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Arsenic inhibits stem cell differentiation by altering the interplay between the Wnt3a and Notch signaling pathways

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ABSTRACT

Millions of people are exposed to arsenic through their drinking water and food, but the mechanisms by which it impacts embryonic development are not well understood. Arsenic exposure during embryogenesis is associated with neurodevelopmental effects, reduced weight gain, and altered locomotor activity, and *in vitro* data indicates that arsenic exposure inhibits stem cell differentiation. This study investigated whether arsenic disrupted the Wnt3a signaling pathway, critical in the formation of myotubes and neurons, during the differentiation in P19 mouse embryonic stem cells. Cells were exposed to 0, 0.1, or 0.5 μM arsenite, with or without exogenous Wnt3a, for up to 9 days of differentiation. Arsenic exposure alone inhibits the differentiation of stem cells into neurons and skeletal myotubes, and reduces the expression of both β-catenin and GSK3β mRNA to ~55% of control levels. Co-culture of the arsenic-exposed cells with exogenous Wnt3a rescues the morphological phenotype, but does not alter transcript, protein, or phosphorylation status of GSK3β or β-catenin. However, arsenic exposure maintains high levels of Hes5 and decreases the expression of MASH1 by 2.2-fold, which are anti- and pro-myogenic and neurogenic genes, respectively, in the Notch signaling pathway. While rescue with exogenous Wnt3a reduced Hes5 levels, MASH1 levels stay repressed. Thus, while Wnt3a can partially rescue the inhibition of differentiation from arsenic, it does so by also modulating Notch target genes rather than only working through the canonical Wnt signaling pathway. These results indicate that arsenic alters the interplay between multiple signaling pathways, leading to reduced stem cell differentiation.

1. Introduction

Arsenic is a contaminant in water systems around the world [37,58]. Although the U.S. EPA and the W.H.O. have set a limit of 10 ppb arsenic, millions of people drink water with higher arsenic levels. In addition, arsenic has also been found at high levels in some foods, such as rice [11,28,41]. Arsenic readily crosses the placental barrier [10,24,72], and *in utero* exposure to arsenic has been linked to an increased incidence of stillbirths, preterm births, and miscarriages starting at drinking water concentrations of ~40 ppb arsenic [7,19,35,51,65].

Human epidemiological studies show that newborn weight and weight gain in early childhood is reduced after arsenic exposure. For example, drinking water containing 40ppb arsenic has been associated with an average 57 g reduction in birth weight [19], while maternal blood levels >5.3 ppb arsenic are associated with a 220 g reduction in birth weight [16]. The mechanisms behind the loss of weight are not fully known. But, studies indicate that arsenic can reduce myoblast differentiation into myotubes by decreasing myogenin, the transcription factor important in muscle cell differentiation [59,73]. Embryonic arsenite exposure can also alter muscle fiber subtype [15].

Additionally, *in utero* and *in vitro* arsenic exposure is linked to impacts on the developing nervous system. For example, arsenic exposure is correlated with reductions in neuronal cell migration and maturation at concentrations of 1–4 μM in embryonic primary rat neuroepithelial cells [55], at concentrations of 5–10 μM arsenic in PC12 and Neuro2a cells [14,68], and in postnatal day 11 pups whose mothers were injected twice with 1 or 2 mg/kg arsenite during pregnancy [12]. Arsenic exposure is also correlated with reduced intellectual function in children at mean drinking water levels ranging between 50 and 185 ppb [52,63,66,69,70]. These studies collectively suggest that arsenic can disrupt muscle and neuronal development. Indeed, in embryonic stem cells, 0.5 μM arsenite reduces the expression and localization of transcription factors such as Myf5, MyoD, myogenin, NeuroD, and neurogenins, all of which are needed to differentiate stem cells into skeletal

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myotubes and sensory neurons [18]. Therefore, signaling pathways upstream of neurons and skeletal myocytes are potential targets of arsenic.

The canonical Wnt/β-catenin signaling pathway is one such pathway that is important during embryogenesis. In the absence of Wnt ligand signaling, the co-transcription factor β-catenin is ubiquitinated and degraded in the proteasome after phosphorylation by casein kinase 1 (CK1α) and glycogen synthase kinase 3 β (GSK3β). These proteins are held together by the scaffolding protein Axin and the adenomatous polyposis coli (APC) protein. When a Wnt ligand is present and bound to the cell surface proteins Frizzled (Fz) and low-density lipoprotein receptor related protein 6 (LRP6), Axin is recruited to the plasma membrane, which inhibits β-catenin's phosphorylation, and leads to its nuclear translocation. β-catenin binds with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to activate downstream target genes (reviewed in Ref. [31]).

Wnt signaling molecules, such as Wnt1, Wnt3, Wnt3a, and Wnt5 [23,26,33], play a critical role in the development of muscle and neurons. Wnt3a is specifically important for both paraxial mesoderm and neural tube development [23,48,60,74]. For example, the Wnt/β-catenin pathway induces early mesodermal markers such as Brachyury and FoxA2 [62] and activates Myf5 in somites, which is one of the early transcription factors needed for skeletal muscle development [1,5]. In human embryonic stem cells, Wnt3a promotes the commitment to myogenic cells [22]. Wnt3a is also needed for neurogenesis, specifically in the growth of the neural tube [40] and in neural crest cell specification [13], which are needed for sensory neuron formation. *In vitro*, the addition of Wnt3a-conditioned medium to P19 mouse embryonic stem cells increased the expression of βIII-tubulin, a specific marker of neuronal cells [36].

Our previous study demonstrated that arsenic exposure inhibited the ability of P19 cells to differentiate into skeletal myotubes and sensory neurons. We hypothesized that a potential mechanism for the reduced differentiation was via the Wnt/β-catenin pathway, since β-catenin protein expression was decreased [18]. Indeed others have shown that arsenic can alter proteins within the Wnt signaling pathway. For example, after an *in utero* exposure to arsenic, the lungs of embryonic day 18 rats had reductions in both β-catenin and GSK3β transcripts [49], while arsenic exposure to SH-SY5Y neuroblastoma cells decreases GSK3β activity [71].

The current study shows that exogenous Wnt3a can effectively rescue the arsenic-mediated inhibition of P19 stem cell differentiation into skeletal myotubes, but not sensory neurons. Although arsenic exposure reduces the expression of β-catenin and GSK3β transcripts, these are not rescued by exogenous Wnt3a. Arsenic exposure reduces levels of the pro-neurogenic transcript Mash1 while maintaining high levels of Hes5. However, the addition of Wnt3a reduces Hes5 levels, and helps to rescue the inhibition of cellular differentiation caused by arsenic. This study underscores the complexity of arsenic-induced changes in cellular differentiation and highlights the cross-talk between the Wnt and Notch signaling pathways.

2. Materials and methods

2.1. P19 cell culture and differentiation

P19 mouse embryonic stem cells (ATCC, Manasass, VA) were cultured in α-MEM containing 7.5% bovine calf serum (Hyclone, Logan, UT), 2.5% fetal bovine serum (Mediatech, Manassas, VA), and 1% L-glutamine (Hyclone) in an incubator at 37 °C with 5% CO_2. Cells were subcultured every 2 days. For differentiation experiments, 1% DMSO was added to the medium, which is a typical compound used with these cells to form skeletal muscle cells [57]. Cells were aggregated for 2 days in hanging drops (500 cells/20 μL drop) [67] with 0, 0.1, or 0.5 μM arsenic as sodium arsenite (Sigma, St. Louis, MO). We have previously shown that these arsenic concentrations do not affect cell viability, but do reduce their ability to differentiate into sensory neurons and skeletal myotubes [18].

After 2 days, each drop was transferred to an individual well in a 96-well ultralow attachment plate for an additional 3 days (day 5) to form a mature embryoid body. To further differentiate the cells, they were transferred to 48 well plates coated with 0.1% gelatin for up to 4 additional days (day 9). The medium was renewed every 2 days until the cells were harvested, and the arsenic exposures continued throughout the differentiation process. When day 5 and 9 cells were harvested, all the wells from each 96-well plate were combined into one replicate (n = 3 plates per arsenic concentration). Cells were harvested in TRI Reagent (Sigma, St. Louis, MO) and stored at −80 °C.

2.2. Exosome isolation

Cell culture medium was also collected from day 5 embryoid bodies to collect exosomes. Briefly, medium was passed through a 0.2 μm filter and incubated with ExoQuick-TC isolation reagent overnight at 4 °C (Systems Biosciences, Mountain View, CA). Exosomes were isolated by centrifugation per manufactuer's instructions and lysed in RIPA buffer with protease and phosphtase inhibitors. Protein concentrations were quantified using Bio-Rad's DC protein assay kit.

2.3. L-cell and wnt3a conditioned medium collection

L-Wnt-3A cells and L-cells (ATCC) were cultured in DMEM containing 10% FBS and 1% L-glutamine. To produce conditioned medium, cells were split 1:10 and cultured for 4 days. The medium was removed and another 10 mL fresh culture medium added for an additional 3 days. The two sets of conditioned medium were combined, filtered, and stored at 4 °C. Once enough medium was obtained, it was filtered through Amicon Pro 30 kD Ultra filtration system (EMD Millipore, Billerica, MA) to concentrate the proteins. The conditioned concentrate was resuspended to its original volume in P19 cell differentiation medium and stored at −20 °C until use.

2.4. Exposure of P19 cells to L- or Wnt3a-conditioned medium

P19 cells were aggregated into embryoid bodies and differentiated into sensory neurons and skeletal myotubes as described above in either L-cell or Wnt3a conditioned medium. Concomitantly, they were exposed to sodium arsenite at 0, 0.1, or 0.5 μM. Embryoid bodies or differentiated cells were collected after 5 or 9 days in either TRI Reagent (Sigma) for qPCR experiments or in RIPA buffer (Pierce Biotechnology, Rockford, IL) containing both phosphatase and protease inhibitors (Pierce) for immunoblotting and immunoprecipitation experiments.

2.5. qPCR

Total RNA was extracted using TRI Reagent, quantitated by spectrophotometry, and cDNA (2 μg) was prepared by reverse transcription. The expression of Wnt3a, Wnt8a, LRP5, glycogen synthase kinase 3β (GSK3β), CK1α, β-catenin, APC, Axin, PTEN, lymphoid enhancer-binding factor 1 (LEF1), Dkk1, Dkk4, Myogenin, Neurogenin1, Hes1, and Hes5 was examined by qPCR. Briefly, 40 ng cDNA were incubated with SYBR Green (Qiagen, Alameda, CA) and gene specific primers (Table S1) to examine levels of transcripts. All samples were run in triplicate. A standard curve (10^{-3}ng–10^{-7}ng)