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Methylmercury modifies temporally expressed myogenic regulatory factors to inhibit myoblast differentiation

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Abstract

Methylmercury (MeHg) is a pervasive environmental toxicant, with known detrimental effects on neurodevelopment. Despite a longstanding paradigm of neurotoxicity, where motor deficits are prevalent among those developmentally exposed, consideration of muscle as a MeHg target has received minimal investigation. Recent evidence has identified muscle-specific gene networks that modulate developmental sensitivity to MeHg toxicity. One such network is muscle cell differentiation. Muscle cell differentiation is a coordinated process regulated by the myogenic regulatory factors (MRFs): Myf5, MyoD, MyoG, and MRF4. A previous study demonstrated that MeHg inhibits muscle cell differentiation *in vitro*, concurrent with reduced MyoG expression. The potential for MeHg to modify the temporal expression of the MRFs to alter differentiation, however, has yet to be fully explored. Using the C2C12 mouse myoblast model, we examined MRF expression profiles at various stages subsequent to MeHg exposure to proliferating myoblasts. MeHg was seen to persistently alter myoblast differentiation capacity, as *myod*, *myog*, and *mrf4* gene expression were all affected. *Myog* exhibited the most robust changes in expression across the various culture conditions, while *myf5* was unaffected. Following MeHg exposure to myoblasts, where elevated p21 expression indicated departure from proliferation, cells failed to subsequently differentiate, even in the absence of MeHg, as reflected by a concurrent reduction in the MRF4 and myosin heavy chain (MHC) markers of terminal differentiation. Our results indicate that within a brief window of exposure MeHg can disrupt the intrinsic myogenic differentiation program of proliferative myoblasts.

Keywords

methylmercury; muscle; myogenic regulatory factors; cell differentiation

Introduction

Methylmercury (MeHg) is a persistent environmental toxicant. Exposure to humans occurs primarily via consumption of MeHg contaminated seafood. Accidental high-dose exposure incidents have underscored the detrimental effects of MeHg in humans, particularly on neurodevelopment (Bakir, Damluji et al. 1973, Harada 1978). As such, MeHg research has predominantly focused on the nervous system as the primary target of toxicity. Some

epidemiological studies in populations that subsist on seafood have found that *in utero* exposed children exhibit neurodevelopmental deficits (Debes, Weihe et al. 2016, Engstrom, Love et al. 2016, Tatsuta, Murata et al. 2017). Among these deficits, poorer motor skills (Grandjean, Weihe et al. 1998) were observed, indicating potential neuromuscular targets for MeHg. The potential for MeHg to disrupt muscle development has received little attention. A previous study in rats demonstrated that MeHg could accumulate in muscle tissue consequent to *in utero* exposure (Cambier, Fujimura et al. 2018). Furthermore, developmental MeHg exposure in *Drosophila* resulted in abnormal muscle morphology (Engel and Rand 2014, Prince and Rand 2017). The precise mechanism by which MeHg elicits toxicity on muscle development, however, remains unexplored.

A genome-wide association study in *Drosophila* revealed several muscle-specific gene networks that associate with tolerance or susceptibility to developmental MeHg exposure. One such network identified in this study was muscle cell differentiation (Montgomery, Vorobjikina et al. 2014). Muscle cell differentiation is a coordinated process, regulated by the myogenic regulatory factors (MRFs): Myf5, MyoD, MyoG, and MRF4 (Zammit 2017). Whereas each of these factor have a unique influence on myogenesis, Myf5 and MyoD are generally thought of as determinants of myogenic potential while MyoG and MRF4 are known to mediate steps toward terminal differentiation of myocytes into myotubes and fibers (Singh and Dilworth 2013). Accordingly, the MRF transcription factors are temporally expressed as differentiation proceeds from myoblasts to myotubes. Myf5 is expressed in myoblasts, and is typically downregulated in response to differentiation cues. MyoD is persistently expressed in myoblasts as well as myocytes (Ishibashi, Perry et al. 2005). As differentiation is induced MyoG is upregulated (Andres and Walsh 1996) under the control of MyoD (Faralli and Dilworth 2012). Finally, MRF4 is expressed as myotubes form, which also propagates expression of myosin heavy chain (MHC) (Hinterberger, Sassoon et al. 1991). The extent to which MeHg can influence MRFs has yet to be fully investigated. Nonetheless, the highly coordinated temporal regulation of these MRFs highlights the possibility that a discrete window of susceptibility for MeHg toxicity may exist where one of these factors is preferentially targeted.

In a prior study, using differentiating mouse C2C12 myoblasts *in vitro*, we demonstrated a robust effect of MeHg in reducing MyoG transcripts that accompanied a strong inhibition of differentiation to myocytes and fusion into myotubes (Prince and Rand 2018). Moreover, this effect correlated with decreased nuclear MyoG (Prince and Rand 2018). Notably, MyoD expression was seen to be little affected by MeHg (Prince and Rand 2018). These findings are consistent with limited studies of MeHg effects on muscle *in vivo* that have demonstrated a decrease muscle fiber size in rats (Usuki, Yasutake et al. 1998) and zebrafish (de Oliveira Ribeiro, Nathalie et al. 2008). While these results support the idea that MeHg might inhibit myoblast differentiation, potentially via select action on MyoG, the influence of MeHg on other MRFs remains unexplored. In addition to the MRFs, the cyclin-dependent kinase inhibitor p21 demonstrates marked change in expression with an increase in myoblasts that are induced to differentiate (Guo, Wang et al. 1995). MeHg effects on p21 in muscle cells has also not been fully examined.

The C2C12 mouse myoblast model offers exceptional control over the study of muscle cell differentiation mechanisms. Proliferative myoblasts can be maintained in high serum (e.g. 20% fetal bovine serum) culture conditions and synchronously induced to differentiate by a shift to low serum containing media (e.g. 2% horse serum). In a prior study we investigated the consequences of the MeHg insult occurring contemporaneously with onset of differentiation, e.g. in a low serum condition (Prince and Rand 2018). In the present work, we aimed to determine if the potent effects of MeHg on myogenic differentiation can be implemented at an earlier stage and in optimal growth conditions (e.g. high serum) that are more representative of myogenic cells in vivo (e.g. in the fetal myotome or in the adult satellite cell niche). Here we show that MeHg exposure to undifferentiated myoblasts uniquely modifies MRF expression and significantly impairs their capacity to subsequently differentiate.

Materials and Methods

Cell culture

C2C12 cells were purchased from ATCC (CRL-1772, Lot # 61633507). Cells were cultured in growth media, Dulbecco's Modified Eagle Medium (DMEM, Gibco #11995-065) supplemented with 20% fetal bovine serum (FBS, Gibco #10082147) and 1% Penicillin/Streptomycin (PenStrep, Gibco #15140-122). Initially, cells were thawed and seeded in T75 cm² flasks (Falcon #353136). After three days, cells reached approximately 60% confluency, and were passaged into appropriate tissue culture ware (~1.25×10⁴ cells/ml) for experiments. Once again, cells reached approximately 60% confluency, and were treated as detailed below. To promote differentiation, cells were cultured in differentiation media, DMEM supplemented with 2% horse serum (HS, ATCC #30-2040), 1 µg/ml insulin (Sigma #I6634), and 1% PenStrep.

Treatment

Methylmercury Chloride (MeHg, Sigma #215465) was prepared as a 50 mM stock in dimethyl sulfoxide (DMSO, Fisher #BP231), and stored at -20°C. Stocks were then diluted in growth media prior to treatment, so that the final DMSO concentration was < 0.01%. Cells were treated in growth media ± MeHg for 24 hr, then either collected (myoblasts) or switched to differentiation media without MeHg for an additional 24 (myocytes) or 48 hr (myotubes) (Figure 1).

Cell viability

Cells were seeded in 6-well plates (Corning #3516) for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma # M2128) assay. A 5 mg/ml MTT stock was made in Hank's Balanced Salt Solution (HBSS, Gibco #14175-095), aliquoted in light sensitive Eppendorf tubes, and stored at -20°C. Stocks were thawed at room temperature prior to experiments. Treatment media was removed, and cells washed with warm HBSS. The appropriate media (growth or differentiation) without MeHg was added back, as well as MTT at a final concentration of 0.25 mg/ml. Cells were incubated for 2 hr at 37°C, the media + MTT removed, and Sorensen's Buffer (0.1 M glycine, 0.1 M NaOH; pH 10.5) +

DMSO added. Absorbance was measured at 570 nm (corrected for background absorbance at 690 nm). Cell viability is reported as percent (%) of control (0 μ M MeHg).

Real-time PCR

Cells were seeded in 60 mm petri dishes (Falcon #353004) for RNA isolation. Total RNA was isolated using the Qiagen RNeasy® Mini Kit (Qiagen #74104), and converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368813). Real-time PCR was done using iTaq Universal SYBR® Green Supermix (Bio-Rad #1725121) and appropriate forward/reverse primers (Supplemental Table 1). Bio-Rad CFX96™ Real-Time PCR System was programmed according to manufacturer instructions, and quantified cycle number (Cq) for genes of interest (*myf5*, *myod*, *myog*, *mrf4*) and a housekeeping gene (*gapdh*) were acquired for subsequent analysis.

Western Blot

Cells were plated in 60 mm petri dishes for whole-cell lysate preparation. Briefly, cells were lysed in Pierce™ RIPA buffer (Thermo #89900) + Halt Protease Inhibitor Cocktail EDTA-free (Thermo #87785), and frozen at -80°C prior to sample preparation. Western blot samples were prepared in 2X Laemmli sample buffer (Bio-Rad #161-0737) + 2-mercaptoethanol (Fisher #BP176) for a final protein concentration of 1 $\mu\text{g}/\mu\text{l}$, and boiled at 100°C . Samples and the Precision Plus Protein™ Kaleidoscope™ (Bio-Rad #161-0375) ladder were run on 12% polyacrylamide gels (30% acrylamide, 1.5 M Tris HCl, 10% SDS, ddH₂O, 10% APS, TEMED). Gels were transferred overnight at 4°C to polyvinylidene difluoride (PVDF, Millipore #IPVH00010) membranes, and stained with Ponceau S (Fisher #BP103-10) to confirm protein transfer. Membranes were blocked in 5% bovine serum albumin (Fisher #BP1600-100), then incubated overnight at 4°C with appropriate primary antibodies. p21 (#556431) and MyoD (#554130) were purchased from BD Pharmigen; MyoG (#F5D) and MHC (#MF-20) were purchased from Developmental Studies Hybridoma Bank; and actin (#A5441) was purchased from Sigma. Membranes were washed, then incubated for 1 hr at room temperature with peroxidase-conjugated goat-anti mouse secondary (Jackson ImmunoResearch #115-035-146). Protein bands were visualized with Clarity™ Western ECL Substrate (Bio-Rad #170-5060), and imaged on the Bio-Rad Chemi-Doc™ MP Imaging System. ImageJ (NIH) was used for band intensity analysis.

Data Analysis

Data are presented as mean \pm standard deviation (SD). For all experiments, at least three biological replicates were completed. Each biological replicate represents a newly thawed from frozen vial of C2C12 cells and one passage prior to treatment and experimentation. To calculate delta Ct (Δ Ct), the Cq value for *gapdh* was subtracted from the Cq value for *myf5*, *myod*, *myog*, or *mrf4*. The $2^{-\Delta\text{Ct}}$ method was used to determine fold change (Livak and Schmittgen 2001). p21, MyoD, MyoG or MHC band intensity was normalized to actin prior to comparison across MeHg concentrations. Shapiro-Wilk Test for Normality and Levene's Test for Equal Variances were applied to all data. One-way analysis of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) or Kruskal-Wallis with Dunn's Multiple Comparisons, Bonferroni correction were determined in R with significance at $p < 0.05$. For delta Ct, untreated myocytes or myotubes were compared to untreated myoblasts

only, while for all other analysis 0.5 μM or 2.5 μM MeHg were compared to untreated (0 μM MeHg) controls.

Results

Temporal profile of myogenic regulatory factor gene expression in C2C12 cells

First, we sought to establish the baseline profile of MRF expression spanning the transition from myoblasts to myocytes to myotubes during differentiation in the absence of MeHg. MRF gene expression patterns were examined by qPCR at time-points according to our culture conditions (Figure 1) and the results are expressed as delta-Ct values in Table 1. Expression of *myf5* in the myocytes was moderately reduced compared to the myoblasts, although not quite significantly ($p = 0.06$). Unexpectedly, *myf5* expression in the myotubes was seen to return to levels near that of myoblasts. *Myod* expression was moderately enhanced in the myocytes ($p < 0.001$) as well as the myotubes ($p < 0.01$) compared to myoblasts. In contrast, *Myog* expression was substantially increased in the myocytes ($p < 0.01$) and in the myotubes, however, a skewed distribution in the myoblasts Ct values resulted in a statistically insignificant change ($p = 0.15$) in the latter. *Mrf4* expression was increased in the myotubes ($p < 0.01$), while showing a moderate insignificant decrease in the myocytes.

C2C12 mouse myoblast cell viability

Next, we assessed cell viability of the C2C12 cells by MTT assay to identify sub-toxic and toxic MeHg concentrations for our experiments. No significant decrease in cell viability at 0.5 μM MeHg in myoblasts ($100.6 \pm 10.5\%$, $n = 3$; $p = 1.00$ (Figure 2A)), myocytes ($102.5 \pm 26.3\%$, $n = 3$; $p = 0.98$ (Figure 2B)), or myotubes ($102.3 \pm 8.6\%$, $n = 3$; $p = 0.98$ (Figure 2C)) was observed. However, 2.5 μM MeHg produced a significant decrease in cell viability in myoblasts ($67.0 \pm 11.7\%$, $n = 3$; $p < 0.05$ (Figure 2A)), myocytes ($43.6 \pm 4.4\%$, $n = 3$; $p < 0.05$ (Figure 2B)), as well as myotubes ($50.5 \pm 19.6\%$, $n = 3$; $p < 0.01$ (Figure 2C)). Therefore, we evaluated 0.5 μM and 2.5 μM MeHg in all our subsequent experiments.

MeHg effects on myogenic regulatory factor gene expression

We then examined MeHg effects on MRF gene expression (Figure 3 and Supplemental Table 2). Relative to the untreated controls, there were no significant effects on MRF gene expression with 0.5 μM MeHg. Further, *myf5* was not significantly altered at 2.5 μM MeHg in the myoblasts, myocytes, or myotubes (Supplemental Table 2). With 2.5 μM MeHg, *myod* (0.67 ± 0.16 , $n = 4$; $p < 0.05$ (Figure 3A)) and *myog* (0.16 ± 0.07 , $n = 4$; $p < 0.001$ (Figure 3B)) expression was significantly decreased in the myoblasts. *myog* expression was also significantly decreased in the myocytes with 2.5 μM MeHg, although to a lesser extent (0.48 ± 0.31 , $n = 5$; $p < 0.01$ (Figure 3D)); whereas *myod* expression was not significantly lower (0.68 ± 0.19 , $n = 4$; $p = 0.26$ (Figure 3C)). With 2.5 μM MeHg *Mrf4* expression was significantly decreased in the myotubes (0.44 ± 0.13 , $n = 3$; $p < 0.01$ (Figure 3E)). Curiously, with 2.5 μM MeHg expression of *myod* and *myog* were not significantly altered in the myotubes (Supplemental Table 2).

Relative protein level of differentiation factors in C2C12 mouse myoblasts

Before assessing MeHg effects on proteins involved in myoblast differentiation, we measured the relative protein level of these factors in our culture conditions without MeHg and the results are seen in Table 2. First, we included examination of the p21 protein as marker of proliferation status. p21 was significantly increased in the myocytes ($p < 0.05$) relative to myoblasts; however, levels in myotubes were nearly equivalent to those in the myoblasts. MyoD protein level was unchanged in the myocytes, yet was decreased in the myotubes compared to the myoblasts, albeit without significance ($p = 0.07$). However, relative to the myocytes, MyoD was significantly decreased in the myotubes ($p < 0.01$). MyoG protein was significantly increased in the myocytes ($p < 0.001$) and myotubes ($p < 0.05$) compared to the myoblasts; however, relative to the myocytes, MyoG was increased to a lesser extent in the myotubes ($p < 0.05$). MHC levels could be determined in the myotubes, but were absent in the myoblasts or the myocytes.

MeHg effects on protein levels of differentiation factors

To compliment transcript expression analyses, we evaluated MeHg effects on corresponding MRF protein levels, together with p21 protein, in myoblasts, myocytes and myotubes (Figures 4, 5 and 6, respectively). In the myoblasts, there were no significant effects on protein levels for any of the factors at 0.5 μM MeHg (Figure 4). With 2.5 μM MeHg, p21 was significantly increased (1.94 ± 0.35 , $n = 3$; $p < 0.01$ (Figs. 4A and B)) in myoblasts, while MyoG was significantly decreased (0.21 ± 0.11 , $n = 3$; $p < 0.05$ (Figs. 4A and D)) compared to untreated myoblasts. MyoD levels were unaltered by MeHg in the myoblasts (Figs. 4A and C). In the myocytes, MyoG was significantly decreased with 0.5 μM (0.66 ± 0.02 , $n = 3$; $p < 0.05$) and 2.5 μM MeHg (0.20 ± 0.12 , $n = 3$; $p < 0.001$) (Figs. 5A and D). p21 was also decreased with 0.5 μM (0.62 ± 0.10 , $n = 3$; $p = 0.22$) and 2.5 μM MeHg (0.46 ± 0.30 , $n = 3$; $p = 0.08$), although not quite significantly (Figs. 5A and B). Again, MyoD levels were unaffected by MeHg in the myocytes (Figs. 5A and C). Interestingly, in the myotubes, p21 (Figs. 6A and B), MyoD (Figs. 6A and C), and MyoG (Figs. 6A and D) were not altered at either MeHg concentration. In contrast, MHC was significantly decreased with 0.5 μM (0.56 ± 0.11 , $n = 3$; $p < 0.01$) and drastically decreased at 2.5 μM MeHg (0.02 ± 0.02 , $n = 3$; $p < 0.001$) (Figs. 6A and E).

Discussion

Despite that motor coordination deficits are common clinical symptoms associated with developmental MeHg exposure, consideration of muscle as a developmental MeHg target has not been extensively explored. Here, we expanded upon emerging evidence that MeHg disrupts normal muscle development, with specific emphasis on dysregulation of a fundamental myogenic differentiation program. First, we established the temporal pattern of MRF expression exhibited in the C2C12 cells under our culture conditions, intended to distinguish myoblasts, myocytes, and myotubes. We confirmed that MeHg has a pointed inhibitory effect on myoblast differentiation. Most striking was that MeHg exposure to the myoblast, while residing in a high-serum growth condition and prior to any differentiation initiating cue, persistently derailed the subsequent ability to differentiate. These results predict that MeHg could have profound effects on muscle development in the fetal

myotome, and alternatively, on the adult satellite cells needed for muscle repair and homeostasis.

The unique MRF expression profiles we observed provide insight into how MeHg may elicits its effects predominantly via MyoG. Curiously, MeHg induced little to no changes in expression levels of the master myogenic regulatory factors Myf5 or MyoD. In contrast, effects on MyoG were the most robust, with substantial reduction in gene expression and protein levels in both myoblasts and myocytes subsequent to MeHg exposure. The MyoG protein level was particularly sensitive showing reduction at the lower (0.5 μ M) MeHg concentration. Equally remarkable is the observation that MyoG transcript and protein levels at the myotube stage were nearly equivalent across all MeHg exposures. This latter profile indicates MyoG expression, while substantially reduced upon MeHg exposure (i.e. in the myoblast), and in the 24 hours after withdrawal of MeHg (i.e. the myocyte), can return to the appropriate level of expression at 48 hours, equal to that seen with the myotubes resulting from untreated myoblasts. Despite this apparent “equilibration” of MyoG levels at the myotube stage, at the higher MeHg exposure MRF4 remains decreased and MHC nearly absent, indicating terminal differentiation has been augmented. Altered expression of MyoG occurs despite the fact that levels of MyoD, a foremost transcriptional regulator of *myog* (Tapscott 2005), are relatively unaffected. One plausible explanation would be that MeHg might act on MyoD cellular distribution, potentially disrupting its nuclear localization. Alternatively, MeHg might act more directly on *myog* expression, possibly altering its transcription by epigenetic mechanisms. For example, H3K27 trimethylation represses *myog* gene expression, and maintains myoblast proliferation (Asp, Blum et al. 2011). MeHg has been demonstrated to enhance H3K27 trimethylation at the brain-derived neurotrophic factor promotor, and repress its expression in the brain (Onishchenko, Karpova et al. 2008). The potential for MeHg to enhance this repressive mark at the *myog* promotor, however, remains to be explored. Regardless of mechanism, the dynamic profile of MyoG in response to MeHg highlights a potential window of susceptibility for MeHg toxicity in myogenic cells.

One limitation to interpreting the MeHg effects in this system is the apparent toxicity profile revealed by the MTT assay. In a prior study, we showed that MeHg treatment up to 3 μ M yielded no decrease in viability by MTT assay (Prince and Rand 2018). Here we demonstrate significant reduction in MTT signal by 2.5 μ M MeHg at all stages. Some discrepancy may be due to the prior study conditions that employed cells at higher density (90% confluence) and differentiation in higher serum levels (i.e. 10% horse serum) than used here (Prince and Rand 2018). Alternatively, it is possible that MTT is simply reflecting an effect on proliferation and not cytotoxicity. In support of this notion, we observe that MeHg at 2.5 μ M induces elevated p21 in myoblasts, which can be interpreted as a direct reflection of cells moving out of the proliferative state. Furthermore, the fact that MyoG protein and transcript levels at the myotube stage resume equivalent levels between MeHg treated and untreated cells indicates the transcription and translation machinery is intact and suggests the MTT values are reflecting a reduced number of cells due to proliferation effects at the myoblast stage. It remains to be determined directly whether or not the C2C12 myoblasts continue to proliferate with MeHg in our culture conditions.

The increase in p21 protein observed in the present work is consistent with MeHg effects in other studies, which demonstrated enhanced p21 in mouse brain (Ou, Thompson et al. 1999), as well as in neural stem cells (Bose, Onishchenko et al. 2012). Interestingly, we observed that MeHg exposure to myoblasts results in decreased p21 protein at the myocyte stage. This latter profile might be consistent with the overall reduction of MyoG at this stage, as a prior report demonstrated MyoG expression precedes that of p21 in C2C12 cells induced to differentiate. Moreover, myocytes were also found to co-express MyoG and p21 (Andres and Walsh 1996). Thus, the significant decrease in MyoG concurrent with increased p21 that occurs in the myoblasts, likely indicates a more complex relationship between these differentiation factors. It remains to be determined whether p21 function in our culture conditions could be independent of its role in myoblast differentiation. Future studies will aim to characterize the proliferative capacity of the C2C12 cells to further illuminate the role of p21 in MeHg-induced myotoxicity.

By comparison, arsenic (As), another ubiquitous environmental toxicant, has also been demonstrated to inhibit cell differentiation and reduce MyoG expression in C2C12 cells (Yen, Tsai et al. 2010, Steffens, Hong et al. 2011). Similar to the present work, As exposure was initiated in the myoblasts; however, in contrast, exposure continued throughout differentiation. Interestingly, C2C12 cells appeared able to recover from As, as MyoG expression and myotube formation were not different from controls after four days of differentiation (Steffens, Hong et al. 2011). In the present study, as well as our previous work (Prince and Rand 2018), C2C12 cells prove to be unable to recover from acute MeHg exposure. It remains to be determined whether chronic MeHg exposure would yield similar results. Intriguingly, zebrafish exposed to cadmium exhibited myotoxicity, but the MyoD and MyoG patterning in the embryos was unaltered; however, comparable to our results, MHC, *myhz* in zebrafish, was reduced (Hen Chow and Cheng 2003). Curiously, MHC expression was not altered in adult zebrafish exposed to MeHg (Cambier, Gonzalez et al. 2010), highlighting that observed effects in the present report need further investigation to distinguish whether MeHg exerts a similar profile of toxicity in developing muscle.

In conclusion, MeHg inhibits C2C12 mouse myoblast differentiation. Based on the present report, as well as our previous work, this effect appears independent of whether cells are acutely exposed prior to differentiation, as myoblasts, or in the earliest phase of differentiation, as myocytes (Prince and Rand 2018). We did not, however, make comparisons of initiating MeHg exposures at later post-differentiation time points. Thus, the relative sensitivity of myoblasts, myocytes, or myotubes to MeHg remains to be explored further. As MyoG was most strikingly impacted by MeHg, compared to the other MRFs, future research should focus on whether MeHg directly or indirectly modifies this pivotal myogenic transcription factor. Importantly, this study sets the stage to determine whether these effects are recapitulated *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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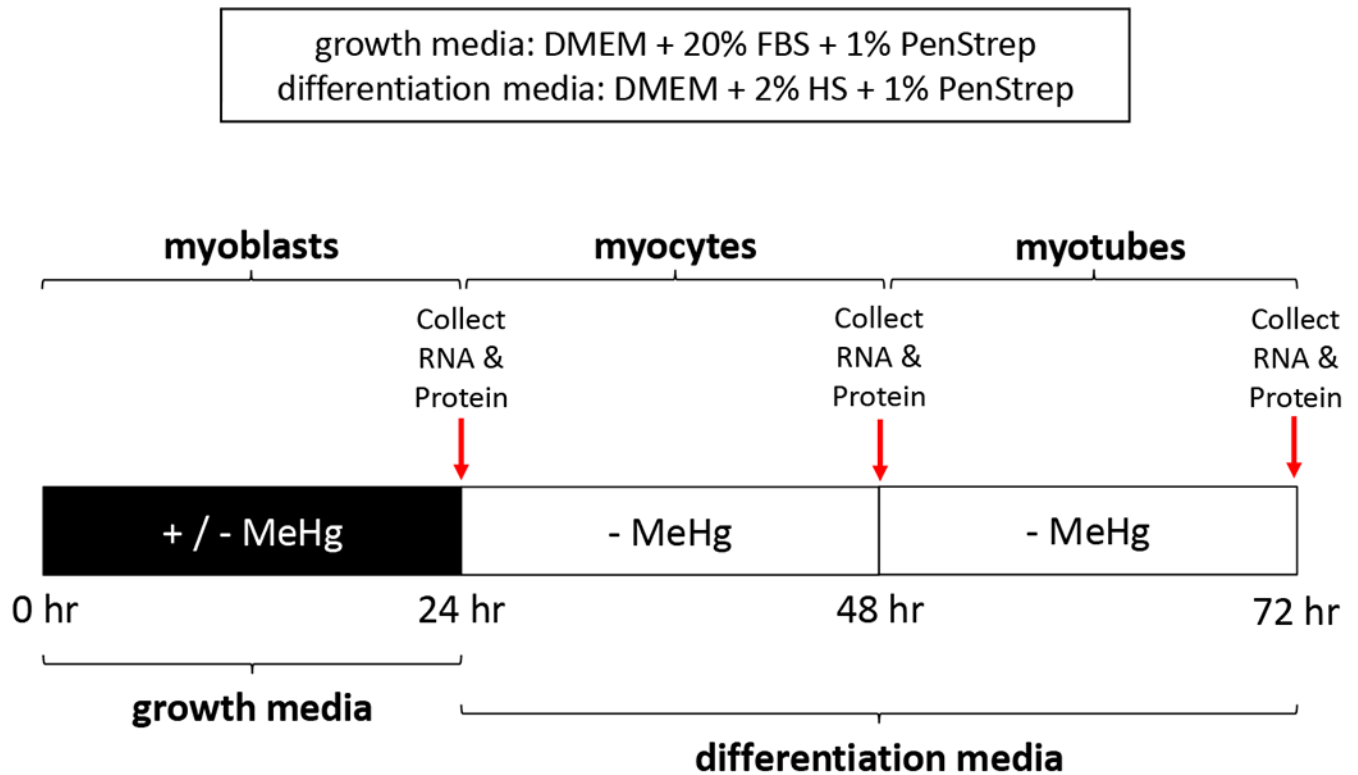


Figure 1. Experimental paradigm.

Culture conditions were designed to evaluate MeHg effects implemented in the growth phase on subsequent C2C12 differentiation stages. In all conditions, cells were exposed in growth media \pm MeHg for 24 hr. Myoblast RNA and protein was immediately collected after this 24 hr exposure. Subsequent to a switch to differentiation media without MeHg for 24 or 48 hours, RNA and protein was collected at the myocyte and myotube stage, respectively.

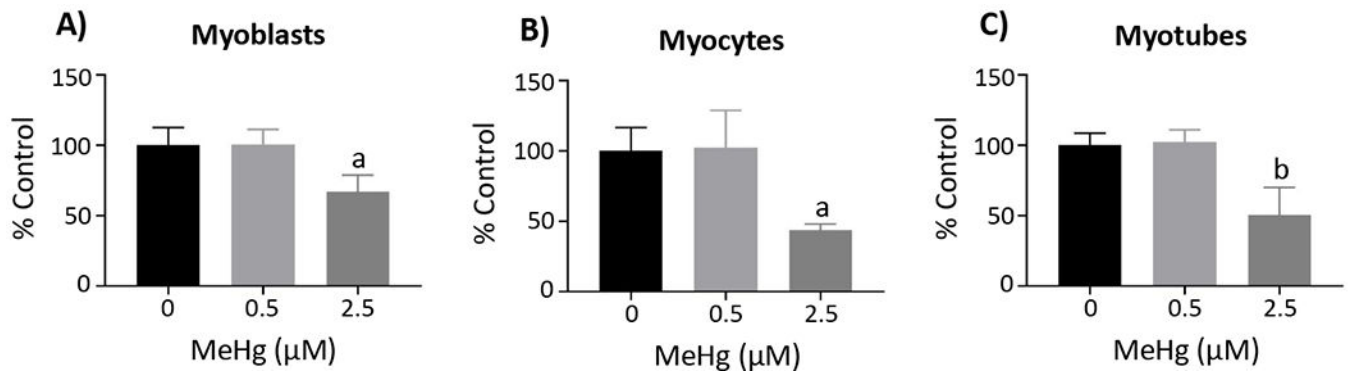


Figure 2. Cell viability.

Cell viability was assessed by MTT assay in the myoblasts (A), myocytes (B), and myotubes (C). Data are reported as percent (%) control, and represent the mean \pm SD; ^aone-way ANOVA, Tukey's HSD, $p < 0.05$; ^bone-way ANOVA, Tukey's HSD, $p < 0.01$

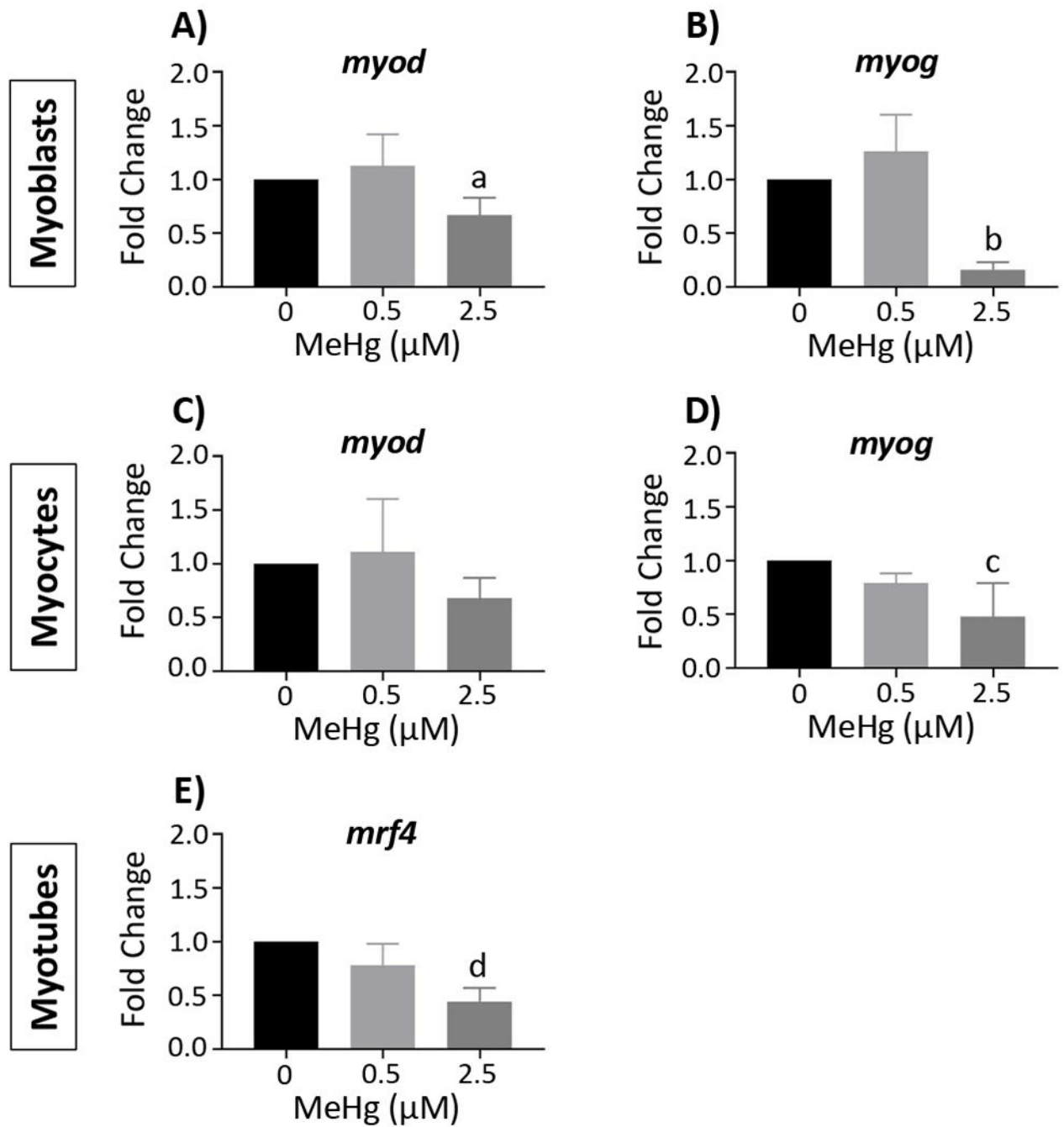


Figure 3. MeHg effects on MRF gene expression.

Gene expression was determined by real-time qPCR. *Myod* and *myog* expression are presented in the myoblasts (A and B) and myocytes (C and D), respectively, while *mrf4* expression is presented in the myotubes (E). Data are reported as fold change, and represent the mean \pm SD; ^aone-way ANOVA, Tukey's HSD, $p < 0.05$; ^bone-way ANOVA, Tukey's HSD, $p < 0.001$; ^cKruskal-Wallis, Dunn's Multiple Comparisons, $p < 0.01$; ^done-way ANOVA, Tukey's HSD, $p < 0.01$ (See also suppl. Table 2)

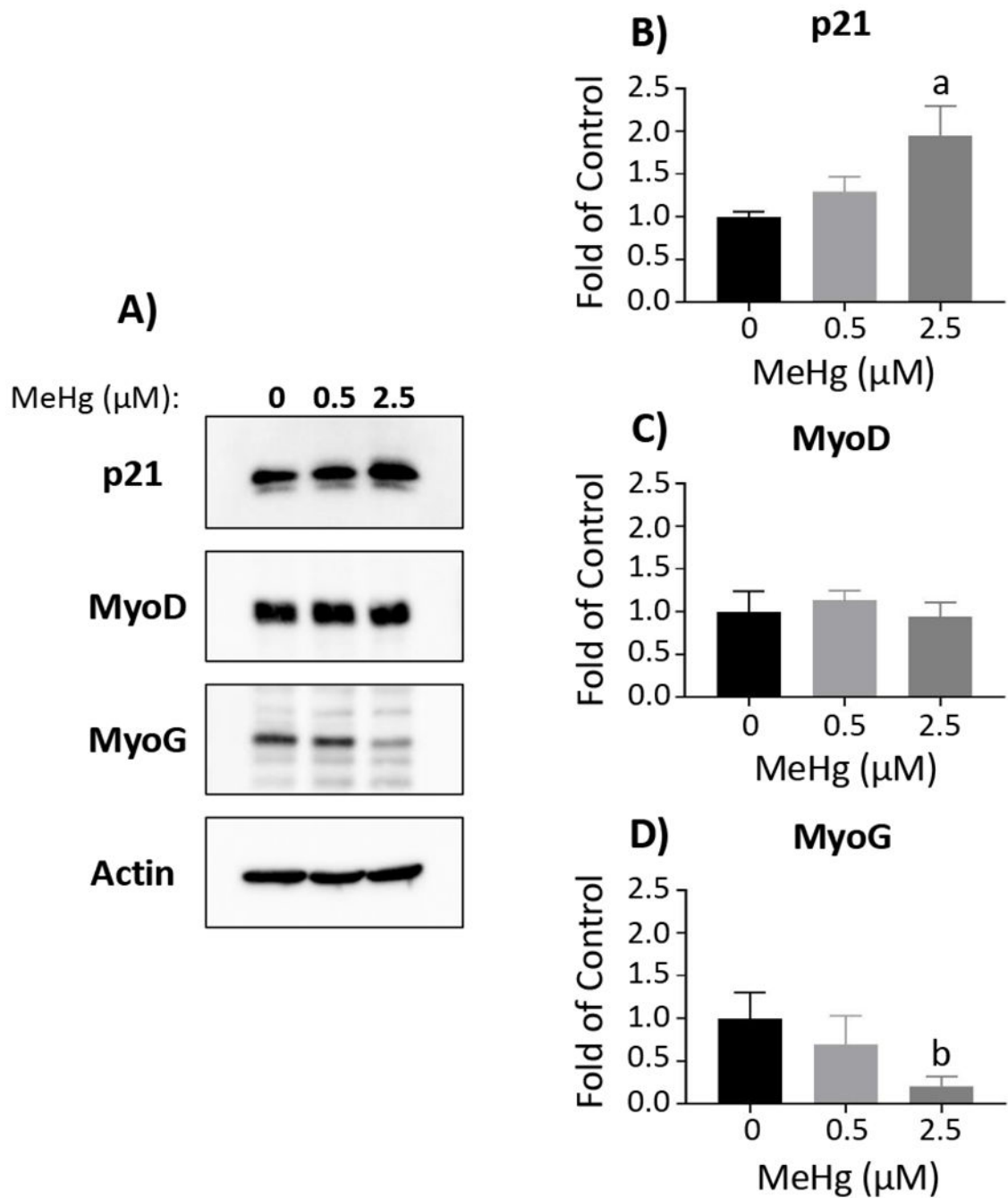


Figure 4. MeHg effects on protein levels of differentiation factors in myoblasts.

Representative Western blots for p21, MyoD, MyoG, and actin are displayed (A). Band intensity was quantified using ImageJ and normalized to actin, prior to comparison between MeHg concentrations. Total protein was quantified for p21 (B), MyoD (C), and MyoG (D). Data are reported as fold of control, and represent mean \pm SD; ^aone-way ANOVA, Tukey's HSD, $p < 0.01$; ^bone-way ANOVA, Tukey's HSD, $p < 0.05$

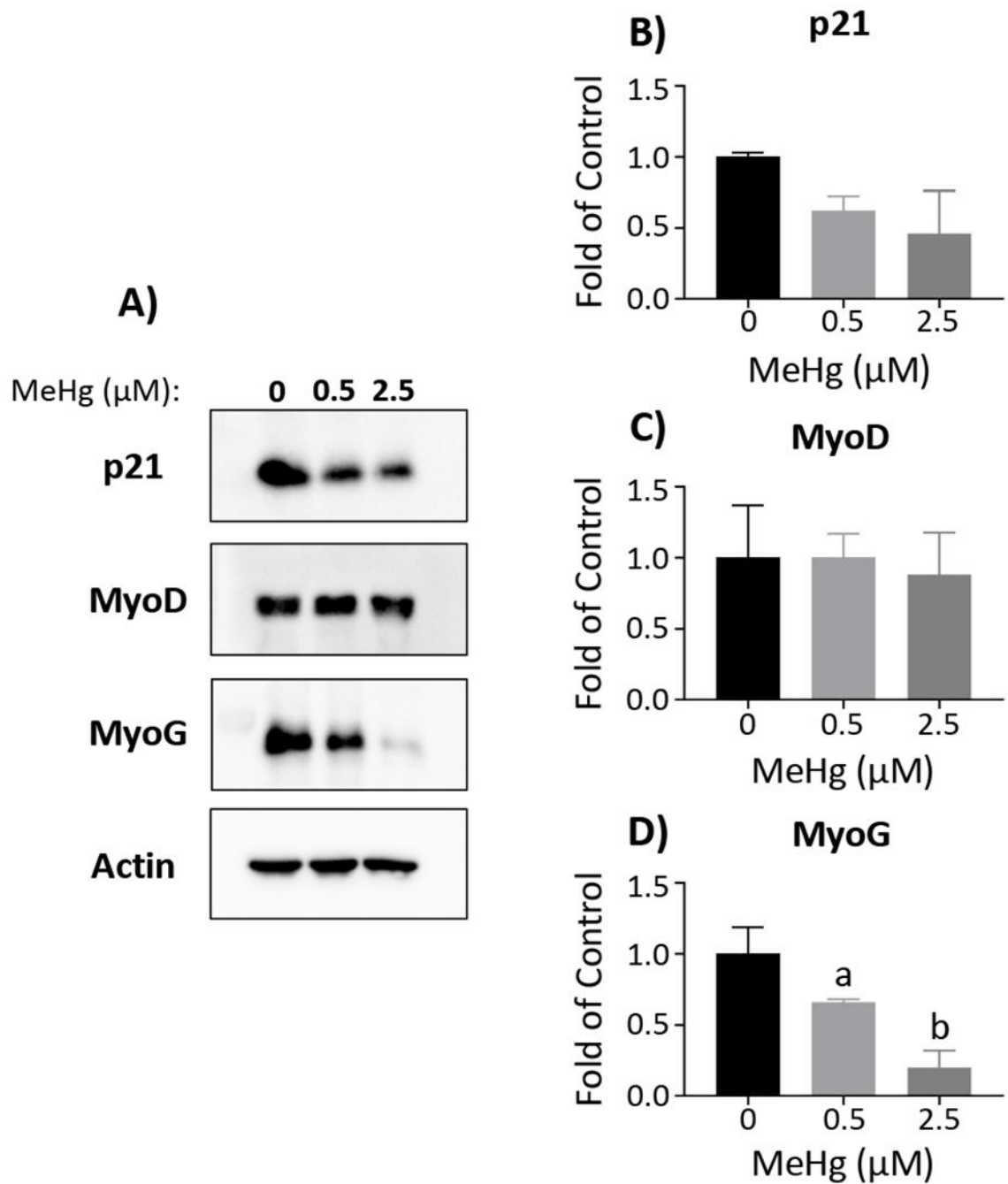


Figure 5. MeHg effects on protein levels of differentiation factors in myocytes.

Representative Western blots for p21, MyoD, MyoG, and actin are displayed (A). Band intensity was quantified using ImageJ and normalized to actin, prior to comparison between MeHg concentrations. Total protein was quantified for p21 (B), MyoD (C), and MyoG (D). Data are reported as fold of control, and represent mean \pm SD; ^aone-way ANOVA, Tukey's HSD, $p < 0.05$; ^bone-way ANOVA, Tukey's HSD, $p < 0.001$

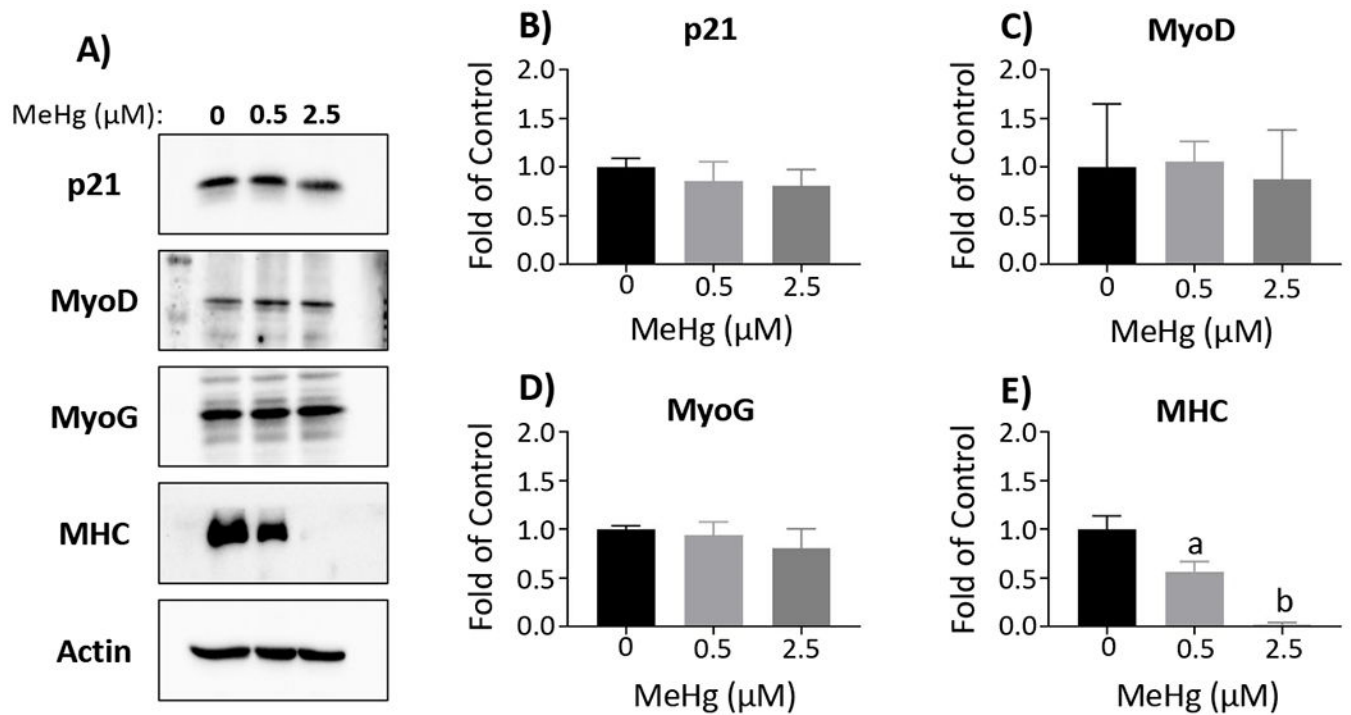


Figure 6. MeHg effects on protein levels of differentiation factors in myotubes.

Representative Western blots for p21, MyoD, MyoG, MHC, and actin are displayed (A).

Band intensity was quantified using ImageJ and normalized to actin, prior to comparison

between MeHg concentrations. Total protein was quantified for p21 (B), MyoD (C), MyoG

(D), and MHC (E). Data are reported as fold of control, and represent mean \pm SD; ^aone-way

ANOVA, Tukey's HSD, $p < 0.01$; ^bone-way ANOVA, Tukey's HSD, $p < 0.001$

Table 1

MRFs delta Ct (Ct) values

Gene	Myoblasts	Myocytes	Myotubes
<i>myf5</i>	5.14 ± 0.66	6.07 ± 0.17	5.27 ± 0.45
<i>myod</i>	3.96 ± 0.26	3.05 ± 0.37 ^a	3.20 ± 0.16 ^b
<i>myog</i>	5.53 ± 2.32	1.59 ± 0.54 ^c	2.10 ± 0.81
<i>mrf4</i>	11.35 ± 0.59	12.36 ± 0.45	9.36 ± 0.96 ^b

mean ± SD; n = 3

^aone-way ANOVA, Tukey's HSD; p < 0.001^bone-way ANOVA, Tukey's HSD; p < 0.01^cKruskal-Wallis, Dunn's Multiple Comparisons; p < 0.01

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Table 2Relative total protein level (normalized to β -actin)

Protein	Myoblasts	Myocytes	Myotubes
p21	0.93 \pm 0.06	1.72 \pm 0.05 ^a	1.03 \pm 0.09
MyoD	1.24 \pm 0.29	1.83 \pm 0.67	0.28 \pm 0.15
MyoG	0.57 \pm 0.17	1.94 \pm 0.37 ^b	1.19 \pm 0.04 ^c
MHC	ND*	ND*	2.03 \pm 0.28

mean \pm SD; n = 3

* ND, not detected

^aKruskal-Wallis, Dunn's Multiple Comparisons; p < 0.05^bone-way ANOVA, Tukey's HSD; p < 0.001^cone-way ANOVA, Tukey's HSD; p < 0.05