differentiation compared to that of the cells exposed to iAs^{III} and DMA^{III} (0.5−1 μM), which showed clear cellular differentiation (Figure 1). We further conducted immunofluorescence using a monoclonal antibody to verify that EBs derived from mouse ES cells express cardiac-specific protein (i.e., α-actinin). We found that the cells treated with iAs^{III} and DMA^{III} (0.5−1 μM) showed significantly higher expression of α-actinin compared to that of those treated with MMA^{III} (0.5−1 μM). Similarly, the protein level of α-actinin was significantly reduced in MMA^{III}-treated cells (Figure 3).

Rhythmically beating EBs differentiate spontaneously into beating cardiomyocytes. Thus, beating frequency was further

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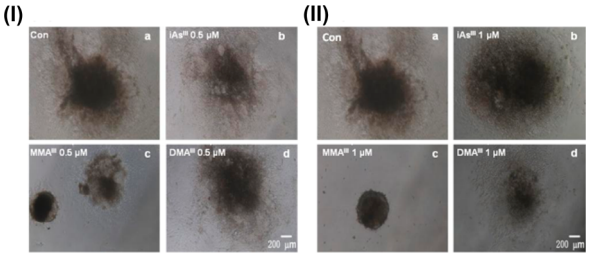

Figure 1. Assessment of morphological changes in EBs after exposure to three arsenicals. EBs were exposed to three arsenic species at 0.5 μM (I) or 1 μM (II) for 3 days. The cell morphology was determined by microscopy.

determined in EBs after exposure to three arsenic species (Figure S2). In iAs^{III}- and DMA^{III}-exposed EBs, the percentage of beating EBs was not affected by either exposure to iAs^{III} or DMA^{III} compared to that of the control. However, there were no beating cells observed following exposure to MMA^{III}, suggesting that MMA^{III} has potent inhibitory effects on the differentiation of mouse ES cells into cardiomyocytes. Moreover, AS3MT is known for the biomethylation of inorganic iAs^{III} to methylated metabolites in the body. Thus, we determined the expression of AS3MT in ES cells, EBs, and mouse liver (positive control). Interestingly, it was found that both ES cells and EBs lack AS3MT expression compared to that in mouse liver (Figure S3A). To further verify whether the EBs are able to generate methylated species of the inorganic iAs^{III}, EBs were exposed to the three arsenic species for 1 and 3 days, and arsenic species in culture medium were determined using HPLC separation on PRP X-100, followed by detection with ICP MS. Interestingly, EBs were incapable of methylating arsenicals to methylated species (Figure S3B).

Inorganic arsenic has been known to accumulate in fetal organs by crossing the human placenta;[13] moreover, arsenic traces have recently been found in newborn meconium.[14] In fact, it has been proposed that *in utero* exposure to arsenic may induce changes such as epigenetic alterations, which may further influence disease conditions.[15] However, to the best of our knowledge, we have for the first time made an attempt to investigate the influence of toxic trivalent arsenic species on mouse embryonic stem cell differentiation into cardiomyocytes. In this study, we demonstrated that methylated MMA^{III} has a strong inhibitory effect on the differentiation of mouse EBs into cardiomyocytes, which was not observed for iAs^{III} and DMA^{III} at 0.5−1 μM (Figure 1).

Moreover, the differentiated cardiac-like cells pretreated with iAs^{III} and DMA^{III} stained positively with an anti-α-actininm antibody, demonstrating their expression of cardiac-specific proteins for sarcomeric structures. However, the cells pretreated with MMA^{III} failed to differentiate the EBs into beating cardiomyocytes, as indicated by the lack of expression of cardiac-specific proteins (Figures 2 and 3). Previous studies showed that arsenic toxicity was most likely due to arsenic uptake and accumulation.[16]

The most interesting point of our current work is that although mouse ES cells and EBs lack AS3MT expression (Figure S3) and thereby are not considered capable of methylating inorganic arsenic to its trivalent metabolites, this does not exclude the possibility that ES cell differentiation will be affected as a result of ES cells and/or EBs being exposed to inorganic arsenic. Because AS3MT is known to be significantly expressed in a majority of mammals, including humans,[17]

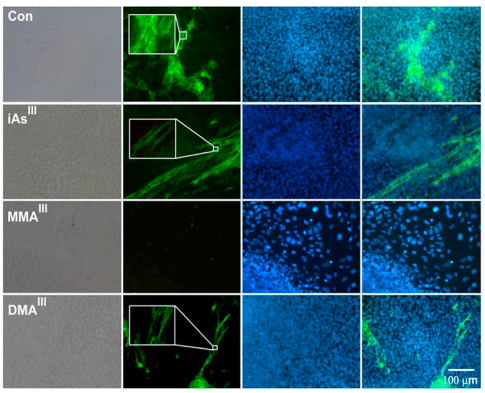

Figure 2. Determination of cardiomyocyte differentiation from mouse embryonic stem cells after exposure to three arsenic species. The cardiomyocytes were identified by the presence of cardiac-specific sarcomeres in EBs after exposure to three arsenic species at 1 μM for 3 days. The larger rectangular frame is the magnification of the area in the small square.

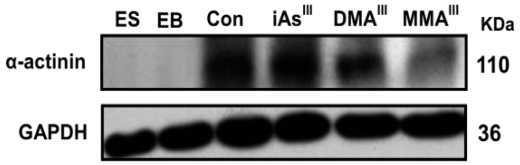

Figure 3. Determination of α-actinin in cardiomyocytes. α-Actinin was determined by western blot of EBs after exposure to 1 μM arsenicals for 3 days.

mothers exposed to inorganic arsenic may form methylated arsenic metabolites and may risk exposing their fetus to the toxic effects of methylated arsenic metabolites (e.g., MMA^{III}), as suggested by the current work.

In conclusion, it has been documented for the first time that MMA^{III} can affect the differentiation of mouse embryonic cells into cardiomyocytes, where MMA^{III} may inhibit the differentiation of ES cells either by inhibiting cell proliferation or by promoting apoptosis. Thus, further studies are required to confirm these effects in humans. Nevertheless, the inhibition of cell differentiation into cardiomyocytes could lead to serious consequences. Therefore, it can be predicted that mothers chronically exposed to arsenic levels that are below the current drinking water standard may have a greater risk of exposing their fetus to cardiac-related anomalies either during fetal development or in later stages of life. However, further investigations are required to validate such correlations.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and methods; schematic of the experimental procedure for EBs formation; determination of percentage of beating cells in mouse embryonic stem cells after exposure to arsenicals; and expression of AS3MT in ES cells, EBs, and mouse liver or determination of arsenic species in EBs culture medium after exposure to arsenicals. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86-571-8820-6736. E-mail: narenman@zju.edu.cn.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

iAs^{III}, arsenite; ES, embryonic stem cells; EBs, embryonic bodies; DMA^{III}, dimethylarsinous acid; DMA^{V}, dimethylarsinic acid; MMA^{III}, monomethylarsonous acid

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