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Lead exposure reduces survival, neuronal determination, and differentiation of P19 stem cells

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ABSTRACT

Lead (Pb) is a teratogen that poses health risks after acute and chronic exposure. Lead is deposited in the bones of adults and is continuously leached into the blood for decades. While this chronic lead exposure can have detrimental effects on adults such as high blood pressure and kidney damage, developing fetuses and young children are particularly vulnerable. During pregnancy, bone-deposited lead is released into the blood at increased rates and can cross the placental barrier, exposing the embryo to the toxin. Embryos exposed to lead display serious developmental and cognitive defects throughout life. Although studies have investigated lead's effect on late-stage embryos, few studies have examined how lead affects stem cell determination and differentiation. For example, it is unknown whether lead is more detrimental to neuronal determination or differentiation of stem cells. We sought to determine the effect of lead on the determination and differentiation of pluripotent embryonic testicular carcinoma (P19) cells into neurons. Our data indicate that lead exposure significantly inhibits the determination of P19 cells to the neuronal lineage by alteration of N-cadherin and Sox2 expression. We also observed that lead significantly alters subsequent neuronal and glial differentiation. Consequently, this research emphasizes the need to reduce public exposure to lead.

1. Introduction

Lead toxicity remains a global public health concern. Significant amounts of environmental lead contamination are still present in sources such as paint and gasoline (Gould, 2009). Public health campaigns and governmental regulations have resulted in a sharp decrease in acute lead poisoning incidents; however, chronic, low-dose lead exposure remains a concern in both developing and industrialized nations (Tong et al., 2000). Acute lead poisoning leads to chronic lead exposure because lead is deposited in the bones, where the toxin is slowly leached into the blood for decades (Korrick et al., 2002). Lead reabsorption from the bones to blood occurs during normal bone remodeling and this process is increased during human pregnancy because of drastic changes in calcium metabolism and lactation (Silbergeld, 1991; Riess and Halm, 2007; Cross et al., 1995). Developing fetuses are particularly sensitive to maternal blood lead levels because lead can freely pass through the placental membrane through passive transport (Goyer, 1990) or possibly through inappropriate binding to the divalent metal transporter-1, a transport protein that is abundantly expressed during human embryo gestation (Bressler et al., 2004; Gundacker and Hengstschläger, 2012). Because the blood-brain barrier is not fully developed in fetuses and newborns (Baynes et al., 2012), lead can easily reach the vulnerable developing brain (Jarup, 2003). Moreover, as the blood-brain barrier develops, lead ions can pass through by substituting for calcium ions (Sanders et al., 2009). Consequently, prenatal exposure to lead has been linked to cognitive defects, neuronal death, decreased neurotransmission, central nervous system (CNS) deformities, and infertility (Lidsky and Schneider, 2003; Gilbert-Barness, 2010).

During development, stem cells that are determined to be neuronal precursor cells (NPCs) form a neural epithelial tube, which proceeds to differentiate into neurons and macroglia such as astrocytes and oligodendrocytes (Kintner, 2002). If this process is disrupted, or proceeds incorrectly, a multitude of defects can occur including the motor and cognitive symptoms associated with spina bifida (Copp et al., 2015). Few studies have shed light on the effect of lead on fetal nervous system development. Previously, lead exposure was shown to reduce neurogenesis and increase apoptosis of neuronal cells in zebrafish embryos (Dou and Zhang, 2011). Moreover, in rat models, lead exposure caused a deficit in the survival and proliferation of neuronal stem cells in various brain regions (Gilbert et al., 2005; Huang and Schneider, 2004).

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Lead also decreases the proliferation and viability of immortalized human neuronal progenitor cells (Breier et al., 2008). These studies document the effect of lead on post-determination stem cells but did not examine lead exposure at different time points across stem cell development despite strong evidence that the timing of lead exposure has a significant effect. For example, epidemiological cohort studies have presented conflicting evidence regarding the relative effect of the timing of maternal lead exposure on the neurodevelopment of the fetus (Cantonwine et al., 2010; Hu et al., 2006; Schnaas et al., 2006). The evidence in these studies requires additional investigation to determine whether the relative timing of lead exposure has a significant effect on development at the stem cell level.

The development of neural progenitor cells (NPCs) requires the coalescence and interaction of stem cells—a process that is largely regulated by the cell-adhesion protein N-cadherin (Pieters and van Roy, 2014).N-cadherin regulates calcium-dependent homophilic interactions and diverse signaling pathways including Akt, Wnt/β-catenin, fibroblast growth factor (FGF)-2, and Rho GTPases (Zhang et al., 2010; Noles and Chenn, 2007; Hansen et al., 2008; Nusser et al., 2002; Li et al., 2002). N-cadherin functioning is critical for maintaining neurogenic stem cell niches (Zhang et al., 2010), axon outgrowth (Riehl et al., 1996), dendritic branching (Yu and Malenka, 2003), synaptogenesis (Benson and Tanaka, 1998), and synaptic plasticity (Okamura et al., 2004). Furthermore, N-cadherin plays a critical role in the formation and expansion of the neural crest and neural tube (Pla et al., 2001; Nakagawa and Takeichi, 1995; Nieto, 2001). Additionally, the development of the nervous system is marked by a number of other proteins including the basic helix-loop-helix (bHLH) transcription factor NeuroD, which is implicated in a variety of developmental functions including cell fate determination, differentiation, and neuron survival (Morrow et al., 1999). The intermediate filament protein Nestin is also considered a marker of neuronal progenitors (Hockfield and McKay, 1985). The transcription factor Sox2 is expressed in the early neuronal development and plays a critical role in both neurogenesis and gliogenesis (Uwanoghoa et al., 1995; Ferri, 2004; Zappone et al., 2000).

Because neural determination and differentiation are closely connected, it is essential to parse apart the relative effects of lead toxicity on each process. The present study investigates how lead exposure affects pluripotent stem cells when introduced during one or both of these two interrelated processes. Of the available treatments for prenatal lead exposure, chelation therapy is typically used only for pregnant women and children with unusually high blood lead levels (Brown, 2013) and, moreover, has been shown to have no improvement on children's neurophysiological functioning (Dietrich, 2004; Rogan et al., 2001). By gaining a more nuanced understanding of the toxic effects of lead on neurogenesis and gliogenesis, additional therapies for prenatal lead exposure could be developed.

Therefore, we investigated the effect of lead exposure on Retinoic acid-induced neurosphere formation (determination) and subsequent differentiation of murine testicular carcinoma pluripotent stem cells (P19 cells) via a neurosphere assay (Hong and Bain, 2012). These free-floating neurospheres are considered more representative of the environment found inside living organisms than other culture methods, especially regarding cell-cell communications and tissue architecture (Zhou et al., 2016). Our studies revealed that lead exposure causes increased cell death by necrosis and, interestingly, lead does not inhibit the proliferation of P19 cells. We also discovered that lead inhibits neuronal determination, neuronal and glial differentiation of P19 cells, and these effects can be attributed to altered N-cadherin and Sox2 expression in the lead-treated P19 cells.

2. Materials and methods

2.1. Materials

P19 cells were purchased from ATCC (CRL-1825). Anti-phosphohistone3 antibody was from Cell signaling technologies, Neuronal tubulin (Tuj1) antibody from Novus Biochemicals. Sox2, Nestin, N-cadherin and NeuroD antibodies were purchased from Genetex and GFAP antibody was from synaptic systems. Alexa flour conjugated secondary antibodies and other reagents were purchased from Thermo Fisher Scientific.

2.2. Cell culture

P19 murine testicular carcinoma stem cells were maintained at 5% CO_2 at 37 °C in complete media (Minimum Essential Medium–Alpha Modification (αMEM) supplemented with 10% fetal calf serum, 5% Glutamax and 5% penicillin/streptomycin).

2.3. Lead treatment

To determine the concentration of lead acetate (Pb^{2+}), we referred to studies performed during the Flint Water Crisis of 2015, that reported some water samples contained lead that exceeded 1000 μg/L (4.93 μM) (Pieper et al., 2018). Additionally, current U.S Occupational Safety and Health Administration (OSHA) standards state that workers are able to return to work when blood lead levels (BLLs) reach < 40 μg/dL (1.93 μM) (CDC, 2018). Therefore, we treated our cells with 1, 2, and 3 μM Pb^{2+} throughout the course of this study.

2.4. Neuronal determination

Neuronal determination was measured by observing neurosphere formation of P19 cells through the use of a neurosphere assay (Negraes et al., 2012). This technique is useful in that it is more similar to the environment of a living organism than other culture methods especially in regard to cell-to-cell communication and structure (Zhou et al., 2016). Furthermore, neurospheres are formed by only the stem cells that have become determined, giving an easy and clear way to measure determination. Neurosphere formation was induced by plating 750,000 cells on bacteriological Petri dishes with 12 mL complete media (αMEM) containing 0.5 μM RA as previously described (Jones-Villeneuve, 1982). After two days, media was replaced with freshly prepared RA containing complete media and cells were allowed to form neurospheres for 2 additional days. When cells are grown in media without adherent substrates they form neurospheres which contain Neuronal Progenitor Cells (NPCs) when induced by RA. These neurospheres are capable of self-renewal and differentiation (Bez et al., 2003). To examine the effect of lead on determination, P19 cells were exposed to 1 μM, 2 μM, or 3 μM of Pb^{2+} at the time of plating. Cells were exposed to consistent amounts of lead throughout the experiment.

2.5. Neuronal differentiation

Neurospheres were removed and subjected to trypsin digestion to obtain single cell suspension of determined NPCs. To induce differentiation, neuronal precursors were plated in 24 well cell culture plates (1000 cells/well) in a neuronal culture media (Neurobasal media supplemented with 2% B27, 5% penicillin/streptomycin, and 5% glutamax). We investigated the effect of lead on three different stages of neuronal development: Condition A; stem cells were exposed to lead during the determination period, washed three times with lead-free media, and then were cultured in the absence of lead throughout differentiation. This condition helped us to determine the effect of lead on neuronal differentiation as stem cells were exposed to lead early in the determination process. Condition B; stem cells which were not exposed to lead during the determination period were cultured in the presence of lead throughout the differentiation process. This condition helped determine the effect of lead on neuronal differentiation as stem cells were exposed to lead during the late development and differentiation processes only. Condition C; stem cells were exposed to lead during

both the period of determination and differentiation. This condition helped us determine the effect of lead on neurogenesis when stem cells are exposed to lead continuously during neuronal development. For conditions B and C, cells were differentiated in the presence of 1, 2, and 3 μM of Pb^{2+} for four days. After completion of the experiment, cells were fixed with 4% paraformaldehyde and immunostained with neuron-specific β-III-tubulin (tuj1) antibody to identify the differentiated neurons. To investigate the mechanism for lead's effect on P19 neuronal differentiation, the cells were immunostained for Sox2 expression and the astroglial marker GFAP.

2.6. Cell death analysis

To determine the effect of lead on stem cell survival, P19 cells were plated (1000 cells/well) on 24-well cell culture plates and were treated with Pb^{2+} for 24, 48, or 72 h. After the lead exposure, one group of cells were incubated with ethidium homodimer 1 for 1 h at 37 °C. After the incubation period, cells were washed with P19 culture media and incubated in culture media for 30 min. Cells were visualized for ethidium homodimer 1 internalization to score the dead cells.

2.7. Cell proliferation analysis

To determine the effects of lead on stem cell proliferation, P19 cells were plated on 24 well cell culture plates (1000 cells/well) and were treated with Pb^{2+} at 24, 48, or 72 h. After completion of the experiment, cells were fixed with 4% paraformaldehyde and immunostained with anti-phospho-histone H3 (ser10) antibody to identify the mitotic cells in different conditions.

2.8. Analysis of neurospheres for neuronal precursor markers

To understand the mechanism of decrease in the number and the area of neurosphere and the decreased neuronal P19 cell population when exposed to P19 cells to 2 μM lead at the time of neural induction. We investigated the expression of NeuroD, Nestin, and N-cadherin in Retinoic acid-induced P19-Neuronal precursor cells (NPCs). Neurospheres produced on bacterial-grade culture plates do not stick to the plastic surface, and because they also form round and large aggregates of the cells, it becomes very difficult to analyze all the cells in the neurospheres by immunofluorescence. Therefore, we utilized a modified method to generate P19 NPCs (Monzo et al., 2012). In brief, P19 cells were plated in 24 well culture plates (60,000 cells/well) in Minimum Essential Medium alpha supplemented with all-trans Retinoic acid (RA) and 2.5% serum, and cultured as a monolayer. Cells were cultured for four days in the presence or absence of 2 μM lead, and these cells formed the aggregates of the induced P19 NPCs (Figs. 7, 8 and 9). Similar to neurospheres cultured on the non-adherent surface, lead treatment resulted in a decrease in the area and number of P19 NPC aggregates. Immunofluorescence studies were performed on the 4 days in vitro (DIV) P19 NPC aggregates for NeuroD, Nestin, and N-cadherin. Images were pseudo-colored, and for the sake of clarity in visualization, Hoechst was colored as magenta. Hoechst images were superimposed with NeuroD, Nestin, or N-cadherin images. Images of 5 random fields per well were used to count NeuroD positive nuclei. Numbers of NeuroD-positive nuclei were counted in the control and lead-treated culture (n = 3 wells per condition from triplicate experiments). To measure Nestin and N-cadherin protein expression, we marked P19 NPC aggregates as regions of interest, and fluorescence intensity was measured after background correction using Image J. Measurements were taken from twenty-five P19 NPC clusters from each experiment (n = 3).

2.9. Differentiation of neurons and glial cells

P19 cells were cultured in the presence of retinoic acid for 4 DIV to induce P19 NPCs. NPCs were trypsinized to obtain a single-cell suspension. Differentiation was induced in adhesion cultures by plating cells for 4 DIV (10,000 cells/well on Poly-D-lysine and laminin-coated 24-well culture plates) in DMEM with 10% FBS. We did not use B27-Neurobasal media for these experiments because B27-Neurobasal media specifically promotes the growth of neuronal cells and nearly inhibits the glial cells (Brewer et al., 1993).

We analyzed the cells in the following experimental conditions: A) P19 cells were not treated with anything and cultured in DMEM with 10% FBS for 4 DIV (on Poly-D-lysine and laminin coated 24 well culture plates). B) P19 cultured in the presence of retinoic acid for 4 DIV to induce P19 NPCs. Adhesion cultures were then started to induce neuronal differentiation. C) P19 cells were cultured in the presence of retinoic acid and 2 μM lead for 4 DIV to induce P19 NPCs. Adhesion cultures were then started to induce neuronal differentiation in the absence of lead. D) P19 cells were cultured in the presence of retinoic acid for 4 DIV to induce P19 NPCs. Adhesion cultures were then started to induce neuronal differentiation in the presence of 2 μM lead. E) P19 cells were cultured in the presence of retinoic acid and lead for 4 DIV to induce P19 NPCs. Adhesion cultures were then started to induce neuronal differentiation in the presence of 2 μM lead.

2.10. Immunocytochemical analysis and analysis of the population of neurons and astrocytes

After 4 DIV, cells in differentiation conditions were fixed with 4% paraformaldehyde and were subjected to immunostaining with antibodies against immature neuronal marker (β-III-tubulin) and astroglial marker (GFAP), transcriptional factor Sox2, and nuclear marker Hoechst. Quantification was performed by counting the number of cells immunoreactive for β-III-tubulin (longer neurites than cell bodies)/GFAP (green astrocytes)/Sox2 (red nuclear staining) in triple immunological analysis, followed by individual calculation of percentage versus total number of cells stained with Hoechst. We utilized the same secondary antibody (green fluorescence) for β-III-tubulin and GFAP. Therefore, before performing the triple labeling, we performed the immunocytochemical analysis with GFAP and Tuj1 in independent culture to ascertain that antibody staining is specific to the neuronal and glial cells.

2.11. Immunostaining

For different immunostaining experiments, cells were permeabilized with 0.2% Triton X-100 in Phosphate buffer. After blocking with 4% BSA, cells were incubated at 4 °C overnight with primary antibodies (1:1000 dilution for β-III-tubulin (Tuj1), 1:500 dilution for Nestin, N-cadherin, NeuroD, Sox2 and GFAP antibodies, and 1:100 dilution for anti-phospho-histone 3 (ser10) antibody). After washing, cells were incubated with Alexa flour conjugated secondary antibodies (1:500) for 2 h.

2.12. Cell visualization

Cells were analyzed by phase contrast and fluorescent microscopy using a Nikon Ti eclipse fluorescence microscope. Cells were imaged by using NIS Element imaging software, a CMOS camera with Lambda 10.3 filter wheel controller, and a Lambda XL Xenon Lamp.

2.13. Quantitative analysis

For cell determination quantitative analysis, we took ten images of each treatment (n = 4) at random locations in each petri dish. The mean number of neurospheres was determined by adding together all of the neurospheres in the images and dividing by the total number of images. In total, 189 neurospheres were counted across all conditions. Neurosphere size was quantified as the diameter using the

measurement tool in ImageJ. The size of every neurosphere was measured unless more than half of the neurosphere was not present in the image area. The mean diameter was calculated for each treatment.

For the proliferation assay, we took ten images of each treatment (n = 3) at random locations in each well. In total, 10,248 cells were counted (609 of which were dividing) across all lead concentrations and time conditions (n = 3). For cell viability assays, we took ten images of each treatment at random locations in each well. Cells were scored as undergoing necrosis by using Ethidium homodimer 1 staining. In total, 41,951 cells were counted (8136 of which were dead). Cells were scored as having undergone apoptosis by the obvious presence of condensed chromatin. In total, 10,248 cells were counted (970 of which were dead) (n = 3).

For cell differentiation quantitative analysis, cells were scored as differentiated by the obvious presence of β-III-tubulin. In total, 17,222 cells were counted.

2.14. Statistical analysis

For the cell determination experiments, we used a one-way ANOVA to analyze counts of neurospheres (Sokal and Rohlf, 2012). We used Tukey's Honest Significant Difference Tests to examine all combinations of sample mean after the ANOVA (Day and Quinn, 1989). For cell differentiation experiments, we used a one-way ANOVA and Tukey' Honest Significant Difference Test after square-root transforming the data to account for lack of normality (Sokal and Rohlf, 2012). After transformation, both normality and homoscedasticity assumptions were met. All means and confidence intervals were backtransformed to raw cell counts for the construction of figures. For cell viability and proliferation experiments, we used a two-way ANOVA and Tukey' Honest Significant Difference Test after square-root transforming the data to account for lack of normality (Sokal and Rohlf, 2012). After transformation, both normality and homoscedasticity assumptions were met. All data were backtransformed and then converted to percent of control for the construction of figures. All effects are expressed as p values and visualized on graphs made in Microsoft Excel. All statistical analyses were performed using R-studio.

All experiments were performed in at least triplicate (n = 3).

3. Results

3.1. Lead exposure alters P19 cell determination into NPCs

We used a neurosphere assay to study the determination process of stem cells because neural stem cells are identified by their ability to form neurospheres (Huang and Schneider, 2004). We tested the effect of lead exposure on the number of neurospheres across three different concentrations (1, 2, and 3 μM). For each treatment, we calculated the mean (± 95% CI) number of neurospheres per petri dish across all replicates (n = 3). The control treatment had a mean of 15.33 ± 8.72 neurospheres (Fig. 1E). Both 1 μM and 2 μM had on average 8.66 ± 5.73 and 8.33 ± 6.25 neurospheres, respectively (Fig. 1E). 3 μM lead treatment resulted in an average of 4.00 ± 2.48 neurospheres (Fig. 1E). One-way ANOVA analysis revealed a significant effect of treatment on the number of neurospheres ($p = 0.005$). Post-hoc analysis reveals a decrease in the mean number of neurospheres with 1 μM and 2 μM Pb ($p < 0.05$) and 3 μM ($p < 0.01$) (Fig. 1E).

Another important aspect of neuronal determination is the size of neurosphere formed because it can indicate the number of cells present to undergo differentiation. We measured the mean diameter of neurosphere in μm (± 95% CI) per treatment using ImageJ. For the lead-free treatment, the mean diameter of neurosphere was 405.56 ± 169.32 μm (Fig. 1A). Neurospheres treated with 1 μM Pb and 2 μM Pb had a mean diameter of 357.90 ± 145.33 μm and 257.80 ± 47.08 μm, respectively (Fig. 1B, C). Treating P19 cells with 3 μM Pb resulted in a drastic reduction in diameter with these neurospheres averaging 183.93 ± 165.00 μm (Fig. 1D). One-way ANOVA analysis revealed a significant effect of lead treatment on neurosphere diameter ($p = 0.003$). Post-hoc analysis reveals a dose-dependent decrease in neurosphere size for the 2 μM Pb treatment ($p < 0.05$) and 3 μM Pb treatment ($p < 0.01$) (Fig. 1F).

3.2. Lead exposure decreases P19 cell viability

To determine if a decrease in stem cell viability caused a decrease in neurosphere number and size, we performed experiments to investigate the effect of lead on stem cell survival. We tested the effect of three different lead concentrations (1, 2, and 3 μM) on P19 cell survival. We exposed the cells to lead for three different periods of time (24, 48, and 72 h). The P19 cell population remained relatively stable across all time points for the control treatment, as indicated by the uniform lack of ethidium homodimer 1 (Fig. 2A). At 24 and 48 h, the number of dead cells increased dramatically with 3 μM Pb treatment (Fig. 2A). At 72 h, the number of dead cells increased only modestly with increasing lead concentration (Fig. 2A). Overall, lead treatment had a significant effect on cell viability at 24 and 48 h ($p = 0.002$) (Fig. 2B). The effect of the timing of lead exposure was not significant.

A decrease in the number and size of neurospheres may also be caused by decreased cell proliferation. Multiple previous studies have found that lead exposure leads to loss of proliferation in neuronal progenitors (Schneider et al., 2005; Engstrom et al., 2015). To examine if lead exposure negatively affects cell proliferation during early P19 cell development, we immunostained both control and lead-exposed P19 cells with Histone-3 Phosphate to visualize cell division. Our data show relatively little variation in the number of cells dividing between lead treatments at each time point (Fig. 3A). ANOVA analysis reveals no significant effect of lead treatment on the number of P19 cells dividing ($p = 0.1973$)(Fig. 3B).

3.3. Lead inhibits differentiation of P19 NPCs

To test the effect of lead exposure on P19 stem cell differentiation into neurons, we transferred the dispersed neurospheres from our determination assay to a 24-well plate so that the P19 cells could differentiate. Because exposure to lead can occur at different time points in embryonic development, we had multiple conditions. We introduced lead at different concentrations (1, 2, 3 μM) during determination only (4DIV, during the process of neurosphere formation), differentiation only (4DIV, during the process, cells were allowed to attached to plastic surface to differentiate), or during both determination and differentiation. We calculated the mean (± 95% CI) number of differentiated neurons (n = 3) per condition. For the condition in which we exposed the P19 cells to lead during determination and not differentiation, the mean number of neurons decreased significantly ($p = 0.0009$) at 2 μM and 3 μM Pb (Fig. 4A). For the condition in which we exposed the P19 cells to lead during differentiation and not determination the mean number of neurons decreased significantly ($p = 0.0018$) at all lead concentrations (Fig. 4B). Finally, for the condition in which we exposed the P19 cells to lead during both determination and differentiation, the mean number of neurons decreased significantly ($p = 0.0159$) at 2 μM and 3 μM Pb (Fig. 4C).

The above analyses demonstrate the negative effect of lead during the determination and differentiation processes but do not discriminate between the relative importance of the timing of lead exposure. To analyze the effect of the timing of lead exposure, we combined the data from all three lead concentrations (1, 2, and 3 μM Pb) and calculated the mean (± SD) such that the timing of lead exposure (e.g., during determination only, during differentiation only, or both) was the variable being manipulated (Fig. 5). When the P19 cells were determined and differentiated in lead-free media, the mean number of differentiated neurons was 840.3 ± 100.8 (Fig. 5). When the P19 cells were treated with lead during determination-only and differentiation-only,

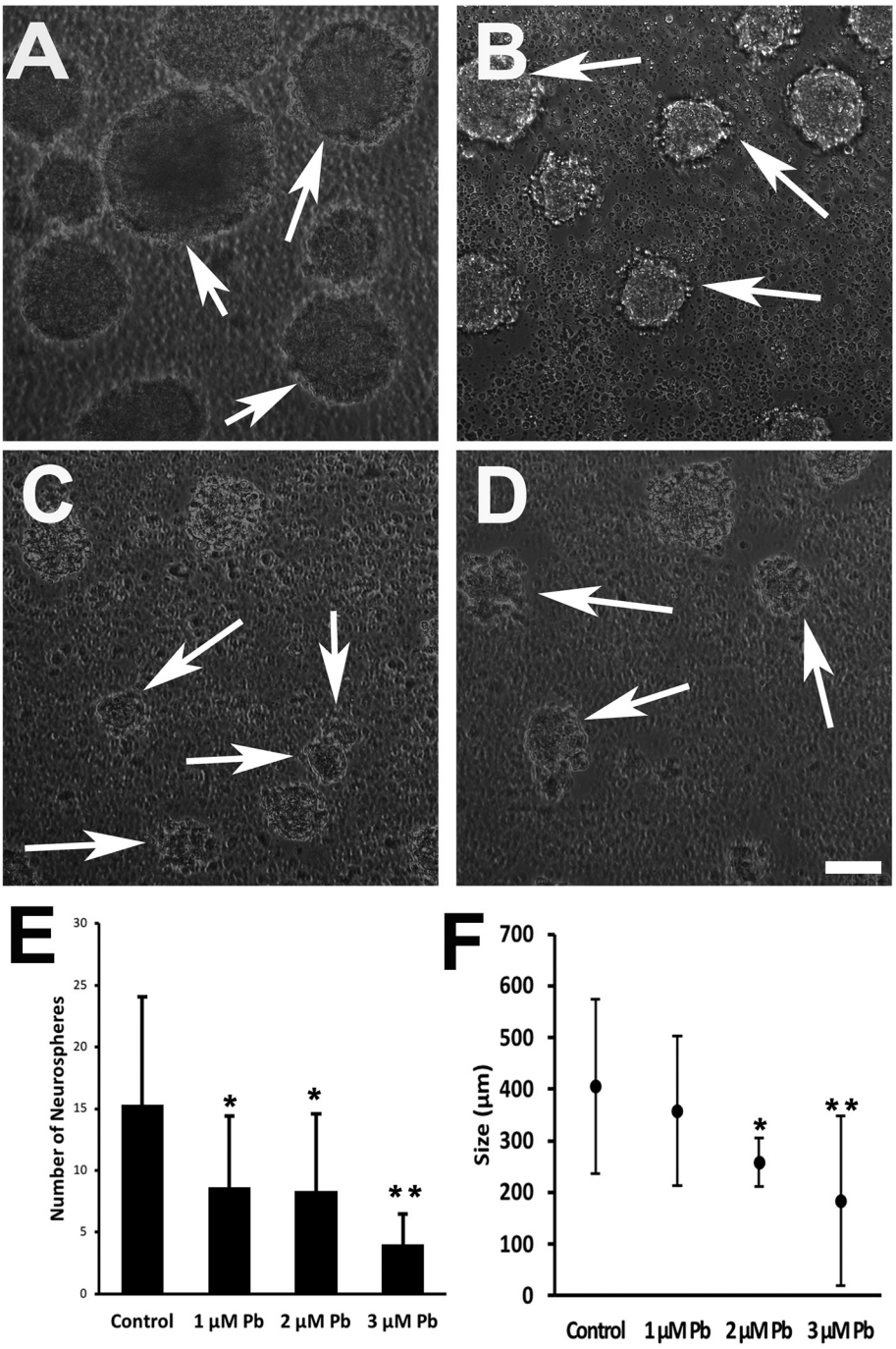

Fig. 1. Neurosphere size and number of neurospheres decrease with increasing lead concentration.

P19 cells were plated in 12 mL media with 0.5 µM RA to induce neurosphere formation. These developing neurospheres were exposed to 0 µM, 1 µM, 2 µM, or 3 µM lead acetate (Pb2+) for 24 h and then imaged by phase-contrast microscopy. Each treatment was replicated three times. A, Control neurospheres. B, Neurospheres treated with 1 µM Pb2+. C, Neurospheres treated with 2 µM Pb2+. D, Neurospheres treated with 3 µM Pb2+. E, Quantitative analysis showing that, with increasing lead concentration, there is a significant decrease in the number of neurospheres ($p = 0.005$). F, Quantitative analysis reveals that, with increasing lead concentration, there is a significant decrease in neurosphere size ($p = 0.003$). Error bars represent 95% confidence intervals in both E and F. *Significantly different from control ($p < 0.05$). **$p < 0.01$.

the mean number of differentiated neurons was 144.2 ± 65.1 and 248.8 ± 58.5, respectively (Fig. 5). When the P19 cells were exposed to lead during both determination and differentiation, the mean number of neurons was 134.3 ± 40.3 (Fig. 5). One-way ANOVA analysis again confirms the significant negative effect of lead ($p = 3.57e\text{-}9$); however, a post-hoc analysis reveals no significant difference between treatments when lead was added at different time points ($p = 0.69, 0.89, 0.64$).

3.4. Lead alters Nestin and N-cadherin expression in retinoic acid induced P19 NPCs

To study the mechanism for the effect of lead exposure on P19 cell determination into neurons, we examined the expression of NeuroD, Nestin, and N-cadherin proteins in Retinoic acid-induced P19 NPCs. Lead treatment did not alter the expression of NeuroD in P19 NPCs (Fig. 6A–G). The data in the figure represents the means ± SEM of three independent experiments ($n = 3$) with 400 nuclear counts from each experiment. Lead treatment did, however, significantly reduce ($p < 0.01$) the expression of Nestin (Fig. 7A–F). Quantitative analysis of nestin fluorescence intensity did reveal a significant decrease in the Nestin protein expression levels in the lead-treated P19 NPCs (Fig. 7G). The data in the figure are means ± SEM of three independent experiments ($n = 3$) with twenty-five P19 NPCs clusters from each experiment. Lead treatment also decreased the protein levels of N-cadherin in the lead-treated P19 NPCs (Fig. 8A–F). Quantitative analysis of N-cadherin fluorescence intensity did reveal a significant decrease in the N-Cadherin protein expression levels in the lead-treated P19 NPCs (Fig. 8G). The data in the figure are means ± SEM of three independent experiments ($n = 3$) with twenty-five P19 NPCs clusters

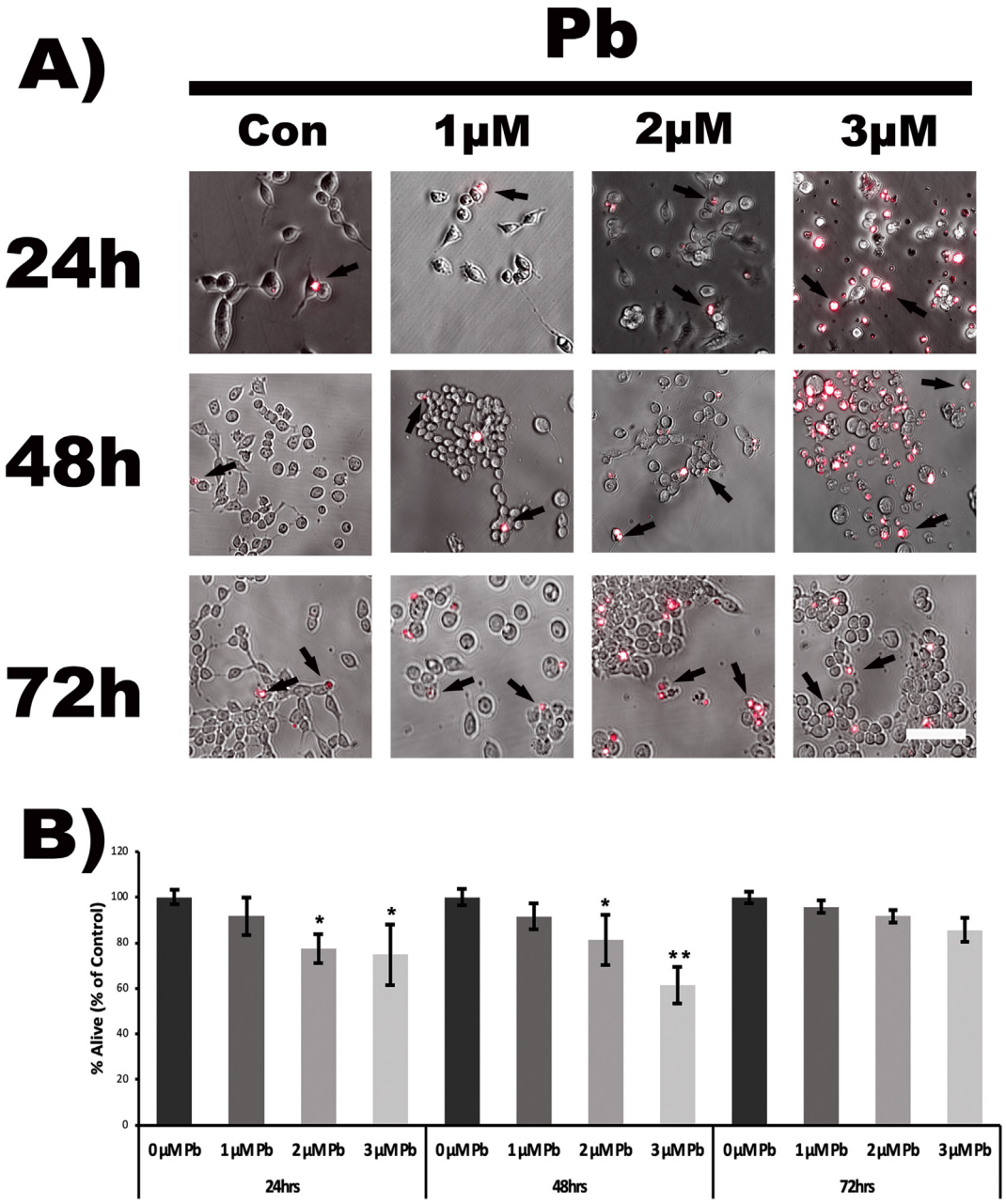

Fig. 2. P19 cells show increase in cell death following lead treatment.
P19 cells were treated with 0 μM, 1 μM, 2 μM, or 3 μM Pb2+ for 24, 48, or 72 h and then incubated with ethidium homodimer 1. Each treatment was replicated three times. Following the experiment, fluorescence and phase-contrast images were taken and overlaid using Photoshop CS4 and counted. A, P19 cells incubated with 0 μM, 1 μM, 2 μM, or 3 μM Pb2+ for either 24, 48, or 72 h. Ethidium homodimer 1 enters the cell if the membrane is damaged. Therefore, all cells with red fluorescence were marked as dead (arrows showing examples). B, Quantitative analysis showing the percent alive relative to the control treatment. ANOVA analysis reveals a significant effect of Pb2+ concentration ($p = 0.002$). Error bars represent 95% confidence intervals. *Significantly different from control ($p < 0.05$) **$p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from each experiment.

3.5. Lead treatment alters the differentiation potential of P19 NPCs

We observed a decrease in the population of differentiated neurons in the lead-treated cultures. To determine whether this decrease in the lead-induced neuronal population is because of selective cell death of the neurons or to altered differentiation potential of P19 NPCs, we investigated the effect of lead exposure on the differentiation potential of P19 cells. We examined the expression of ß-III-tubulin, GFAP, and Sox2 during the different stages of neuronal development (Fig. 9). P19 cells that were not subjected to determination or differentiation processes did not show expression of ß-III-tubulin or GFAP (Fig. 9A). Lead exposure significantly decreased ($p < 0.01$) the total number of surviving cells during the period of determination to 47 ± 8.7% and to 75 ± 11.45% when lead was present during the period of differentiation (Fig. 10A). However, when lead was present throughout the duration of the experiment, the total number of surviving cells decreased to 16 ± 5.45%. We then calculated the number of different cell types in the remaining live cells. Lead treatment significantly decreased the number of neurons (ß-III-tubulin expressing cells, identifiable morphologically with a long neurite and cell body). These neurons exited the cell cycle and they stopped expressing Sox2 in all the culture conditions (Fig. 9, F, I, L & O). Quantitative analysis of cells expressing

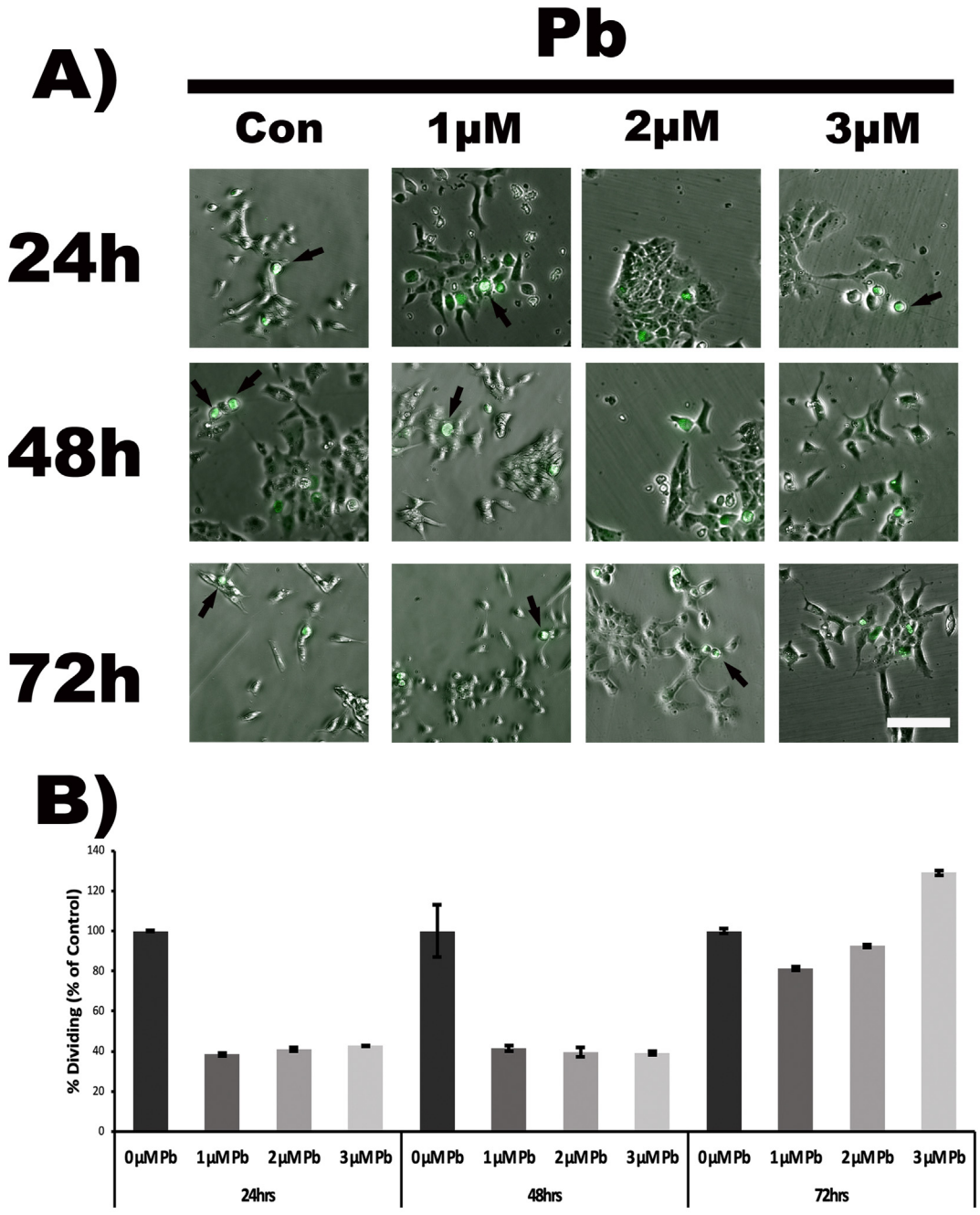

Fig. 3. Lead treatment has no significant effect on P19 cell proliferation.
P19 cells were treated with 0 μM, 1 μM, 2 μM, or 3 μM Pb2+ for 24, 48, or 72 h. After incubation, cells were fixed and immunostained with Histone-3 Phosphate and imaged using both fluorescence and phase-contrast microscopy. After the experiment, images were overlaid in Photoshop CS4 and counted. A, P19 cells incubated with 0 μM, 1 μM, 2 μM, or 3 μM Pb2+ for 24, 48, or 72 h. Histone-3 Phosphate staining shows cell division taking place (arrows showing examples). B, Quantitative analysis showing the number of cells dividing for each condition. ANOVA analysis reveals that lead concentration did not have a significant effect on P19 cell proliferation ($p = 0.197$). Error bars represent 95% confidence intervals. *Significantly different from control ($p < 0.05$) **$p < 0.01$.

ß-III-tubulin clearly showed the significant decrease in the number of neurons in the lead-treated culture (Fig. 10B). We next analyzed the number of cells expressing the stem cell marker Sox2-expressing cells (cells that have not exited the cell cycle). The number of Sox2-expressing cells was significantly altered by lead treatment (Fig. 9B, E, H, K & N). Quantitative analysis revealed that the number of Sox2-positive cells increased significantly in the cultures that were exposed to lead at the time of determination. The cultures exposed to lead at the time of determination also showed a significant decrease in GFAP-positive cells (Fig. 10B) (Fig. 9B). Thus, lead exposure at the time of the determination process inhibits the differentiation of P19 cell to glial cells. Interestingly, exposure to lead during the period of differentiation resulted in the expression of Sox2 in the GFAP positive cells (Fig. 9J, K, L). Quantitative analysis revealed a significantly higher percentage of cells showing expression of Sox2 in the GFAP positive cells (Fig. 10B). Thus, lead exposure at the time of differentiation induces the expression of Sox2 in the glial cells. Although we observed a decrease in the percentage of neurons and increase in the Sox2-expressing glial cells in the P19 cells those were exposed to lead during the process of both determination and differentiation, (Fig. 9M, N, O and Fig. 10B) this data may be misleading because lead exposure in these cells significantly decreased the number of surviving neurons to only 16% of the control.

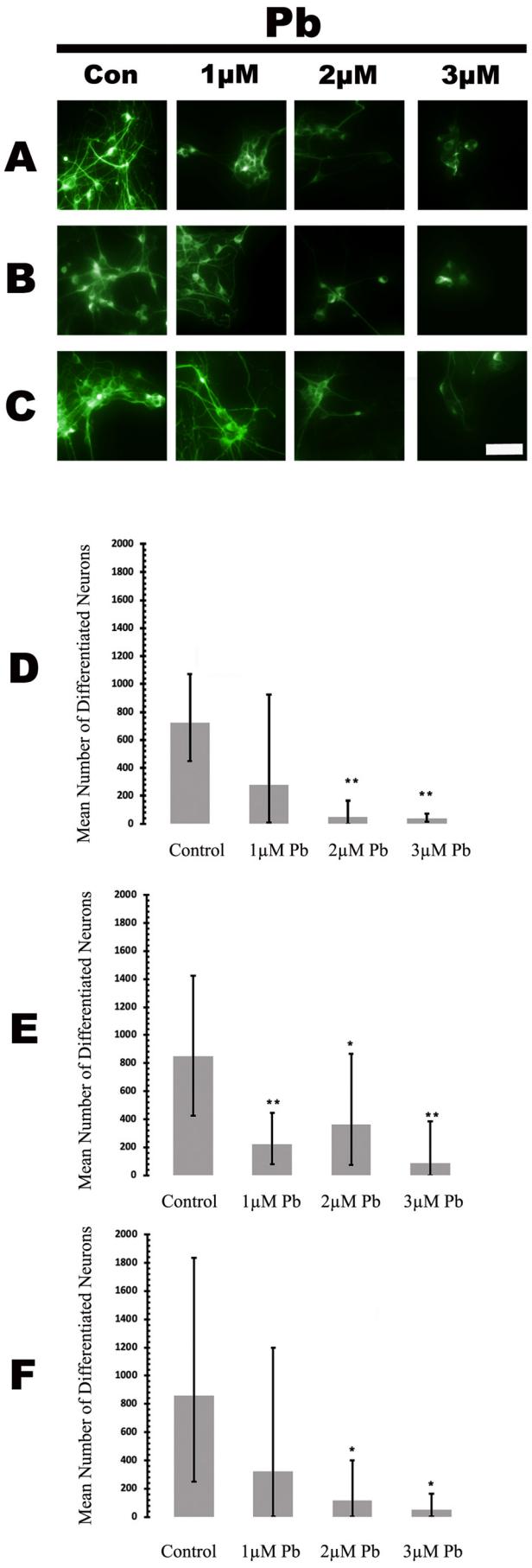

Fig. 4. Lead treatment significantly decreases the number of differentiated P19 cells.

Three experiments were performed to determine the effect of lead during determination and differentiation. Condition A: stem cells were exposed to lead during determination and underwent differentiation in lead-free media. Condition B: stem cells were determined in lead-free media and exposed to lead during differentiation. Condition C: stem cells were exposed to lead during the periods of both determination and differentiation. The images labelled A–C represent differentiated P19 cells after treatment with 0 μM, 1 μM, 2 μM, or 3 μM Pb2+. Graphs A–C represent the quantitative analysis for each of the conditions described above. For each condition, the number of differentiated neurons significantly decreased when compared to the control. A, $p = 0.000003$. B, $p = 0.000584$, C, $p = 0.0000031$. p values represent One-way ANOVA and Tukey's post hoc tests. Error bars represent 95% confidence intervals. *Significantly different from control ($p < 0.05$) **$p < 0.01$.

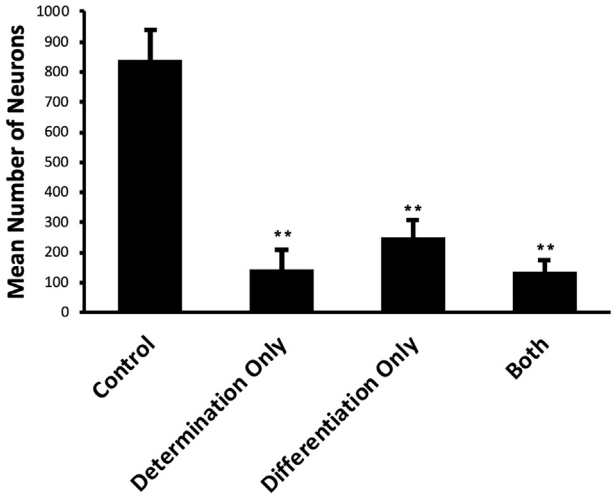

Fig. 5. Neuronal differentiation is adversely affected regardless of the timing of lead exposure.

We combined the data from all three lead concentrations (1, 2, and 3 μM Pb) and calculated the mean such that the timing of lead exposure (e.g., during determination only, during differentiation only, or during both) was the variable being manipulated. All of these conditions are significantly different from the control. *Significantly different (One-way ANOVA and Tukey's post hoc analysis, $p < 0.05$) from control neurons, **significantly different (One-way ANOVA and Tukey's post hoc analysis, $p < 0.01$) from control group of neurons. Error bars represent 95% confidence intervals.

The data in Fig. 10 represent means ± SEM of three independent experiments (n = 3).

4. Discussion

We examined the effect of lead toxicity on P19 stem cell determination into NPCs using Retinoic acid. We found that lead has an adverse effect on both the number of neurospheres formed and their mean diameter. Previous literature has identified the formation and closure of the neural tube during development (the primary hallmark of in vivo neuronal determination) as crucial in avoiding various congenital birth defects such as hydrocephaly (Gilbert et al., 1986). Although there are many reasons why the neural tube may fail to close, various studies have shown that altered cell-adhesion molecules, such as N-cadherin, can cause this failure (Moase and Trasler, 1991; Deak et al., 2005; Hu et al., 2011). N-cadherin regulates the formation of neurospheres and subsequent differentiation. Previous studies have demonstrated that the restriction of N-cadherin functioning inhibits the formation of neurospheres and the differentiation of the precursor cells from the subventricular zone (Yagita et al., 2009). In P19 cells, upregulation of N-cadherin enhances neurosphere formation and promotes neuronal

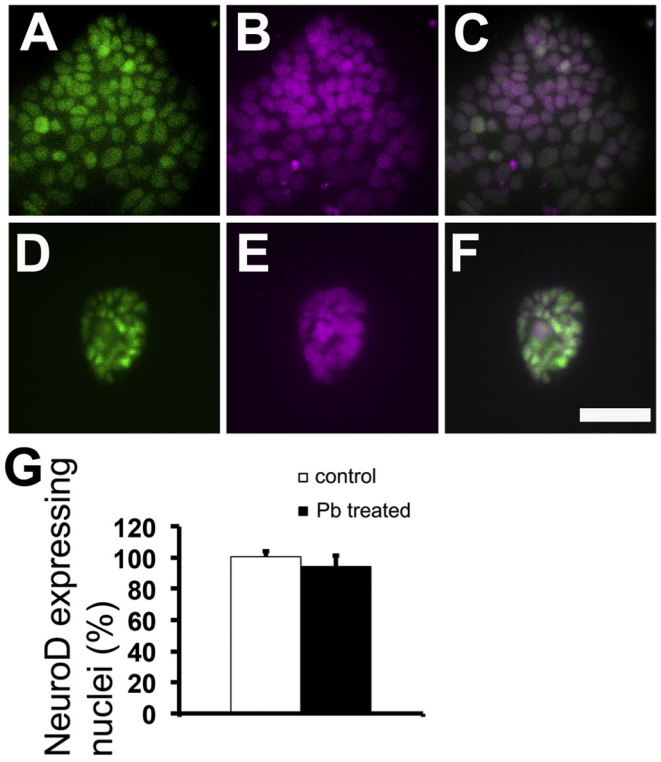

Fig. 6. Lead treatment does not alter NeuroD expression in P19 NPCs. P19 cells were cultured in the presence of Retinoic acid for 4 days in vitro (DIV) to induce P19 NPCs in the absence or presence of lead. P19 NPCs were immunostained for the expression of NeuroD. Immunofluorescence of NeuroD (A, D), nuclear staining (B, E), and NeuroD expression and nuclear stained merged images depicting the expression of NeuroD in the P19 NPCs clusters (C, F). Quantitative analysis of fluorescence intensity did not indicate any difference in the number of NeuroD positive cells in control and lead-treated P19 NPCs (G). Values are the means ± SEM of three independent experiments (n = 3) with 400 nuclear counts from each experiment. Scale bar, 50 μm.

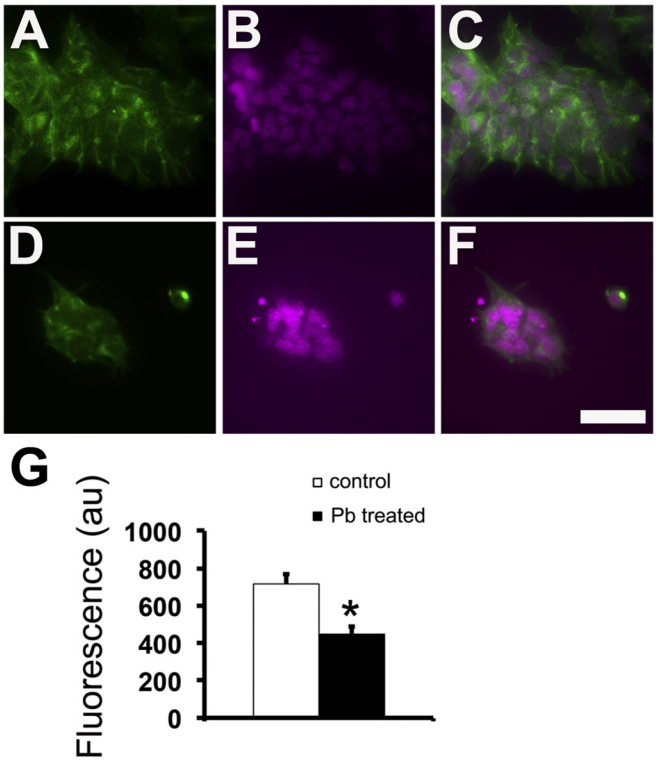

Fig. 7. Lead treatment reduces Nestin protein expression in P19 NPCs. P19 cells were cultured in the presence of Retinoic acid for 4 DIV to induce P19 NPCs in the absence or presence of lead. P19 NPCs were immunostained for the expression of Nestin. Immunofluorescence of Nestin (A, D), nuclear staining (B, E), and Nestin expression and nuclear stained merged images depicting the expression of Nestin in the P19 NPCs clusters (C, F). Quantitative analysis of fluorescence intensity did reveal a significant decrease in the Nestin protein expression levels in the lead-treated P19 NPCs (G). Values are the means ± SEM of three independent experiments (n = 3) with twenty-five P19 NPCs clusters from each experiment. Scale bar, 50 μm. * significantly different from control value ($p < 0.01$).

differentiation (Noh et al., 2012). Our data demonstrate that lead treatment inhibits the expression of N-cadherin in P19 NPCs. Thus, the loss of N-cadherin in the lead-treated P19 cells may be the causal factor for the smaller neurosphere formation in the lead-treated cultures. Because N-cadherin also regulates neuronal differentiation, this may be a possible reason for the lower number of neurons in the group of P19 cells that were treated with lead at the time of neuronal determination.

Growth of neurospheres requires cell division and maintenance of the number of neuronal progenitors in the growing neurospheres. Our data contradict previous work that found that lead exposure alters the proliferation in neuronal progenitors (Engstrom et al., 2015; Schneider et al., 2005). However, these studies focused on adult neural progenitor cells (aNPCs) in the hippocampal region of the brain. These studies corroborate the idea that lead exposure alone may be sufficient enough to severely impair many aspects of adult neurogenesis and memory. Our data on cell death agree with the previous study by Engstrom et al. (2015) which found that lead exposure caused an increase in cell death of aNPCs. We observed a moderate but a significant loss in cell survival when the P19 cells were exposed to lead for 24 h or 48 h. However, when we exposed P19 cells to lead for 72 h, we did not observe a significant loss in the cell survival. We speculate that proliferation of the surviving P19 cells may have compensated the loss of P19 cells at 72 h. Interestingly when we treated the P19 cells for 4 days during the process of determination in the presence of retinoic acid and subsequently plated these cells for differentiation process in an adhesion culture, we observed 76 ± 11.5% surviving cells in the presence of lead. However when the P19 NPCs were exposed to lead during the period of differentiation, we observed only 47 ± 8.6% surviving cells. This difference in the survival percentage in the two conditions can be attributed to the differentiation stage of the cells. During the process of determination, the cells are still dividing and, moreover, the lead-treated cell culture had a higher percentage of Sox2 expressing cells compared to the control culture. Interestingly, 100% of the cells in P19 cultures exhibited the expression of Sox2. In the culture where we treated the cells during the process of differentiation, we observed greater cell loss because these cells were differentiating and lost Sox2 expression and thus came out of the cell cycle (Sox2 + ve cells in control cultures (1.8 ± 0.90) vs Sox2 + ve cells in lead treatment culture during the period of differentiation -sox2 + ve cells (11 ± 1.7%) vs Sox2 + ve cells in the cultures of lead treatment during the period of determination -sox2 + ve cells (63 ± 5.6%)).

NeuroD expression is observed during neurogenesis and is believed to regulate neuronal survival and maturation (Lee et al., 1995; Lee, 1997; Miyata et al., 1999; Kim et al., 2001; Kim, 2012). Previous studies have reported the expression of NeuroD in Retinoic acid induced neuronal induction of P19 NPCs (McCormick et al., 1996; Bressler et al., 2004). We found that while RA-induced neural induction is associated with NeuroD expression, lead treatment has no effect on its expression in RA-induced aggregates of P19 NPCs. Thus, the lead-induced decrease in the neuronal population may not be due to the alteration in NeuroD expression in P19 cells. We observed a smaller mean diameter and fewer NPC-containing neurospheres; therefore, we tested the expression of Nestin in the lead-treated neurons and discovered that the lead

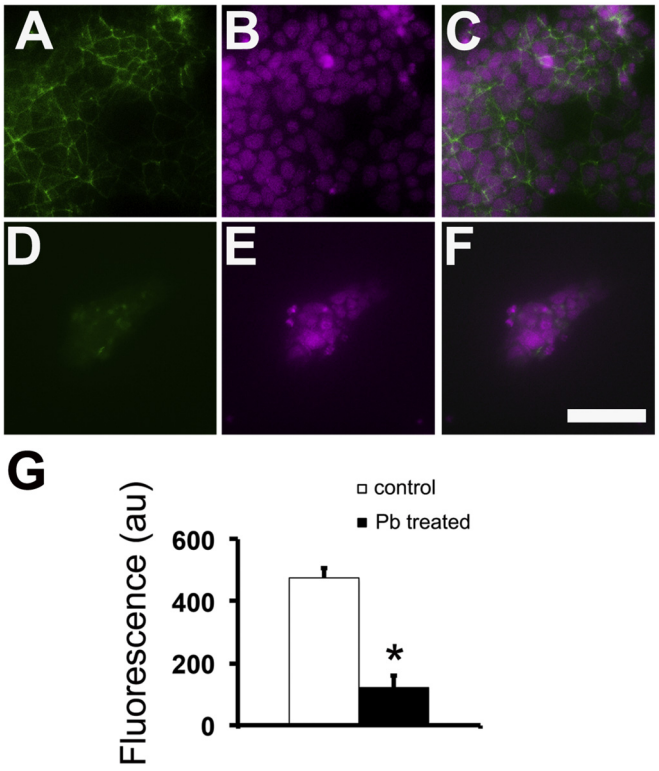

Fig. 8. Lead treatment reduces N-cadherin protein expression in P19 NPCs.
P19 cells were cultured in the presence of Retinoic acid for 4 DIV to induce P19 NPCs in the absence or presence of lead. P19 NPCs were immunostained for the expression of N-cadherin. Immunofluorescence of N-cadherin (A, D) nuclear staining (B, E) and N-cadherin expression and nuclear stained merged images depicting the expression of N-cadherin in the P19 NPCs clusters (C, F). Quantitative analysis of fluorescence intensity did reveal a significant decrease in the N-cadherin protein expression in the lead-treated P19 NPCs (G). Values are the means ± SEM of three independent experiments (n = 3) with twenty-five P19 NPCs clusters from each experiment. Scale bar, 50 μm. * significantly different from control value ($p < 0.001$).

treatment significantly decreased Nestin protein expression in the lead-treated NPCs. Nestin-expressing cells possess the characteristic features of stem cells such as multipotency and self-renewal. Nestin expression is downregulated in differentiated cells (Lendahl et al., 1990; Kachinsky et al., 1995).

We observed that Sox2 is expressed in P19 pluripotent stem cells; however, we did not detect Sox2 expression in the differentiated neurons or glial cells of control culture. This is in accordance with the fact that Sox2 is expressed throughout the neural stem cells comprising the neural tube, and its expression is down regulated when the neuronal precursors exit the cell cycle and differentiate into neurons or glial cells. Uwanoghoa et al. (1995) found that Sox2 expression in the adult nervous system is present in the areas of neuronal stem cell niches in the sub-ventricular zone (SVZ) and sub-granular zone (SGZ) of the dentate gyrus (Avilion, 2003). Interestingly, the group of cells that were treated with lead at the time of neuronal determination exhibited an increase in the number of Sox2-positive cells in the differentiation cultures. Sox2 is expressed in the stem cells and its expression is down regulated when the neuronal precursor exit the cell cycle and differentiate in to neurons (Uwanoghoa et al., 1995). Tight cell to cell interactions are required for the differentiation of pluripotent stem cells (Pieters and van Roy, 2014). It is plausible that a decrease in N-Cadherin expression in the lead-treated culture prevented these types of cell-cell interactions from occurring effectively, and this may be the reason for the expression of Sox2 in a greater number of cells in culture treated with lead during the process of determination. Thus, because of continued Sox2 expression, most of the cells do not come out of the cell cycle to terminally differentiate into neurons.

In neural progenitors, expression of Sox2 inhibits neurogenesis and Sox2-expressing cells are considered glial progenitors that lead to the formation of oligodendrocytes (Brazel et al., 2005).

Intriguingly, Sox2 expression is also present in nervous system tumors in astroglial, oligodendroglial, and ependymal cells (Garros-Regulez et al., 2016; Phi et al., 2008). We observed an increase in the population of Sox2-expressing cells in the group where P19 cells were exposed to lead at the time of determination. Our studies hint that lead exposure may alter the differentiation potential for the neuronal precursors by altering the expression pattern of the Sox2 transcription factor. It is also possible that the presence of lead on its own may induce the expression of Sox2 in undifferentiated cells, and this may be the reason for the expression of Sox2 in GFAP-expressing astrocytes in the culture where lead was introduced during the period of differentiation. Sox2 expression is also linked to oligodendrocyte progenitor cells, and Sox2 expression induces the differentiation of oligodendrocytes (Zhao et al., 2015). Although more long-term studies are required, our data also suggest that lead exposure may alter the neuronal population in the developing embryo by promoting the differentiation of neuronal precursors into oligodendrocytes.

In the experimental condition where lead was introduced to the cultures at the time of neuronal differentiation, we found a significant increase in the cells expressing both Sox2 and GFAP + together. Astrocytes expressing both Sox2 and GFAP together are observed in the adult stem cell niches (Avilion, 2003). Interestingly, Sox2 expression is upregulated in gliomas and the inhibition of Sox2 can prevent the proliferation and tumorigenicity of glioblastoma tumor-initiating cells (Schmitz et al., 2007; Gangemi et al., 2009). Although further studies are required, our data also suggest that lead exposure may be linked to gliomas.

Our experiments demonstrate that lead detrimentally alters the differentiation potential of P19 cells into neurons and glial cells. We found that the negative effect of lead exposure on P19 cell neuronal differentiation did not significantly differ when we exposed the cells to lead during determination only or differentiation only. However, the effects of lead on glial cell differentiation are stage specific. Overall, when examining our data, we conclude that lead toxicity can cause nervous system defects through different mechanisms depending on the stage of development. Early on in the stages of determination and differentiation process, necrosis and alteration in the Sox2 and N-cadherin expression may be the primary mechanism driving the decrease in the size of neurospheres, number of differentiated neurons, and alterations in glial populations. Thus, lead exposure during the development process may alter the differentiation potential and survival of the neuronal stem cells and these may affect the overall development of the nervous system in the developing embryos.

Conflict of interest

Authors have no conflict of interests.

Transparency document

The Transparency document associated with this article can be found, in online version.

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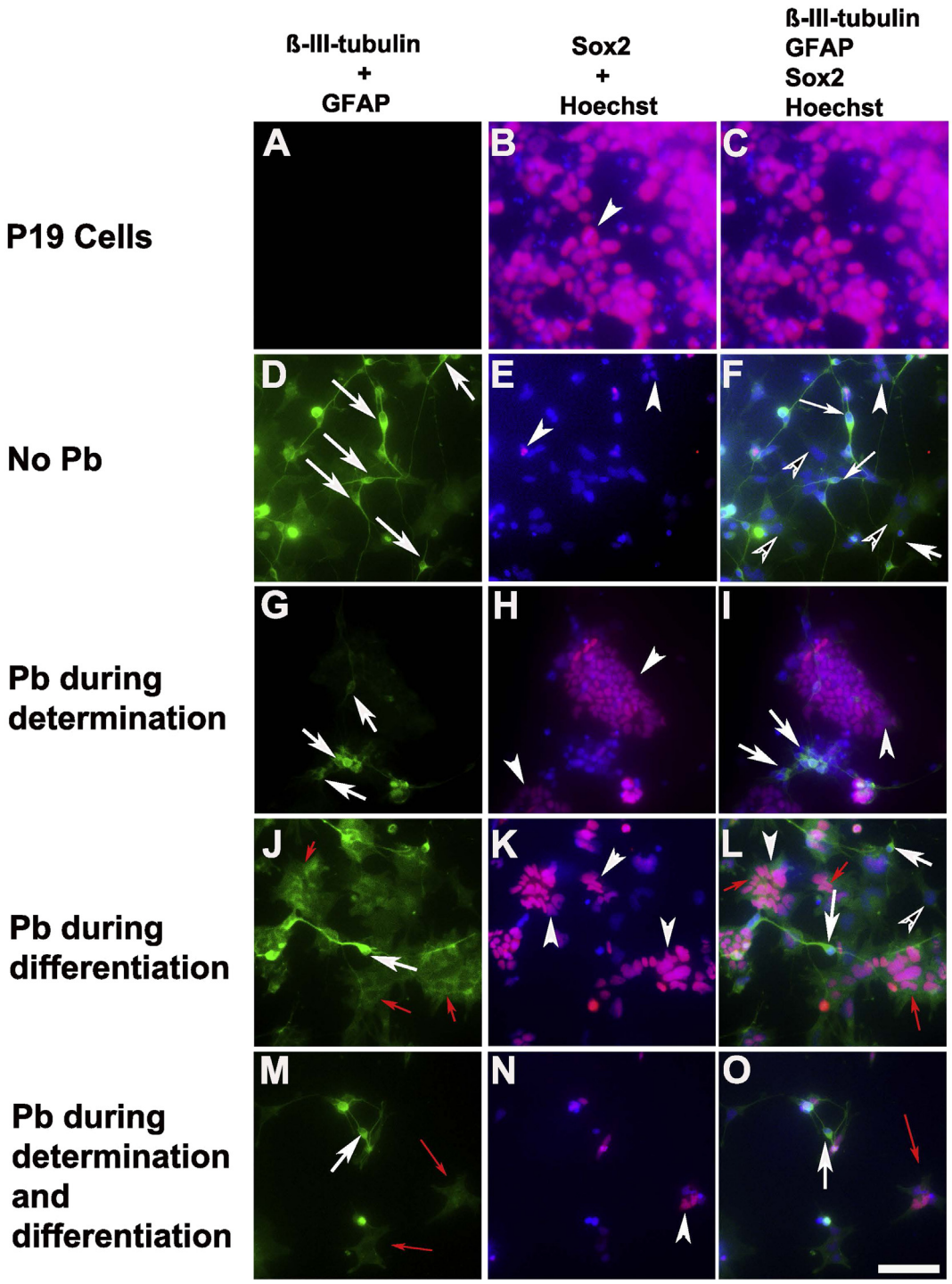

Fig. 9. Lead treatment alters the differentiation potential of P19 NPCs.

P19 cells which were not subjected to determination or differentiation processes did not show the expression of ß-III-tubulin or GFAP (A). Nuclear Hoechst staining (C) and superimposed images of nuclear staining and Sox2 expressing cells (purple nuclei) showing the expression of Sox2 in these cells (arrowheads) (B).

P19 cells were cultured in the presence of Retinoic acid for 4 DIV to induce P19 NPCs. Adhesion cultures were started at 4 DIV to induce neuronal differentiation (D, E, F). P19 cells were cultured in the presence of Retinoic acid and lead for 4 DIV to induce P19 NPCs. Adhesion cultures were started at 4 DIV to induce the neuronal differentiation in the absence of lead (G, H, I). P19 cells were cultured in the presence of Retinoic acid for 4 DIV to induce P19 NPCs. Adhesion cultures were started at 4 DIV to induce the neuronal differentiation in the presence of lead (J, K, L). P19 cells were cultured in the presence of retinoic acid and lead for 4 DIV to induce P19 NPCs. Adhesion cultures were started at 4 DIV to induce neuronal differentiation in the presence of lead (M, N, O).

Differentiated cells were immunostained for the expression of ß-III-tubulin and astroglial marker (GFAP) (A, D, G, J, M). White arrows represents differentiated neuron like cells with longer neurites than diameter of cell body (D, G, J, M) and red arrows show GFAP positive cells (J, M). Immunofluorescence of Sox2 and Hoechst-stained superimposed images (B, E, H, K, N) showing Sox2 positive (purple) and Sox2 negative (blue) nuclei. Superimposed images of ß-III-tubulin, GFAP, Sox2, and Hoechst nuclear staining (F, I, L,O) showing Sox2 negative mature neurons (white arrows) in various conditions (F, I, L, O) and clumps of Sox2 positive cells (red arrowheads) in the group of P19 cells treated with lead during the determination process (I). Lead treatment during differentiation (J, K, L) showing Sox2 positive GFAP cells (red arrows in L) and Sox2 negative GFAP cells (black and white arrowheads). Scale bar, 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

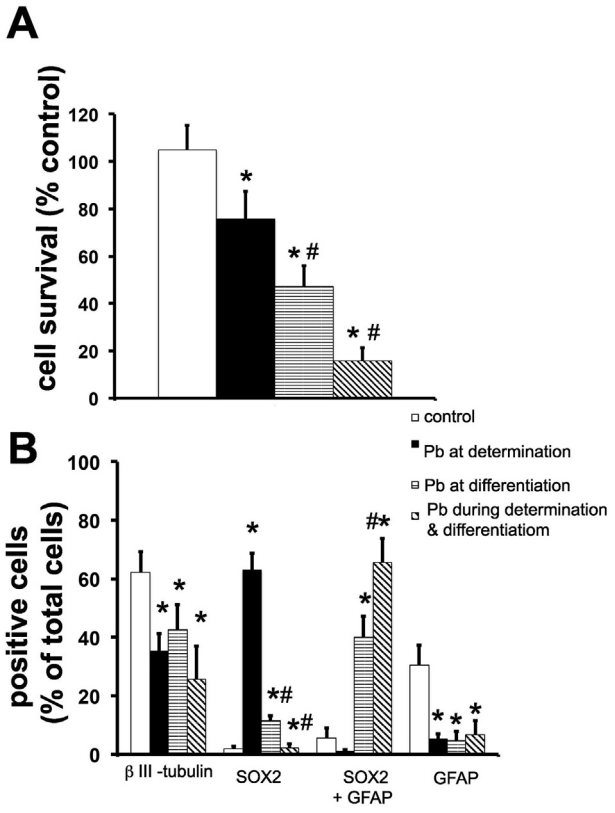

Fig. 10. Lead treatment decreases cell survival and alters the differentiation potential of P19 NPCs.

(A) Differentiated cells were immunostained for various markers and total numbers of surviving cells were counted in each experimental condition. * indicates statistically different ($p < 0.001$) from control, # indicates statistically different ($p < 0.05$) from culture treated with lead during the period of determination. Data are normalized to control culture, error bars = ± SEM.

(B) The number of cells positive for each marker were counted using Image J and normalized by the number of Hoechst positive cells. * indicates statistically different ($p < 0.001$) from control, # indicates statistically different ($p < 0.05$) from culture treated with lead during the period of determination. Error bars = ± SEM.

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