Investigation of the Effects of ZEB2 in Neural Differentiation of SH-SY5Y Neuroblastoma Cell Line

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INTRODUCTION & OBJECTIVES

Neuroblastoma is a malignant solid tumor commonly arisen in childhood term (1). The occurrence of neuroblastoma is involved in failure in neural differentiation (2). Drug-like Retinoic Acid (RA) is commonly used to induce differentiation of neuroblastoma cells and as well as in neuroblastoma treatment (3). Many cellular process have been related and changed during RA induced differentiation. Affects of RA on cell cycle is well known and RA induces cell cycle arrest (4). Despite the usage of RA in neuroblastoma treatment, still need to learn more about the downstream mediators of RA to improve treatment. Therefore, to learn and identify the effector protein and downstream elements of the RA signaling pathway will shed the light on our way in treatment. In this study, we aimed to assess the role of ZEB2, one of the fundamental Epithelial-Mesenchymal Transition (EMT) transcription factors, in RA induced neuronal differentiation of neuroblastoma cell line, SH-SY5Y. ZEB2 has a differential expression during embryonic development and absence of ZEB2 expression causes the defects of neural tube closure. However, the knowledge about function of ZEB2 in neuronal differentiation and RA pathway largely unknown except one literature that showed downregulation of ZEB2 during mouse neuronal differentiation. To this aim, we used lentiviral shRNA to generate control and ZEB2 knockdown clones of SH-SY5Y cells. Differential expression of ChIP-seq targets of ZEB2 in that cell clones by RT-qPCR analysis. Selected genes were also analyzed in differentiated and control SH-SY5Y cells to identify the potential target genes of ZEB2 during neuronal differentiation.

MATERIALS & METHODS

In the first step of lentiviral shRNA knockdown of ZEB2, we generated lentiviral particles in HEK293T cells by co-transfection of validated lentiviral ZEB2 shRNA (#0883, SIGMA) or pLKO.1 (#8453 Addgene) plasmids with lentiviral packaging mix (SHP001, Sigma), in presence of Lipofectamine 3000 transfection reagent. After 48 h, the supernatant of the HEK293T cells were collected lentiviral particles and stored at -80°C until its used. SH-SY5Y cells were seeded into 6-well plates at 40% confluency. The next day, cells were transduced with 1 mL of viral particles in the presence of 8 µg/mL Polybrene (Thermo Fisher Scientific). After 24 h, cells were split into 60 mm Petri dishes and stable cell clones were generated by selection with 0,5 µg/mL puromycin (InvivoGen) for 2 weeks. In knockdown clones, gene and protein levels of ZEB2 were validated by Western Blotting (WB) and RT-qPCR analysis, respectively. ChIP-seq targets of ZEB2 were sorted out according to their relationship with differentiation, development/morphogenesis, cell signaling, and proliferation. The ZEB2 dependent expression of the selected genes were evaluated in ZEB2 knockdown and control clones of SH-SY5Y cells by RT-PCR analysis. In the second step, SH-SY5Y cells were seeded into 6-well plates at 20% confluency. The next day, SH-SY5Y cells were treated with all-trans retinoic acid (ATRA or RA) for 3rd and 6th days. Untreated SH-SY5Y cells were used as undifferentiated control cells. In differentiated and undifferentiated SH-SY5Y cells, ZEB2 expression and differentiation were analyzed by both WB and RT-qPCR. In final step, ZEB2 dependent expression of selected genes were assessed by RT-qPCR in these cells.

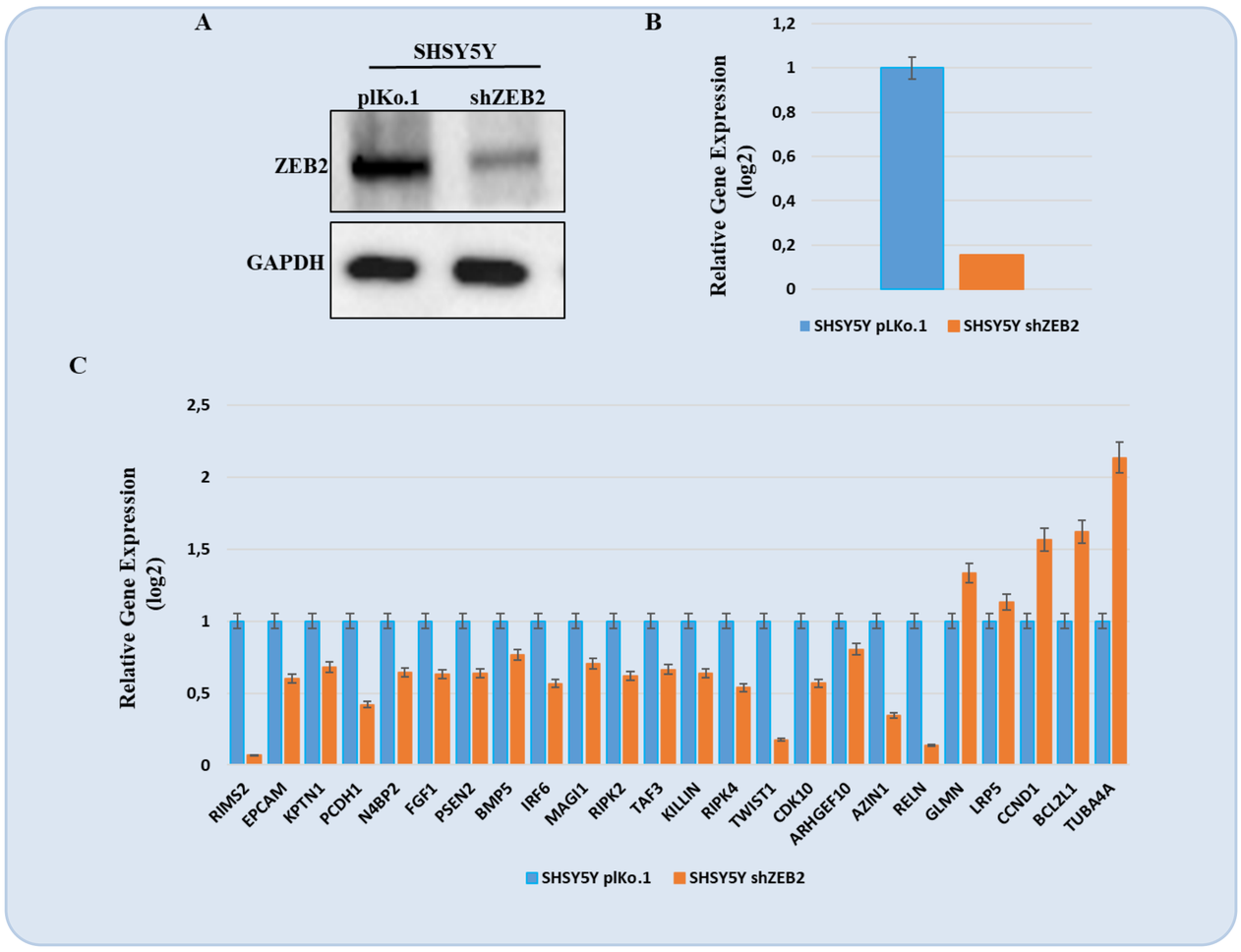


Figure 1: Screening of ZEB2-dependent expression of differentiation-related genes in shZEB2 and control clones of SH-SY5Y. A. Western blotting analysis of ZEB2 expression in human neuroblastoma cell line SH-SY5Y control and shRNA clones. Validation of shRNA ZEB2-knockdown in SH-SY5Y cell line. GAPDH was used as a loading control. **B.** Expression of ZEB2 genes in mRNA levels were analyzed in ZEB2-knockdown clones and calibrated to control clones by RT-qPCR. Relative fold change values (log2) were normalized to GAPDH. **C.** Expression of differentiation-related genes in mRNA levels were analyzed in ZEB2-knockdown clones and calibrated to control clones by RT-qPCR. Relative fold change values (log2) were normalized to GAPDH.

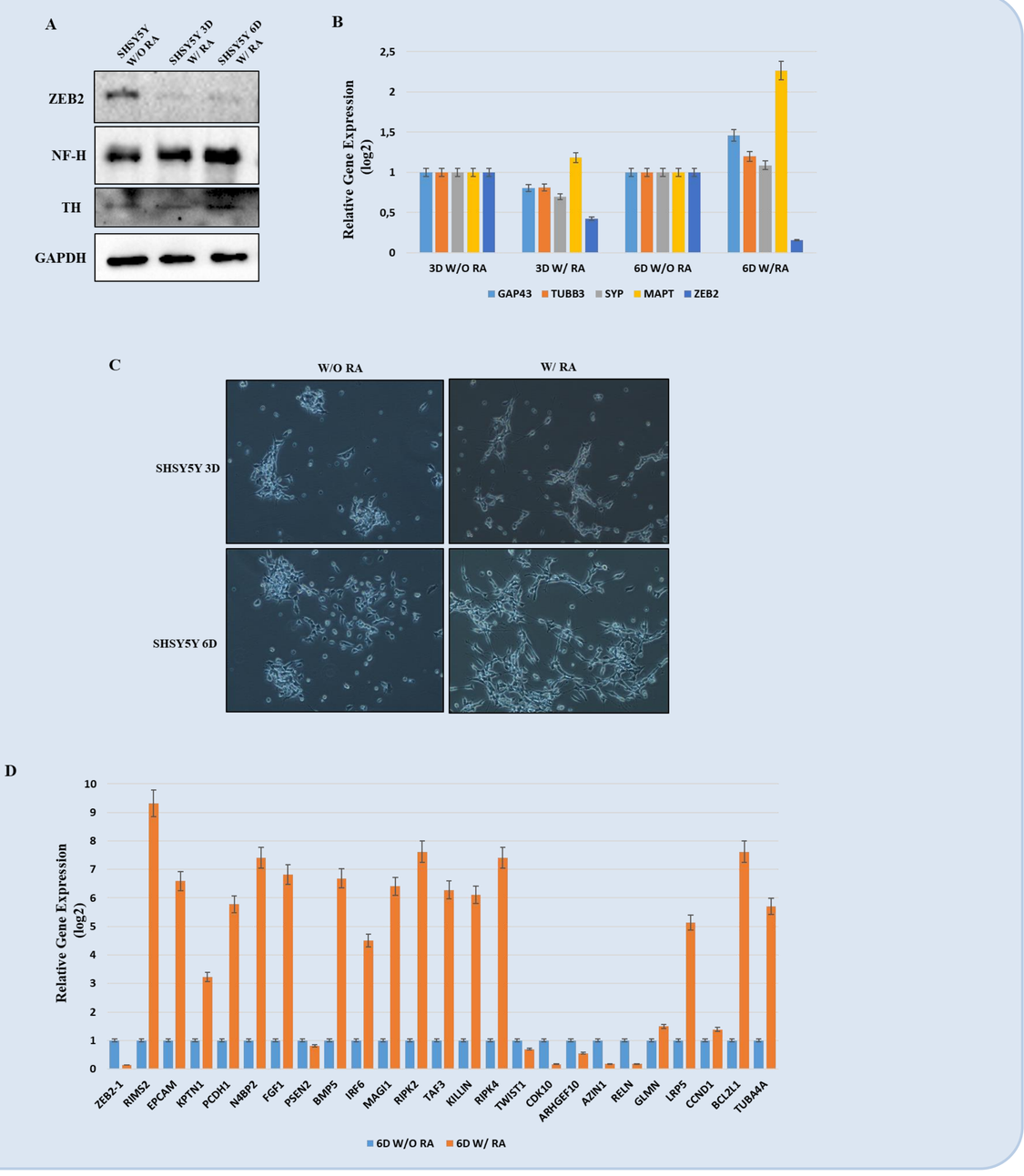
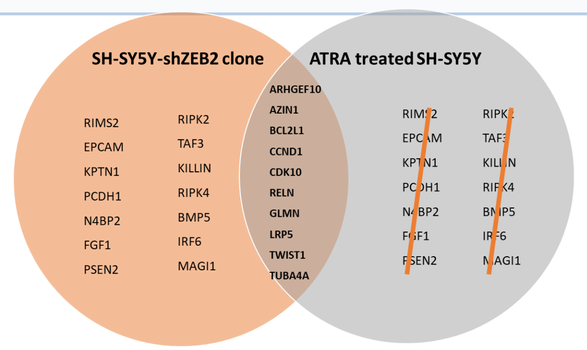


Figure 2: Quantitative screening of ZEB2 dependent genes in cells that were induced by retinoic acid. A. Western blotting analysis of ZEB2 and differentiation markers expression in retinoic acid-induced SH-SY5Y and non-induced cells at 3rd day and 6th day. GAPDH was used as a loading control. **B.** Expression of differentiaton markers in mRNA levels were analyzed in retinoic acid-induced SH-SY5Y and calibrated to non-induced cells by RT-qPCR. Relative fold change values (log2) were normalized to GAPDH. **C.** Morphological observation of 3rd and 6th day of SH-SY5Y cells treated with retinoic acid by light microscope. **D.** Expression of differentiation-related genes in mRNA levels were analyzed in retinoic acid-induced cells and calibrated to non-induced cells by RT-qPCR. Relative fold change values (log2) were normalized to GAPDH.

RESULTS and CONCLUSIONS

We determined upregulation and downregulation with regard to common changes at the end of these two RT-PCR analyzes. By this way, we think that ZEB2 may be associated with differentiation of SH-SY5Y cells at the gene level. Taken advantages of our results, we obtained molecular knowledges about ZEB2's role in complexity of neuroblastoma differentiation and its cell-based therapy potential. In our study, firstly, ZEB2 knockdown SH-SY5Y cells were successfully obtained. In these cells, differentiation-related genes found in ZEB2 CHIP-seq targets obtained in previous studies were screened and a significant change was observed in 24 genes depending on the ZEB2 gene. Secondly, retinoic acid was applied to parental SH-SY5Y cells, and changes in ZEB2 and differentiation markers were examined. In these cells, 24 genes were screened. In both cases, genes that show common significant changes were presented as possible ZEB2-dependent genes in the differentiation pathway.



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*No potential conflict of interest