Regulatory Role of Insulin on Endogenous L1 ORF1 and NEFM Gene Expression through PI3K Signaling Pathway Specifically in Neuroblastoma Cell Line

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Abstract

Background: One of the most important endogenous factors causing genomic instability in human cells is L1s retrotransposons. In this study, we assume that increased activity of L1 retrotransposons (specifically L1 expression) might be induced by hyperglycemia and hyperinsulinemia in neuroblastoma cell line.

Methods: Two different cell lines (BE (2)-M17 and HEK293) were treated with insulin and its PI3K signaling pathway inhibitor under three conditional media including hyperglycemic and retinoic acid treatment in the department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran in 2018. The expression of *L1 ORF1*, as well as genes involved in insulin signaling pathway and neuronal stress and structure were measured at RNA level.

Results: Insulin could significantly down regulate the expression of *L1 ORF1* and *NEFM* genes. Hyperglycemia result in severe decrease in expression of all candidate genes in control neuroblastoma but not HEK293 cells. Retinoic acid as the concentration used in this study cause increase stemness in neuroblastoma but not HEK293 cells. We could not find significant correlation between expression pattern of other genes tested in our study and *L1 ORF1* expression.

Conclusion: Total regulatory effect of insulin on *L1 ORF1* RNA expression as well as *NEFM* markedly in BE (2)-M17 cell line. Although these results could not be interpreted as L1 retrotransposition, expression of L1 RNA during stress conditions might be considered following inhibition of the insulin pathway. The result of this study also confirms the impotence of insulin on human evolution.

Keywords: Retroelement; Regulatory subunit 3; Neurofilament protein NF-M; Neuroblastomas

Introduction

Neurodevelopmental disorders (NDDs) are conditions that are generally associated with delay in developmental abilities including brain and motor skills. Although some cases of a specific of these disorders are caused by mutations in specific genes, most NDDs are caused by the effects of environmental factors on the regulation of genes during fetal nervous system development (1). Different lines of studies suggest a wide range of environmental risks such as maternal diabetes impact on the brain and finally could lead to NDDs by causing genomic instability (2).

Genomic instability could be induced by different inter/intra cellular factors. Mobile DNA elements have been shown to be one of the main causes of genomic instability, which among them L1 retrotransposons are prominent. L1s are members of ancient mobile DNA families that still have the ability to retrotranspose through the human genome autonomously and result in genomic instability (3, 4). The activity of these elements are mostly inhibited through evolution; however lines of evidence have been shown that de novo L1s activity is increased in human embryonic stem cells, neural progenitor cells (NPCs) as well as cancer cells (5, 6), when the evolutionary locks up happen mostly by global genomic demethylation. However, transcription factors that could recognize L1s internal promoter driven by RNA polymerase II (7, 8) might help increase L1 activity during embryonic development (9, 10). Although the influence of various environmental agents on L1 expression and retrotransposition has been studied extensively (11-13), the effect of hyperinsulinemia and hyperglycemia on L1 activity remains to be elucidated. Increasing maternal glucose indices and resultant hyperinsulinemia known as pregnancy diabetes have been shown to increase the rate of neurodevelopmental disorder in newborns (14).

In this study, we assumed that the activity of L1 retrotransposons (specifically L1 expression) might be induced by hyperglycemia and hyperinsulinemia. To test the idea, we assayed the effect of different concentrations of insulin (physiological and toxic) and insulin inhibitor for PI3K signaling pathway on endogenous *L1 ORF1* expression, in association with expression of neuronal differentiation markers (*NEFM*: mature neural marker, and *TUBB3*: immature neural marker), and genes involved in insulin signaling pathway and development in neuronal and non-neuronal cell lines (*FoxO3:* oxidative stress that mediate multiple transcriptional targets and *SOX2:* predominantly expressed in the immature, undifferentiated cells of the neural epithelium).

Materials and Methods

Cell culture and treatments

The BE (2)-M17 human neuroblastoma cell line (NB) (ATCC Cat No. CRL-2267) as a case cell line and human embryonal kidney cells (HEK293, ATCC Cat No. CRC-1573) as a control cell line were cultured in a standard F12: DMEM (1:1) medium (DNA biotech, Cat No: LM-D1221/500) and RPMI 1640 (BioIdea, Cat No: BI-1006-05), respectively supplemented with 20% heat-inactivated fetal bovine serum (FBS) (DNA biotech, Cat No: DB9723-100ml), and 1% penicillin-streptomycin (Gibco/Sigma Source, Cat No: AC16-025). The selected cell lines were then treated with conditions as can be seen from Table 1.

Table 1: Treatment strategies (at different concentrations of insulin: 5, 50, and 100 nM) used in study

Cells Time	Control (NB/HEK)	NB treated	HEK293 treated
	-	Insulin	Insulin
	Inhibitor	Insulin+ Inhibitor	Insulin+ Inhibitor
	High glucose	High glucose+ Insulin	High glucose+ Insulin
	High glucose+ Inhibitor	High glucose+ Insulin+ Inhibitor	High glucose+ Insulin+ Inhibitor
48 hours &1 week	RA	RA+ Insulin	RA+ Insulin
	RA+ Inhibitor	RA+ Insulin+ Inhibitor	RA+ Insulin + Inhibitor

NB: BE (2)M17, HEK: HEK293, RA: Retinoic acid (sigma Prod. No. R 2625), Insulin (Sigma-Aldrich-I5500), Inhibitor LY294002 (Sigma, Cat No. 1130) high glucose DMEM (Gibco, Cat No: DB9696), all treatment has been done in culture with confluency of 60% cells

Retinoic acid (RA) was dissolved in fresh dimethyl sulfoxide (DMSO) (Sigma-Aldrich-276855) and used at a final concentration of 1μM as described previously (15). Induction was done on exponentially growing cells using 1μM RA for 48 hours. All treatments were performed in dark. Afterward, washed once in phosphate-buffered saline (PBS) (DNA biotech, Cat No: DB0011).

MTT assay

Cells were plated in 96-well plates at a density of 3000 cells/well and allowed to adhere overnight (6 wells for each concentration). After overnight incubation, the medium was removed, and a fresh medium containing various concentrations of insulin were added to each well (5, 50, and 100nM) for both experimental times (48 h, and one week). Afterward, viable cell counting was performed by the MTT (Cat No: T-030-1) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye colorimetric test, based on the reduction of the number of metabolically active cells as previously described (16, 17).

Propidium iodide (PI) cell cycle assay

The BE (2)-M17 human neuroblastoma cell line (NB) and human embryonal kidney cells (HEK293) (5×10^5) were seeded and incubated in the presence of insulin at concentrations of 5, 50, and 100 nM. Forty-eight hours later, the cells were collected and fixed in 4.5 ml cold 70% ethanol. After 2 h, cells were pelleted by centrifugation at $600 \times g$ for 5 min at room temperature (RT). Cell pellets were washed once with PBS and centrifuged at $600 \times g$ for 5 min at RT. Pellets were resuspended in a solution containing 200 μl PI dye (cat No: 75002) (1 mg/ml, biolegend, 421301), 10 mL Triton X-100 (Promega, H5141) and 2 mg RNase (Bioneer, Cat. No. KB-0101). Cells were incubated for 30 min in 37 ºC. PI emission was then read at 617 nm wavelength by means of flow cytometry (Partek-Germany).

Gene expression analysis

Treated Cells were harvested using 0.25% trypsin–EDTA and incubated for 3-4 min. Then the cells were transferred to a new tube containing 5 ml of fresh medium, centrifuged 5 min at 3000rpm with subsequent supernatant removal. Total RNA was extracted using a total RNA extraction mini kit (Favorgen company, cat No: FABRK001) and treated with DNase I (Qiagen, Cat. No: 79254). RNA (1 μg) was reverse-transcribed by the reverse transcription (RT) Kit (YTA, Cat No: YT4500) with random primers. For each of the six candidate genes, pairs of primers were designed (Table 2). qRT-PCR was carried out by using the Amplicon SYBR green master mix (Denmark, Cat No: A323402) in triplicate for each sample. The reaction was run in Light-Cycler® 96 system as follows: 500 ng of cDNA, 25 μl of SYBR green, 300 nM final concentration for forward and reverse primers, up to 50 μl double-distilled water. Data were analyzed with the ΔΔCT equation. Beta-2-microglobulin was used as an internal control. The fold change for each PCR product was calculated in treated vs. non-treated samples in each group of treatment.

Statistical analysis

The data obtained from all experiments were expressed as the mean ± SD of three independent experiments with each experiment performed in triplicate. Statistical analyses among multigroup were conducted using a one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test and two-way analysis using GraphPad Prism software version 8 and the values of $P<0.05$ were considered statistically significant for all analyses. The MTT assay was done in three independent experiments, each time as hexad; and the PI test in two independent assays, each time in duplicate.

Table 2: Primers used in this study

| Gene			Length	Tm	GC%	Self-complementary	Self-3' complementary	Product size
FoxO3	F	TCAAGGA-TAAGGGCGACAGC	20	59.82	55	2	2	90
	R	GGACCCG-CATGAATCGACTA	20	59.61	55	4	2	
NEFM	F	TGAAGGAAATCCGCTCCCAG	20	59.75	55	4	1	81
	R	CGTAGCGGCATTT-GAACCAC	20	60.18	55	3	0	
SOX2	F	CCAGAAAAACAGCCCG-GACC	20	61.52	60	4	3	164
	R	GCTTCTCCGTCTCCGACAAA	20	60.04	55	3	1	
TUBB3	F	GAGATGGAGTTCAC-CGAGGC	20	60.18	60	3	2	107
	R	TCGTCTTCG-TACATCTCGCC	20	59.62	55	4	1	
L1ORF1	F	gaatgattttgacgagctgagagaa	25	58	55	3	2	67
	R	aattactctgagctacgggaggac	24	60	55	3	1	
B2-M	F	GAGGCTATCCAGCG-TACTCCA	21	61.09	57	4	0	248
	R	CGGCAGGCAT-ACTCATCTTTT	21	58.71	47	2	0	

Results

Different concentrations of insulin had no effects on cell cycle and cell viability

We first checked the effect of different concentrations of insulin in the cell cycle and toxicity by PI and MTT test, respectively (Fig. 1 A and B).

Insulin could decrease L1 ORF1 as well as RNA expression prominently in neuroblastoma cell line through PI3K signaling pathway

We first grew BE (2)-M17 and HEK293 cells under a medium containing a selected concentration of insulin (5, 50, and 100 nM), and PI3K signaling inhibitor (LY294002) for two-time lines. The results indicated the statistically significant ($P<0.05$) decrease in the expression of *L1 ORF1, FOXO3, SOX2, NEFM,* and *TUBB3* genes in comparison to their untreated controls in BE (2)-M17 cell line at all concentrations. In this regard, PI3K kinase inhibitor could significantly

($P<0.05$) increase the expression of *L1 ORF1* and *NEFM* even more than its condition in control cells. However, this expression induction more than control could not be seen for *FOXO3* and *TUBB3* genes showing that the effect of insulin through the PI3K signaling pathway is high for *L1 ORF1* and *NEFM* genes (Fig. 2A). The effect of insulin inhibitor on *SOX2* expression did not result significant effect showing independent of PI3K pathway. Similar results have been obtained in both timelines. Although somehow similar results could be obtained in HEK293 treated cells showing the impact of insulin through the PI3K signaling pathway (Fig. 2B); the regulation between control and treated cells are not as regulated as BE (2)-M17 cells showing that neuroblastoma cell lines could be affected more specifically by insulin signaling pathway than other cell line.
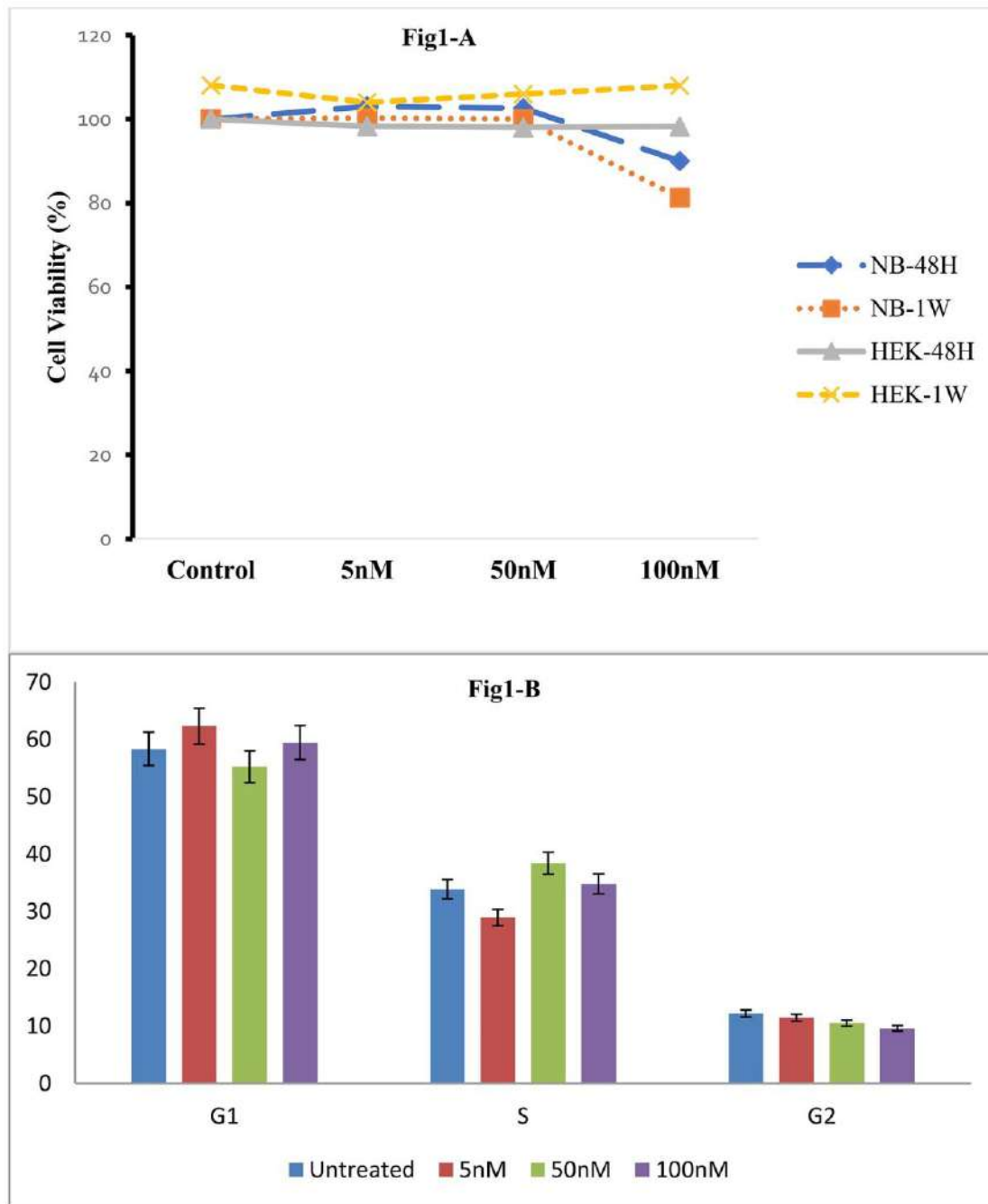
Fig. 1: Study the effect of three concentrations of insulin on cell cycle and toxicity in BE (2)-M17 and HEK293 cell lines. A) The MTT assay also showed that different insulin concentrations used in our study did not have significant effect on cell viability. B) Our data showed that there is no significant change ($P < 0.05$) in cell-cycle phases between untreated and insulin-treated neuroblastoma cells at concentrations of 5, 50, and 100 nM
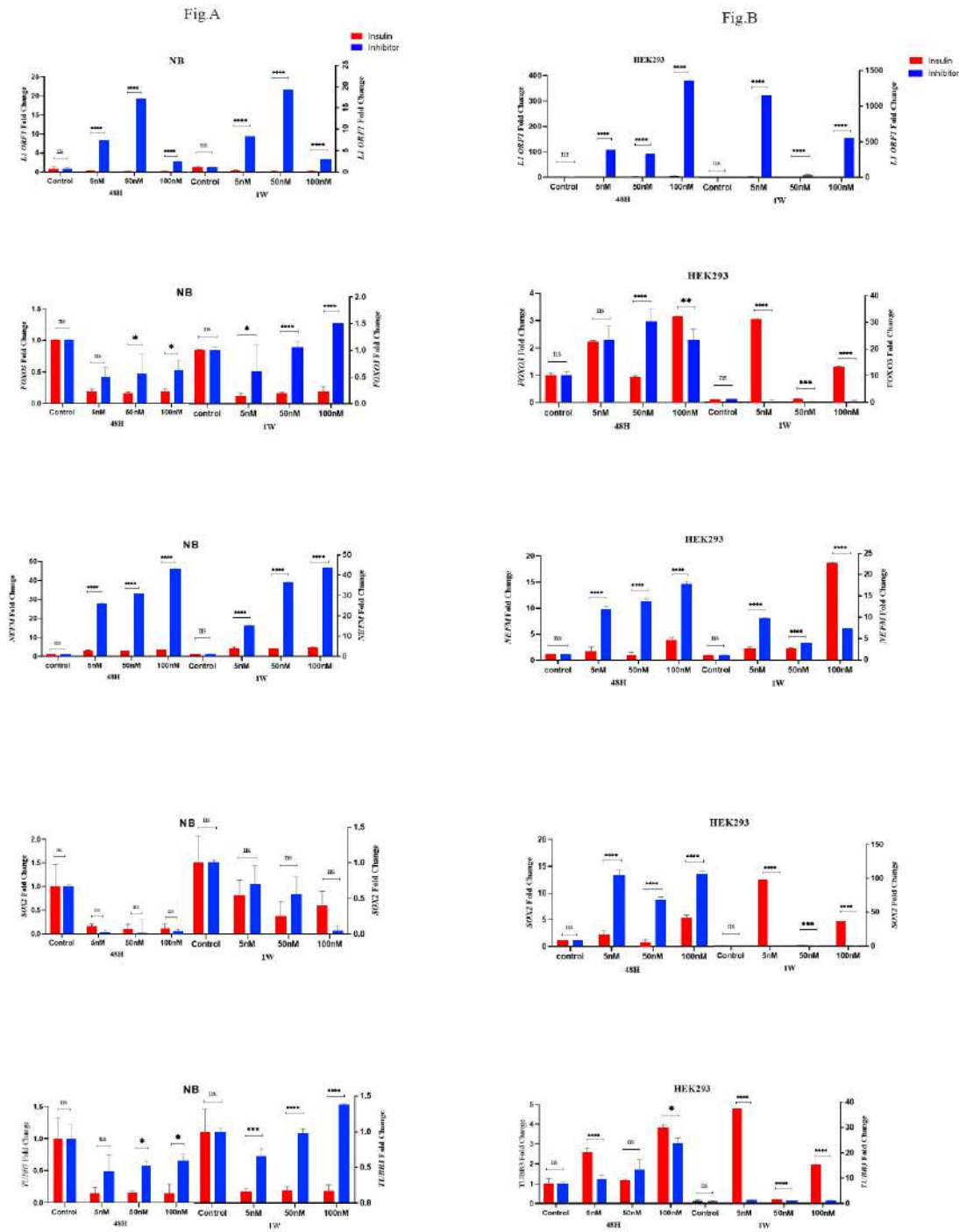
Fig. 2: Candidate genes expression in BE (2)-M17 and HEK293 cell lines after treatment with insulin and its PI3K signaling pathway inhibitor. A) Insulin significantly decreases the expression of all selected genes. Inhibitor treatment result in increasing the expression of *L1 ORF1* and *NEFM* even more than controls B) the similar result could be seen in HEK293, however their effect are not as regulate as the result seen in BE (2)-M17 cells. NB: neuroblastoma, BE (2)-M17; SM: simple medium; ns: non-significant. Expression levels were measured by qRT-PCR using $2^{(-\Delta\Delta Ct)}$ method. * *P* -value < 0.05; **P< 0.001, ****P < 0.0001 vs. control group. Error bars represent SEM of the values obtained from three independent experiments
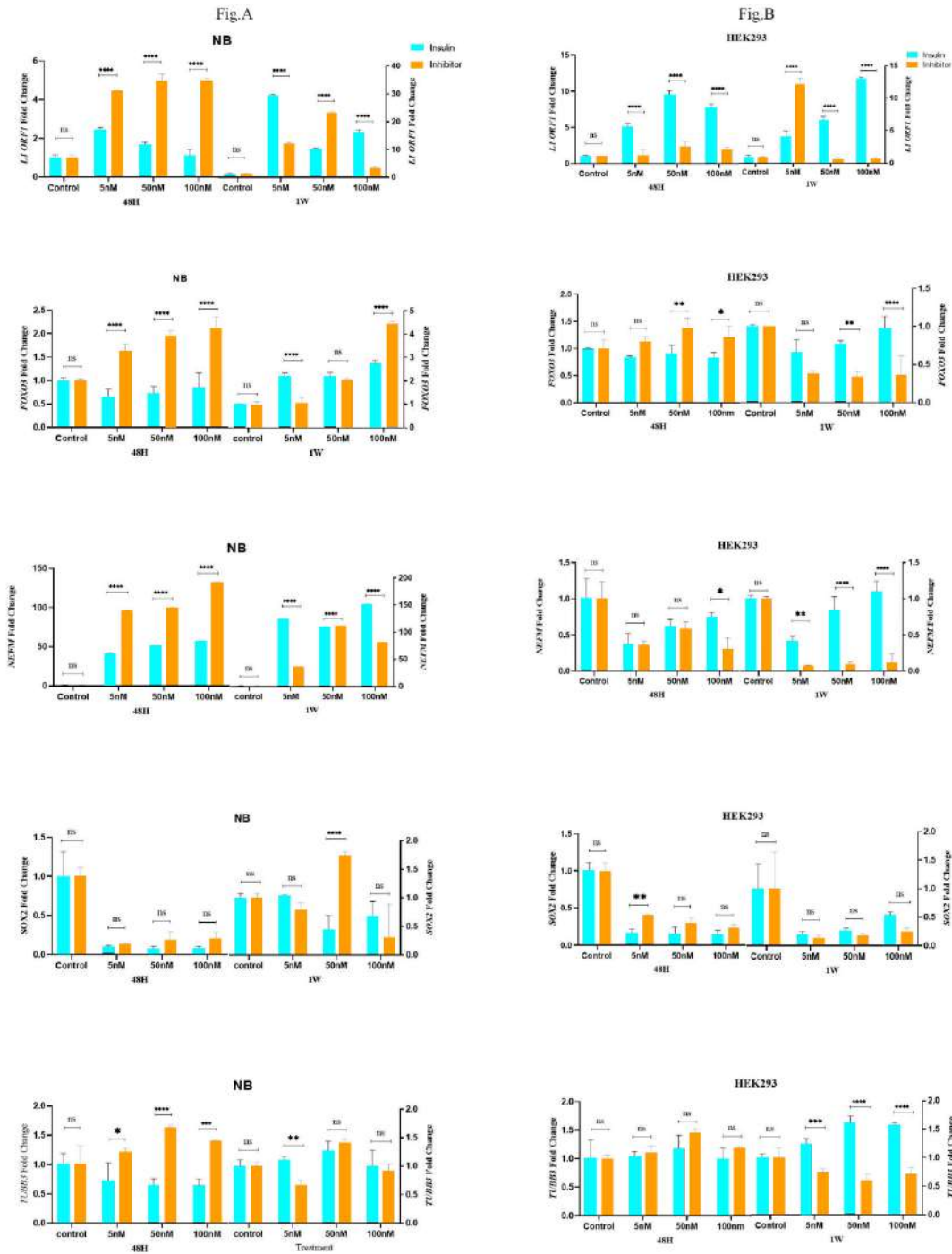
Hyperglycemia induces the expression of L1 ORF1 and NEFM genes in BE (2)-M17 cells in the presence of insulin and its PI3K inhibitor

BE (2)-M17 cells grown under hyperglycemic conditions (control) showed an unexpectedly decrease in expression of almost all genes (*L1 ORF1, FOXO3, NEFM, and TUBB3*) in comparison to non-treated cells (Fig 1). This unexplained result could just be seen in BE (2)-M17 cells and was not prominent in the HEK293 cell line. However, within the hyperglycemic treated group, our data showed that insulin at all concentrations could significantly (*P*<0.05) increase the expression of *L1 ORF1* as well as *NEFM* in comparison to hyperglycemic control. However, this up-regulation is still not as high as when we used PI3K pathway inhibitor, which may reflect some regulatory roles of insulin even in hyperglycemic conditions through this pathway in neuroblastoma cell line. This PI3K dependent event could not be seen for the same conditions in the HEK293 cell line (Fig. 3 A and B). A decrease in expression of *FOXO3, SOX2,* and *TUBB3* induced by insulin has been continued for these genes and this event seems to be independent of PI3K signaling pathway for *SOX2* genes mainly in neuroblastoma cell line compared to HEK293 (Fig. 3 A and B).

Down regulatory effect of insulin on L1 ORF1 and NEFM expression after treatment of BE (2)-M17 cells with retinoic acid

We treated our cells with RA and measured SOX2 expression. Comparing the expression of SOX2 in RA vs non-treated cells, we find that 1μM RA treatment for 48h resulted in increased stemness of BE (2)-M17 but not HEK293 cell line (Suppl. Fig. 1). Therefore under this circumstance insulin showed a significant (*P*<0.05) down regulatory effect on the expression of *L1 ORF1* in BE (2)-M17 and all selected genes but not in HEK293 cells (Fig. 4A). Our data also revealed that inhibition of the PI3K signaling pathway in the presence of insulin could not retrieve *L1 ORF1* and NEFM expression; However, expression of *TUBB3* and *FOXO3* significantly increased following treatment of neuroblastoma cells with PI3K inhibitor. Similar results could not be obtained for the HEK293 cell line (Fig. 2 A).

This data might propose that the regulatory effect of insulin on L1 retrotransposon activity following retinoic acid treatment might have occurred through other pathways as well (Fig. 4 A and B).

Fig. 3: Candidate genes expression in BE (2)-M17 and HEK293 cell lines under hyperglycemic conditions and treatment with insulin and its PI3K signaling pathway inhibitor. A) Insulin helps increase expression of *L1 ORF* and *NEFM* under hyperglycemic conditions but still has down-regulatory role through PI3K signaling pathway. Insulin inhibitory role on *FoxO3*, *SOX2* and *TUBB3* expression is the same as non- hyperglycemic conditions. B) Insulin helps increasing the expression of *L1 ORF1* but no other genes in HEK293 cell line and showed any down-regulatory effect through PI3K signaling pathway in these cells. NB: neuroblastoma, BE (2)-M17; HG: high glucose; ns: non-significant. Expression levels were measured by qRT-PCR using $2^{(-\Delta\Delta Ct)}$ method. * *P* -value < 0.05; ** *P* < 0.001, **** *P* < 0.0001 vs. control group. Error bars represent SEM of the values obtained from three independent experiments
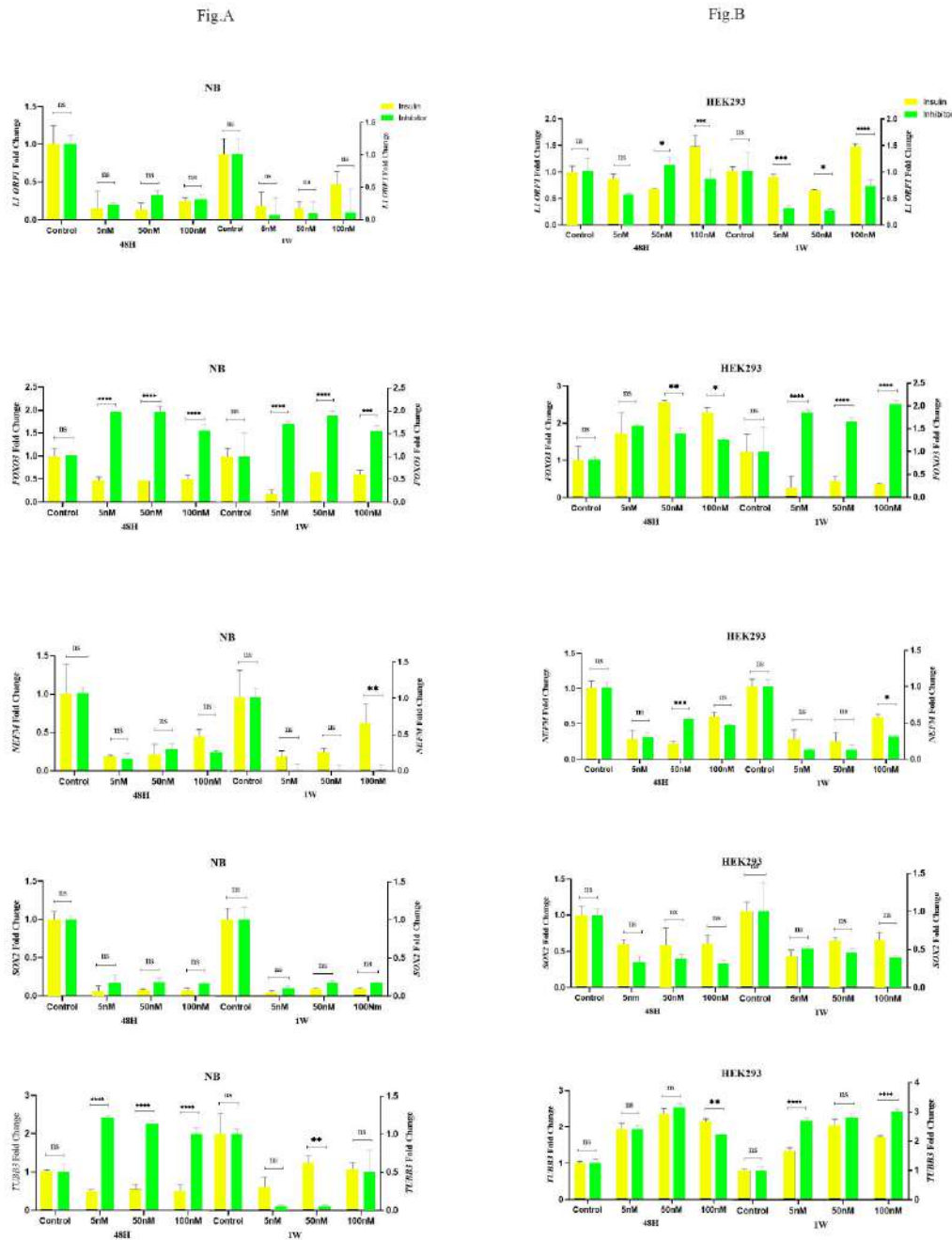
Fig. 4: Candidate genes expression in BE (2)-M17 and HEK293 cell lines after RA-induction of differentiation and treatment with insulin and PI3K signaling pathway inhibitor. A) Insulin could decrease the expression of *L1 ORF1* and *NEFM* genes that might partially be dependent of PI3K signaling pathway in neuroblastoma cell line. The regulatory effect of insulin on expression of *FoxO3* and *TUBB3* genes in RA-treated NB cell line is highly dependent on PI3K signaling pathway in compare to undifferentiated conditions. B) Down regulatory effect of insulin in RA treated HEK293 cell line on *L1 ORF1* and *NEFM* genes is not as effective as BE (2)-M17 cells. The expression pattern of *FoxO3* and *TUBB3* genes has not been affected through RA treatment in these cells. NB: neuroblastoma, BE (2)-M17; RA: Retinoic acid; ns: non-significant. Expression levels were measured by qRT-PCR using $2^{(-\Delta\Delta Ct)}$ method. * *P* -value < 0.05; **P < 0.001, ****P < 0.0001 vs. control group. Error bars represent SEM of the values obtained from three independent experiments

Discussion

One of the most important factors causing genomic instability in human cells is L1 retrotransposons. Although L1 expression is suppressed in most peripheral somatic cells, recent evidence has convincingly shown that neuronal progenitor cells (NPCs) in humans and mice are capable of high levels of L1 expression and insertion during neurogenesis that can lead to subsequent somatic mosaicism, as de novo form of mutagenesis, and can promote neuronal CNVs and genomic instability (18-20). Therefore, regulation of L1 activity during all human developmental stages would be important for human health specifically at the embryonic stage.

We obtained results revealing insulin base regulation of *L1 ORF1* and *NEFM* expression remarkably in the neuroblastoma cell line. This regulation has occurred through PI3K signaling pathway (Fig. 5). To best of our knowledge, this is the first report showing the inhibitory effect of insulin on *L1 ORF1* as well as *NEFM* expression. Within this study, we could not explain concurrency between the results obtained for *L1 ORF1* and *NEFM* expression in BE (2)-M17 cell line; however, both gene expressions have been shown to be increased in cells following stress conditions. This correlation could not be found with other genes (*FOXO3, TUBB3, and SOX2*).
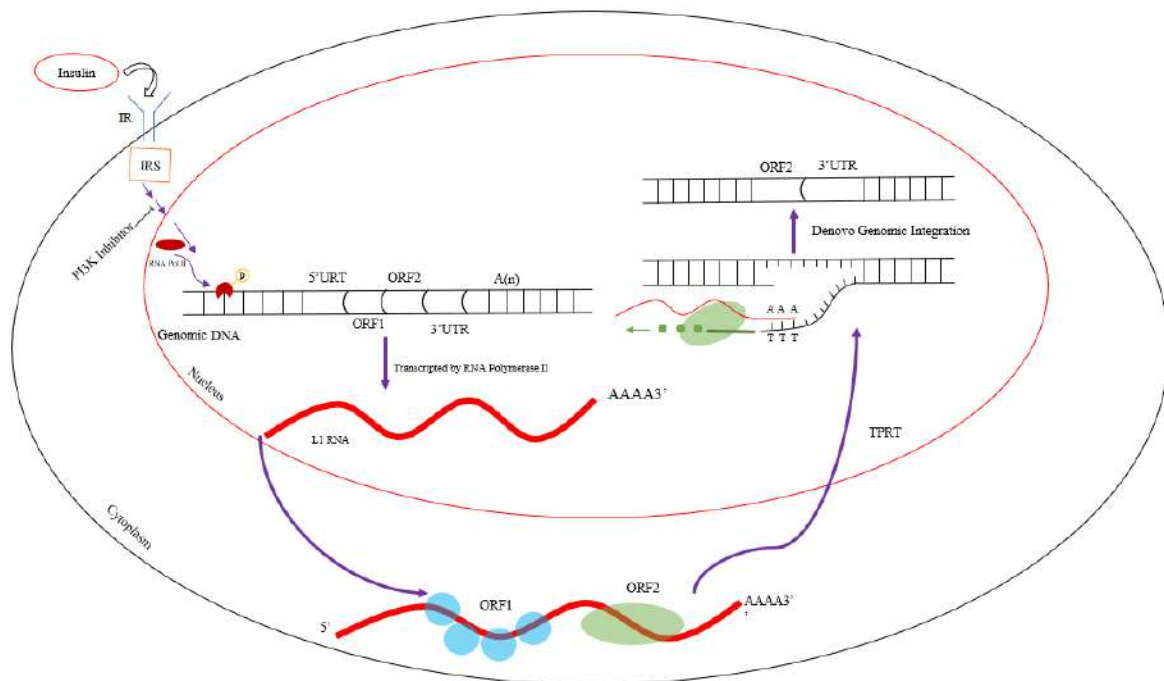
Fig. 5: A graphical illustration of regulatory pathway in the present study. IR: Insulin Receptor, IRS: Insulin Receptor Substrate, PI3K: Phosphoinositide 3-kinases, ORF: Open Reading Frame, TPRT: Target-Primed Reverse Transcription

Hyperglycemia in critical morphogenesis stages seems to be the main teratogen (21), and several studies reveal that the offspring of diabetic mothers presents more often NDDs (22-24). In vitro studies represent a paradigm shift in understanding the relationship between epigenetic change and hyperglycemic memory (25). Diabetes

in vivo or high glucose in vitro suppresses specific histone deacetylases expression (*SIRT2* and *SIRT6*) through oxidative stress pathway, and this down/up regulation is might be induced by epigenetic changes (26). Mimicking hyperglycemic conditions in vitro at control cells level revealed unexpected data, where we found a significant

decrease in expression of all genes in our study following growing BE (2)-M17 but not HEK293 cells in this condition. However, insulin regulatory effect through PI3K signaling pathway on *L1 ORF1* and *NEFM* expression could still be found in comparison to controls in our study. The intense inhibitory effect of hyperglycemia in neuroblastoma cell line remains to be elucidated.

One of the goals of our research was to study the effect of insulin on L1 expression in differentiated cells. Therefore, we used 1μM RA to induce differentiation in neuroblastoma cell line as described elsewhere (15). However, we found increasing stemness following 48h treating BE (2)-M17 with RA and measuring *SOX2* expression (Suppl. Fig. 1). This effect of RA has been described for other cell lines as well (27). Regarding our controls insulin revealed its down regulatory independent of PI3K signaling pathway for *L1 ORF1* and *NEFM* genes. Moreover, neuroblastoma cells can differentiate toward a neuronal phenotype upon RA treatment (28-31), which is sometimes used in high-risk neuroblastoma therapy (32,33). Therefore, RA has a major role in differentiation of brain cells and is used in various therapeutics targeted for nerve regeneration (34,35) but is toxic at higher concentrations.

Due to sanctions and financial problems, we could not study on L1 retrotransposition; therefore; further studies are required to evaluate changes in global DNA methylation as well as L1 retrotransposition that may be a main part of various diseases like cancers.

Conclusion

The result of our study showed a total regulatory effect of insulin on *L1 ORF1* RNA expression as well as *NEFM* markedly in BE (2)-M17 cell line. Although these results could not be interpreted as L1 retrotransposition, expression of L1 RNA during stress conditions might be considered following inhibition of the insulin pathway. The result of this study also confirms the impotence of insulin on human evolution.

Journalism Ethics considerations

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interest.

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