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Arsenic Targets Neural Plate Border Specifier Cells In P19 Cells

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ARSENIC TARGETS NEURAL PLATE BORDER SPECIFIER CELLS IN P19 CELLS

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ABSTRACT

Epidemiological studies have shown that arsenic exposure during early embryogenesis can cause reduced weight gain and neurological deficits later on in life. In addition, *in vitro* and *in vivo* studies have indicated that arsenic suppresses neurogenesis and myogenesis. The exact mechanism of how arsenic causes these undesired developmental outcomes is poorly understood, however both skeletal muscle and sensory neuron development require the Wnt/β-catenin signaling pathway to initiate the specific differentiation of precursor cells. We were interested in determining the target cell population of arsenic and its metabolites. Arsenic's metabolites were of interest because they have been shown to be more toxic than arsenic itself.

We found that arsenic and its metabolites, monomethylarsonous (MMA III) acid and dimethylarsinous (DMA III) acid, target a specific population of progenitor cells termed the neural plate border specifier (NPBS) cells by reducing the expression of signals required for neurogenesis (Pax3, Sox10, and NeuroD1) and myogenesis (Msx1, MyoD, and Myogenin). Pluripotent P19 embryonic stem cells were differentiated into embryoid bodies (EBs) in the presence of 0.1μM and 0.5μM sodium arsenite, 0.01μM and 0.05μM MMA III, or 0.001μM and 0.005μM DMA III. The expression of myogenic and neurogenic signals was determined by immunohistochemistry in EBs after 2-5 days of differentiation. Starting on day 2, in the neurogenic pathway, and day 3 in the myogenic pathway, arsenic is targeting the neural plate border specifier (NPBS) cells, which resulted in reduced transcription factor expression of Pax3 and Msx1. Arsenic also

altered nuclear localization of Msx1 (day 2), MyoD (days 2-5), NeuroD1 (days 4-5), and altered co-localization patterns in both the neurogenic and myogenic lineages.

After exposure to arsenic's metabolites, MMA III and DMA III, more drastic patterns were seen. In the neurogenic pathway, it appears that MMA III is targeting the neural plate border specifier cells on day 3, while DMA III does not affect transcription factor expression until day 5. Co-localization patterns were again changed after exposure. After 3 days of MMA III and DMA III exposure, co-localization patterns were significantly changed in the myogenic pathway. In regards to nuclear localization MyoD's nuclear localization was significantly decreased on days 3 and 5 in both the MMA and DMA treatments. In the neurogenic pathway, NeuroD1's nuclear localization was significantly decreased in day 5 EBs after exposure to both MMA and DMA.

Overall, these results suggest that arsenic and its metabolites are targeting the precursor cells to skeletal muscles and sensory neurons, and are therefore suppressing neurogenesis and myogenesis.

DEDICATION

To my family and most importantly my mother, Dyanna Lynn McCoy, who is and always will be my source of inspiration.

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CHAPTER ONE

LITERATURE REVIEW

Arsenic Sources and Location

Arsenic is naturally occurring element that can be found in water, soil, and rocks. Arsenic also has no odor, tasteless, and colorless (Ambrosio et al., 2014) giving it the potential to be very dangerous. Even though it is natural, it is a known carcinogen and toxicant that is posing many threats to human health. It is the 53rd most abundant element on Earth, and varies in concentration from 1 ppm to 10 ppm in soil, and between 1.5-1.6 ppm in seawater (Nickson et al., 2000). However, arsenic contamination in our environment, especially groundwater, has become a worldwide concern and is becoming an epidemic in many developing countries. It has been such an issue that the World Health Organization (WHO) has put arsenic on its list of the top 10 chemicals of major health concern (WHO).

Even though arsenic is not found in a huge abundance in earth's crust (Wedepohl, 1995) it is easily solubilized by ground water (Smedley and Kinniburgh, 2002) making it very easy for arsenic contaminate drinking water. Anthropogenic arsenic contamination is very common due to arsenic's historical use in pesticides, mineral extraction, smelting, and extraction of fossil fuels (Edelstein, 1985). It has also been used in recent times for industrial, medicinal, and homicidal purposes (Ambrosio et al., 2014).

In the past few decades, it has been estimated that over 140 million people worldwide have arsenic in their drinking water and food sources, with over 4 million of those individuals living in the United States (Ambrosio et al., 2014). In 2013, the U.S.

Food and Drug Administration found that common beverages such as beer could have on average 2.1 µg/serving (serving: 8 fl oz) of inorganic arsenic, and found that some brands of brown rice contain levels of 160 ppb of arsenic (FDA, 2013). What is also interesting to note is that what many consider to be a healthier option in brown rice, had much higher levels of arsenic than did the white instant rice brands (FDA, 2013).

In many parts of the world, a big reason for arsenic's contamination of drinking water, which is eventually used on crops, is because of contaminated well water. A lot of these wells are drilled in areas where natural arsenic deposits have leached into the water (Ambrosio et al., 2014). Two areas that have a true epidemic of arsenic poisoning in its groundwater and wells is Bangladesh and West Bengal (Kirchner and Weil, 1998). In Bangladesh, a country of 125 million, the estimate of people exposed to toxic levels of arsenic range from 35 to 77 million people (Smith et al., 2000). There, the Ganges delta aquifer that is used by the public for water is contaminated with arsenic (Acharyya et al., 1999; Kirchner and Weil, 1998). Many of the wells were originally drilled in conjunction with UNICEF to try and provide these areas with water, and to prevent water-borne diseases like cholera and typhus (Alvarez, 2001). The WHO recommends a maximum concentration of arsenic in drinking water of 10µg/L. However, 35% of the wells in Bangladesh have concentrations above 50µg/L, and 8.4% of the wells have concentrations above 300µg/L (Smith et al., 2000). When levels exceed these thresholds, negative physical symptoms can arise.

Adverse Health Effects

Undesired health effects from arsenic exposure include fever, diarrhea, and skin rashes (Dakeishi et al, 2006). In one study, researchers examined infants that were exposed to Morinaga dried milk in Japan that was accidently contaminated with arsenic. The 381 infants examined had clinical symptoms ranging from fever, diarrhea, vomiting, cough, eye discharge, skin pigmentation and rash, edema, and abdominal distension (Dakeishi et al., 2006).

Many diseases and conditions can develop later in life due to chronic arsenic exposure. These include cardiovascular disease, diabetes, hearing loss, and anemia (Chappell et al., 1997). Some exposed people also develop Blackfoot disease, which is a disease of the blood vessels that can lead to gangrene, and eventual amputation of the foot (Tseng et al., 2005). More serious diseases like skin, bladder, and lung cancer develop as well due to arsenic exposure (Tchounwou et al., 2003).

Although arsenic has been linked to causing cancer in adulthood, it has been used to cure cancer. In China, researchers used arsenic trioxide in conjunction with chemotherapy in patients with acute promyelocytic leukemia (APL). This resulted in remission rates ranging from 70-90% in newly diagnosed cases, and 65-90% in patients who had previously relapsed (Sun et al., 1992). In a follow up study of 32 patients, 16 of them survived more than 5 years with some living more than 17 years after the initial treatment (Sun et al., 1992). Similar results were seen in the United States when twelve APL patients were treated with 0.06-0.2 mg/kg/body weight of arsenic trioxide. The patients were treated until the leukemic blasts and promyelocytes were eradicated from

the bone marrow (Soignet et al., 1998). Of the 12 patients treated, 11 of them went into remission, and the only adverse side effects seen were rashes, slight fatigue, and musculoskeletal pain. More recently, arsenic trioxide was used in conjunction with dichloroacetate to induce cell death in breast cancer cells (Sun et al., 2011). Arsenic trioxide was seen to inhibit complex IV of the electron transport chain leading to a decrease in ATP production, and apoptosis of the cells (Sun et al., 2011).

Developmental Health Effects of Arsenic

Arsenic can cause many undesired developmental outcomes as well. Many epidemiological studies have shown that *in utero* exposure can lead to miscarriages, infantile death, decreased weight gain, cardiovascular diseases, muscular issues and neurological issues (Concha et al., 1998; Raqib et al., 2009). Since arsenic easily crosses the placental barrier, and can come in contact with the embryo (Jin et al., 2006, Concha et al., 1998) this may explain some of these developmental effects on the newborn.

One town that has become a natural experiment regarding chronic arsenic exposure is Antofagasta, Chile. The city and its 130,000 residents have exceedingly high levels of arsenic in their water supply. In the 60's the arsenic was estimated to be around 800 ppb in some wells and many dermatological and cardiovascular issues were being seen in the children (Borgono et al., 1977). During this time, one study found that over 70% of the children (n=37, 13-14 years) who grew up drinking the contaminated water had cutaneous lesions (Borgono et al., 1977). However, more serious developmental health effects have been seen. In one study, the authors examined over 400,000 residents in Northern Chile. They found that the mortality for bladder cancer among men in this

region was six times than that of the rest of Chile (Smith et al., 1998). In addition, lung cancer, skin cancer, and kidney cancer mortality was all significantly increased (Smith et al., 1998)

In West Bengal, India researchers studied over 200 women's pregnancies. Amount of arsenic exposure was measured throughout the pregnancies and it was found that some wells had more than 200 µg/L of arsenic (Ehrenstein et al., 2005). Women who were exposed to these wells during their pregnancy then had a 6-fold increase of stillbirths (Ehrenstein et al., 2005). Even if the child is born to a mother who has been exposed to these high levels of arsenic, they often times have very low birth weights. Birth weight is a known to be very good indicator of a newborn's overall health and physical and psychological development (Biswas et al., 2006). In one area in West Bengal, an epidemiological study looked at birth weight in newborns and found 31% of the newborns had low birth weight (< 2.5 kg, n=487) in the Puruliya region (Biswas et al., 2006). These newborns with low birth weight then have a much higher chance of dying, with 80 percent of neonatal deaths and 50 percent of infant deaths occur in conjunction with a low birth weight (Paul et al., 2002). Even in areas where arsenic exposure is not nearly as high (<50µg/L), the children born there are about 60g lighter than a child not exposed to arsenic (Hopenhayn et al., 2003). These results have been replicated in animal studies as well. When mice were exposed *in utero* to 10µg/L of arsenic, the arsenic cohort had low birth weights, weighing significantly less than the mice that were born to mothers not exposed to arsenic (Kozul-Horvath et al., 2012).

In adolescence, other issues from arsenic exposure are often seen including many neurological deficits and lower intelligence quotients (IQ). For example, in a follow-up study on the infants in Japan who had been exposed to arsenic-contaminated powered milk found that even fourteen years later, the poisoned individuals had higher incidences of abnormal electroencephalograms and lower intelligence quotients when compared to the control group (Dakeishi et al., 2006). In Bangladesh, children who have been chronically exposed to arsenic levels of 50µg/L or greater had significantly lower IQ's, scoring up to 10 points lower than children who grew up drinking water that contained less than 5µg/L of arsenic (Wasserman et al., 2004). Another study done in South Carolina found increased mental retardation and developmental disability in children whose mothers were pregnant while living on land that had higher than average levels on arsenic in the soil (Liu et al., 2010). Of the 6048 mother child pairs, 1490 cases (24%) of mental retardation or developmental disability were seen (Liu et al., 2010). These neurological issues may be explained by arsenic's ability to accumulate in the brain (Koehler et al., 2014; Xi et al., 2010). One study exposed astrocytes, a glial cell found in the brain and spinal cord that help form the blood-brain barrier and repair the brain and spinal cord following an injury, to arsenite and arsenate. After just 8 hours of exposure to 1mM of arsenite, the astrocytes already had altered cell morphology and increase in lactate dehydrogenase, which is released in response to tissue damage (Koehler et al., 2014). In addition arsenite was found inside the viable astrocytes after arsenite and arsenate exposure (Koehler et al., 2014).

In addition to these neurological deficits and lower IQ's, sensorimotor deficits, impaired gaits, and muscle weakness is seen as well. One study found that 35-85% of people in regions like West Bengal and parts of Bangladesh where arsenic contamination is rampant have some sort of neuropathy and muscle weakness (Chakraborti et al., 2003). After an electromyographic test they found around 10% of the people chronically exposed to high levels of arsenic developed sensorimotor deficits and had impaired gait (Chakraborti et al., 2003; Mukherjee et al., 2003).

P19 Cells

The mouse P19 cell line is derived from a teratocarcinoma in mice and provides a good model to examine arsenic's effects on cell lineage formation. They are pluripotent stem cells that can be induced to differentiate into any of the three germ layers, and show similar signaling pathways seen during early mouse embryogenesis (Kultima et al. 2010; Marikawa et al., 2009; McBurney, 1993). One of these crucial developmental pathways is the Wnt/ β-catenin pathway. This pathway is involved in the regulation of neural crest development and somitogenesis (Clevers et al., 2012; Schmidt et al., 2008). When mouse embryonic stem cells are deficient in β-catenin they exhibit self-renewal (Lyashenko et al., 2009); however, when β-catenin is overexpressed the stem cells will differentiate into muscle and neuronal cell lineages (Otero et al., 2004). We can use these muscle and neuronal lineages to examine arsenic's effect on early embryogenesis.

Our earlier studies have shown when P19 cells are induced to form embryoid bodies (EBs) while being exposed to 0.1μM, 0.5μM, and 1.0μM sodium arsenite, neurogenesis and myogenesis was suppressed (Hong and Bain, 2012). The delayed

development in the cells exposed to arsenic was shown to be due the repression of many transcription factors (TFs), including Pax3, MyoD, myogenin, and NeuroD (Hong and Bain, 2012). The reduced expression of these TFs was thought to be due the repression of the Wnt/β-catenin pathway during early embryogenesis, due to reduced β-catenin expression (Hong and Bain, 2012). Additional *in vitro* studies using different cells lines have shown similar degeneration to neuronal and muscle cells due to arsenic exposure. Reduced myelination of axons was seen when rats had 10mg of arsenite added to their drinking water daily (Garcia-Chavez et al., 2007), and when Neuro-2a cells were exposed to arsenic trioxide, neurite outgrowth was inhibited (Wang et al., 2010). Also, exposure of 1-2ppm of arsenic trioxide in mice's drinking water resulted in degeneration of their neuronal cells in the cerebellum and cerebrum (Piao et al., 2005).

Neural Plate Border Specifiers

With these findings, it is of interest to identify the target cell population of arsenic. One potential target is the progenitor cells of the neural crest (NC). The NC is a migratory cell population that is unique to vertebrates and will form the peripheral nervous system and glia, many elements of the craniofacial skeleton, and muscle progenitors (Hong and Saint-Jeannet, 2007; Le Douarin and Kalcheim, 1999). Just after gastrulation, the ectoderm has three distinct regions including the non-neural ectoderm, neural plate, and the neural plate border (NPB) which is found in the middle (Hong and Saint-Jeannet, 2007; Milet and Mosoro-Burq, 2012). Numerous signals, including the Wnt signaling pathway (Garnett et al., 2012), are then sent into the NPB which induces the presumptive NC cells to become competent to the neural crest specifier signals

(Betancur et al., 2010). These cells will then undergo an epithelial to mesenchyme transition (EMT), delaminate, and migrate away from the neural tube (Trainor, 2005). The signals that induce the NC come from genes that are collectively known as the neural plate border specifiers (NPBS) (Betancur et al., 2010). These genes include many muscle cell lineage TFs Msx1, MyoD, and Myogenin, which are required for myogenesis, and neuronal cell lineage TFs Pax3, Sox10, and NeuroD1, which are required for neurogenesis during early embryonic development. Not much is known about the temporal-spatial aspects of these signals (Garnett et al., 2012). However, it is well known from previous studies that arsenic disrupts EB formation (Stummann et al., 2008), myogenesis (Garcia-Chavez et al., 2007), and neurogenesis (Piao et al., 2005; Wang et al., 2010) in mouse embryonic stem cells.

Methylated Metabolites

In addition to inorganic arsenic, organic arsenic compounds are of major concern as well. Arsenic can be found in four oxidation states, +V, +III, 0, and –III, with pentavalent arsenate (As^{V}) and trivalent arsenite (As^{III}) being the most common species found (Sharma and Sohn, 2009). Once ingested, 80-90% of the arsenic is absorbed in the gastrointestinal tract (Freeman et al., 1995; Pomroy et al., 1980) and arsenic's biotransformation processes create much more toxic methylated compounds. First, inorganic arsenic is reduced between pentavalent and trivalent forms (Vahter, 2002) and a methyl group is added from S-adenosylmethionine (SAM) through a SAM-dependent As^{III} methyltransferase (Lin et al., 2002). This leads to the production of the methylated metabolites monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Vahter,

2002; Styblo et al., 2002) which are the primary metabolites excreted in urine (Styblo et al., 2002).

The most toxic methylated species produced are monomethylarsonous acid (MMA III) and dimethylarsinous acid (DMA III) and have been found to increase in concentration during pregnancy (Gardner et al., 2011) which can exert toxic effects on the developing fetus. Epidemiological studies have shown that pregnant women in chronically exposed areas like Bangladesh have increased concentrations of arsenic in their urine. In Matlab, Bangladesh urinary arsenic levels were as high as 84µg/L at 30 weeks gestation (Tofail et al., 2009). Similar results were found in Chilean women whose urinary arsenic levels increased from 36µg/L at 20 weeks gestation, to 47µg/L at 36 weeks gestation (Hopenhayn et al., 2003).

In vitro studies have examined the effects of these methylated metabolites on cells. Cultured human and rat hepatocytes were exposed to As^{III} and As^{V} and methylated species as well at concentrations ranging from 0.4-20µM (Styblo et al., 2000). The pentavalent species were not found to be cytotoxic; however, the trivalent species significantly decreased cell viability in a concentration dependent manner (Styblo et al., 2000). Another study found the even levels of MMA (III) as low as 1µM stopped the differentiation of mouse embryonic stem cells into cardiomyocytes (Wang et al., 2014).

Thesis Goals and Objectives

The goal of this thesis was to determine if arsenic affects the number and/or location of the neural plate border specifier cells. With previous studies showing arsenic reduces neuronal and skeletal differentiation during early embryogenesis through the

Wnt/β-catenin signaling pathway, we were interested in determining the target cell population of arsenic. We hypothesized that arsenic was targeting the neural plate border specifier cells, and its downstream targets, by reducing the signals required for neurogenesis and myogenesis. An additional area of research of this study is to examine if a mono-methylated or di-methylated species of arsenic is more or less toxic to the cells. My goals for this thesis were two-fold:

1) To determine if arsenic affects the number and/or location of neural plate border specifier cells. Pluripotent P19 cells will be cultured and induced to from embryoid bodies. The embryoid bodies will be exposed to sodium arsenite and immunohistochemistry will be used to determine expression levels of the transcription factors involved.

2) Examine the level of developmental toxicity of a mono-methylated and di-methylated species of arsenic. The same methods described from above will be used, but the embryoid bodies will be cultured with a mono-methylated and di-methylated species of arsenic.

References

1. Acharyya, S. K., Chakraborty, P., Lahiri, S., Raymahashay, B. C., Guha, S., Bhowmik, A. 1998. Arsenic poisoning in the ganges delta. Nature 401: 546.

2. Alvarez, C. 2001. Del diablo - Las aguas del infierno inundan el delta del ganges. Planeta Humano Magazine.

3. Ambrosio, F., Brown, E., Stolz, D., Ferrari, R., Goodpaster, B., Deasy, B., Distefano, G., Roperti, A., Cheikhi, A., Garciafigueroa, Y., Barchowsky, A. 2014. Arsenic induces sustained impairment of skeletal muscle and muscle progenitor cell ultrastructure and bioenergetics. Free Radical Biology and Medicine. 74:64-73.

4. Arques, Chicote, Tenbaum, Puig, Palmer. 2012. Standardized relative quantification of immunofluorescence tissue staining. Nature Protocol Exchange. doi:10.1038/protex.2012.008.

5. Betancur, P., Bronner-Fraser, M., Sauka-Spengler, T. 2010. Assembling neural crest regulatory circuits into a gene regulatory network. Annu. Rev, Cell Dev. Biol. 26: 581-603.

6. Biswas R., Dasgupta, A., Sinh, R.N., Chaudhuri, R. N. 2008. An Epidemiological Study of Low Birth Weight Newborns in the District of Puruliya, West Bengal. Indian Journal of Public Health 52(2): 65–71.

7. Borgono, J. M., Vicent, P., Venturino, H., Antonio, I. 1977. Environmental Health Perspectives. 19:103-105.

8. Chappell, Beck , Brown , North, Thornton, Chaney, Cothern, R., Cothern, C., North, D., Irgolic, Thornton I, Tsongas T. 1997. Inorganic arsenic: A need and an opportunity to improve risk assessment. Environ Health Perspect 105: 1060–1067.

9. Chakraborti, D. Mukherjee, S.C. Pati, S.Sengupta, M.K., Rahman, M.M., Chowdhury, U.K., Lodh, D., Chanda, C.R., Chakraborti, A.K., Basu, G.K. 2003. Arsenic groundwater contamination in middle ganga plain bihar, india: a future danger?.Health Perspect. 111:1194–1201.

10. Clevers, H., Nusse, R. 2012. Wnt/ β-catenin signaling and disease. Cell 149: 1192-1205.

11. Concha, G., Vogler, G., Lezcano, D., Nermell, B., and Vahter, M. 1998. Exposure to inorganic arsenic metabolites during early human development. Toxicol. Sci. 44: 185–190.

12. Dakeishi, M., Murata, K., and Grandjean, P. 2006. Long-term consequences of arsenic poisoning during infancy due to contaminated milk powder. Environ. Health 5: 31.

13. Davis, M.A., Mackenzie, T.A., Cottingham, K.L., Gilbert-Diamond, D., Punshon, T., Karagas, M.R., 2012. Rice consumption and urinary arsenic concentrations in U.S. children. Environ. Heal. Perspect. 120, 1418–1424.

14. Edelstein, D. L. 1985. In Mineral Facts and Problems; US. Department of the Interior: Washington, DC, 1985; Bulletin 675:1.

15. Ehrenstein, Mazumder, Hira-Smith, Ghosh, N., Yuan, Windham, Ghosh, A., Haque, Lahiri, Kalman, Das, Smith A. H. 2006. Pregnancy outcomes, infant mortality, and arsenic in drinking water in west bengal, india. Am. J. Epidemiol. 163(7):662-669.

16. Etchevers, H. C., Vincent, C., Le Douarin, N. M., Couly, G. F. 2001. The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development 128: 1059-1068.

17. Farzan, S.F., Korrick, S., Li, Z., Enelow, R., Gandolfi, A.J., Madan, J., Nadeau, K., Karagas, M.R., 2013. In utero arsenic exposure and infant infection in a United States cohort: a prospective study. Environ. Res. 126: 24-30.

18. Ferreccio, C., Gonzalez, C., Milosavlijevic, V., Marshall, G., Sancha, A. M., Smith, A. H. 2000. Lung cancer and arsenic concentrations in drinking water in Chile. Epidemiology 11(6): 673–679.

19. Food and Drug Administration. 2013. Analytical results from inorganic arsenic in rice and rice products sampling.

20. Freeman, Schoof, Ruby. 1995. Bioavailability of arsenic in soil and house dust impacted by smelter activities following oral administration in cynomolgus monkeys. Fundamental and Applied Toxicology. 28(2):215–222.

21. García-Chávez, E., Segura, B., Merchant, H., Jiménez, I., and Del Razo, L. M. 2007. Functional and morphological effects of repeated sodium arsenite exposure on rat peripheral sensory nerves. J. Neurol. Sci. 258: 104–110.

22. Garnett, A. T. 2012. Bmp, wnt and fgf signals are integrated throμgh evolutionarily conserved enhancers to achieve robust expression of pax3 and zic genes at the zebrafish neural plate border. Development 139(22): 4220-4231.

23. Hamadani, Grantham-McGregor, Tofail, Nermell, Fangstrom, Huda, Yesmin, Rahman, Vera-Hernandez, Airfeen, Vahter. 2010. Pre- and postnatal arsenic exposure and child development at 18 months of age: a cohort study in rural bangladesh. Int. J. Epidemiol. 2010: 1-10.

24. Hong, G. M., Bain, L. J. 2012. Arsenic exposure inhibits myogenesis and neurogenesis in p19 stem cells through repression of the β-catenin signaling pathway. Toxicological Sciences 129(1): 145-156.

25. Hong, C. S., Saint-Jeannet, J. P. 2005. Sox proteins and neural crest development. Cell and Developmental Biology 16: 694-703.

26. Hopenhayn, Huang, Christian, Peralta, Ferreccio, Atallah, Kalman. 2003. Profile of urinary arsenic metabolites during pregnancy. Environmental Health Perspectives 111(16): 1888-1891.

27. Hughes, M. F., Beck, B. D., Chen, Y., Lewis, A. S., Thomas, D. J. 2011. Arsenic exposure and toxicology: a historical perspective. Toxicological Sciences 123(2), 305-332.

28. Jin, Y., Xi, S., Li, X., Lu, C., Li, G., Xu, Y., Qu, C., Niu, Y., and Sun, G. 2006. Arsenic speciation transported through the placenta from mother mice to their newborn pups. Environ. Res. 101: 349–355.

29. Kirchner, J. W., Weil, A. 1998. Arsenic poisoning of Bangladesh groundwater. Nature 395: 338.

30. Koehler, Y., Luther, E. M., Meyer, S., Schwerdtle, T., Dringen, R. 2014. Uptake and toxicity of arsenite and arsenate in cultured brain astrocytes. J. Trace Elements in Medicine and Biology. 28(3):328-337.

31. Kultima, K., Jergil, M., Salter, H., Gustafson, A. L., Dencker, L., and Stigson, M. (2010). Early transcriptional responses in mouse embryos as a basis for selection of molecular markers predictive of valproic acid teratogenicity. Reprod. Toxicol. 30: 457–468.

32. Le Douarin, N., & Kalcheim, C. 1999. The neural crest. Cambridge University Press. No 36.

33. Lin, S., Shi, Nix, B. 2002. A novel S-adenosyl- L-methionine:arsenic(III) methyltransferase from rat liver cytosol. J. Biological Chemistry. 277(13):10795–10803.

34. Lyashenko, N., Winter, M., Migliorini, D., Biechele, T., Moon, R. T., and Hartmann, C. (2011). Differential requirement for the dual functions of ß-catenin in embryonic stem cell self-renewal and germ layer formation. Nat. Cell Biol. 13: 753–761.

35. Marikawa, Y., Tamashiro, D. A., Fujita, T. C., and Alarcón, V. B. (2009). Aggregated P19 mouse embryonal carcinoma cells as a simple in vitro model to study the molecular regulations of mesoderm formation and axial elongation morphogenesis. Genesis 47: 93–106.

36. McBurney, M. W. (1993). P19 embryonal carcinoma cells. Int. J. Dev. Biol. 37: 135–140.

37. Milet, C., Monsoro-Burq, A. H. 2012. Neural crest induction at the neural plate border in vertebrates. Developmental Biology 366: 22-33.

38. Mukherjee, S.C., Rahman, M.M., Chowdhury,U.K., Sengupta, M.K., Lodh, D., Chanda, C.R., Saha, K.C., Chakraborti, D. 2003. Neuropathy in arsenic toxicity from groundwater arsenic contamination in west bengal india. J. Environ.Sci. Health, Part A:Toxic/Hazard.Subst.Environ.Eng. 38:165–183.

39. Nickson, R.T., McArthur, J.M., Ravenscroft, P., Burgess, W.G., Ahmed, K.M. 2000. Mechanism of arsenic release to groundwater, bangladesh and west bengal. Applied Geochemistry 15: 403-413.

40. Nordstrom, S., Beckman, L., and Nordenson, I. 1979. Occupational and environmental risk in and around a smelter in northern Sweden: V. Spontaneous abortion among female employees and decreased birth weight in their offspring. Hereditas 90: 291-296.

41. Ohira, M., Aoyama, H. 1973. Epidemiological studies on the Morinaga powdered milk poisoning incident. Jpn. J. Hyg. 27:500-531.

42. Otero, J. J., Fu, W., Kan, L., Cuadra, A. E., and Kessler, J. A. 2004. Beta-catenin signaling is required for neural differentiation of embryonic stem cells. Development 131: 3545–3557.

43. Paul, Deorari, Singh. 2002. Management of Low Birth Weight Babies; in IAP Text Book of Pediatrics, 2nd edition, Jaypee Brothers Medical Publishers Pvt.

Ltd., p- 60-61.

44. Piao, F., Ma, N., Hiraku, Y., Murata, M., Oikawa, S., Cheng, F., Zhong, L., Yamauchi, T., Kawanishi, S., and Yokoyama, K. 2005. Oxidative DNA damage in relation to neurotoxicity in the brain of mice exposed to arsenic at environmentally relevant levels. J. Occup. Health 47: 445–449.

45. Pomroy, Charbonneau, McCullough, Tam. 1980. Human retention studies with 74As. Toxicology and Applied Pharmacology. 53(3):550–556.

46. Raqib, R., Ahmed, S., Sultana, R., Wagatsuma, Y., Mondal, D., Hoque, A. M., Nermell, B., Yunus, M., Roy, S., Persson, L. A., et al. 2009. Effects of in utero arsenic exposure on child immunity and morbidity in rural Bangladesh. Toxicol. Lett. 185: 197–202.

47. Schmidt, C., McGonnell, I., Allen, S., and Patel, K. (2008). The role of Wnt signaling in the development of somites and neural crest. Adv. Anat. Embryol. Cell Biol. 195: 1–64.

48. Sharma, V.K., Sohn, M. 2009. Aquatic arsenic: toxicity, speciation, transformations, and remediation. Environment International. 35:743–759.

49. Smedley, P. L., Kinniburgh, D. G. 2002. Appl. Geochem. 17:517.

50. Smith, A. H., Lingas, E. O., Rahman, M. 2000. Contamination of drinking-water by arsenic in bangladesh: a public health emergency. Bulletin of the World Health Organization 78: 1093-1103.

51. Smith, A. H., Goycolea, M., Haque, R., Biggs, M. L. 1998. Marked increase in bladder and lung cancer mortality in a region of northern chile due to arsenic in drinking water. Am. J. Epidemiol. 147(7):660-669.

52. Soignet, S. L., Maslak, P., Wang, Z-G., Jhanwar, S., Calleja, E., Dardashti, L. J., Corso, D., DeBlasio, A., Gabrilove, J., Scheinberg, D. A., Pandolfi, P. P., Warrell, R. P., Jr. 1998. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N. Engl. J. Med. 339: 1341–1348.

53. Stummann, T. C., Hareng, L., and Bremer, S. 2008. Embryotoxicity hazard assessment of cadmium and arsenic compounds using embryonic stem cells. Toxicology 252: 118–122.

54. Styblo, Del Razo, Vega, Germolec, LeCluyse, Hamilton. 2000. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. Arch Toxicol 74:289–299.

55. Styblo, Drobna, Jaspers, Lin, Thomas. 2002. The role of biomethylation in toxicity and carcinogenicity of arsenic:a research update. Environ Health Perspect 110(5):767–771.

56. Sun, H. D., Ma, L., Hu, X-C., and Zhang, T-D. 1992. Ai-Lin 1 treated 32 cases of acute promyelocytic leukemia. Chinese Journal of Integrated Chinese and Western Medicine 12: 170–172.

57. Sun, R. C., Board, P. G, Blackburn A. C. 2011. Targeting metabolism with arsenic trioxide and dicholoroacetate in breast cancer cells. Molecular Cancer 10:142.

58. Tchounwou, P. B., Patolla, A. K., Centeno, J. A. 2003. Carcinogenic and systemic health effects associated with arsenic exposure. Toxicologic Pathology. 31: 575-588.

59. Tsai, S. Y., Chou, H. Y., The, H. W., Chen, C. M., and Chen, C. J. 2003. The effects of chronic arsenic exposure from drinking water on the neurobehavioral development in adolescence. Neurotoxicology 24:747–753.

60. Tseng, C. H., Huang, Y. K., Huang, Y. L., Chung, C. J., Yang, M. H., Chen, C. J., Hsueh, Y. M. 2005. Arsenic exposure, urinary arsenic speciation, and peripheral vascular disease in blackfoot disease-hyperendemic villages in Taiwan. Toxicology and applied pharmacology 206(3), 299-308.

61. Vahter, M. 2002. Mechanisms of arsenic biotransformation. Toxicology. 181-182:211-217.

62. Wang, X., Meng, D., Chang, Q., Pan, J., Zhang, Z., Chen, G., Ke, Z., Luo, J., and Shi, X. 2010. Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway. Environ. Health Perspect. 118: 627–634.

63. Wedepohl, K. H. 1995. Geochim. Cosmochim. Acta 59:1217.

64. Xi, S., Guo, L., Qi, R., Sun, W., Jin, Y., and Sun, G. 2010. Prenatal and early life arsenic exposure induced oxidative damage and altered activities and mRNA expressions of neurotransmitter metabolic enzymes in offspring rat brain. J. Biochem. Mol. Toxicol. 24: 368–378.

CHAPTER TWO

ARSENIC TARGETS NEURAL PLATE BORDER SPECIFIER CELLS IN P19 CELLS

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Abstract

Epidemiological studies have shown correlation between arsenic exposure and adverse developmental outcomes. Exposure to arsenic and its metabolites during early embryogenesis can cause reduced weight gain and neurological deficits later on in life. Additionally, *in vitro* and *in vivo* studies have shown that arsenic suppresses neurogenesis and myogenesis through Wnt/β-catenin signaling pathway by stopping the differentiation of precursor cells. This study used P19 mouse embryonic stem cells to determine the target cell population of arsenic its metabolites. Pluripotent P19 cells were exposed to 0.1μM and 0.5μM sodium arsenite, 0.01μM and 0.05μM MMA III, and 0.001μM and 0.005μM DMA III. On day 2, in the neurogenic pathway, and day 3 in the myogenic pathway, arsenic is targeting the differentiation of neural plate border specifier (NPBS) cells, which resulted in a 1.2-fold reduction of Pax3 and a 1.5-fold reduction in Msx1 protein. Arsenic also altered the nuclear localization of Msx1 on day 2 by 1.5-fold, resulting in reductions in both MyoD nuclear expression on days 2-5, and in NeuroD1 nuclear expression on days 4-5. Arsenic exposure also altered co-localization patterns in both the neurogenic and myogenic lineages.

After exposure to arsenic's metabolites, MMA III and DMA III, MMA III was seen to target the neural plate border specifier cells on day 3 in both the neurogenic and myogenic pathways, while DMA III does not affect transcription factor expression until day 5. MyoD's nuclear localization was significantly decreased on day 3 and 5 in both the MMA and DMA treatments. In the neurogenic pathway, NeuroD1's nuclear localization was significantly decreased by1.4-fold in day 5 EBs after exposure to both

MMA and DMA. In conclusion, these results suggest that arsenic and its metabolites are targeting the precursor cells to skeletal muscles and sensory neurons, and are therefore suppressing neurogenesis and myogenesis.

Key Words: arsenite, dimethylarsinous acid, P19 cells, monomethylarsonous acid, myogenesis, neurogenesis

Introduction

Arsenic is a known toxicant that poses many threats to human health. It is a naturally occurring element found in bedrock, and its weathering has resulted in arsenic contamination of groundwater in many parts of the world (Kirchner and Weil, 1998; Yang et al., 2009). In the West Bengal region of India, it is estimated that more than 26 million individuals have been exposed to drinking water contaminated with arsenic (As) (Mondal et al., 2010). Although the World Health Organization recommends a maximum concentration of arsenic in drinking water of 10 µg/L, some wells in West Bengal have up to 531 µg/L As (Desbarats et al., 2014). Arsenic has also been found as a contaminant in food, such as rice, with levels ranging from 0.15-0.36 mg/kg (Zavala and Duxbury, 2008). For an average adult consuming 400 g of rice per day containing 0.25 mg/kg, their intake is approximately 100 µg As, which is equivalent to five times the amount of arsenic an adult would get from drinking 2L of water at the 10 µg/L limit (Zavala et al., 2008). In particular, increased arsenic levels in the urine of pregnant women and children have been seen due to the consumption of rice products (Davis et al., 2012).

Once inside the body, arsenic can be methylated by arsenic methyltransferases in a series of metabolism steps, with monomethylarsonous acid (MMA) and dimethylarsinous acid (DMA) being the primary species excreted in the urine (Styblo et al., 2002). It is known that the concentrations of MMA and DMA increase during pregnancy (Concha et al., 1998; Hopenhayn et al., 2003; Gardner et al., 2011). For example, a study of Chilean pregnant women whose drinking water contained at least 40

µg/L determined that at ~20 weeks of gestation, average urinary arsenic levels were 36 µg/L, while at ~36 weeks of gestation, urinary arsenic levels had increased to 54 µg/L (Hopenhayn et al., 2003). Furthermore, DMA levels increased from 30 µg/L to 47 µg/L.

In vitro studies have shown that some arsenic metabolites, especially the trivalent species, are more toxic than arsenic itself. For example, MMA (III) at concentrations between 0.5-1 µM inhibited the differentiation of mouse ES-D3 stem cells into cardiomyocytes more strongly than arsenic trioxide or DMA (III) (Wang et al., 2014). In another study, MMA (III) caused a decrease in myeloid progenitor colonies at concentrations as low as 0.13µM (Ferrario et al., 2008). Since there is an increase in arsenic biotransformation and methylated metabolites during pregnancy, continuous exposure to these metabolites could also have adverse effects on the developing fetus.

Epidemiological studies have shown that *in utero* exposure to arsenic can lead to increased occurrence of neonatal death, low birth weight, and miscarriages (Concha et al., 1998; Raqib et al., 2009). It has been show that during pregnancy, exposure to even moderate levels of arsenic in drinking water (<50 µg/L) can result in a 57g reduction in birth weight (Hopenhayn et al., 2003), and levels 67-85 µg/L are correlated with a 3-fold increase in miscarriages (Ogata et al., 2014). Mice exposed to 10µg/L As *in utero* and during the postnatal period weighed significantly less than control mice (Kozul-Horvath et al., 2012). These outcomes are thought to result from arsenic's ability to cross the placental barrier, and come in contact with the embryo (Jin et al., 2006, Concha et al., 1998). For example, cord blood and placenta contained an average of 9 and 34 µg/L arsenic, respectively, in women drinking water containing 200 µg/L of arsenic, compared

to a placental concentration of 7µg/kg in women who were not exposed to arsenic (Concha et al., 1998).

Embryonic arsenic exposure has also been correlated with many neurological deficits, such as decreased pattern memory (Tsai et al., 2002) and mental retardation in adolescents (Dakeishi et al., 2006, Liu et al., 2010). A follow-up study fourteen years after infants were exposed to As-contaminated dry milk found that the adolescents had lower intelligence quotients when compared to the control group (Dakeishi et al., 2006). Similar results were found in children from Bangladesh. When their drinking water contained >50 µg/L arsenic, they scored significantly worse on IQ tests than children who drank water from wells with <5 µg/L (Wasserman et al., 2004) Since arsenic has the ability to accumulate in the brain, which has been shown in rats (Xi et al., 2010), this may explain the connection between increased embryonic exposure to arsenic and neurological deficits. Collectively, these studies suggest that arsenic impacts the development of neurons and skeletal muscle.

In vitro studies have also shown similar impacts of arsenic on cellular development and differentiation. For example, when Neuro-2a cells were exposed to 3µM arsenic trioxide, neurite outgrowth was inhibited (Wang et al., 2010). Our lab has previously showed that neurogenesis and myogenesis was suppressed when P19 mouse stem cells were exposed to 0.5µM arsenite, due to reductions of many developmentally-important transcription factors (TFs), such as Pax3, MyoD, Myogenin, and NeuroD (Hong and Bain, 2012). Since arsenic appears to impact skeletal muscle and neurons

during embryogenesis, there is likely a common progenitor cell type that is a target of arsenic.

One potential target cell type is the progenitors of the neural crest (NC), termed neural plate border specifier (NPBS) cells. These cells send out signals into the neural plate border (NPB) region to induce neural crest (NC) formation and also delineate the location of the somites (Sauka-Spengler and Bronner-Fraser, 2008.) Numerous signals, including the Wnt signaling pathway (Garnett et al., 2012), are then sent into the NPB region, which induces the presumptive NC cells to become competent to the neural crest specifier signals (Betancur et al., 2010). These cells will then undergo an epithelial to mesenchyme transition (EMT), delaminate, and migrate away from the neural tube (Trainor, 2005). The signals that induce the NC come from genes that are collectively known as the neural plate border specifiers (NPBS) (Betancur et al., 2010). The signals produced include many transcription factors that are required for neurogenesis, such as Pax3, Sox10, and NeuroD (Howard, 2005), and required for myogenesis, such as Msx1, MyoD, and Myogenin (Yokoyama and Asahara, 2011).

The objective of this study was to determine whether arsenic specifically targets the neural plate border specifier cells during embryogenesis, thereby impairing appropriate differentiation into sensory neurons and skeletal muscle cells. Further, we wanted to examine whether the metabolites of arsenic differed in their ability to reduce cell differentiation. Our results suggest that early exposure to arsenite and its methylated metabolites target the differentiation of neural plate border specifier cells by reducing the expression of Pax3, Sox10, and NeuroD1 in the neurogenic pathway, and Msx1 in the

myogenic pathway.

Materials and Methods

P19 cell culture and differentiation of embryoid bodies

The mouse P19 mouse embryonal carcinoma cell line (ATCC, Manassas, VA) was maintained in α-MEM containing 7.5% bovine calf serum (Hyclone, Logan, UT), 2.5% fetal bovine serum (Mediatech, Manassas, VA), 1% L-glutamine, and 1% penicillin/streptomycin (designated as growth medium) at 37°C in a humidified incubator containing 5% CO_2. The medium was changed every 48 hours.

To form embryoid bodies and induce differentiation, P19 cells were aggregated using the hanging drop method (Wang and Yang, 2008). Briefly, P19 cells were trypsinized and suspended in differentiation medium (growth medium with 1% dimethyl sulfoxide) with 0, 0.1, and 0.5 µM sodium arsenite at a density of 500 cells/20 µl drop. These concentrations correspond to 7.5 and 37.5 µg/L arsenic. Each replicate contained 96 drops (n=3 replicates per concentration and per day). The hanging drops were allowed to form into embryoid bodies (EBs) for 2 days, after which, each drop was transferred to a 96-well ultralow attachment plate containing 70 µl of fresh differentiation medium with 0, 0.1, or 0.5µM arsenite. The EBs were collected at days 2, 3, 4, and 5, fixed in 10% neutral buffered formalin (NBF) overnight at 4^{o}C, dehydrated in ethanol, cleared in xylene, embedded in paraffin, and then sectioned for use in immunohistochemistry.

Synthesis of monomethylarsonous acid ($As^{III}(CH_3)(OH)_2$, MMA(III))

Synthesis of the MMA(III) precursor $[As(CH_3)O]_n$ was performed as reported (Cullen et al., 1989) with minor modifications. Arsenic trioxide (20 g, 101.1 mmol) was dissolved in aqueous sodium hydroxide solution (100 mL, 0.01 M) before adding methyl iodide (126 mL) and heating to reflux for 24 h. After cooling, ethanol (100 mL) was added to precipitate a white solid that was then filtered and dried, yielding disodium methylarsonate (16.48 g). Disodium methylarsonate was then dissolved in warm water (60 mL), and sulfur dioxide (generated *in situ*[4] as described below) was bubbled through the solution for 15-20 min. The resulting solution was heated to boiling for 2 min, cooled to 0 ^oC, and neutralized with sodium carbonate until bubbling ceased. The neutralized solution was evaporated to dryness and was extracted with benzene (other similar solvents such as toluene did not work for this extraction). Benzene was removed *in vacuo* to yield $[As(CH_3)O]_n$ as a white solid (7.5 g, 69.5% yield). The ^1H NMR ($CDCl_3$) spectrum was consistent with reported values (Cullen et al., 1989), and the MALDI mass spectrum showed a single peak at 106.8 m/z for $[As(CH_3)O + H^+]$. The desired MMA(III) product was formed by dissolving $[As(CH_3)O]_n$ in water (Cullen et al., 1989; Mass et al., 2001).

Synthesis of dimethylarsonous acid ($As^{III}(CH_3)_2OH$, DMA(III))

Synthesis of this compound was performed as reported (Burrows and Turner, 1920) with minor modifications. Cacodylic acid (12.5 g, 90.5 mmol) and potassium iodide (40 g, 240.9 mmol) were dissolved in water (50 mL), and sulfur dioxide

(generated *in situ*) (Descriptive Inorganic Chemistry, 2006) was bubbled through the solution for 10 min. A 1:1 solution of concentrated hydrochloric acid and water (25 mL) was then added periodically to the reaction mixture over the course of 30 min until a yellow oil formed and elemental sulfur precipitated. The oil layer was separated, dried over $CaCl_2$, and distilled to afford dimethylarsonous acid (16.8 g, 80% yield). The melting point of the purified compound was -35 ˚C, consistent with the reported value (Burrows and Turner, 1920). The ^1H NMR spectrum of DMA(III) in $CDCl_3$ showed a single resonance at δ 1.97, and the MALDI mass spectrum showed a single peak at 123 m/z for $[As^{III}(CH_3)_2OH + H^+]$.

In situ synthesis of SO_2

Sodium metabisulfite ($Na_2S_2O_5$,10 g) was added to a two-neck, round-bottomed flask (250 mL) and concentrated sulfuric acid (100 mL) was added dropwise periodically as effervescence ceased. From this flask, a vacuum adaptor attached to tubing with a glass pipette at the end was used to bubble SO_2 through the reaction solutions as described (Descriptive Inorganic Chemistry, 2006).

Embryoid body exposure to MMA and DMA

To derive appropriate monomethylarsonous acid (MMA) and dimethylarsinous acid (DMA) concentrations, dose-response experiments were carried out using varying MMA (0-0.1μM) and DMA (0-0.01μM) concentrations. The P19 cells were aggregated into embryoid bodies as described above and allowed to differentiate for 12 days,

changing the medium every 48 hours. Cells were examined visually under the microscope for viability and differentiation (data not shown). Concentrations of 0.01 and 0.05µM MMA, and 0.001 and 0.005µM DMA were chosen as appropriate levels that inhibited differentiation at the highest concentration without causing overt cellular death. P19 cells were cultured and differentiated as described above. On days 3 and 5, the EBs were collected, fixed in 10% NBF, and then used for immunohistochemical analysis as described above.

Immunohistochemistry

The fixed and embedded EBs were cut in 5 µm sections, placed on slides, deparaffinized, and rehydrated in graded ethanol washes. Antigen retrieval was carried out with citric acid buffer (pH=6) and then microwaved. The slides were blocked (1X PBS, 5%BSA, 0.05% Tween-20) for 1 hr. Primary antibodies were incubated at a 1:200 dilution overnight at 4^{o}C, and included Pax3 (Gene Tex no. GTX100663), Sox 10 (Abnova no. H00006663-M01), NeuroD1 (Abcam no. AB60704), Msx1 (Sigma-Aldrich no. SAB2500650), MyoD (Santa Cruz no. SC304), and Myogenin (Imgenex no IMG131). The secondary antibodies (1 µg/ml) conjugated to Alexa Fluor 488 (anti-goat), Alexa Fluor 488 (anti-mouse), Alexa Fluor 594 (anti-rabbit) or Alexa Flour 647 (anti-mouse) (Invitrogen) were incubated with the slides, which were counterstained with DAPI (Invitrogen). Alexa Fluor 488 (anti-goat), Alexa Fluor 594 (anti-rabbit) and Alexa Fluor 647 (anti-mouse) were multiplexed together for Msx1, MyoD and Myogenin staining. Alexa Fluor 594 (anti-rabbit) and Alexa Fluor 647 (anti-mouse) were

multiplexed together for Pax3 and NeuroD1 staining. Alexa Fluor 488 (anti-mouse) was used for Sox10 staining. Slides were examined by conventional immunofluorescence on a Nikon Ti Eclipse Inverted Microscope.

Analysis of protein expression

To determine the overall expression of each transcription factor, its intensity was calculated using ImageJ following the protocol developed Arques et al., 2012. Briefly, a region of interest (ROI) was defined around each embryoid body in the blue (DAPI) channel and an integrated density value (IDV) calculated. This was then repeated for each channel of interest. Next, ten representative nuclei covering different sizes and intensities throughout the blue channel ROI were marked using the elliptical selection tool, and an average IDV was calculated. Next, the blue channel IDV was divided by the mean nucleus value, resulting in the average number of cells present in each ROI. Then, to calculate individual protein content per EB, each respective channel IDV (green, red, far red) was divided by the average number of cells.

To examine nuclear localization of specific transcription factors, and co-localization of multiple transcription factors, a 50 µm x 100 µm grid was overlaid in the bottom right-hand corner of each image. Approximately 100 cells were counted per image and scored as to whether each transcription factor was localized in the nucleus of the cell, and whether two or more transcription factors were co-localized together.

Statistical analysis

For image intensity, the replicates (n=3) of each treatment, day, and transcription factor were averaged together and statistical significance determined by ANOVA followed by Tukey's ($p \leq 0.05$). Nuclear localization and co-localization numbers were converted into percentages for each treatment, day, and transcription factor(s) (n=3) and statistical significance determined by ANOVA followed by Tukey's ($p \leq 0.05$).

Results

Time course of transcription factor expression and nuclear translocation in P19 cell-derived embryoid bodies

To determine if arsenic or its metabolites targeted the neural plate border specifier (NPBS) cells and impacted their differentiation, a time-course of transcription factor expression during embryoid body formation was first determined. Key protein markers of NPBS cells include Msx1 and Pax3 (Sauka-Spengler and Bronner-Fraser, 2008; Betancur et al., 2010). Along the neurogenic lineage, NPBS cells differentiate into neural crest (NC) progenitor cells by expressing the transcription factor Sox10 (Sauka-Spengler and Bronner-Fraser, 2008). The NC progenitor cells then differentiate into NC cells, expressing the transcription factor NeuroD1 before developing into sensory neurons (Betancur et al., 2010). Along the myogenic lineage, NPBS cells differentiate into myogenic progenitor cells, expressing the transcription factor MyoD (Miner and Wold; 1991; Ridgeway et al., 2000). The myogenic progenitor cells then differentiate into myocytes, expressing Myogenin, and then finally develop into Myotubes (Ridgeway et

al., 2000). To determine the temporal aspects of each transcription factor, its expression was examined in day 2, 3, 4, and 5 embryoid bodies (EBs).

In the neurogenic lineage, Pax3, a marker of neural plate border specifier (NPBS) cells, and Sox10, a marker of neural crest progenitors, had steady, medium expression throughout days 2-5 (Table 1). NeuroD1, a neural crest cell marker, is not expressed on days 2-3, but has high expression on day 4, and medium expression on day 5 (Table 1).

In the myogenic lineage Msx1, a NPBS cell marker had low expression on day 2, high expression days 3-4, and medium expression on day 5 (Table 1). MyoD, a myogenic progenitor marker, expression has medium expression throughout days 2-5 (Table 1). Myogenin, which is a marker for myocytes, is not expressed on days 2-4, and has low expression on day 5 (Table 1).

When looking at the percent nuclear localization, on day 2 only Pax3 has at least 50% nuclear localization (Table 1; Figure 1C). Sox10, Msx1, and MyoD are mainly found in the cytoplasm with 1-10% nuclear localization on day 2 (Table 1; Figure 1C). Sox10 and Msx1 stay this way throughout days 2-5 (Figure 1C-4C), while MyoD's translocation to the nucleus increases on days 3-5 (Table 1; Figure 2C-4C). NeuroD1 is not expressed until day 4, but once expressed, it is predominantly expressed in the nucleus on days 4 and 5 (Table 1; Figure 3 and 4C). Myogenin, which is not expressed until day 5, is predominantly found in the cytoplasm (Table 1; Figure 4C).

Arsenic targets neural plate border specifier cells

To determine whether neural plate border specific cells are the target cell population of arsenic, embryoid bodies (EBs) were exposed to 0, 0.1, and 0.5μM sodium

arsenite and expression of transcription factors along the muscle and neural lineages examined. After 2 days of exposure, the 0.1 and 0.5µM treated cells showed a significant 15-19% decrease in Pax3 intensity (Figure 1 A and B). After 3 days of arsenic exposure, Sox10 expression was also significantly decreased by 30% and 51% in the 0.1 and 0.5 µM treatments, respectively (Figure 2). After 4 and 5 days of arsenic exposure, NeuroD1 expression was decreased by 23% in the 0.5 µM treatment (Figure 3A and B; Figure 4A and B). In the neurogenic pathway, it appears that arsenic is targeting the neural plate border specifier cells starting on day 2, which results in reduced transcription factor expression indicative of both neural crest progenitor cells and neural crest cells (Table 2). Within the muscle lineage, only Msx1 expression was significantly decreased (34%) in the 0.5 µM treatment on day 3 (Figure 2 A and B). No other transcription factor levels were altered on any of the remaining days due to arsenic exposure (Table 2).

Monomethylarsonous acid (MMA III) and dimethylarsinous acid (DMA III) targets neural plate border specifier cells

Since methylated arsenical metabolites often have higher toxicity than that of the inorganic species (Ferrario et al., 2008; Wang et al., 2014), P19 cells were exposed to MMA(III) and DMA(III) for either 3 or 5 days, and the expression of transcription factors along the muscle and neural lineages were examined. After 3 days of exposure to MMA , Msx1, MyoD, Pax3 and Sox10 expression were significantly decreased in the 0.05 µM treatment by 22%, 11%, 7%, and 26%, respectively (Figure S1; Figure 5 A). After 5 days of MMA exposure, only the neurogenic lineage was affected with Pax3,

Sox10 and NeuroD1 showing a significant reduction in expression of 10%, 11%, and 13%, respectively in the 0.05 µM treatment (Figure S2; Figure 6A).

None of the transcription factor levels were reduced after 3 days of DMA exposure (Figure S3; Figure 7 A). However, after 5 days of DMA exposure, Msx1, MyoD and Myogenin were all significantly reduced by 17%, 12%, and 22%, respectively (Figure S4; Figure 8 A). In the neurogenic pathway, Sox10 and NeuroD1 showed a significant decrease in the 0.005 µM treatment by 13% and 9% (Figure S4; Figure 8 A).

In the neurogenic pathway, it appears that MMA is targeting the neural plate border specifier cells on day 3 while DMA does not affect transcription factor expression until day 5 (Table 4).

Arsenic alters nuclear localization of transcription factors

In addition to examining overall transcription factor expression, their cellular localization was also examined. In the myogenic pathway after 2 days of arsenic exposure, Msx1 nuclear localization was significantly decreased by 1.5-fold, while MyoD nuclear expression was reduced by 3.4-fold in the 0.5µM treatment (Figure 1C). On days 3, 4, and 5, MyoD nuclear localization continues to be reduced in the 0.5 µM treatment by 2.2, 1.2-, and 1.2-fold, respectively (Figure 2-4C). On both days 4 and 5, NeuroD1 nuclear expression was significantly reduced in the 0.5 µM treatment by 1.1- and 1.4-fold, respectively (Figure 3-4C).

Monomethylarsonous acid (MMA III) and Dimethylarsinous acid (DMA III) alters nuclear localization of transcription factors

After 3 days of exposure to MMA III (0.05 µM) and DMA III (0.005 µM), MyoD nuclear localization was significantly decreased by 2-fold and 1.5-fold (Figure 5B and 7B; Table 5). After 5 days of exposure to MMA III and DMA III, MyoD nuclear localization was again significantly decreased by 1.1-fold and 1.3-fold, at the highest concentrations (Figure 6B and 8B). In addition, NeuroD1 nuclear localization was significantly decreased in both the MMA III and DMA III treatments at the highest concentrations after 5 days of exposure by 1.4-fold (Figure 5C and 7C).

Arsenic alters co-localization patterns

In addition to looking at overall intensity and nuclear localization of the transcription factors, their co-localization patterns within the cells were examined as well. In the myogenic pathway, Msx1 and MyoD co-localized together predominately in the cytoplasm on days 2-4. However, the frequency of them being together in the same cell drops from 98% on day 2, to 73% on day 3, and only 38% on day 4 (Figures 1-3). When treated with arsenic, on day 2 of differentiation, the overall co-localization of these two transcription factors is decreased by 1.5-fold (Figure 1). By differentiation day 4, arsenic exposure does not alter the overall percentage of cells co-expressing Msx1 and MyoD, however, arsenic does significantly increase the percentage of cells expressing these transcription factors exclusively in the cytoplasm from 34% to 42% (Figure 3).

In the neurogenic pathway, Pax3 and NeuroD1 are expressed in the same cells 82-84% of the time on days 4 and 5. Their co-expression is predominately located in the

nuclei (Figure 3-4), although this drops from 77% on day 4 to 66% on day 5. When cells are exposed to arsenic, there is a significant shift towards the co-expression being in the cytoplasm rather than in the nuclei, such that on day 5, co-localization of Pax3 and NeuroD in the nucleus drops from 66% of cell to 48% of cells.

Monomethylarsonous acid (MMA III) and dimethylarsinous acid (DMA III) alters co-localization patterns

After exposure to MMA, similar co-localization patterns were seen in the myogenic pathway. For example, the frequency of Msx1 and MyoD being expressed in the same cell on day 3 of differentiation decreases from 78% in the controls to 66% in the 0.05 µM MMA group (Figure 5). With DMA, while there is not an overall reduction in Msx1 and MyoD expression in the same cell, there is a shift in cellular localization patterns. DMA exposure reduces the nuclear co-expression of Msx1 and MyoD by 3.6-fold (Figure S3). Interestingly, neither MMA nor DMA alter the co-localization of Pax3 and NeuroD on day 5 of differentiation (Figure S2 and S4).

Discussion

The results from this study show that arsenic and its methylated metabolites (MMA and DMA) inhibit neurogenesis and myogenesis in P19 cells by targeting the neural plate border specifier cells. In addition, arsenic and its metabolites were shown to alter nuclear localization, and co-localization patterns of the key markers involved in neurogenesis and myogenesis.

Starting on day 2 of sodium arsenite exposure, it was already apparent that the neural plate border specifier (NPBS) cells were being targeted. In the neurogenic lineage, in both the 0.1 and 0.5 µM treated cells, a significant decrease in Pax3 intensity was seen. Similar findings have been found in our lab. After P19 cells were exposed to 0.1 and 0.5 µM sodium arsenite Pax3 protein was reduced in day 2 EBs (Hong and Bain, 2012). It is known that during embryogenesis, Pax3, a marker of NPBS cells, is activated by the Wnt/β-catenin pathway (Marikawa et al., 2009). However, in order for neural crest specification to occur, Zic1 need to be activated at the same time as Pax3 (Betancur et al., 2010). Pax3 alone is only capable of causing moderate induction of the neural crest program followed by migration and differentiation (Milet et al., 2013). Zic1 expression alone activates snail1, an early neural crest specifier (Plouhinec et al., 2014). Once activated, the Pax3/Zic1 combination activates a cascade of genes involved in neural plate border formation, including snail2, another neural crest specifier, and Sox10 activation later on during neurulation (Milet et al., 2013). The co-activation of these TFs causes additional NPBS expression and the neural crest to begin its EMT, migration, and differentiation (Milet et al., 2012). The Pax3/Zic1 combination then helps regulate the differentiation of neurons and skeletal muscle by activating further transcription factors required for myogenesis and neurogenesis (Ridgeway et al., 2000). In addition to the Pax3/Zic1 co-activation, Pax3 has recently been found to target itself. It was found that there are Pax3 binding sites upstream of its promoter (Plouhinec et al., 2014), indicating that there is a positive feedback loop in addition to the Pax3/Zic1 co-expression that is driving the neural crest gene regulatory network.

The reduction in Pax3 expression may help explain the decrease in Sox10, a marker of neural crest progenitors, on day 3, and a reduction in NeuroD1, a neural crest cell marker, on days 4 and 5. As mentioned before, the Pax3/Zic1 co-expression, in addition to the positive feedback loop of Pax3 on itself, activates a cascade of genes involved in neurogenesis, including Sox10 (Milet et al., 2013). Studies have shown that Pax3 and Sox10 physically interact in complex signaling pathways and protein-protein interactions (Lang and Epstein, 2002). Once Pax3 is bound to the DNA, it interacts with Sox 10 via the Pax3 paired domain (PD) (Lang and Epstein, 2002) resulting in increased transcriptional activity. Normally, Sox10, a marker of neural crest progenitors, then activates Islet1 (Isl1) whose role is to carry out the terminal differentiation of neurons involving activation of basic helix-loop-helix genes such as NeuroD (Radde-Gallwitz et al., 2004).

Normally, Sox10 expression begins when neural crest progenitors start to migrate from the neural tube and its expression starts to decrease as the cells differentiate into neural crest cells, which express NeuroD1 (Britsch et al., 2001). It makes sense that if there is a reduction on Pax3 on day 2, that there would be a reduction in subsequent transcription factors, including Sox10 and NeuroD1 leading to a reduction in neurogenesis.

Regarding the muscle lineage, the NPBS cells were again being targeted. This was apparent due to the significant decrease in the expression of the NPBS cell marker Msx1 on day 3. It is interesting to note that no other transcription factor levels were altered on any of the remaining days due to arsenic exposure (Table 2). One possible

explanation is the protein-protein interaction between Msx1 and Pax3. Normally, Msx1 expression overlaps the expression of Pax3, as shown in migrating limb muscle precursors (Bendall et al., 1999). Pax3 expression is then downregulated once myogenic regulatory transcription factors are expressed (Kuang et al., 2006; Goulding et al., 1994; Williams and Ordahl, 1994), such as MyoD. These muscle precursors are committed to form myoblasts, which express markers such as MyoD. Pax3 then promotes the delamination and migration of these muscle precursors from the neural tube (Buckingham, 2007; Bajard et al., 2006). In our study, there was a decrease in Msx1 intensity on day 3 with no decrease of subsequent markers such as MyoD or Myogenin. This could due to the fact that since Msx1 expression has decreased, there is a decrease in the Msx1-Pax3 protein-protein interaction, and Pax3 is still able to activate MyoD expression, as seen in previous studies (Buckingham, 2007; Bendall et al., 1999).

With arsenic's methylated metabolites; monomethylarsonous acid (MMA III) and dimethylarsinous acid (DMA III), some differences between the treatments were seen. In the neurogenic pathway, there was an early decrease in Pax3 and Sox10 on day 3 after MMA exposure, but not after 3 days of DMA exposure. It seems that MMA targets the NPBS cells early on, where DMA does not. There was then a subsequent decrease in expression of NeuroD1 on day 5 in both the MMA and DMA treatments, indicating that they were targeting the neural crest cells. With the myogenic pathway's response to MMA III and DMA III treatment, there was an early decrease in Msx1 and MyoD expression seen on day 3 in the MMA treatment, but not the DMA treatment, potentially indicating again that MMA is targeting the NPBS cells much earlier than DMA, and

possibly that the DMA concentrations were too low to cause an effect. There were then no subsequent decreases in any markers on day 5 in the MMA treatment, but all of the myogenic markers (Msx1, MyoD, Myogenin) were decreased in the DMA treatment on day 5. These findings, first, show that methylated metabolites are more toxic to the cells at a much lower concentration than sodium arsenite, which is consistent with other studies (Wang et al., 2014; Ferrario et al., 2008). The methylated metabolites have been shown to have a high binding affinity to thiol groups in proteins and are very good at inhibiting enzymes (Styblo etl., 1997) It is also interesting to note that P19 appear to have arsenic methylation capabilities. Arsenite methyltransferase (As3MT) is the enzyme that transfers the methyl group from SAM turning arsenite into MMA (Albores et al., 1992), and at least its transcript is present in stem cells both before and after differentiation into embryoid bodies (Supplementary Figure 5). Second, these results suggest that the neural lineage seems to be affected much more than the myogenic lineage after exposure to MMA III and DMA III. This is also confirmed when looking at the sodium arsenite exposures.

Finally, nuclear localization and co-localization of the markers, NeuroD1 and MyoD are altered during the differentiation process in exposure to sodium arsenite (Table 3), MMA III and DMA III (Table 5). It appears that arsenic and its metabolites are inhibiting the translocation of the markers to the nucleus. Arsenic itself could be targeting the transcriptional complex of these markers. Normally, all of the basic helix-loop-helix proteins (bHLH) bind to a consensus E-box sequence (CANNTG) (Berkes and Tapscott, 2005). MyoD forms a heterodimer with E-protein, a sub-family of bHLH proteins, by

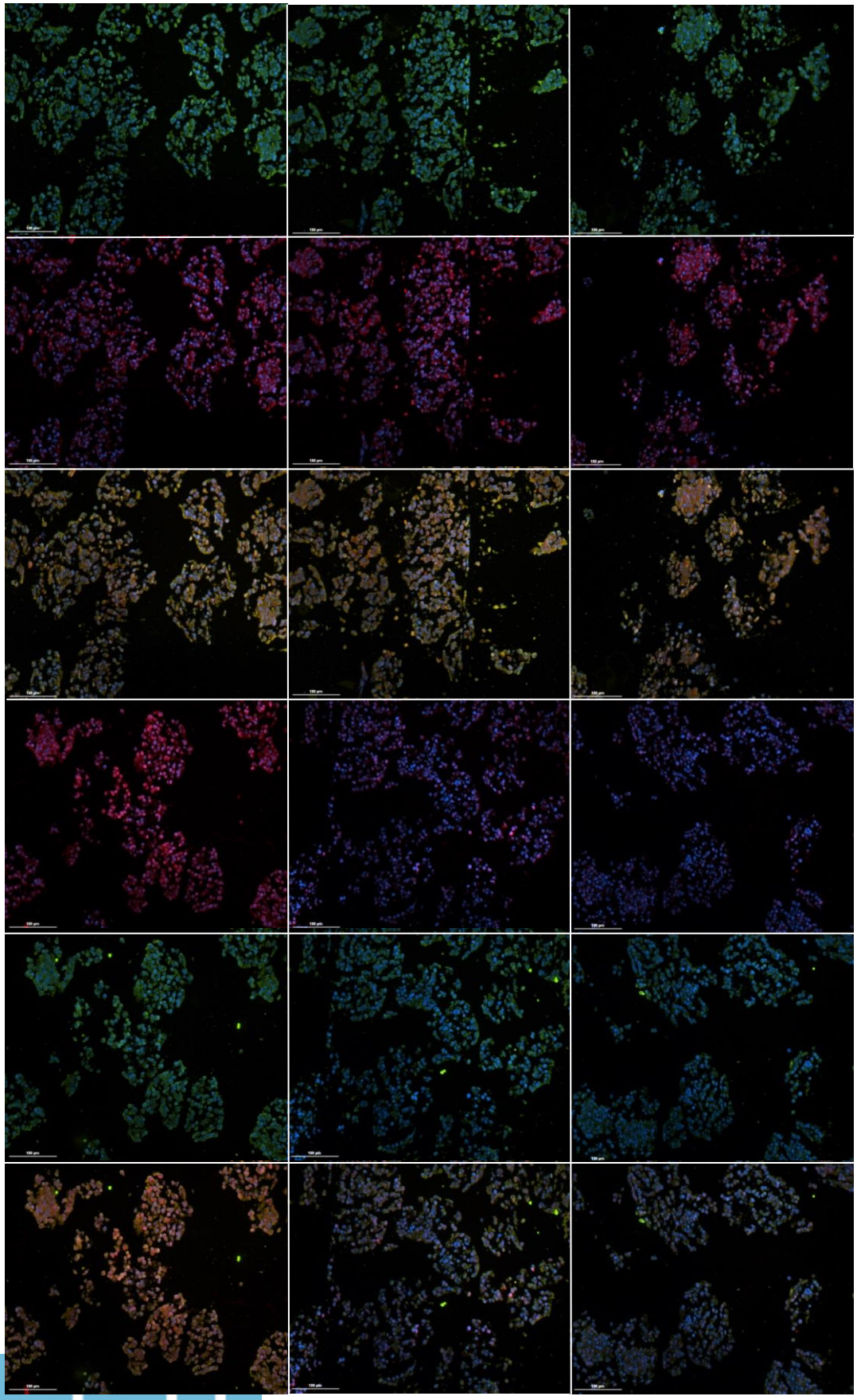
interacting with the HLH domains (Lassar et al., 1991; Murre et al., 1989) which mediate additional bHLH dimerization (Berkes and Tapscott, 2005). MyoD then activates further gene transcription by binding to E-boxes in the promoter region of the skeletal muscle genes (Tapscott, 2005). Many of these binding sites recognize sub-families of the bHLH proteins like NeuroD (Tapscott, 2005). Arsenic then could be blocking the MyoD and E-box transcriptional complex by interfering post-translational modifications of MyoD.

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) both interact with MyoD, switching it from a repressor state, to an activator (Berkes and Tapscott, 2005). HATs transfer an acetyl group from acetyl-coA, which has been shown to increase during the differentiation stage of myogenesis (Polesskaya et al., 2001), which then causes the transfer of histones H2A/H2B from DNA to chaperone proteins (Ito et al., 2000). This then allows transcription factors, like MyoD, to get to the DNA and bind with the E-box forming the transcriptional complex (Bergstrom et al., 2002). In myotubes, the complex formed includes MyoD, and HAT-proteins p300 and PCAF which then allows MyoD to stably bind to the E-box of additional myogenic genes (Puri et al., 1997). Arsenic then could be interfering with HATs, and stopping the formation of these complexes. Without MyoD translocation to the nucleus and binding to the E-box, no further signal transduction is occurring, which then results in the decrease in the differentiation of the precursors into myotubes and sensory neurons.

In conclusion, our results indicate that As, MMA III, and DMA III all suppresses skeletal muscle and sensory neuron formation by targeting NPBS cells during embryogenesis in P19 mouse embryonic stem cells. MMA III was shown to be the most

toxic to the cells, followed by DMA III, and sodium arsenite. The metabolites toxicity is of great concern due to the fact that they are increased during pregnancy and can cause miscarriages, and many detrimental neurological and physical conditions later on in life. This study also sheds light on some of the temporal and spatial aspects on the neurogenic and myogenic signals involved after exposure to arsenic and its metabolites. In addition, it provides insight on how exposure to arsenic and its metabolites can affect differentiation of skeletal muscle and sensory neurons, and shows the effectiveness of using embryonic stem cells to study the determination of cell fates.

A.



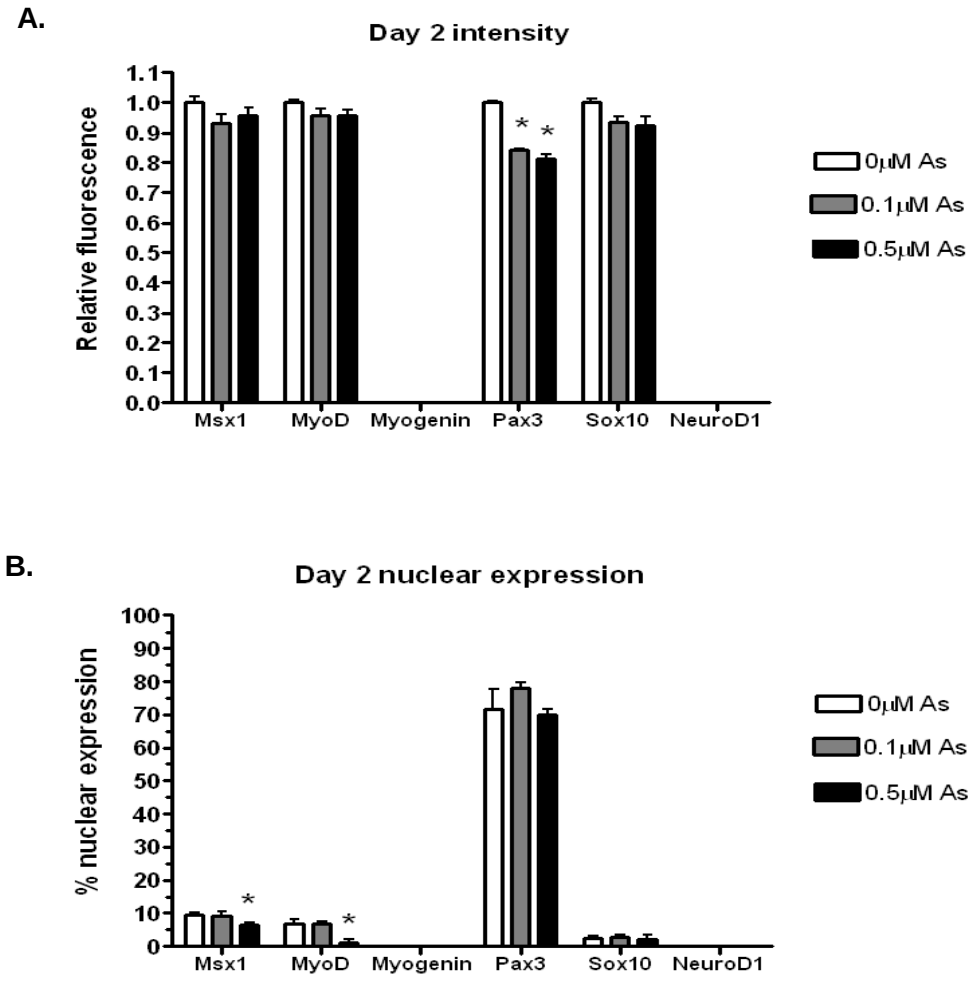
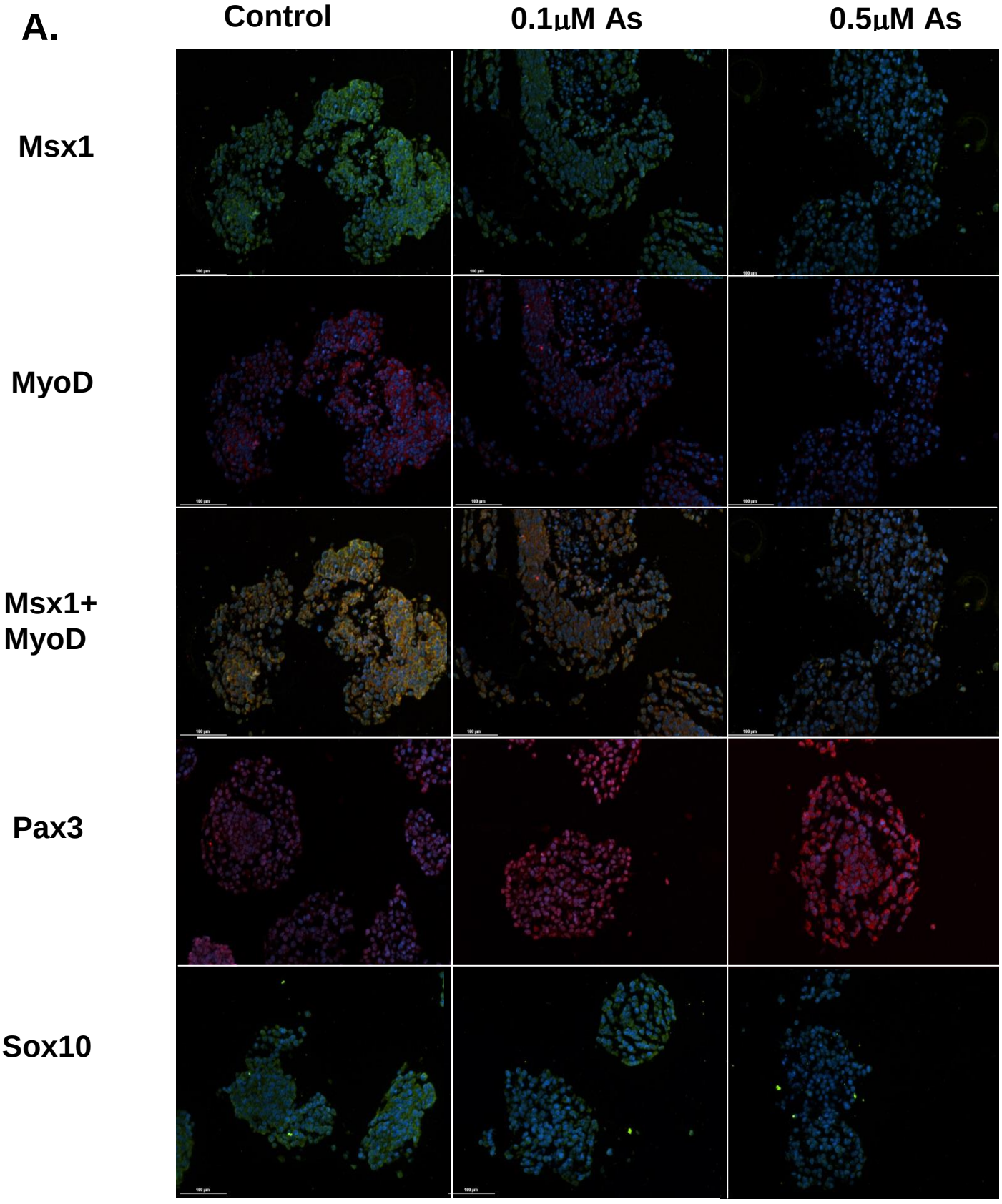


Figure 1. Arsenic decreases Pax3 intensity, and Msx1 and MyoD nuclear localization in day 2 embryoid bodies

After exposure to 0, 0.1, and 0.5 µM arsenic for two days, embryoid bodies were fixed, embedded, and stained for transcription factor expression. Representative images are shown (A; n=3 per group). Intensity values were averaged, and are presented as relative fluorescence after normalization to the control group ± standard deviation (B). Nuclear localization was determined by placing a 50µm x 100µm grid on each image. The cells (n=100) where the counted and examined for nuclear localization of each marker. Nuclear localization is presented as % nuclear localization ± standard deviation (C). Statistical differences were determined by ANOVA followed by Tukey's (*; $p<0.05$). Although myogenin and NeuroD1 expression were examined, neither of the transcription factors are expressed on day 2.

A.



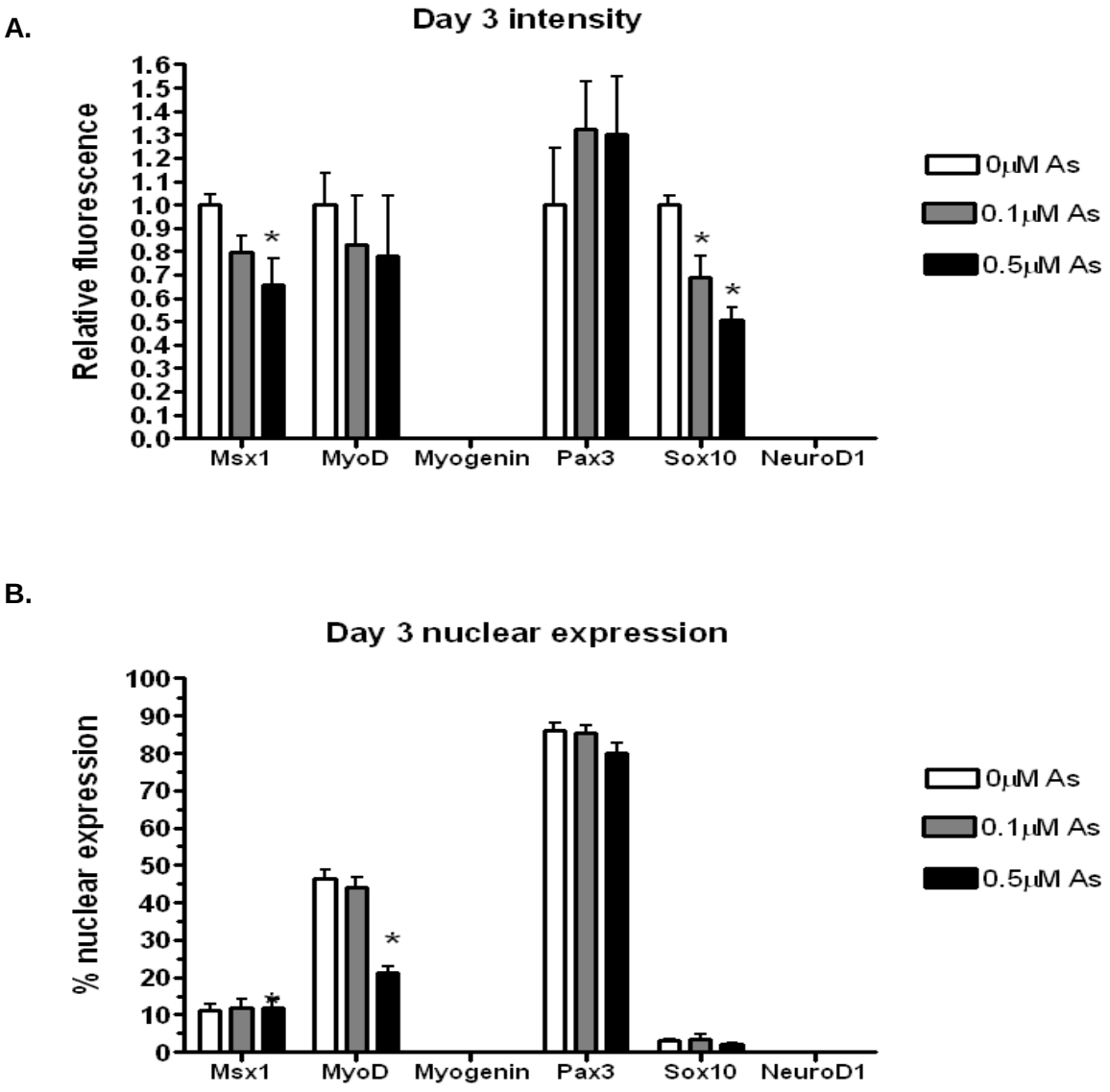
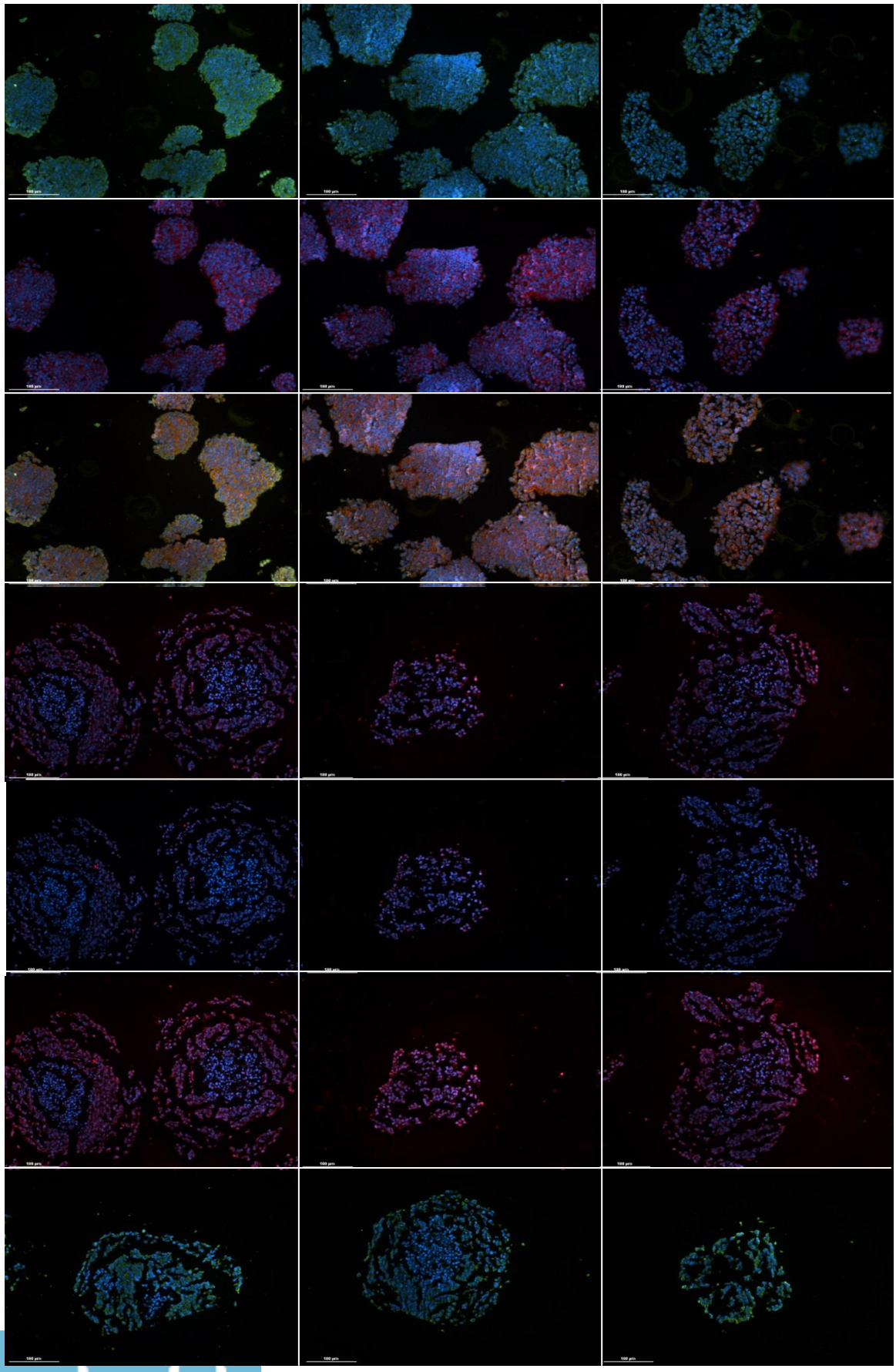


Figure 2. Arsenic decreases Sox10 intensity, and MyoD nuclear localization in day 3 embryoid bodies

After exposure to 0, 0.1, and 0.5 μM arsenic for three days, embryoid bodies were fixed, embedded, and stained for Msx1, MyoD, Pax3, and Sox10 expression. Representative images are shown (A; n=3 per group). Intensity values (B) and nuclear localization (C) were calculated as in Figure 1. Statistical differences were determined by ANOVA followed by Tukey's (*; $p<0.05$).

A.



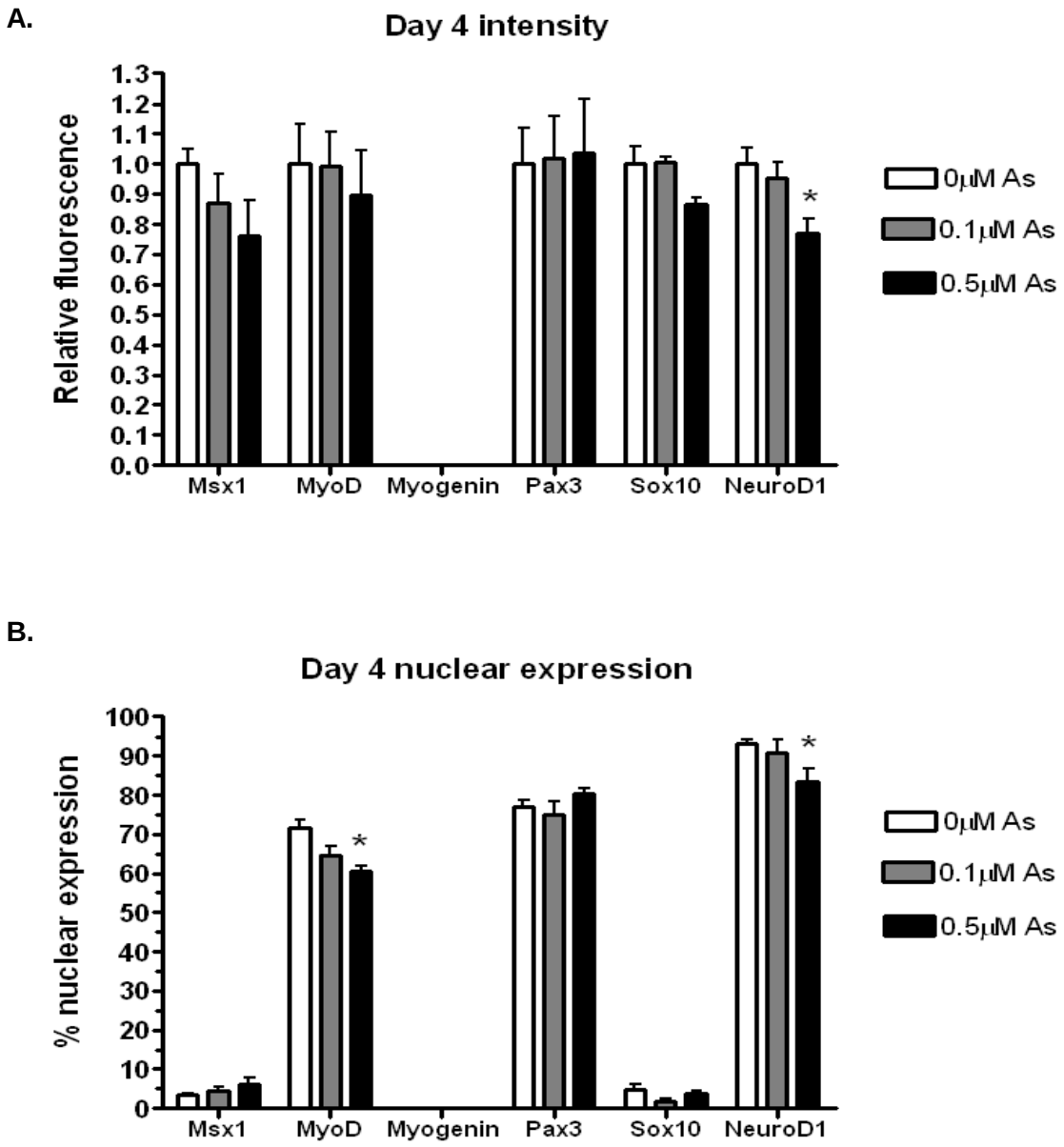
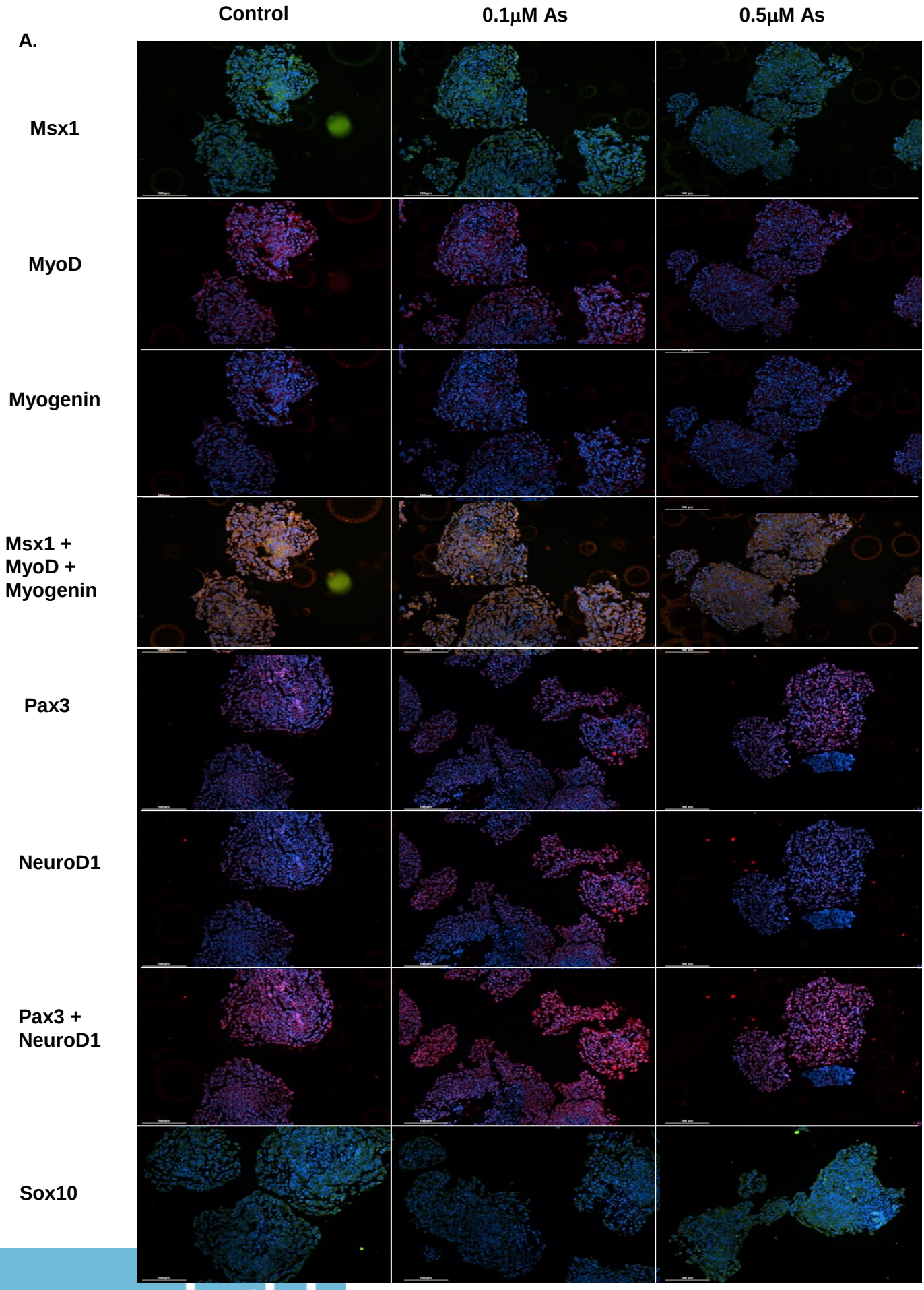


Figure 3. Arsenic decreases NeuroD1 intensity, and MyoD and NeuroD1 nuclear localization in day 4 embryoid bodies

After exposure to 0, 0.1, and 0.5 μM arsenic for four days, embryoid bodies were fixed, embedded, and stained for transcription factor expression. Representative images are shown (A; n=3 per group). Intensity values (B) and nuclear localization (C) were calculated as in Figure 1. Statistical differences were determined by ANOVA followed by Tukey's (*; $p<0.05$).



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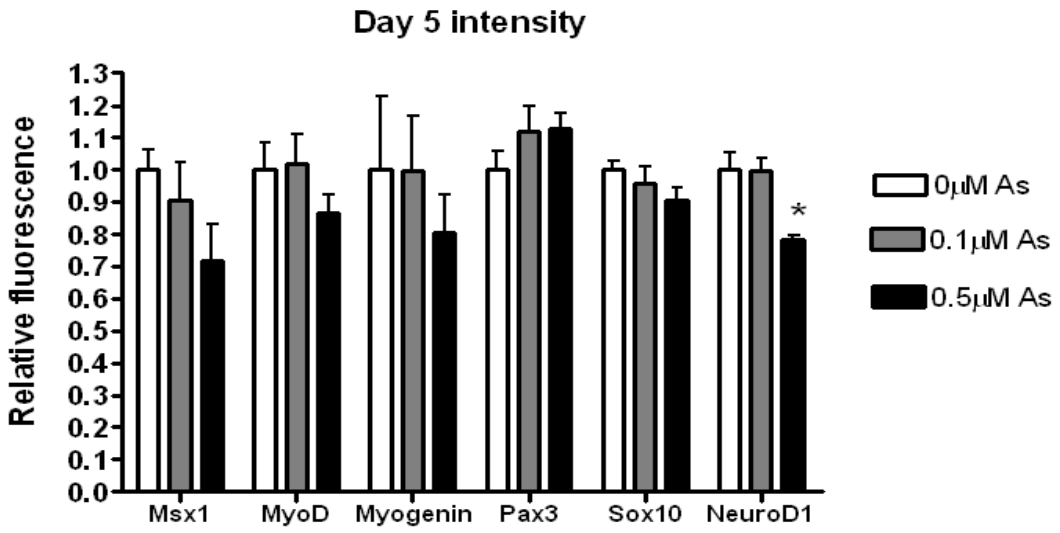


B.

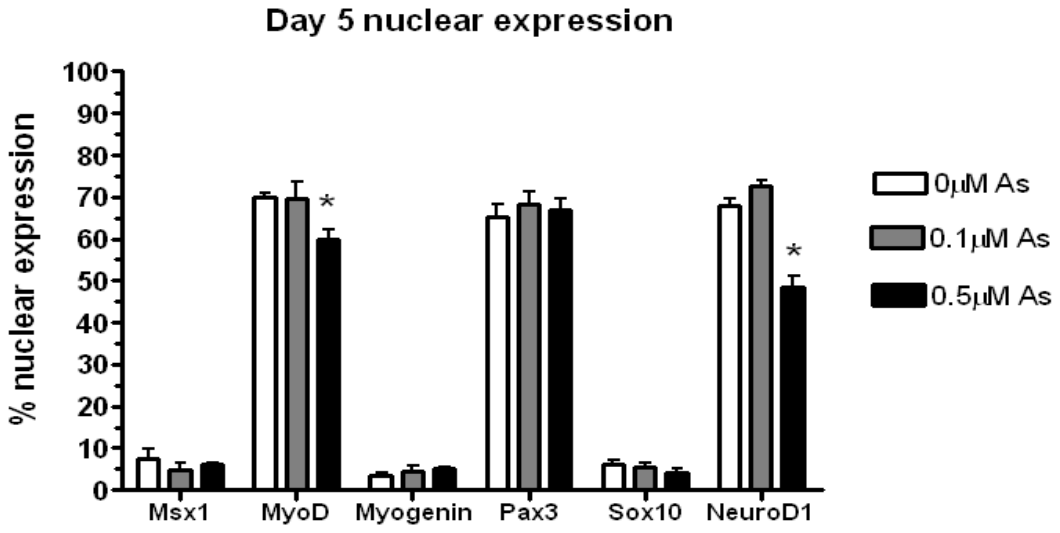


Figure 4. Arsenic decreases NeuroD1 intensity and nuclear localization in day 5 embryoid bodies

After exposure to 0, 0.1, and 0.5 μM arsenic for five days, embryoid bodies were fixed, embedded, and stained for Msx1, MyoD, Myogenin, Pax3, Sox10, and NeuroD1 expression. Representative images are shown (A; n=3 per group). Intensity values (B) and nuclear localization (C) were calculated as in Figure 1. Statistical differences were determined by ANOVA followed by Tukey's (*; $p<0.05$).

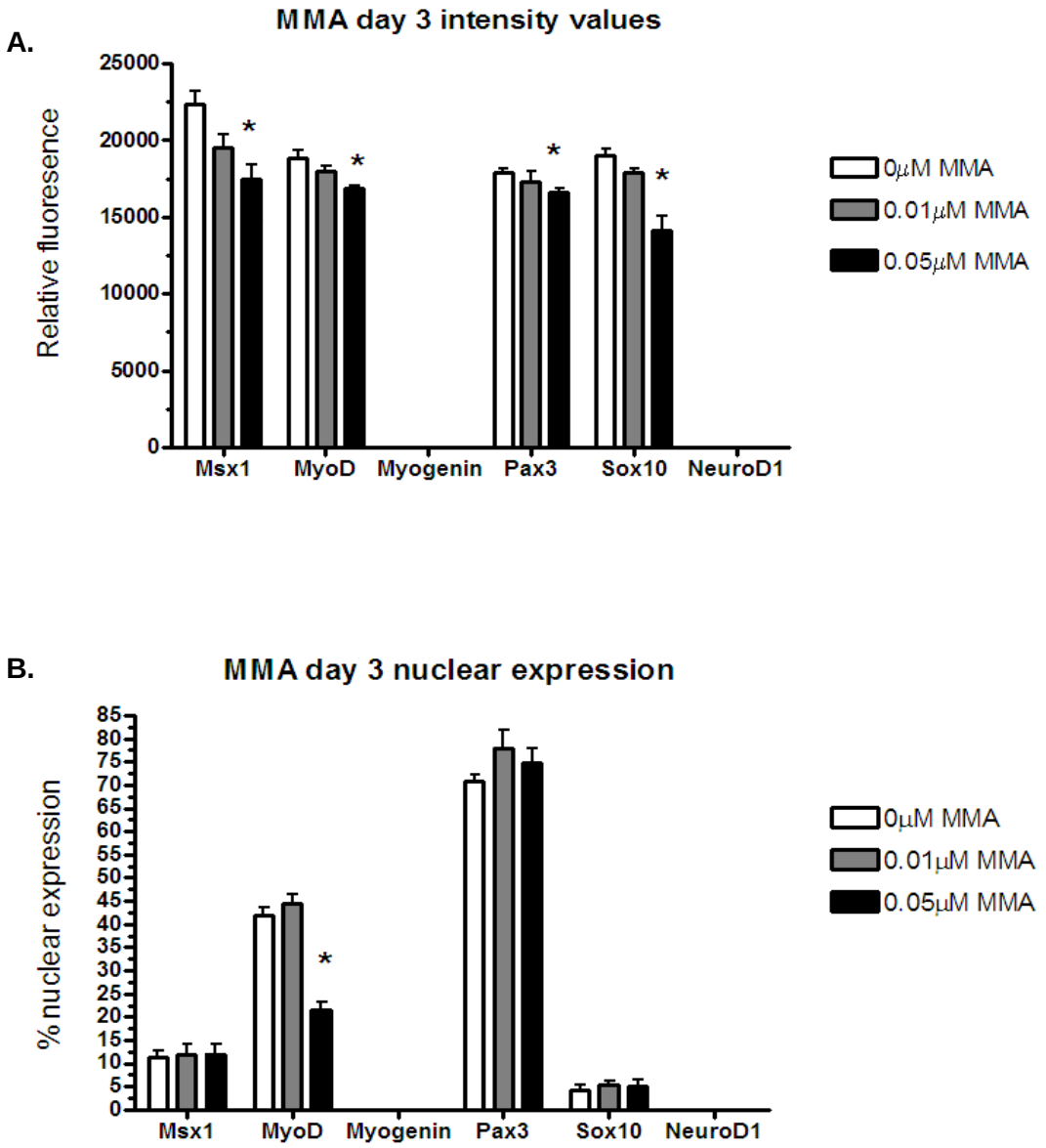


Figure 5. MMA(III) MyoD and Pax3 nuclear localization in day 3 embryoid bodies

After exposure to 0, 0.01, and 0.05 μM MMA for three days, embryoid bodies were fixed, embedded, and stained for transcription factor expression. (Representative images are shown in Figure S1, n=3 per group). Intensity values (A) and nuclear localization (B) were calculated as in Figure 1. Statistical differences were determined by ANOVA followed by Tukey's (*; $p<0.05$).

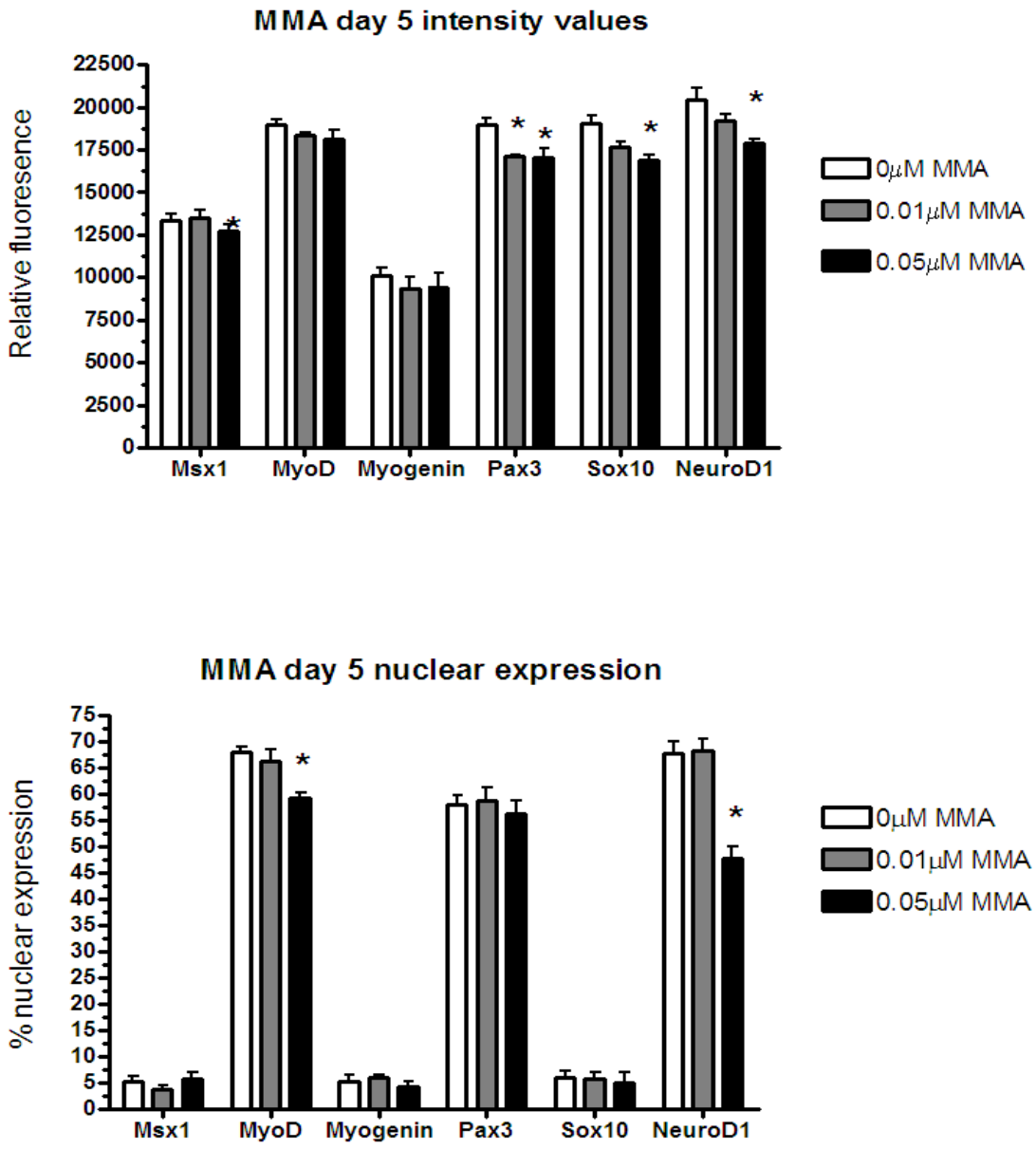


Figure 6. MMA(III) decreases MyoD and Pax3 nuclear localization in day 5 embryoid bodies

After exposure to 0, 0.01, and 0.05 μM MMA for five days, embryoid bodies were fixed, embedded, and stained for Msx1, MyoD, Myogenin, Pax3, Sox10, and NeuroD1 expression. (Representative images are shown in Figure S2, n=3 per group). Intensity values (A) and nuclear localization (B) were calculated as in Figure 1. Statistical differences were determined by ANOVA followed by Tukey's (*; $p<0.05$)

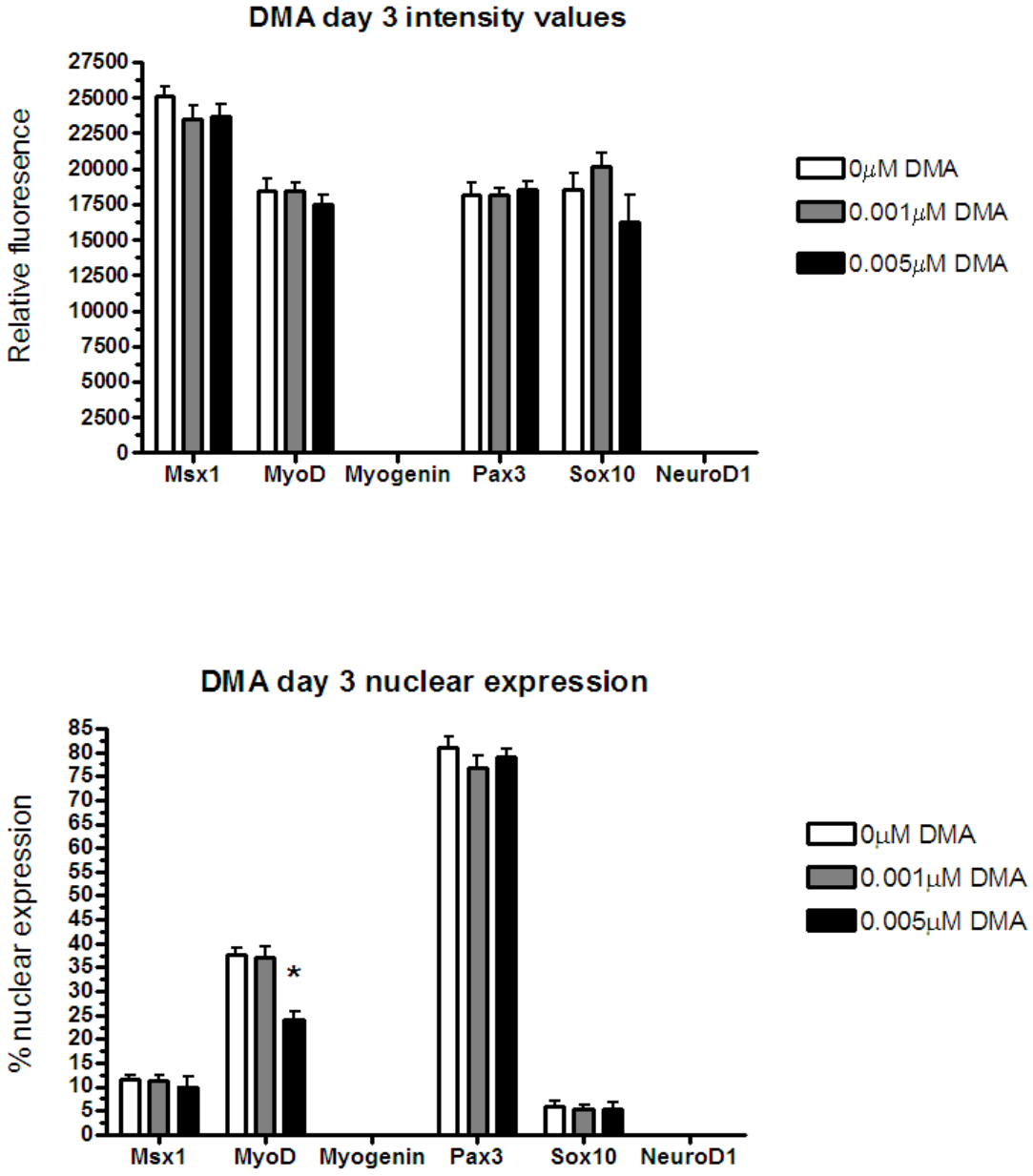


Figure 7. DMA(III) decreases MyoD nuclear localization in day 3 embryoid bodies

After exposure to 0, 0.001, and 0.005 μM DMA for three days, embryoid bodies were fixed, embedded, and stained for transcription factor expression. (Representative images are shown in Figure S3, n=3 per group). Intensity values (A) and nuclear localization (B) were calculated as in Figure 1. Statistical differences were determined by ANOVA followed by Tukey's (*; $p<0.05$).

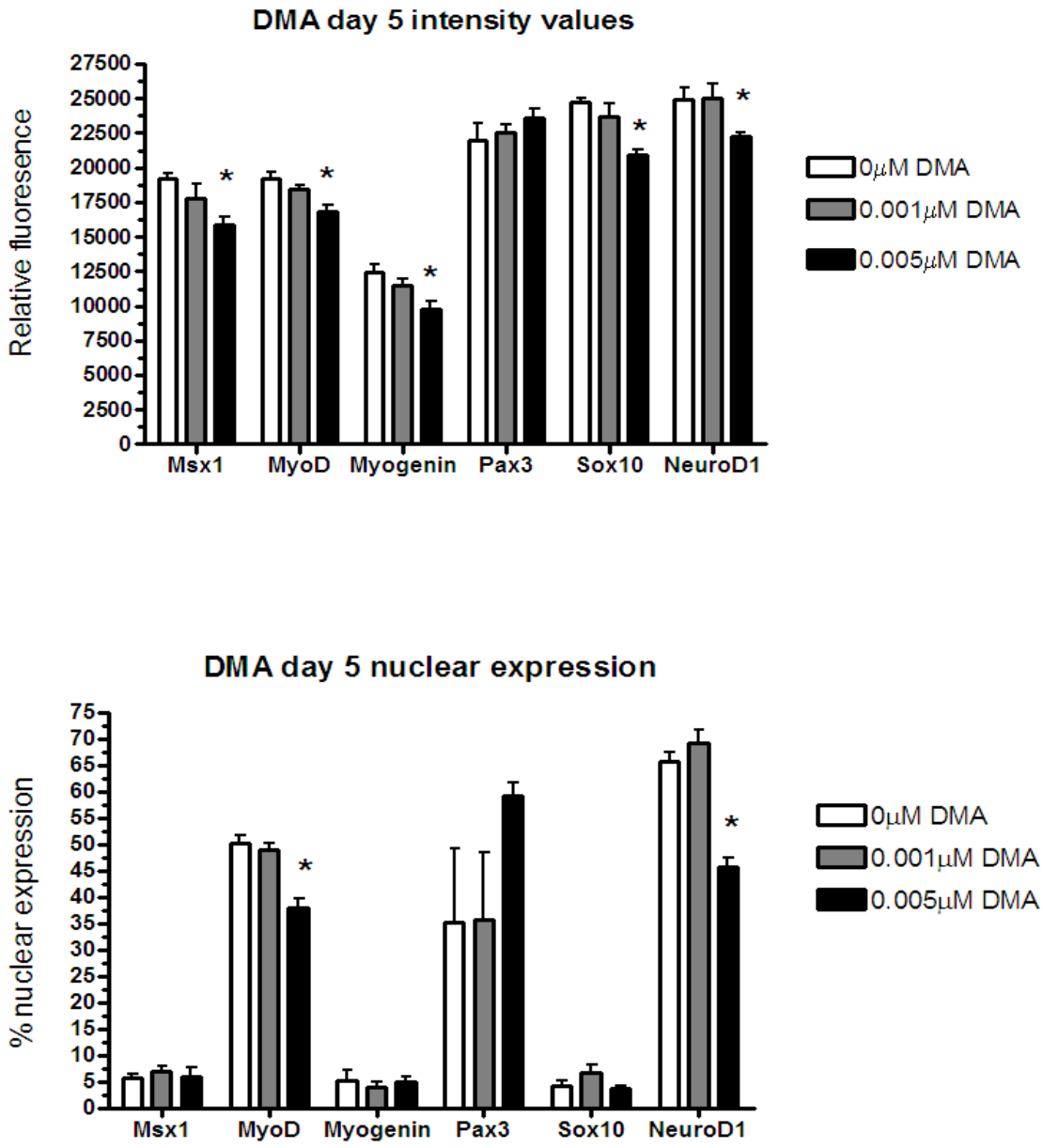


Figure 8. DMA(III) decreases MyoD and NeuroD1 nuclear localization in day 5 embryoid bodies

After exposure to 0, 0.001, and 0.005 μM DMA for five days, embryoid bodies were fixed, embedded, and stained for Msx1, MyoD, Myogenin, Pax3, Sox10, and NeuroD1 expression. (Representative images are shown in Figure S4, n=3 per group). Intensity values (A) and nuclear localization (B) were calculated as in Figure 1. Statistical differences were determined by ANOVA followed by Tukey's (*; $p<0.05$).

Table 1. Time course of transcription factor expression and nuclear translocation in P19 cell-derived embryoid bodies.

	Expression (days)[a]					Nuclear localization (days)[b]			
	2	3	4	5		2	3	4	5
Neural lineage									
Pax3 (neural plate border cells)	++	++	++	++		+++	++++	++++	+++
Sox10 (neural crest progenitors)	++	++	++	++		+	+	+	+
NeuroD1 (neural crest cells)	-	-	+++	++		-	-	++++	+++
Muscle lineage									
Msx1 (neural plate border cells)	+	+++	+++	++		+	+	+	+
MyoD (myogenic progenitor)	++	++	++	++		+	++	++++	+++ +
Myogenin (myocytes)	-	-	-	+		-	-	-	+

[a]Relative expression values; " –": not expressed; ""+": low expression; "++": medium expression; "+++": high expression.

[b]Average percent nuclear localization: " –": not expressed; ""+": 1-10% nuclear localization; "++": 11-50% nuclear localization; "+++": 51-70% nuclear localization; "++++": >71% nuclear localization.

Table 2. Changes in overall transcription factor expression after exposure to 0.1 and 0.5 µM arsenic during P19 cell differentiation.

	Day 2	Day 3	Day 4	Day 5
Neural Lineage				
Pax3 (neural plate border cells)	↓	—	—	—
Sox10 (neural crest progenitors)	—	↓	—	—
NeuroD1 (neural crest cells)	X	X	↓	↓
Muscle Lineage				
Msx1 (neural plate border cells)	—	↓	—	—
MyoD (myogenic progenitor)	—	—	—	—
Myogenin (myocytes)	X	X	X	—

An arrow indicates reduced expression, a dash indicates no change in expression, and an X indicates that the TF was not expressed on that day.

Table 3. Changes in overall transcription factor expression after exposure to monomethylarsonous acid (MMA) and dimethylarsinous acid (DMA) during P19 cell differentiation.

	Day 3			Day 5		
	iAs	**MMA**	**DMA**	**iAs**	**MMA**	**DMA**
Neural Lineage						
Pax3 (neural plate border cells)	—	↓	—	—	↓	—
Sox10 (neural crest progenitors)	↓	↓	—	—	↓	↓
NeuroD1 (neural crest cells)	X	X	X	↓	↓	↓
Muscle Lineage						
Msx1 (neural plate border cells)	↓	↓	—	—	—	↓
MyoD (myogenic progenitor)	—	↓	—	—	—	↓
Myogenin (myocytes)	X	X	X	—	—	↓

An arrow indicates reduced expression, a dash indicates no change in expression, and an X indicates that the TF was not expressed on that day.

Table 4. Changes in the nuclear translocation of transcription factors after exposure to monomethylarsonous acid (MMA) and dimethylarsinous acid (DMA) during P19 cell differentiation.

	Day 3			Day 5		
	iAs	**MMA**	**DMA**	**iAs**	**MMA**	**DMA**
Neural Lineage						
Pax3 (neural plate border cells)	—	—	—	—	—	—
Sox10 (neural crest progenitors)	—	—	—	—	—	—
NeuroD1 (neural crest cells)	X	X	X	↓	↓	↓
Muscle Lineage						
Msx1 (neural plate border cells)	—	—	—	—	—	—
MyoD (myogenic progenitor)	↓	↓	↓	↓	↓	↓
Myogenin (myocytes)	X	X	X	—	—	—

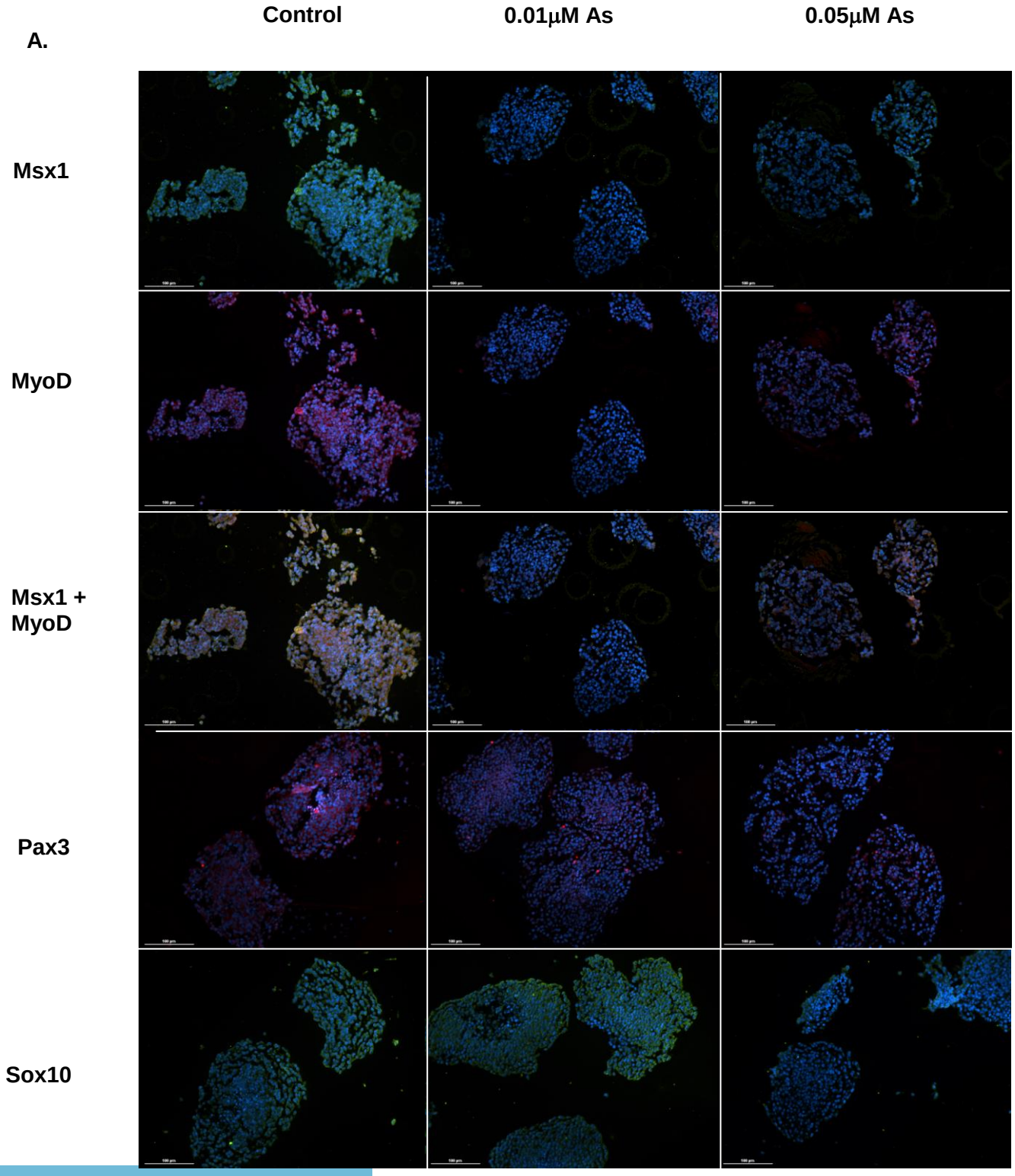
An arrow indicates reduced expression, a dash indicates no change in expression, and an X indicates that the TF was not expressed on that day.

Table 5. Changes in the nuclear translocation of transcription factors after exposure to monomethylarsonous acid (MMA) and dimethylarsinous acid (DMA) during P19 cell differentiation.

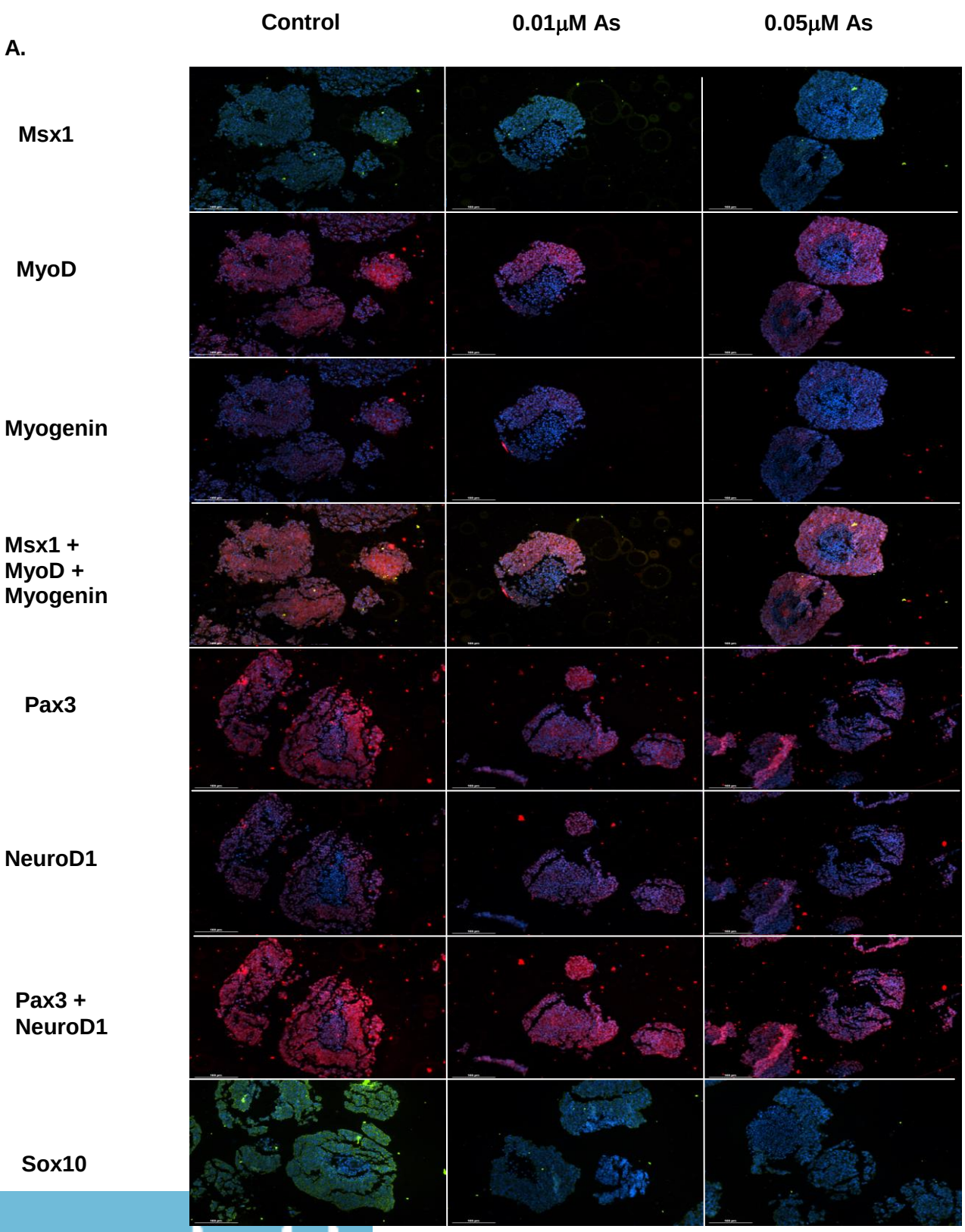
	MMA		DMA	
	Day 3	**Day 5**	**Day 3**	**Day 5**
Neural Lineage				
Pax3 (neural plate border cells)	—	—	—	—
Sox10 (neural crest progenitors)	—	—	—	—
NeuroD1 (neural crest cells)	X	↓	X	↓
Muscle Lineage				
Msx1 (neural plate border cells)	—	—	—	—
MyoD (myogenic progenitor)	↓	↓	↓	↓
Myogenin (myocytes)	X	—	X	—

An arrow indicates reduced expression, a dash indicates no change in expression, and an X indicates that the TF was not expressed on that day.

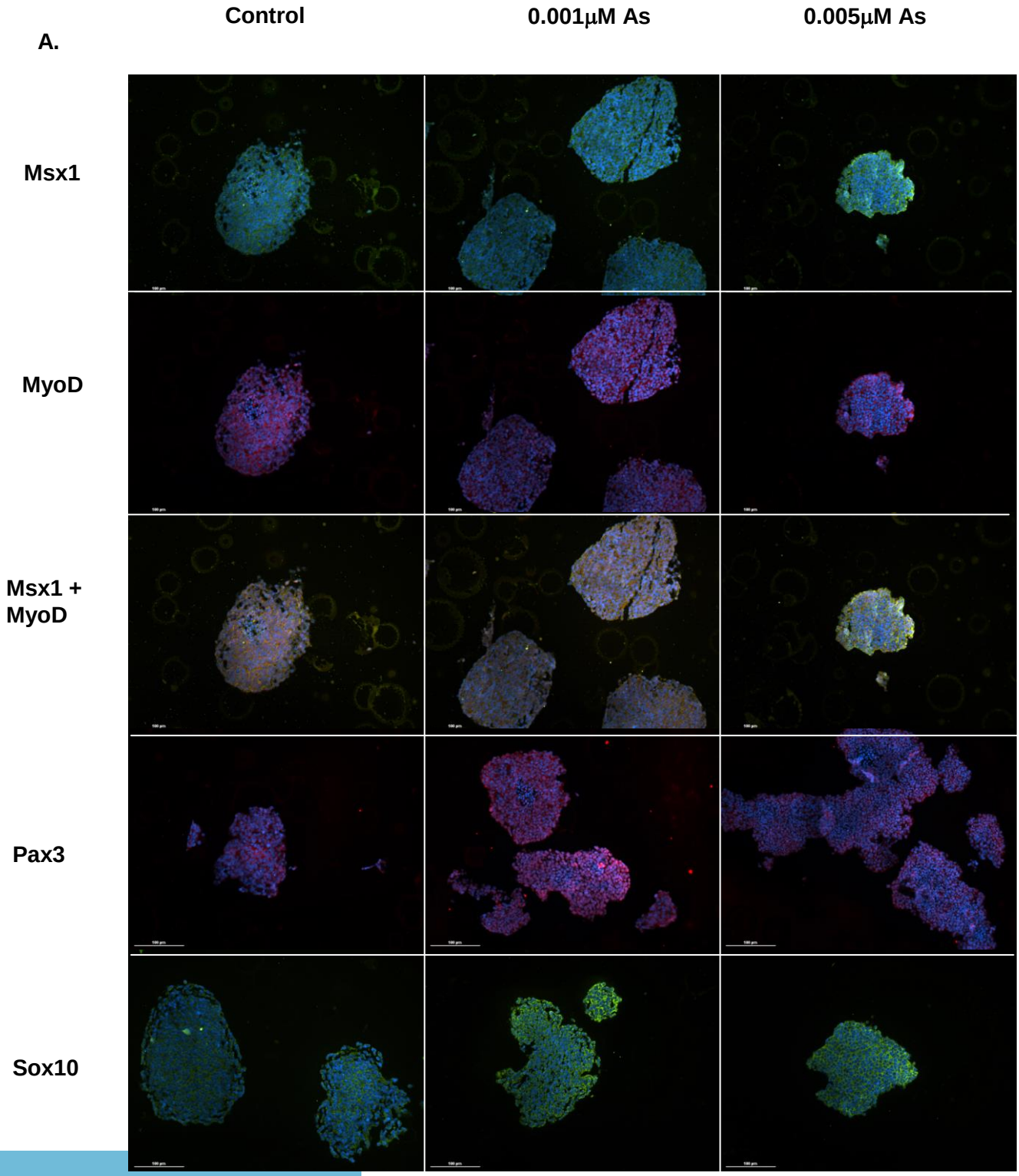
Supplementary Figure 1. MMA (III) decreases Msx1, MyoD, Pax3, Sox10 intensity in day 3 embryoid bodies.



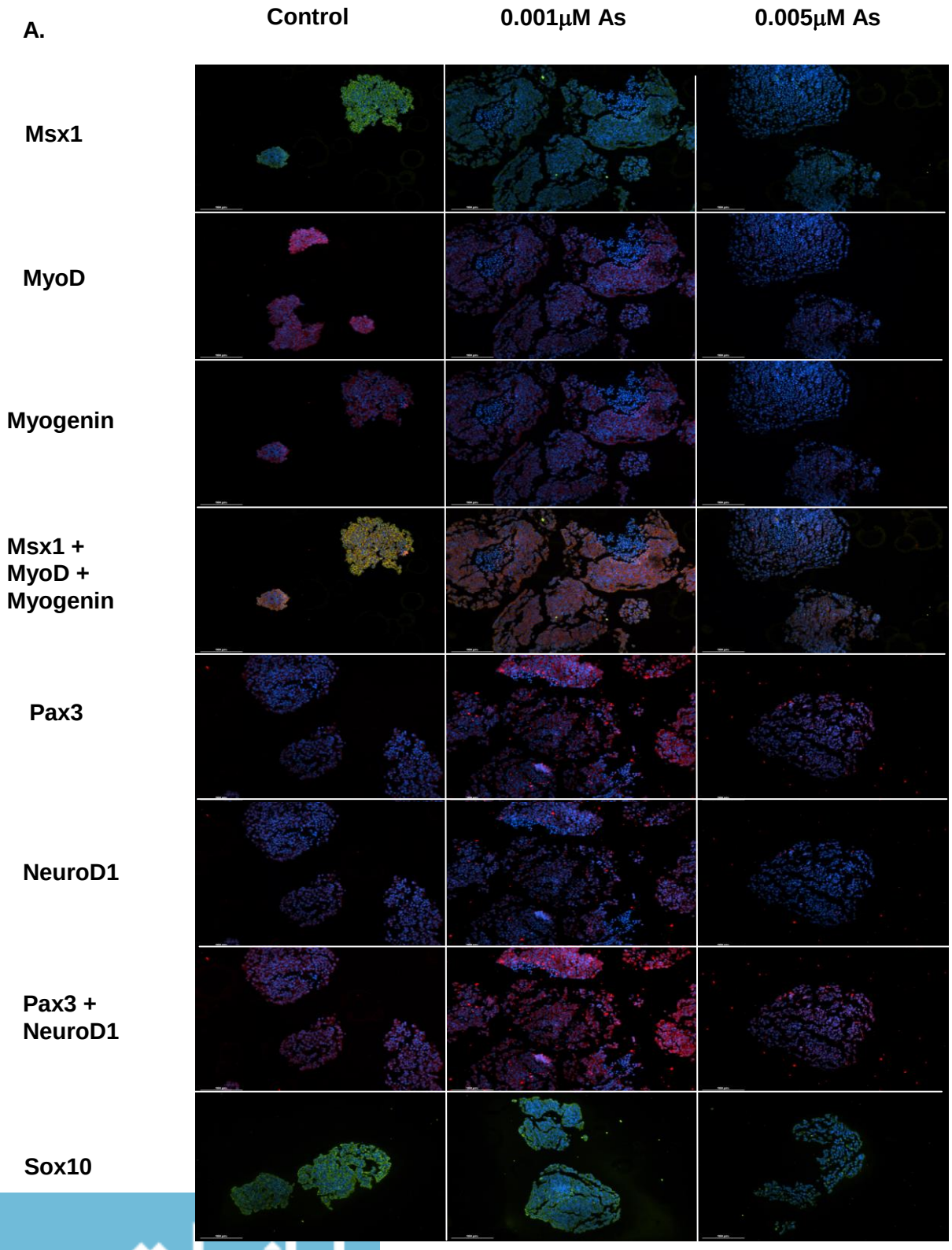
Supplementary Figure 2. MMA(III) decreases Pax3, Sox10 and NeuroD1 intensity in day 5 embryoid bodies.

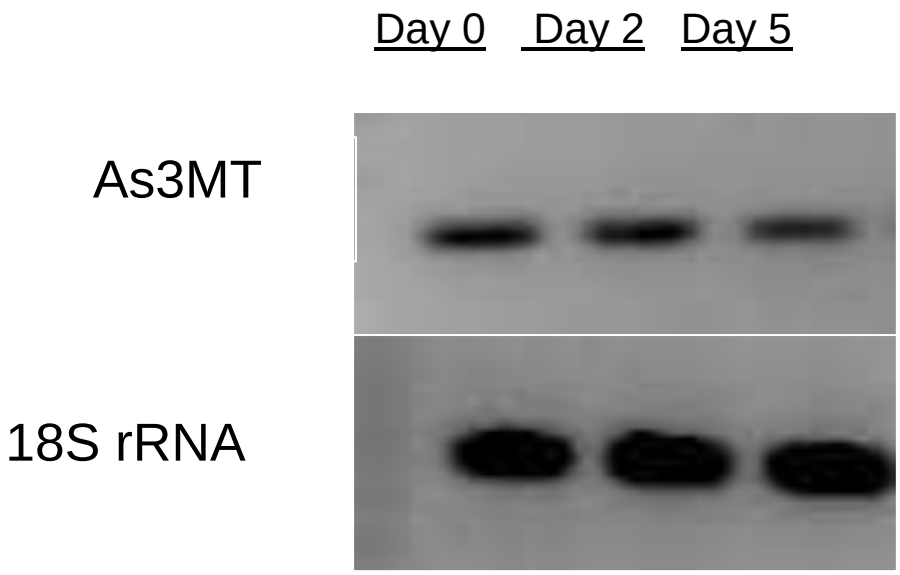


Supplementary Figure 3. DMA(III) caused no marked decreases in transcription factor intensity in day 3 embryoid bodies.



Supplementary Figure 4. DMA(III) decreases Msx1, MyoD, Myogenin, Sox10 and NeuroD1 intensity in day 5 embryoid bodies.





Supplementary Figure 5. Differentiating P19 cells contain arsenic methyltransferase.

Arsenic methyltransferase (As3MT) transcript levels in differentiating P19 cells, which suggests that they are fully capable of methylating arsenic.

References

1. Acharyya, S. K., Chakraborty, P., Lahiri, S., Raymahashay, B. C., Guha, S., Bhowmik, A. 1998. Arsenic poisoning in the ganges delta. Nature 401: 546.

2. Albores, Koropatnick, Cherian, Zelazowski. 1992. Arsenic induces and enhances rat hepatic metallothionein production in vivo Chem.–Biol. Interact. 85:127–140.

3. Arques, Chicote, Tenbaum, Puig, Palmer. 2012. Standardized relative quantification of immunofluorescence tissue staining. Nature Protocol Exchange. doi:10.1038/protex.2012.008.

4. Bendall, Ding, Hu, Shen, Abate-Shen. 1999. Msx1 antagonizes the myogenic activity of pax3 in migrating limb muscle precursors. Development. 126:4965-76.

5. Betancur, P., Bronner-Fraser, M., Sauka-Spengler, T. 2010. Assembling neural crest regulatory circuits into a gene regulatory network. Annu. Rev, Cell Dev. Biol. 26: 581-603.

6. Bajard, Relaix, Lagha, Rocancourt, Daubas, Buckingham. 2006. Genes Dev. 20(17): 2450–2464.

7. Bergstrom, Penn, Strand, Perry, Rudnicki, Tapscott. 2002. Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. Mol. Cell. 9:587–600

8. Berkes, C. A., Tapscott, S. J. 2005. MyoD and the transcriptional control of myogenesis. Seminars in Cell and Developmental Biology 16:585-595.

9. Britsch, D.E. Goerich, D. Riethmacher, R.I. Peirano, M. Rossner, K.A. Nave, C. Birchmeier, M. Wegner. 2001. The transcription factor sox10 is a key regulator of peripheral glial development, Genes Dev. 15:66–78

10. Buckingham, M. 2007. Skeletal muscle progenitor cells and the role of pax genes. C. R. Biol. 330, 530–533.

11. Burrows, G. J.; Turner, E. E. 1920. A new type of compound containing arsenic. J. Chem. Soc., Trans. 117:1373-138.

12. Concha, G., Vogler, G., Lezcano, D., Nermell, B., and Vahter, M. 1998. Exposure to inorganic arsenic metabolites during early human development. Toxicol. Sci. 44: 185–190.

13. Cullen, W. R.; McBride, B. C.; Manji, H.; Pickett, A. W.; Reglinski, J. 1989. The metabolism of methylarsine oxide and sulfide. Appl. Organomet. Chem. 3:71-78.

14. Dakeishi, M., Murata, K., and Grandjean, P. 2006. Long-term consequences of arsenic poisoning during infancy due to contaminated milk powder. Environ. Health 5: 31.

15. Davis, M.A., Mackenzie, T.A., Cottingham, K.L., Gilbert-Diamond, D., Punshon, T., Karagas, M.R., 2012. Rice consumption and urinary arsenic concentrations in U.S. children. Environ. Heal. Perspect. 120, 1418–1424.

16. Desbarats, A. J., Koenig, C. E. M., Pal, T., Mukherjee, P. K., Beckie R. D. Groundwater flow dynamics and arsenic source characterization in an aquifer system of west bengal, india. Water Resources Research. 5 (6): 4974-5002.

17. Descriptive Inorganic Chemistry, 4th ed.; Rayner-Canham, G.; Overton, T., Eds. W. H. Freeman and Company: New York, 2006, p 422.

18. Ferrario, Croera, Brustio, Collotta, Bowe, Vahter, Gribaldo. 2008. Toxicity of inorganic arsenic and its metabolites on haematopoietic progenitors "*in vitro*": Comparison between species and sexes

19. García-Chávez, E., Segura, B., Merchant, H., Jiménez, I., and Del Razo, L. M. 2007. Functional and morphological effects of repeated sodium arsenite exposure on rat peripheral sensory nerves. J. Neurol. Sci. 258: 104–110.

20. Gardner, Nermell, Kippler, Grander, Li, Ekstrom, Rahman, Lonnerdal, Hoque, Vahter. 2011. Arsenic methylation efficiency increases during the first trimester of pregnancy independent of folate status. Reproductive Toxicology. 31(2):210-218.

21. Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. and Gruss, P. 1991. Pax3, a novel murine DNA binding protein expressed during early neurogenesis. EMBO J. 10: 1135-1147.

22. Hong, G. M., Bain, L. J. 2012. Arsenic exposure inhibits myogenesis and neurogenesis in p19 stem cells through repression of the β-catenin signaling pathway. Toxicological Sciences 129(1): 145-156.

23. Hopenhayn, Huang, Christian, Peralta, Ferreccio, Atallah, Kalman. 2003. Profile of urinary arsenic metabolites during pregnancy. Environmental Health Perspectives 111(16): 1888-1891.

24. Hopenhayn, Ferreccio, Browning, Huang, Peralta, Herman, Hetrz-Picciotto. 2003. Arsenic exposure from drinking water and birth weight. Epidemiology. 14(5)-593-602.

25. Howard, M. J. 2005. Mechanisms and perspectives on differentiation of autonomic neurons. Dev. Biol. 277, 271–286.

26. Hughes, M. F., Beck, B. D., Chen, Y., Lewis, A. S., Thomas, D. J. 2011. Arsenic exposure and toxicology: a historical perspective. Toxicological Sciences 123(2), 305-332.

27. Ito, Ikehara, Nakagawa, Kraus, Muramatsup. 2000. p300-mediated acetylation facilitates the transfer of histone H2A-H2B dimers from nucleosomes to a histone chaperone. Genes Dev. 14 (15):1899–1907

28. Jackson, B. P., Taylor, V. F., Karagas, M. R., Punshon, T., and Cottingham, K. L. 2012. Arsenic, organic foods, and brown rice syrup. Environ Health Perspect. 120(5): 623–626.

29. Jin, Y., Xi, S., Li, X., Lu, C., Li, G., Xu, Y., Qu, C., Niu, Y., and Sun, G. 2006. Arsenic speciation transported through the placenta from mother mice to their newborn pups. Environ. Res. 101: 349–355.

30. Kirchner, J. W., Weil, A. 1998. Arsenic poisoning of Bangladesh groundwater. Nature 395: 338.

31. Kozul-Horvath, C. D., Zandbergen, F., Jackson, B. P., Enelow, R. I., Hamilton, J. W. 2012. Effects of low-dose drinking water arsenic on mouse fetal and postnatal growth and development. Plos one. 7(5): e38249. doi:10.1371/journal.pone.0038249

32. Kuang, Charge, Seale, Huh, Rudnicki. 2006. Distinct roles for pax7 and pax3 in adult regernative myogenesis. J Cell Bio. 172(1):103-113.

33. Lang, D., Jonathan, A. E. 2002. Sox10 and pax3 physically interact to mediate activation of a conserved c-ret enhancer. Human Molecular Genetics. 12(8):937-945.

34. Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell 66:305 -315.

35. Liu, Y., McDermott, S. 2010. The relationship between mental retardation and developmental delays in children and the levels of arsenic, mercury and lead in soul samples taken near their mothers residence during pregnancy. Int. J. Hygiene Environ. Health 213(2):116-123.

36. Marikawa, Y., Tamashiro, D. A., Fujita, T. C., and Alarcón, V. B. 2009. Aggregated P19 mouse embryonal carcinoma cells as a simple *in vitro* model to study the molecular regulations of mesoderm formation and axial elongation morphogenesis. *Genesis* 47**:** 93–106**.**

37. Milet, C., Maczkowiak, F., Roche, D., Monsoro-Burq, A. H. 2013. Pax3 and zic1 drive induction and differentiation of multipotent, migratory, and functional neural crest in xenopus embryos. PNAS 110(14):5528-5533.

38. Milet, C., Monsoro-Burq, A. H. 2012. Neural crest induction at the neural plate border in vertebrates. Dev. Biol. 366:22-33.

39. Miner, J. H., Wold, B. J. 1991. C-myc inhibition of myod and Myogenin-initiated myogenic differentiation. Mol. Cell Biol. 11(5):2842-2851.

40. Mondal, D., Banerjee, M., Kundu, M. 2010. Comparison of drinking water, raw rice and cooking of rice as arsenic exposure routes in three contrasting areas of West Bengal, India. Environ Geochem Health 32:463–477.

41. Mass, M. J.; Tennant, A.; Roop, B, C.; Cullen, W. R.; Styblo, M.; Thomas, D. J.; Kligerman, A. D. 2001. Methylated trivalent arsenic species are genotoxic. Chem. Res. Toxicol. 14:355-361.

42. Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537 -544.

43. Ogata, T., Nakamura, Y., Endo, G., Hayashi, T., Honda. Y. 2014. Subjective symptoms and miscarriage after drinking well water exposed to diphenlyarsinic acid. Japanese journal of public health. 61(9):556-564.

44. Piao, F., Ma, N., Hiraku, Y., Murata, M., Oikawa, S., Cheng, F., Zhong, L., Yamauchi, T., Kawanishi, S., and Yokoyama, K. 2005. Oxidative DNA damage in relation to neurotoxicity in the brain of mice exposed to arsenic at environmentally relevant levels. J. Occup. Health 47: 445–449.

45. Plouhinec, J., Roche, D., Pegoraro, C., Figueiredo, A. L., Maczkowiak, F., Brunet, L., Milet, C., Vert, J., Pollet, N., Harland, R., Monsoro-Burq, A. H. 2014.

Pax3 and zic1 trigger the early neural crest gene regulatory network by the direct activation of multiple key neural crest specifiers. Dev. Biol. 386:461-472.

46. Polesskaya, Naguibneva, Fritsch, Duquet, Ait-Si-Ali, Robin. 2001. CBP/p300 and muscle differentiation: no HAT, no muscle. EMBO J. 20: 6816–6825.

47. Puri, Sartorelli, Yang, Hamamori, Ogryzko, Howard. 1997. Differential roles of p300 and PCAF acetyltransferases in muscle differentiation. Mol. Cell. 1(1):35–45.

48. Radde-Gallwitz, Pan, Gan, Lin, Segil, Chen. 2004. Expression of islet1 marks the sensory and neuronal lineages in the mammalian inner ear. J. Comparative Neurology. 477:412-421.

49. Raqib, R., Ahmed, S., Sultana, R., Wagatsuma, Y., Mondal, D., Hoque, A. M., Nermell, B., Yunus, M., Roy, S., Persson, L. A., et al. 2009. Effects of in utero arsenic exposure on child immunity and morbidity in rural Bangladesh. Toxicol. Lett. 185: 197–202.

50. Ridgeway, A. G., Petropoulos, H., Wilton, S., Skerjanc, I. S. 2000. Wnt signaling regulates the function of myod and myogenin. J. Biol. Chem. 275(42):32398-32405.

51. Sauka-Spengler, T. and Bronner-Fraser, M. 2008. A gene regulatory network orchestrates neural crest formation. Nature Reviews Molecular Cell Biology 9:557-568.

52. Schoof, R. A., Yost, L. J., Eickhoff, J., Crecelius, E. A., Cragin, D. W., Meacher, D. M., Menzel, D. B. 1999. A market basket survey of inorganic arsenic in food. Food and chemical toxicology. 37(8): 836-846.

53. Sinczuk-Walczak, H., Szymczak, M., Halatek, T. 2010. Effects of occupational exposure to arsenic on the nervous system: clinical and neurophysiological studies. Int. J. Occupational Medicine and Environ. Health. 23(4):347-355.

54. Smith, A. H., Lingas, E. O., Rahman, M. 2000. Contamination of drinking-water by arsenic in bangladesh: a public health emergency. Bulletin of the World Health Organization 78: 1093-1103.

55. Styblo, Del Razo, Vega, Germolec, LeCluyse, Hamilton. 2000. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. Arch Toxicol 74:289–299.

56. Styblo, Drobna, Jaspers, Lin, Thomas. 2002. The role of biomethylation in toxicity and carcinogenicity of arsenic:a research update. Environ Health Perspect 110(5):767–771.

57. Styblo, Serves, Cullen, Thomas. 1997. Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. Chem. Res. Toxicol. 10:27-33.

58. Suzuki, K.T., Katagiri, A., Sakuma, Y., Ogra, Y., Ohmichi, M. 2004. Distributions and chemical forms of arsenic after intravenous administration of dimethylarsinic and monomethylarsonic acids to rats. Toxicol. Appl. Pharmacol. 198 (3):336–344.

59. Tapscott, S. J. 2005. The circuitry of a master switch: myod and the regulation of skeletal muscle gene transcription. Development. 132:2685-2695.

60. Tsai, S. Y., Chou, H. Y., The, H. W., Chen, C. M., and Chen, C. J. 2003. The effects of chronic arsenic exposure from drinking water on the neurobehavioral development in adolescence. Neurotoxicology 24:747–753.

61. Wang, Q. Q., Lan, Y. F., Rehman, K., Jiang, Y. H., Maimaitiyiming, Y., Zhu, D. Y., Naranmandura, H. 2014. Effect of arsenic compounds on the in vitro differentiation of mouse embryonic stem cells into cardiomyocytes. Chemical Research in Toxicology.

62. Wang, X., Meng, D., Chang, Q., Pan, J., Zhang, Z., Chen, G., Ke, Z., Luo, J., and Shi, X. 2010. Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway. Environ. Health Perspect. 118: 627–634.

63. Wasserman, G. A., Liu, X., Parvez, F., Ahsan, H., Factor-Litvak, P., A. van Geen. 2004.Water arsenic exposure and children's intellectual function in araihazar, bangladesh. Environ Health Perspect. 112 (2004):1329–1333.

64. Williams, B. A. and Ordahl, C. P. 1994. Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. Development 120:785-796.

65. Xi, S., Guo, L., Qi, R., Sun, W., Jin, Y., and Sun, G. 2010. Prenatal and early life arsenic exposure induced oxidative damage and altered activities and mRNA expressions of neurotransmitter metabolic enzymes in offspring rat brain. J. Biochem. Mol. Toxicol. 24: 368–378.

66. Yang, Q., Jung, H. B., Culbertson, C., Marvinney, R., Loiselle, M., Locke, D., Cheek, H., Thibodeau, H., Zheng, Y. 2009. Spatial pattern of groundwater arsenic

occurrence and association with bedrock geology in greater augusta, maine. Environ. Sci. Technol. 43(8): 2714–2719.

67. Yokoyama, S. and Asahara, H. 2011. The myogenic transcriptional network. Cell Mol. Life Sci. 68, 1843–1849.

68. Zaval, Y. J., Duxbury, J. M. 2008. Arsenic in rice: I. estimating normal levels of total arsenic in rice grain. Environ. Sci. Technol. 42(10):3856-60.

69. Zaval, Y. J., Gerads, R., Gurleyuk, H., John, M. D. 2008. Arsenic in rice II. Arsenic speciation in usa grain and implications for human health. Environ. Sci. Technol. 42(10):3861-3866

CHAPTER THREE

CONCLUSION

Even with our knowledge that chronic arsenic exposure has a negative effect on development, the exact mechanisms of how arsenic causes these undesired developmental outcomes is still poorly understood. We already knew that arsenic disrupts embryoid body formation (Stummann et al., 2008), myogenesis (Garcia-Chavez et al., 2007), and neurogenesis (Piao et al., 2005; Wang et al., 2010), but hopefully my study has now shed some light on the temporal and spatial aspects of the NPBS signals involved during neurogenesis and myogenesis. It appears that arsenic targets the neural plate border specifier cells. Since these cells arise early during embryogenesis and give rise to cell types such as the neural crest (Hong and Saint-Jeannet, 2007; Le Douarin and Kalcheim, 1999), this targeting could account for why arsenic can impact structures as diverse as the elements of the craniofacial skeleton, muscle progenitors, melanocytes, and parts of the nervous system and glia.

This study has also demonstrated the harmful effects that arsenic and its metabolites can exert on cells and the usefulness of using cell lines to examine cell fates. It is particularly alarming that arsenic's metabolites MMA III and DMA III can target the NPBS cells at much lower concentrations than arsenic itself. With these metabolites increasing during pregnancy (Davis et al., 2012) this is very worrying for any expecting parents, especially those who live in areas with very high levels of arsenic contamination in their water supply including areas in Mexico, Chile, India, and Bangladesh. Some of the wells in these countries can have to 500 µg/L (Desbarats et al., 2014), which is 50

times the limit set by the World Health Organization. In addition, Chilean pregnant women whose drinking water had arsenic levels around 40 µg/L had increased urinary arsenic levels (Hopenhayn et al., 2003). By gestational week 36 urinary arsenic levels had increased to 54 µg/L and urinary DMA levels had increased to 47 µg/L (Hopenhayn et al., 2003). Studies in Matlab, Bangladesh have shown urinary arsenic levels up to 89µg/L as early as gestational week 8 in pregnant women exposed to water with >50 µg/L arsenic (Rahman et al., 2010). These urinary arsenic levels translate to an odds ratio for a spontaneous abortion of 1.31 for women <20, but for women >40 years old, the odds ratio jumps to 4.5 (Rahman et. al, 2010).

In addition to a rise in urinary arsenic levels, blood arsenic levels have been to increase as well. In Argentina, pregnant women whose water contained 200 µg/L of arsenic had 9 µg/L of arsenic in their cord blood, which was almost as high as the maternal blood which contained 11 µg/L of arsenic (Concha et al., 1998). Another study had similar findings. In Bangladesh, pregnant women who were exposed to water containing 90.5 µg/L had cord blood arsenic levels of 23.1 µg/L, with over 31% of it being in the form of MMA (Hall et al., 2007). Both of these studies show that arsenic is easily transported from the placenta to the fetus. This was confirmed when looking at the placental arsenic levels and the baby's urine. There was 34µg/kg of arsenic in the placenta late in gestation in exposed women, but only 7µg/kg for non-exposed women (Concha et al. 1998). Regarding the baby's urine, it was found that it contained on average 80µg/L of arsenic during the first two days, and was still elevated as high as 30µg/L at 4.5 months of age (Concha et al., 1998). This is particularly worrying due to

the fact that in our study we found that levels as low as 0.005µM DMA, or 0.375µg/L has adverse effects on cells *in vitro*.

Research now needs to focus on identifying exactly what thresholds these harmful effects start to appear. In addition, much more resources need to be dedicated to looking at arsenic's metabolites, and carrying our more *in vivo* studies examining what diseases appear at certain thresholds. We aren't sure at exactly what threshold things like cutaneous lesions, Blackfoot disease, and cancer start to appear. Many genetic factors including methylation and metabolism efficiency comes into play. It has been shown that the rate of excretion of As and its metabolites increases as methylation efficiency increases (Vahter, 1999). However, methylation efficiency is known to vary between children and adults, with women having much higher methylation rates than men, especially during pregnancy (Vahter, 1999).

However, what we do know is that arsenic exposure around the world is an epidemic. Even in the United States over 4 million people have harmful levels of arsenic in their drinking water (Ambrosio et al., 2014). People in countries such as Bangladesh, India, Chile and Mexico are in need of help. This help could be as simple as education about arsenic contamination, painting contaminated wells red and non-contaminated ones green, and even installing filtering systems to remove the arsenic from the water. It is also very important, even in the United States, to keep on monitoring arsenic levels in our food and drinks to help establish more robust safety standards.

References

1. Ambrosio, F., Brown, E., Stolz, D., Ferrari, R., Goodpaster, B., Deasy, B., Distefano, G., Roperti, A., Cheikhi, A., Garciafigueroa, Y., Barchowsky, A. 2014. Arsenic induces sustained impairment of skeletal muscle and muscle progenitor cell ultrastructure and bioenergetics. Free Radical Biology and Medicine. 74:64-73.

2. Concha, G., Vogler, G., Lezcano, D., Nermell, B., and Vahter, M. 1998. Exposure to inorganic arsenic metabolites during early human development. Toxicol. Sci. 44: 185–190.

3. Davis, M.A., Mackenzie, T.A., Cottingham, K.L., Gilbert-Diamond, D., Punshon, T., Karagas, M.R., 2012. Rice consumption and urinary arsenic concentrations in U.S. children. Environ. Heal. Perspect. 120, 1418–1424.

4. Desbarats, A. J., Koenig, C. E. M., Pal, T., Mukherjee, P. K., Beckie R. D. Groundwater flow dynamics and arsenic source characterization in an aquifer system of west bengal, india. Water Resources Research. 5 (6):4974-5002.

5. García-Chávez, E., Segura, B., Merchant, H., Jiménez, I., and Del Razo, L. M. 2007. Functional and morphological effects of repeated sodium arsenite exposure on rat peripheral sensory nerves. J. Neurol. Sci. 258: 104–110

6. Hall, M., Gamble, M., Slavkovich, V., Liu, X., Levy, D., Cheng, Z., van Geen, A., Yunus, M., Rahman, M., Pilsner, J. R., Graziano, J. 2007. Determinants of arsenic metabolism: blood arsenic metabolites, plasma folate, cobalamin, and homocysteine concentrations in maternal-newborn pairs. Env. Health. Perspect. 115(10):1503-1509.

7. Hong, C. S., Saint-Jeannet, J. P. 2005. Sox proteins and neural crest development. Cell and Developmental Biology 16: 694-703.

8. Hopenhayn, Huang, Christian, Peralta, Ferreccio, Atallah, Kalman. 2003. Profile of urinary arsenic metabolites during pregnancy. Environmental Health Perspectives 111(16): 1888-1891.

9. Le Douarin, N., & Kalcheim, C. 1999. The neural crest. Cambridge University Press. No 36.

10. Piao, F., Ma, N., Hiraku, Y., Murata, M., Oikawa, S., Cheng, F., Zhong, L., Yamauchi, T., Kawanishi, S., and Yokoyama, K. 2005. Oxidative DNA damage in relation to neurotoxicity in the brain of mice exposed to arsenic at

environmentally relevant levels. J. Occup. Health 47: 445–449.

11. Rahman, Perrson, Nermell, Arifeen, Ekstron, Smith, Vahter. 2010. Arsenic exposure and risk of spontaneous abortion, stillbirth, and infant mortality. Epidemiology 21:797-804.

12. Stummann, T. C., Hareng, L., and Bremer, S. 2008. Embryotoxicity hazard assessment of cadmium and arsenic compounds using embryonic stem cells. Toxicology 252: 118–122.

13. Vahter, M. 1999. Methylation of inorganic arsenic in different mammalian species and population groups. Science Progress. 82:69-88.

14. Wang, X., Meng, D., Chang, Q., Pan, J., Zhang, Z., Chen, G., Ke, Z., Luo, J., and Shi, X. 2010. Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway. Environ. Health Perspect. 118: 627–634.