

Original Paper

# As-Cd-Pb Mixture Induces Cellular Transformation via Post-Transcriptional Regulation of Rad51c by miR-222

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## Key Words

microRNA 222 • Metals mixture • DNA damage • DNA repair • Carcinogenesis

## Abstract

**Background/Aims:** Exposure to heavy metals is today a threat to society. The understanding of the molecular processes related to diseases related to exposure to metals mixture involve changes in the expression of microRNAs. Changes on microRNAs expression may alter several cellular processes, among them, DNA repair inhibition has been described as an essential event leading to the initiation of metal-induced carcinogenesis. **Methods:** We evaluate the miR-222 expression in the two-stage transformation Balb/c 3T3 cell assay treated with As-Cd-Pb mixture. **Results:** We could appreciate that up-regulation of miR-222 reduces the expression both gene and as a protein expression of Rad51c by RT-PCR and immunoblot, respectively. **Conclusion:** Here, we demonstrate that the mixture of As-Cd-Pb at epidemiologically relevant concentrations induces miR-222 up-regulation, which directly negatively regulates Rad51c expression and impairs homologous recombination of DNA during the initiation stage of cell transformation. This inhibition triggers morphological transformation in a murine two-stage Balb/c 3T3 cell assay, suggesting that this small RNA acts as an initiator of the carcinogenesis process.

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## Introduction

Heavy metals and metalloids mixtures such as arsenic (As), cadmium (Cd), and lead (Pb), are considered a major category of globally-distributed pollutants according to the Agency for Toxic Substances and Disease Registry (ATSDR). Exposure to complex mixtures of metals in the workplace or environment is more likely to occur than exposure to a single metal alone. Additionally, the US Agency for Toxic Substances and Disease Registry (ATSDR) has associated metal mixtures of As, Cd and Pb with haematological, hepatic, renal, neurological,

reproductive, immunological diseases and Cancer [1]. Nevertheless, several mechanisms may exist by which heavy metals exert their effects, such as DNA repair inhibition, in which base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and homologous recombination (HR) are attenuated or inhibited [2]. DNA repair inhibition has been described as an essential event leading to the accumulation of DNA damage and subsequent genomic instability, which are considered hallmarks of cancer [3, 4].

Recently, knowledge about the molecular background of heavy metal-associated illness has been enriched by the inclusion of experimental data suggesting altered microRNA (miRNA) expression under metal mixture exposure [5].

MicroRNAs constitute an RNA-polymerase II (RNA Pol II)-transcribed family of evolutionarily conserved, non-coding RNAs that recognize the 3' untranslated regions (UTRs) of specific messenger RNA (mRNA) populations [6]. Main function of miRNAs is to down-regulate the expression of protein-coding genes [7] and their interaction is mediated by partial sequence homology. In spite of their relatively small number, computational and experimental studies have indicated that miRNAs can control the expression of most, if not all, human protein-coding genes [8-10]. Hence, miRNAs function is indissolubly linked to their targets and, because each miRNA can bind and modulate hundreds of targets, any cell function is in fact regulated by miRNAs. miRNAs are key regulators of numerous biological functions. Deviation from their normal expression has been involved in human diseases [11-16].

In a previous work, we reported that upon As-Cd-Pb mixture exposure at epidemiologically relevant concentrations, these small RNAs may regulate gene expression, leading to alterations in several cellular processes, including DNA repair, which have previously been related to the metal-induced carcinogenesis process [17-19].

In this context, the negative regulation of DNA repair gene expression through miRNA is poorly understood; however, there is evidence that these RNAs modulate the process [20-32]. Homologous Recombination (HR), which recognized DNA lesion double strand breaks (DSBs), is of great interest due to its ability to maintain genomic instability and its important role in the development of diverse types of cancer [33]. Particular attention is paid to the Rad51 recombinase protein family implicated in this DNA repair mechanism and more specifically to the Rad51c member in light of the experimental data demonstrating its mutation or inactivation as a susceptibility factor of various types of cancer, including head and neck, breast and ovarian, and colorectal [33-36].

miR-222 is known to be up-regulated under As-Cd-Pb mixture exposure [3] and in established cancers [37-40] which have been related to these metals [1]. As commented before these metals are considered a major category of globally-distributed pollutants, and several human populations are exposed to chemical mixtures including this metal mixture [41, 42]. In light of this fact and given the previous studies of miR-222, the aim of the present study is to determine the role of miR-222 as an initiator stimulus of cellular transformation through the modulation of Rad51c protein expression under the As-Cd-Pb mixture treatment in the murine two-stage Balb/c 3T3 model.

## Materials and Methods

### *In silico identification of miR-222 target and Rad51c interaction*

Prediction of complementary binding sites between the mature mmu-miR-222 (seed sequence: 5'-aGCUACAU-3') and the 3'UTR of Rad51c mRNA (RefSeq ID: NM\_053269) sequences was made using the miRWalk [43], microRNA [44] and miRbase [45] algorithms.

### *Chemical compounds*

Sodium meta-arsenite ( $\text{NaAsO}_2$ , purity 100%) and cadmium chloride ( $\text{CdCl}_2$ , purity 99.5%) were purchased from the Aldrich Chemical Company (WI, USA). Lead acetate ( $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$ , purity 99.9%) was obtained from J. T. Baker (México).

## Cell lines

The Balb/c 3T3 clone A31-1-1 is a *Mus musculus* (mouse) non-tumorigenic and non-immunosuppressed embryo fibroblast widely used for carcinogenicity test purposes. The strain was acquired in 2009 from the ATCC (American Type Culture Collection; VA, USA) and it has been cryopreserved in freeze medium (complete growth medium supplemented with 5% (v/v) DMSO (dimethyl sulfoxide)) on liquid nitrogen. All of the experiments were carried out using an early passage number of the subculture (passage 4) and employing a positive (known initiator and promoter) and negative (basal conditions culture) control for cellular transformation assays.

## Two-stage Balb/c 3T3 cell assay

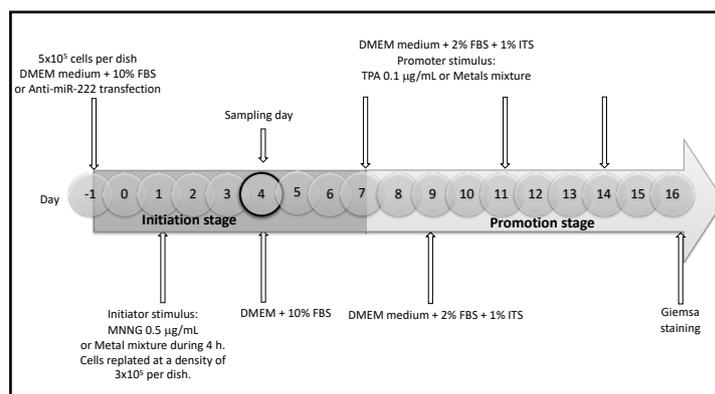
The two-stage Balb/c 3T3 cell assay was performed as described previously with slight modifications [5, 18]. The transformation protocol consisted of 16 days, divided in two phases: initiation between days 1 to 7 and promotion between days 7 to 16. Cells were plated at a density of  $5 \times 10^5$  cells per 100-mm dish in DMEM (Dulbecco's modified eagle medium) supplemented with 10% FBS (fetal bovine serum). On day 1 of the assay, subconfluent cells were exposed to the initiator stimuli during 4 h; MNNG (N-methyl-N-nitrosoguanidine) (0.5  $\mu\text{g}/\text{mL}$ ) as a positive control; metal mixture (2  $\mu\text{M}$   $\text{NaAsO}_2$ , 2  $\mu\text{M}$   $\text{CdCl}_2$ , and 5  $\mu\text{M}$   $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$ ), and untreated cells were used as a negative control (Fig. 1). After such initiator treatment, cells were harvested and replated at a density of  $3 \times 10^5$ /dish. On day 4, cultures were replenished with fresh DMEM medium supplemented with 10% FBS. On day 7 the cells were filled with medium supplemented with 1% ITS-A (insulin transferrin selenium-A) and 2% FBS, and the metal mixture ( $\text{NaAsO}_2$  2  $\mu\text{M}$ ,  $\text{CdCl}_2$  2  $\mu\text{M}$  and  $\text{Pb}$  5  $\mu\text{M}$  ( $\text{C}_2\text{H}_3\text{O}_2$ )  $2 \cdot 3\text{H}_2\text{O}$ ) or TPA (12-O-tetradecanoylphorbol-13-acetate) (0.1  $\mu\text{g}/\text{ml}$ ) (positive control) was added as promoter stimulus, on day 9 it was replenished with medium without promoter stimuli, at day 11 and 14 cells were replenished with metal mixture or TPA (positive control) as second and third promoter stimulus (Fig. 1) On day 16, cells were fixed with ethanol, stained with 10% aqueous Giemsa and scored for foci formation (Fig. 1). Transformed foci were scored according to criteria that discriminate transformed foci on the basis of 3 morphological characteristics: (1) basophilic staining; (2) a dense layer formation; and (3) random orientation of cells at the edges of foci [46]. Foci less than 2-mm diameter were not scored. Relative Colony Formation Efficiency (RCFE) was calculated as the number of foci per dish in experimental conditions/number of foci per dish in control conditions. The experimental conditions were as follows: control (untreated cells); Metal/Metal to test initiator and promoter capacities of the metal mixture (Fig. 1).

## Cell viability assay

Cell viability was measured by the dual stain FDA/EtBr method [47]. FDA is taken up by cells, which through esterase activity transform the non-fluorescent FDA into the green fluorescent metabolite. Meanwhile, nuclei of death cells are ethidium bromide stained and visualized as red fluorescence. Cells were then analyzed under a fluorescence microscope (Olympus BMX-60 with a UM61002 filter). One hundred randomly chosen cells per condition were evaluated and the results are expressed as percentages.

## RNA isolation

On day 4 of the Balb/c 3T3 transformation assay, during the initiation stage, control and



**Fig. 1.** Scheme of the conventional two-stage Balb/c 3T3 cellular transformation assay with the metal mixture. Metal mixture: 2  $\mu\text{M}$   $\text{NaAsO}_2$ , 2  $\mu\text{M}$   $\text{CdCl}_2$ , and 5  $\mu\text{M}$   $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$ ; MNNG: positive initiator; TPA: positive promoter. Modified from Tsuchiya et al., 2005 and Silva-Aguilar et al., 2011.

treated cells were harvested with 0.2% PBS-EDTA (phosphate buffered saline-ethylenediaminetetraacetic acid) and centrifuged to remove the medium. Total RNA was immediately extracted from  $2 \times 10^5$  cells using the ZR RNA MicroPrep (Zymo Research; CA, USA) isolation kit according to the manufacturer's protocol for miRNA analysis and with the Maxwell 16 LEV Simply RNA cells kit (Promega; WI, USA) on a Maxwell 16 Instrument (Promega) for mRNA evaluation. Total RNA was analyzed on a Nanodrop 1000 (Thermo Fisher Scientific; DE, USA) spectrophotometer for mRNA integrity evaluation and sample quantification; samples were then aliquoted and stored at  $-80^\circ\text{C}$ .

#### *miR-222 RT-qPCR*

*Mus musculus* miR-222 expression was determined by RT-qPCR (reverse transcription-quantitative polymerase chain reaction), and all of the reagents were purchased from the same supplier (Applied Biosystems; CA, USA). For miRNA cDNA (complementary DNA) synthesis, RNA was reverse transcribed with the TaqMan MicroRNA Reverse Transcription kit and TaqMan MicroRNA Assay hsa-miR-222 primer, and real time PCR was performed using TaqMan Universal Master Mix II (no UNG (uracil-N-glycosylase)) along with labeled TaqMan MicroRNA Assay hsa-miR-222 primers. The mammalian U6 non-coding small nuclear RNA (U6 snRNA) was used for data normalization, and the  $2^{-\Delta\Delta\text{CT}}$  comparative method (described on the Applied Biosystems ABI Prism User Bulletin No. 2, and explained by Livak and Schmittgen [48]) was applied to calculate relative changes in gene expression determined from quantitative experiments.

#### *Rad51c mRNA RT-PCR*

Rad51c gene expression was assessed by endpoint RT-PCR using the Access RT-PCR System kit (Promega) and mouse Rad51c recombinase primers (IDT; IA, USA) in cells treated with the metal mixture; we employed the mouse Hprt1 gene as an endogenous control across all experiments. The RT-PCR products were resolved on a 2.5% agarose gel containing EtBr (0.5 mg/mL), visualized under UV (ultraviolet) light on the MiniBIS Pro Imaging System (DNR; JRS, Israel) and quantified by means of band intensity with the Kodak 1D Image Analysis v3.5 software (Kodak; NY, USA).

#### *Protein extraction and Rad51c immunoblot*

Whole cell protein was extracted with Radio Immunoblot Precipitation Assay buffer (RIPA) and conventionally treated for specific immunodetection by western blot technique [49] of mouse Rad51c protein in cells treated with the metal mixture. We used a mouse-specific rabbit anti-Rad51c polyclonal antibody (Cat. ab95201, Abcam; Cams., UK) and detected  $\beta$ -tubulin protein as an endogenous control for the experiments with a mouse anti- $\beta$ -tubulin monoclonal antibody (Cat. 322600, Invitrogen, Camarillo, CA, USA). Horseradish Peroxidase (HRP)-coupled goat anti-rabbit IgG (immunoglobulin G) monoclonal (Cat. 816129, Invitrogen) and goat anti-mouse IgG monoclonal (Cat. 626520, Invitrogen) secondary antibodies were utilized for Rad51c and  $\beta$ -tubulin radiographical detection, respectively, with the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore; MA, USA). Protein quantitation was performed in terms of band intensity with the Kodak 1D Image Analysis v3.5 software.

#### *Neutral single cell gel electrophoresis assay*

To evaluate DNA DSBs induced by the up-regulation of miR-222 expression, the neutral comet assay was performed in Balb/c 3T3 cells as described previously [50]. Slides were prepared per duplicate; 50,000 cells were mixed with  $75\ \mu\text{L}$  of 0.7% LMP (low melting point) agarose solution and loaded onto microscope slides prelayered with  $150\ \mu\text{L}$  of 0.5% normal melting point agarose, after which a third layer of LMP agarose was added. After incubation with lysis buffer for 24 hours (cold EDTA sodium salt 30 mM and SDS 0.5% pH 7), the slides were subjected to a 2 h unwinding and 25V, 20mA (0.8 V/cm) electrophoresis for 25 min (boric acid 90 mM, EDTA 200 mM and Tris Base 117 mM pH 7.8), dehydrated with 96% ethanol, stained with EtBr and visualized under a fluorescence microscope (20x) to determinate the Tail length (microns) of 100 comets/slide with the Komet 5 software (Andor Technology Ltd. UK). As positive control to challenge the DSB DNA repair mechanism, a 3-Gy gamma radiation was applied [51].

#### *Statistical analysis*

All of the data were analyzed using a Welch-corrected unpaired two-tailed *t*-test to determine differences between experimental conditions using the Prism 6 (GraphPad; CA, USA) statistics package.

Results with a *p*-value < 0.05 were considered statistically significant. A One-way ANOVA was performed for determine transformation capacity, and Tukey post-hoc (95% CI (confidence interval)) test was applied for comparisons between groups. Results with a *p*-values <0.05 were considered as statistically significant.

## Results

### Prediction of miR-222 and Rad51c interaction

We identified the possible direct binding of the miR-222 seed sequence on five different sites throughout the 3'UTR of Rad51c mRNA using the miRWalk, microRNA and miRBase databases. The miRWalk algorithm allowed us to predict the regulation of this gene target with a *p*-value < 0.05 (*p*=0.0413), strengthening the possibility of its negative regulation by miR-222.

### miR-222 expression is up-regulated when cells are treated with the metal mixture

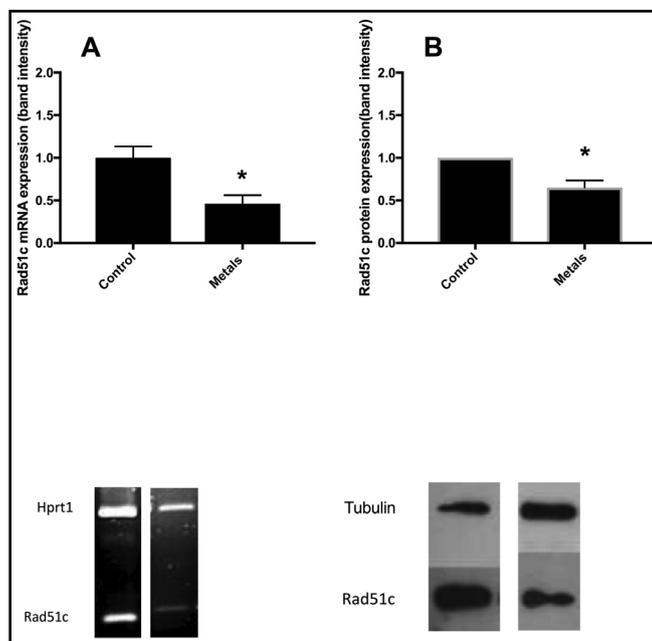
Viability determination revealed that metal mixture (2 μM NaAsO<sub>2</sub>, 2.4 μM CdCl<sub>2</sub>, and 4.8 μM Pb(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>·3H<sub>2</sub>O) exposure had no cytotoxic effect (92.66%±0.88) relative to the control condition (95%±0.57) (Table 1). In a previous work [5], we demonstrated miR-222 over-expression on day 4 of the Balb/c 3T3 transformation assay in cells treated with the As-Cd-Pb metal mixture. Here, we corroborate the result by RT-qPCR and observed a significant elevation in the levels of mmu-miR-222 (2<sup>-ΔΔCT</sup> = 29.04±0.55) relative to the control condition (2<sup>-ΔΔCT</sup> = 1.0±0.10) (Table 1).

### Rad51c mRNA and protein expression in cells treated with the metal mixture

Once we confirmed the up-regulation of miR-222 during metal-induced cellular initiation, we investigated the expression levels of its predicted target.

**Table 1.** Viability and miR222 expression. Percentage of cellular viability, measured using the FDA/EtBr method, of Balb/c 3T3 cells on day 4 (initiation phase) of the transformation assay. Cells were treated with metal mixture or transfected with anti miR-222 and treated with the metal mixture; n=3, two-tailed unpaired t-test. Relative expression of mmu-miR-222 in Balb/c 3T3 cells on day 4 (initiation phase) of the transformation assay. Cells initiated with the metal mixture. The results of RTqPCR are represented in terms of 2<sup>-ΔΔCT</sup>; endogenous control: snRNA U6, n=3, mean±s.e., two-tailed unpaired t-test, \*\*\**p*<0.001

	Viability	miR-222 expression
Control	95 ± 0.5	1.0 ± 0.10
Metals	93 ± 0.9	29.04 ± 0.55 ***
AntimiR-222	95 ± 0.5	0.006 ± 0.001



**Fig. 2.** Changes in Rad51c expression on day 4 (initiation phase) of the transformation assay. (A) Relative gene expression of Rad51c assessed by RT-PCR in Balb/c 3T3 cells on day 4 (initiation phase) of the transformation assay. Cells treated with the metal mixture,. Band intensity was normalized against the Control or siPORT (transfection reagent) condition; endogenous control: Hprt1; n=3, mean±s.e., two-tailed unpaired t-test, \**p*<0.05. (B) Relative protein expression of Rad51c measured by immunoblot in Balb/c 3T3 cells on day 4 (initiation phase) of the transformation assay. Cells treated with the metal mixture,. Band intensity was normalized against the Control or siPORT (transfection reagent) condition; endogenous control: β-tubulin; n=3, mean±s.e., two-tailed unpaired t-test, \**p*<0.05.

First, by end-point RT-PCR, we measured the expression of Rad51c and observed the down-regulation of its mRNA by approximately 50% ( $0.46 \pm 0.16$ ) compared to the control ( $1.0 \pm 0.09$ ) (Fig. 2A). Second, immunoblotting of the Rad51c protein was reduced 35% ( $0.65 \pm 0.08$ ) compared with the control condition ( $1.0 \pm 0.08$ ) (Fig. 2B).

#### DNA DSBs in cells over-expressing miR-222

Having demonstrated that metals induce miR-222 and the subexpression of Rad51c at both gene and protein level, we attempted to investigate the consequence of such regulation on HR-mediated DNA damage repair. Therefore, we evaluated the presence of DNA DSBs in cells treated with metals and irradiated with gamma radiation at a dose of 3 Gy to challenge the HR mechanism response. The distribution of damage (Fig. 3A) clearly shows DNA damage accumulation in cells treated with metals. Also these cells present a delay in DNA-DSB's repair with respect to wild type cells (Fig. 3B). A representative comet images are show in Fig. 3C.

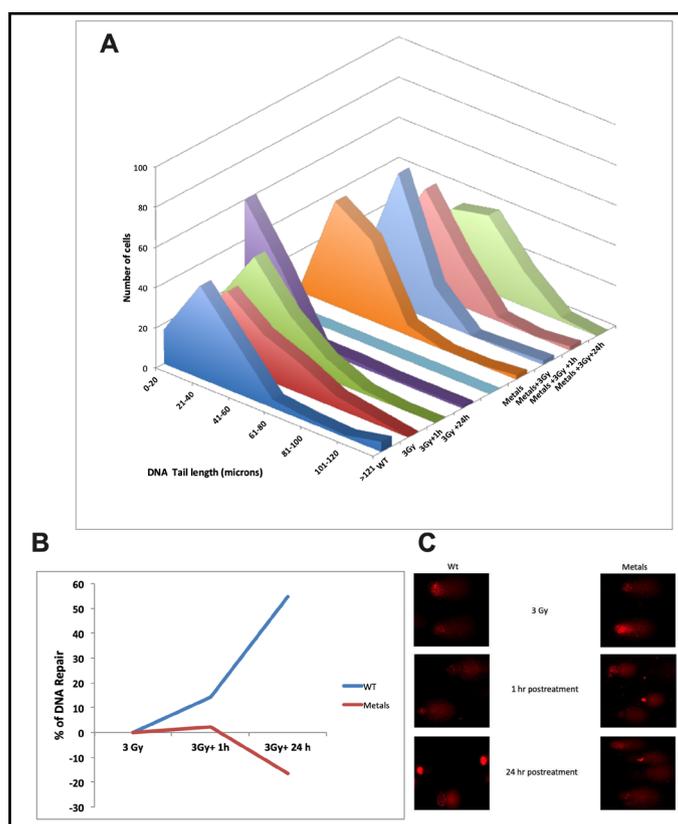
#### Anti miR-222 molecule transfection

Balb/c 3T3 cells were transfected with 5 pmol of the anti miR-222 molecule for 24 h using the siPORT NeoFX transfection solution (all reagents from Ambion; MA, USA) to induce the down-regulation of miR-222 expression, which was confirmed by RT-qPCR. Subsequently the cells were treated with metals as described in the previous section

#### Balb/c3T3 morphological transformation after metals treatment.

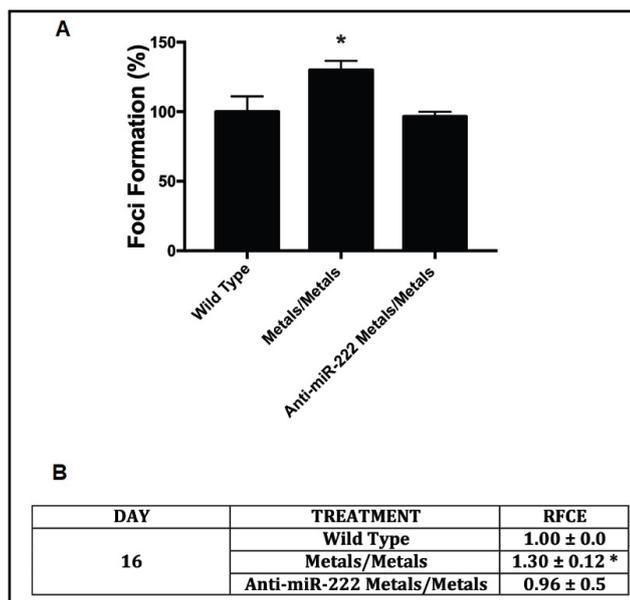
Finally, we evaluated the role of Rad51c regulation through miR-222 in the Balb/c 3T3 morphological transformation assay by replacing the initiator and/or promoter by metal-mixture stimulus or anti-miR-222 and following the cultures until transformation. We observed that percentage of *foci* formation increases in the Metals/Metals condition and with anti-miR-222 transformation was blocked (Fig. 4A).

Then, we calculated the RCFE (No. *foci*/dish of each experimental condition/No. *foci* per dish of the control of day 16) (Fig. 4B). As expected, we observed an increase in the foci number ( $1.3 \pm 0.12$ ) initiated and promoted by the As-Cd-Pb mixture (Metals/Metals) on day 16.



**Fig. 3.** (A) Distribution of DNA damage from metals exposed cells treated with gamma-radiation (3Gy), 1 and 24 h post-exposure, analyzed by neutral comet assay (B) DNA DSBs in irradiated Balb/c 3T3 cells previously exposed to metal mixture at 5 min (3 Gy), 1 h and 24 h post-exposure and evaluated with the neutral comet assay protocol. Results are presented in terms of Tail length (microns); dose of gamma radiation=3 Gy, n=2 slides/condition, 50 comets/slide, mean±s.e., two-tailed unpaired t-test, \*p<0.05. (C) A representative neutral comet images (20X).

**Fig. 4.** Percentage of foci generation (Foci %) in the Balb/c 3T3 cellular transformation assay (RCFEs were divided over the Control/Control condition of the respective day and multiplied by 100). (A) Cellular transformation foci analysis on day 16 of the assay from cultures treated with the metal mixture as an initiator and promoter stimulus, compared to the control condition (Control/Control), n=2, mean±s.e., one-way ANOVA F(5,6)=49043 \*p<0.0001, Tukey post-hoc comparisons of the groups 95% CI. (B) Relative colony formation efficiency (RCFE) of Balb/c 3T3 cells on day 16, for cells treated with the metal mixture as initiator and promoter stimulus, n=2, mean±s.e., two-tailed unpaired t-test, \*p<0.05.



## Discussion

The cell viability of the Balb/c 3T3 cultures during the initiation stage of the transformation assay did not exhibit differences upon metal exposure relative to the control, (Table 1), thereby indicating that the results presented in this manuscript are not due to a the cytotoxic effects of the metal mixture (2  $\mu$ M NaAsO<sub>2</sub>, 2  $\mu$ M CdCl<sub>2</sub>, and 5  $\mu$ M Pb (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>·3H<sub>2</sub>O) treatment, similar behavior to what we found in our previous work exposing the human hepatocyte WRL-68 cells to the same metal-mixture [52].

The expression of microRNAs can be regulated on multiple levels. At the transcriptional level, expression of microRNA genes can change together with (intragenic miRNAs), or independently of (intergenic miRNAs), their host genes. On the post-transcriptional level the expression of microRNAs can be downregulated due to changes in the activity of key miRNA biogenesis enzymes, such as Dicer and Drosha. Moreover, regulation of microRNA expression is dependent on cell type, physiological conditions and external factors [53]. However little is known about the induction of miRNA transcription in response to metals [5].

Nevertheless, our finding of the ability of the metal mixture to induce miR-222 expression in the initiation stage of the Balb/c 3T3 cell transformation assay (Table 1) may be explained in part by the significant evidence regarding how these metals may regulate mRNA expression. Some of the recognized mechanisms include genetic mutation, transcription factor and cell signaling deregulation, and epigenetic events [54, 55]. Because the genomic background of miR-222 is intergenic and has its own regulatory sequences [56], and since this kind of miRNA behaves like protein-coding genes [57], we suggest that the metal-associated mRNA regulation mechanisms may apply. Furthermore, Cd favors MAPK (mitogen-activated protein kinases) signaling [58] and is known to induce miR-222 expression [59]. Recently, Wang et al. [60] also observed that miR-222 play an important role in arsenic-transformed cells via Arid1A down-regulation. Likewise, it has been observed in occupationally exposed persons who work in an electric furnace steel plant, that miR-222 expression correlates with exposure to lead [61]. We observed a decrease in the mRNA (Fig. 2A) and protein (Fig. 2B) levels of Rad51c in cells treated with the metal mixture; this result is consistent with the literature data demonstrating that As and Pb inhibit Rad51-dependent HR repair of DNA DSBs [2, 62].

After confirming the miR-222 up-regulation and Rad51c down-regulation, we tested for DSBs DNA repair in Balb/c 3T3 under those conditions by inducing DSBs with gamma

radiation and testing its presence or absence at 5 min, 1 h after the challenge (Fig. 3). We observed an increase in DNA damage in cells over-expressing the miRNA alone, indicating that miR-222 plays a determinant role during the initiation of the transformation process through Rad51c inhibition and the consequent HR repair inhibition. This inhibition may lead to the characteristic damage accumulation and genomic instability of this stage of the process [3, 4, 7, 8]. This effect was also detected in the cells 5 min and 24 h post-irradiation, confirming the role of miR-222 as demonstrated by the exacerbation on DNA damage.

Finally, cellular transformation endpoint was assessed by morphological changes and *foci* formation in Balb/c 3T3 model [5, 18, 19], where we have observed transformation on day 16 with metals mixture. Trying to confirm the role of miR-222 in this process, we decide to use the anti-miR-222 to inhibit this miRNA. In this situation the metal mixture treatment was unable to induce miR-222 and cellular transformation was blocked.

Our results suggest that a metals mixture treatment induce miR-222 and play an important role as an initiator of the carcinogenesis, and is not merely a marker of various established cancers [37-40]. Together, our data further our understanding of metal mixture carcinogenesis.

## Conclusion

In summary, mixture of As-Cd-Pb induces miR-222 up-regulation in the initiation process of the Balb/c 3T3 *in vitro* model of transformation and directly regulates the mRNA and protein expression of the recombinase Rad51c, which participates in DNA DSB repair through the homologous recombination to maintain genomic stability. Our results suggest that an increase in miR-222-mediated Rad51c inhibition contributed to the loss of genomic stability, initiating the carcinogenesis process.

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## Disclosure Statement

The authors declare they have no conflict of interests.

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