Effect of Heavy Metals on Silencing of Engineered Long Interspersed Element-1 Retrotransposon in Nondividing Neuroblastoma Cell Line

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ABSTRACT

Background: L1 retrotransposons are the most active mobile DNA elements in human genome. Unregulated L1 retrotransposition may have deleterious effect by disrupting vital genes and inducing genomic instabilities. Therefore, human cells control L1 elements by silencing their activities through epigenetic mechanisms. It has been shown that cell division and heavy metals stimulate the frequency of L1 activities. Removal of silencing by L1 motivators may restart L1 element functions. Here, we have proposed that weather neurotoxic environmental heavy metals (as L1 stimulating factors) have a role in removing L1 silencing and restating its activities in nondividing neuronal cells. **Methods:** L1-RP green fluorescent protein (GFP)-tagged knock-in human neuroblastoma clones were prepared. Single-cell clone was treated with mitomycin-c combined with nontoxic and toxic concentrations of iron (Fe), copper (Cu), and mercury (Hg). Silencing status of engineered L1 elements in dividing and nondividing cells was determined through measuring the amount of GFP expressing cells with flow cytometry. The cytotoxic effect of mitomycin-c combined with metals was measured by MTT assay. **Results:** Hg in nondividing cells and Fe, Cu, and Hg in dividing neuroblastoma cells could significantly remove L1 silencing. Also, mitomycin-c treatment did not have any effect on metal toxicity status in neuroblastoma cells. **Conclusion:** Totally, our findings have shown that cell division has a role in removing L1 silencing as well as L1 retrotransposition induced by environmental heavy metals. It has been also indicated that Hg at all concentrations could remove silencing of engineered L1 element regardless of cell cycle state. *Iran. Biomed. J. 17 (4): 171-178, 2013*

INTRODUCTION

Human long interspersed elements-1 (LINE-1 or L1 retrotransposons), which is classified as autonomous transposable element [1], occupies 17% of DNA and still shows some activities in the human genome [2]. A typical active L1 retrotransposon possesses regions, including 5'UTR [3] that has promoter activity, ORF1 that encodes RNA chaperone [4], ORF2 that codes for one protein with two domains and function as endonuclease and reverse transcriptase [5], and 3'UTR [6]. These regions enable L1 retrotransposons to copy and paste themselves throughout DNA by a mechanism called target-primed reverse transcription [7, 8].

LINE-1 retrotranspositions contribute to genomic evolution [1] as well as creating new genes [9] and influencing gene expression [10]. L1 activities could also result in deleterious effects such as genetic disorders [11, 12] and increasing DNA instabilities [13, 14]. Therefore, human cells coordinate epigenetic mechanisms, which can silence L1 element activities up to basal level [15, 16]. Despite this epigenetic inhibition of L1 functions, a few studies have been shown that stress stimulation of cells could increase frequency of L1 retrotransposition beyond this silencing force [17-19]. In this regard, El-Sawy *et al.* [17] have demonstrated that treating HeLa cells by nickel chloride after 48 hours could result in a 2.5-fold increase in L1 retrotransposition by influencing normal cellular events involved in retrotransposition process. On the other hand, Giorgi *et al.* [18] focused on the effect of damage induced by oxidative stress on the amount of L1 retrotransposition; however unlike *El-*

Sawy et al. [17], they did not clarify the mechanism involved in this process.

In another point of view, some studies have shown that cell cycle status influences L1 retrotransposition frequency [20-22]. Little is known about the effect of heavy metals on removing L1 silencing in nondividing cells.

In this study, we have tried to check the role of heavy metals, that specifically have effect on central nervous system, on L1 silencing in nondividing neuroblastoma cell line. Some of these heavy metals have been shown to affect L1 retrotransposition frequency in HeLa cells [17]. Therefore, we created L1-GFP (green fluorescent protein)-tagged knock-in neuroblastoma cell line [BE (2)-M17] clones. The cells from clone 1 were arrested at G_2 phase of cell cycle using mitomycin-c and treated with different concentrations of iron (Fe), copper (Cu), and mercury (Hg). The silencing effect of the metals was determined by measuring the amount of GFP expressing cells.

MATERIALS AND METHODS

L1 retrotransposon vectors. To study the effect of extracellular stimulation on removing L1 retrotransposon, two L1-specific vectors including 99-RPS EGFP-Puro (L1-RP GFP) and 99 JM111 EGFP-Puro (JM111) (Kazazian HH lab, McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University, USA) were used. As shown in Figure 1A, L1-RP GFP vector contains a full-length L1 retrotransposon, which is disrupted by GFP gene located at 3'UTR end and is oriented in reverse direction regarding L1 element. After entrance of the exogenous L1-RP vector to the cell and following reverse transcription process, GFP gene could be expressed in cells that support at least one round of retrotransposition (Fig. 1A). JM111 vector contains a full-length L1 retrotransposon that possesses critical mutations in ORF1 region of L1 element and consequently cannot move. Therefore, this vector cannot support L1 retrotransposition, and cells transfected with this vector do not express any GFP gene (Fig. 1B). JM111 was used as control vector during transfection and flow sorting experiments.

Creating L1-RP GFP tagged knock-in neuro-blastoma clone. BE(2)-M17 neuroblastoma cell line (ECACC Cat No: 95011816; Iranian Cell Bank, Pasteur Institute of Iran, Tehran) was transfected with L1-RP GFP-tagged (99-RPS EGFP-Puro) and JM111 (99 JM111 EGFP-Puro) (control vector) following Lipofectamine protocol. Cells were selected with 2 µg/ml puromycin 72 hours post transfection. After ten

days, GFP-positive cells were selected and separated using flow sorter (BD FACSAria, USA). JM111-transfected cells were used as negative control during flow sorting. GFP-positive cells were grown as single cells under 20% FBS, 1% nonessential amino acid, and DMEM:F12 (1:1). Five clones (C1-C5) survived and were grown in 6-well plates. To confirm L1-RP GFP-tagged knocked-in clones, PCR was performed for the GFP gene using forward (5′ GCACCATCTTCTTCAA GGACGAC-3) and reverse primers (5′-TCTTTGC TCAGGGCGGACTG-3) [23] with the following protocol: 250 ng DNA (from C1-C5 cells), 300 nM final concentration for forward and reverse primers and 10 µl PCR 2× master mix (Vivantis, Malaysia). PCR cycles included 94°C (2 min), 94°C (30 s), 59°C (30 s), 72°C (2 min) for 35 cycles and finally 72°C (10 min). Presence of GFP gene in these clones was confirmed by obtaining the 342-bp PCR product size on agarose gel and furthermore by sequencing the purified PCR product. Clone C1 of transfected BE(2)-M17 with L1-RP GFP-tagged vector was named BE (2)-M17-L1RP-C1 and used in the experiments.

Arresting BE (2)-M17-L1RP-C1 cells with mito-mycin-c and propidium iodide (PI) test. BE (2)-M17-L1RP-C1 was treated with different concentrations of mitomycin-c, including 1, 3, and 4 µg/ml for 48 hours. Cells were collected and fixed in 4.5 ml cold 70% ethanol. After two hours, cells were pelleted by centrifuging at 600 ×g at room temperature for 5 min, and ethanol was discarded thoroughly. Cell pellets were washed once with PBS 1× and centrifuged at 600 ×g at room temperature for 5 min. Pellets were re-suspended in solution containing 200 µl PI dye (1 mg/ml, Sigma, Germany), 10 mL Triton X-100 and 2 mg RNase. Cells were incubated at 37°C for 30 min. PI emission was then read at red wavelength with flow cytometry (Partec, Germany).

Treatment of arrested BE (2)-M17-L1RP-C1 cells with metals and flow cytometry. Different concentrations of Fe, Cu, and Hg were prepared in fresh media (Table 1). Arrested and nonarrested (dividing) BE (2)-M17-L1RP-C1 cells were treated with media containing the metals for 48 hours. The treated cells were trypsinized and transferred on ice for flow cytometry. Flow cytometry was carried out for the number of GFP expressing cells.

MTT assay. Arrested and nonarrested BE (2)-M17-L1RP-C1 cells (2×10^5) were seeded in 96-well plates (6 wells for each concentration) and incubated overnight. A volume of 200 µl of media containing selected metals was added subsequently. After 24-hour incubation at 37°C with 5% CO_2, supernatant was
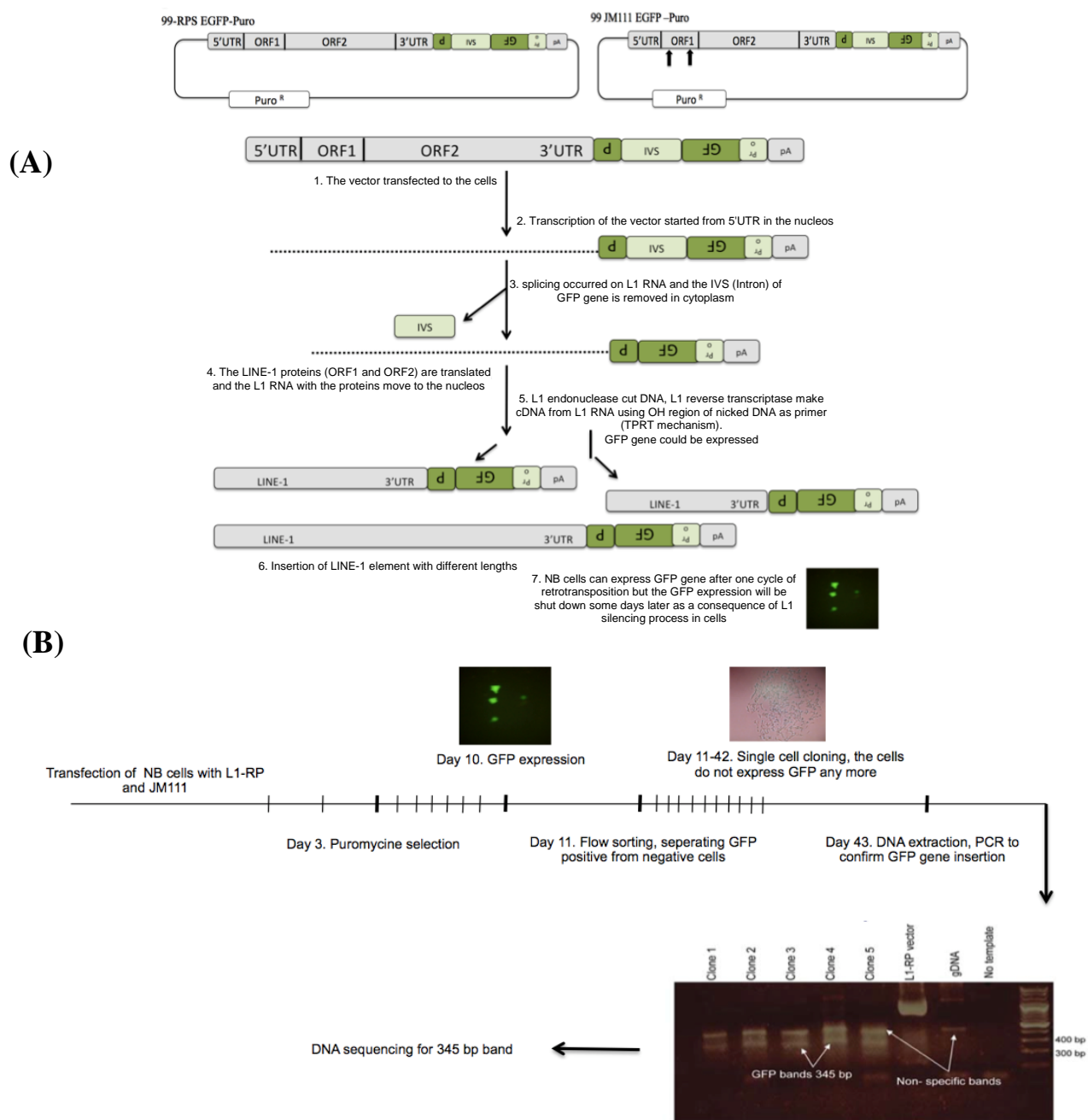
Fig. 1. Mechanism of vector functions, GFP expression in neuroblastoma cell, and schematic view of experiments used to make L1 knock-in neuroblastoma cells. **(A)** 99-RPS EGFP-Puro (L1-RP GFP tagged) vector contains full-length active L1 element and a GFP gene that is inserted in reverse orientation at 3'UTR end of L1 element. The GFP gene contains a CMV promoter (pro) and an intron (IVS). Once the vector enters the cell, it can be transcribed by host transcription machinery. Following transcription, L1 RNA undergos splicing process in cytoplasm; therefore, the intron located in GFP gene will cut out in this stage. Then, L1 RNA is translated and proteins required for reverse transcription process are provided. L1 RNA together with its encoded proteins moves to the nucleus. In the nucleus, L1 retrotransposon combined with GFP gene is reverse transcribed and pasted to the genome. GFP gene can now be expressed (as its intron has been spliced out) and functions as a marker of completion of one cycle of L1 retrotransposition in the cell. In another word, just the transfected cells that support one cycle of retrotransposition (target-primed reverse transcription mechanism) can express GFP gene. JM111 is similar to L1-RP GFP vector, but the two mutations in ORF1 region (the arrows) cause the vector not to be able to retrotranspose. Therefore, the cells transfected with this vector cannot express GFP. The products of this mechanism are L1 elements with different lengths. Therefore, approximately almost all copies of L1 are not full length and not be able to retrotranspose again (a cellular defend mechanism). **(B)** Neuroblastoma cells were transfected with L1-RP GFP-tagged vector and JM111 vectors. Totally, A few amounts (5%) of cells could support retrotransposition and subsequently express GFP. GFP-positive cells were separated from negative cells by flow sorting. After this time, the cells did not express GFP anymore. This phenomenon indicated silencing of L1 retrotransposon in neuroblastoma cells. Just 5 single cells were survived and expanded colonies after one month (clone 1-5). PCR analysis showed the insertion of spliced GFP gene (345 bp), and the presence of GFP gene was confirmed following sequencing of 345-bp band extracted from agarose gel.

Table 1. Metals, their sources and the selected concentrations used in this study

Metals	Sources of metals	Nontoxic concentrations		Toxic concentrations		Ref
		Low	High	Low	High	
Iron	$FeCl_3(H_2O)_6$	0.276 mg/L	2.76 mg/L	10.8 mg/L	21.6 mg/L	29
Copper	$CuSo_4$	3 mg/L	2 mg/L	60 mg/L	100 mg/L	29
Mercury	$HgCl_2$	1 µg/L	5 µg/L	10 µg/L	15 µg/L	29

removed and 100 µl of 0.5 mg/ml MTT dye (Sigma, Germany) was added to the cells. The plate was incubated at 37°C for 4 hours and MTT dye was replaced by isopropanol and incubated at 37°C. The plate was shaked and read by an ELISA reader (Biohit, Finland) at 545 nm versus 630 nm.

Statistical analysis. Flow cytometry, PI test, and MTT assay for each concentration of metals were carried out in three independent experiments. In each experiment, MTT assay was performed hexed, PI test duplicate and GFP flow cytometry just once. After checking the symmetry coefficient using ANOVA (analysis of variance), Post Hoc, Dunnett (two-sided), and LSD tests, data were analyzed using SPSS 16.0 software.

RESULTS

Effects of different heavy metals on silencing of L1 retrotransposon in nondividing neuroblastoma cells. L1-RP knock-in neuroblastoma cells were created

following the procedure depicted in Figure 1. Neuroblastoma cells containing retrotransposition region (GFP gene) (Fig. 1A) were separated from GFP negative cells using flow sorter. GFP expression in retrotransposition-positive cells was shut down after flow sorting as a cause of epigenetic silencing of engineered L1 retrotransposon inserted into the DNA of neuroblastoma. This phenomenon is a part of a natural defense mechanism against entrance of exogenous L1 retrotransposon and inhibits excessive L1 activity. GFP-positive single cells were grown to make colonies. Presence of GFP gene was confirmed by PCR [23] and by sequencing in subsequent colonies (Fig. 1B). Neuroblastoma cells from clone 1 [BE (2)-M17-L1RP-C1] were selected and treated with different concentrations of mitomycin-c. PI test showed that mitomycin-c at concentration of 3 µg/ml effectively arrested neuroblastoma cells at the G_2 phase of cell cycle without affecting cell survival. Mitomycin-c at concentration of 1 µg/ml did not effectively arrest cells at the G_2 phase of cell cycle (Fig. 2). When
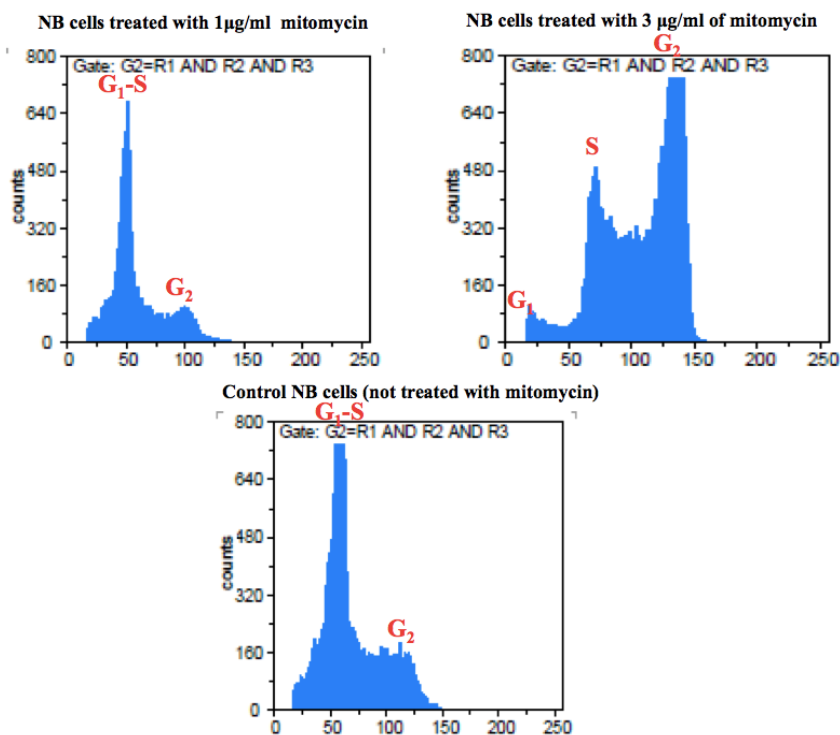

Fig. 2. Neuroblastoma cells arrested at G_2 phase of cell cycle by mitomycin-c. Neuroblastoma cells were treated with 3 µg/ml of mitomycin-c for 48 hours. Mitomycin-c at concentration of 1 µg/ml could not effectively arrest neuroblastoma cells at G_2 phase of cell cycle. S, synthesis

Table 2. *P* values obtained in order to show the amount of GFP positive cells after treating arrested NB cells with metals

Stressors	Concentrations			
	Low nontoxic	**High nontoxic**	**Low toxic**	**High toxic**
Hg	0.0001	0.001	0.002	0.001
Cu	0.9930	0.117	0.201	0.251
Fe	0.9910	0.851	0.878	0.907

treated with 4 μg/ml mitomycin-c, neuroblastoma cells did not survive for 48 hours. Arrested (nondividing) and nonarrested (dividing) BE (2)-M17-L1RP-C1 cells were treated with Fe, Cu, and Hg for 48 hours. Mitomycin-c (3 μg/ml) for nondividing group was added to ensure that the cells stopped at G_2 phase at the time of metal treatment. Flow cytometry analysis for treated cells showed that the number of GFP-positive cells did not significantly increase (Table 2) after treating G_2-arrested cells with Fe and Cu. However, Hg at all concentrations significantly ($P<0.05$) increased the number of GFP-positive cells. Dividing neuroblastoma cells grown under media containing metals showed significantly ($P<0.05$) increased GFP expressing cells after Hg (up to 20%), Cu, and Fe treatment (up to 13-15%) (Fig. 3).

Toxicity effect of metals and mitomycin-c on flow cytometry results. We performed cytotoxicity assays to check if the toxicity of metals combined with mitomycin-c caused the differences between flow cytometry data from dividing and nondividing neuroblastoma cells. MTT assay showed that mitomycin-c did not have significant effect on toxicity of selected metals in neuroblastoma cells. On the other hand, Hg and Cu at toxic concentrations in dividing and nondividing neuroblastoma cells had significant ($P<0.001$) toxic effect on cell survival (Fig. 4).

DISCUSSION

L1 retrotransposons are the most active mobile DNA element in human genome [1]. The activity of these elements may result in genomic plasticity [1] as well as different disorders in human, including Duchene muscular dystrophy [24], hemophilia [25], neurofibromatosis [26], and cancer [27]. Therefore, human cells have evolved different epigenetic mechanisms including DNA methylation and histone modifications to silence the activity of these elements [15, 16]. However, some internal and external factors including cell cycle status and heavy metals have been shown to influence the frequency of L1 retrotransposition in HeLa cells [17, 20-22].

In this study, we have examined the role of cell division and the effect of neurotoxic environmental factors, such as Fe, Cu, and Hg in removing the silencing of engineered L1 retrotransposon in neuroblastoma cell line. Although the effect of some heavy metals on L1 retrotransposition had been studied elsewhere [17], our findings show that the silencing effect could be removed when nondividing neuroblastoma cells are treated with Hg. However, all metals could effectively remove silencing of L1 element when the cells are continuously dividing. Nevertheless, arresting the cell cycle by mitomycin-c did not affect both cell surveillance in metal-treated cells and the interpretation of data for silencing effect of metals. Hg, a neurotoxic agent, removed the silencing effect of L1 retro-transposon in nondividing as well as dividing cells but not with the same strength. Removing silencing from L1 elements in nondividing neuroblastoma by Hg could reactivate L1 elements and result in harmful effects, such as increasing double-strand breaks or mutagenesis in these cells and predisposing them to degeneration. Since we have checked the effect of Hg simultaneously on arrested neuroblastoma, our study might reflect the conditions of affecting Hg on neurons (as nondividing cells) in our body. In this case, because our findings did not show similar effects for other metals including Fe and Cu, which have also been shown to have neurotoxic effects [27, 28], we cannot expand our hypothesis and data to all neurotoxins.

The results of this study showed the role of cell division in L1 silencing that could be complementary to the other reports which have been shown the effect of cell cycle on L1 retrotransposition process [20-22]. Additionally, it has been shown here that the effect of L1 silencing removal is not associated with toxicity of mitomycin-c, and it is specifically correlated with metal toxicity in neuroblastoma cells.

Conclusively, our data pave the way to search for one of the mechanisms that may be involved in silencing of L1 retrotransposons rather than histone modification [16].
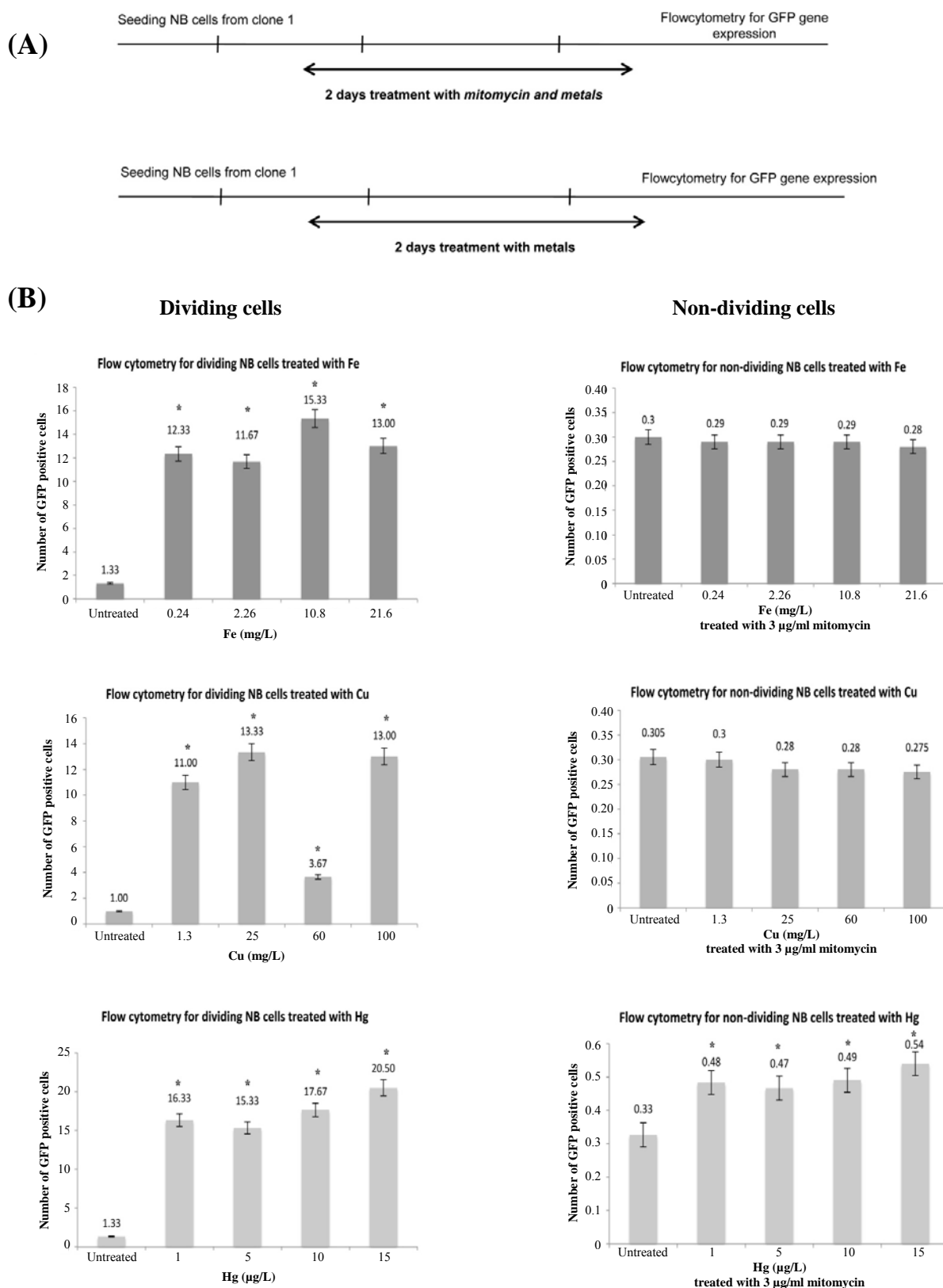
(A)

(B)

Fig. 3. Removing the silencing of engineered L1 retrotransposon in dividing and nondividing neuroblastoma cells treated with metals. (**A**) Schematic view of the assay. (**B**) Hg at all concentrations could significantly remove silencing of engineered L1 retrotransposon in nondividing neuroblastoma cells. Fe and Cu did not have such effect in nondividing neuroblastoma cells. However, all metals could significantly increase GFP-expressed cells at all concentrations in dividing neuroblastoma cells. Cell division has a positive role in removing L1 silencing in neuroblastoma cells. However, Hg is more powerful and could remove silencing of engineered L1 regardless of cell cycle stage in NB cells. *P<0.05

(A)

Seeding NB cells from clone 1   MTT test

24 hours treatment with *mitomycin and metals*

Seeding NB cells from clone 1   MTT test

24 hours treatment with metals

(B)

Dividing cells   **Non-dividing cells**

MTT assay for dividing NB cells treated with Fe

MTT assay for non-dividing NB cells treated with Fe

MTT assay for dividing NB cells treated with Cu

MTT assay for non-dividing NB cells treated with Cu

MTT assay for dividing NB cells treated with Hg
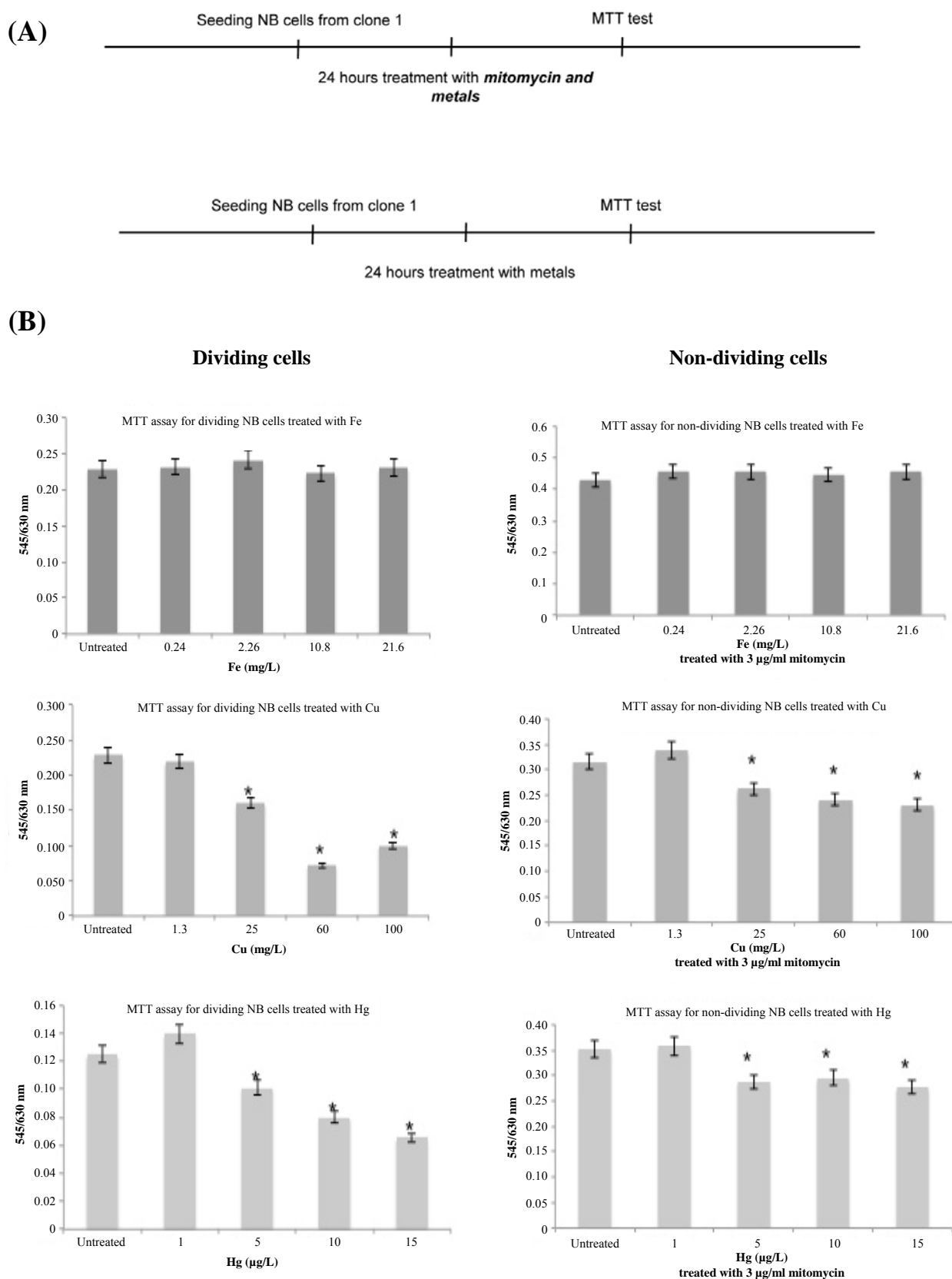
MTT assay for non-dividing NB cells treated with Hg

Fig. 4. Determining toxic effect of metals on dividing and nondividing NB cells by MTT assay. **(A)** Schematic view of the MTT assay. **(B)** Hg and Cu at toxic concentrations have significant toxic effect on both dividing and nondividing NB cells. It seems that mitomycin-c treatment does not have any significant effect on toxicity of selected metals in these cells. Also, Fe seems not to have toxic effect in dividing and nondividing NB; *$P<0.001$

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