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RNA-seq based transcriptome analysis of EHMT2 functions in breast cancer

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ABSTRACT

For breast cancer treatment, hormone therapy is effective for hormone receptor-positive breast cancer but not for TNBC (triple-negative breast cancer). Thus, many researchers have attempted to identify more effective therapeutic candidates for all subtypes of breast cancer. In this study, we established an RNA-seq analytical pipeline to analyze the subtype-specific functions of EHMT2 in the MB231 and MCF7 cell lines. After EHMT2 knockdown, we identified subtype-specific DEGs (differentially expressed genes) and overlapping DEGs. Through GO (Gene Ontology) analysis, GSEA (gene set enrichment analysis), and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis using the DEGs, we identified the subtype-specific functions of EHMT2 in the MB231 and MCF7 cell lines. Therefore, herein, we suggest that EHMT2 is an attractive therapeutic target for the treatment of all types of breast cancer.

1. Introduction

To effectively treat breast cancer, the subtypes of breast cancer must be confirmed because treatment decisions depend on these subtypes [1]. Breast cancer is divided into the hormone receptor-positive (65%~75%), HER2-positive (15%~20%), and triple-negative (15%) subtypes [2]. Although hormone therapy can be performed on hormone receptor-positive breast cancer, the TNBC (triple-negative breast cancer) subtype does not respond to hormone therapy [3]. Therefore, for the treatment of breast cancer, more effective therapeutic candidates have been continuously needed.

EHMT2 (euchromatic histone-lysine N-methyltransferase 2) is a histone methyltransferase for histone H3 lysine 9 mono-, di-methylation that is involved in the repression of gene expression [4]. In various cancers, EHMT2 is overexpressed and recognized as a prognostic and diagnostic marker [5–10]. Moreover, nonhistone protein methylation by EHMT2 is also involved in cancer proliferation [11]. Recently, our group reported that EHMT2 is overexpressed in breast cancer cohorts from publicly available data (TCGA (The Cancer Genome Atlas) data portal), and EHMT2 knockdown suppressed the growth and metastasis of breast cancer cell lines [7,8]. However, in several subtypes of breast cancer cell lines, the subtype-specific functions of EHMT2 are not yet fully understood.

Therefore, in this study, we established an RNA-seq analytical pipeline to analyze the subtype-specific functions of EHMT2 in the MB231 and MCF cell lines. After treatment with EHMT2 siRNA and a specific inhibitor (BIX-01294), we performed RNA-seq analysis and selected DEGs (differentially expressed genes) based on three criteria (MB231 only, MCF7 only, and MB231 and MCF7 overlapping genes). We used various transcriptome analytical programs (GluGO, DAVID (Database for Annotation, Visualization and Integrated Discovery), and GSEA (gene set enrichment analysis)) to analyze the DEGs and identified the subtype-specific functions of EHMT2. Based on the results, we suggest EHMT2 as an attractive therapeutic marker for the treatment of several types of breast cancer.

2. Materials and methods

2.1. Cell culture and reagents

The human breast cancer cell lines MCF7 and MDA-MB-231 were cultured in DMEM supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 [12]. BIX-01294 (EHMT2 inhibitor) was

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purchased from Abcam (ab141407).

2.2. Cell viability assay

For crystal violet staining, the cells that were treated with BIX-01294 (3 μM) for 24 h were washed twice with PBS (phosphate-buffered saline) and fixed with cold 100% methanol for 5 min at −20 °C. After being washed twice with PBS, the cells were stained with 0.1% crystal violet solution (C0775; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 5 min at room temperature [13]. The cells were then washed 5 times with distilled water and observed under a microscope (CELENA® S; Logos Biosystems; Anyang, Korea).

2.3. siRNA transfection

siRNA duplexes against EHMT2 (siEHMT2; 5′-GCAAAUAUUU-CACCUG CCATT-3′, 5′-UGGCAGGUGAAAUAUUUGCTT-3′) were purchased from Bioneer (Daejeon, South Korea). Negative control siRNA (siCont; 5′-AUGAACGUGAAUUGC UCAATT-3′, 5′-UUGAG-CAAUUCACGUUCACTT-3′) was used for the control treatments. The siRNAs (100 nM) were transfected into the cancer cell lines using RNAiMax (Invitrogen, Carlsbad, CA) for 72 h [14].

2.4. Migration and invasion assays

Transwell inserts were coated with a 2% gelatin solution and incubated at room temperature for 4 h for the migration assay. The gelatin-coated transwell inserts (353097, BD Falcon, Bedford, MA) and invasion chambers (354480, Corning, Corning, NY) were rehydrated in serum-free medium. Complete medium with 20% FBS (700 μl) served as a chemoattractant and was placed in the bottom chamber. Approximately 1×10^5 cells/well were incubated in the plates for 48 h at 37 °C with 5% CO_2. At the end of the incubation period, the migrated and invaded cells were fixed with methanol for 5 min and stained with 0.1% crystal violet.

2.5. RNA-seq and analysis

Using TruSeq RNA Sample Preparation Kit V2, purification and library construction were carried out with total RNA, and Illumina HiSeq2500 machines (Illumina, San Diego, CA, USA) was used for sequencing with a read length of 2×100 bases. FastQC v.0.11.4 was used for the quality of the paired-end reads. Cutadapt v.1.15 and Sickle v. 1.33 was used for filtering of low quality reads and adaptors. Cufflinks version 2.2.1 was used for calculation of FPKM (Fragments Per Kilobase of transcripts per Million mapped reads) values. The cuffdiff was carried out for selection of differentially expressed genes (DEGs) (fold change>1.5). All the Gene Ontology and KEGG pathway enrichment analyses were performed with DAVID ver 6.8 and CluGO ver 2.5.5 in cytoscape ver 3.7.1. GSEA analysis was performed with GSEA ver 4.0.1. Heat maps and hierarchical clustering were performed with R scripts.

3. Results

3.1. Research strategy for EHMT2 based on RNA-seq analysis in breast cancer cell lines

To study the functions of EHMT2 in several types of breast cancer, we selected two types of breast cancer cell lines (MDA-MB231 and MCF7). Comparing the two cell lines, MB231 is a basal-type and TNBC cell line that presents highly aggressive and invasive characteristics [3]. On the other hand, MCF7 is a luminal-type and ER/PR-positive cell line that is more sensitive to drug treatment than MB231 [15]. After knocking down EHMT2 in breast cancer cell lines, we performed RNA-seq analysis and selected the DEGs in each cell line only or the DEGs overlapping both cell lines. Finally, we predicted the function of EHMT2 in two types of breast cancer via GO (Gene Ontology) analysis, KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis, and GSEA (Fig. 1A).

3.2. EHMT2 facilitated cell growth and cell migration/invasion in breast cancer cell lines

To verify the RNA-seq results, after EHMT2 knockdown, we assessed the EHMT2-related effects on cancer in MB231 and MCF7 cells via a cell growth assay and migration/invasion analysis. Fig. 1B shows that treatment with BIX-01294 significantly suppressed cell growth. Moreover, because MB231 is a more invasive cell line than MCF7, after siCont treatment as a negative control, the rate of migration and invasion was critically increased in MB231 cells compared to MCF7 cells. And EHMT2 knockdown by EHMT2-specific siRNA also reduced cell migration and invasion (Fig. 1C). Thus, we suggest that EHMT2 is a very important regulator for proliferation and invasion/migration in the two types of breast cancer cell lines.

3.3. EHMT2 mainly controlled the cell cycle process in MB231 and MCF7 cells

After RNA-seq analysis of EHMT2 knockdown in MB231 and MCF7 cells, we selected 2-fold DEGs with siEHMT2 and siCont in MB231 and MCF7 cells and identified 1208 DEGs (MB231 only), 1055 DEGs (MCF7 only), and 557 DEGs (MB231 and MCF7) (Fig. 2A). To assess the effect of EHMT2 on both cell lines, we first performed GO analysis (biological processes) using the CluGO plug in of Cytoscape with the 557 DEGs. Fig. 2B shows that EHMT2 knockdown enriched the mitotic cell cycle, cell cycle process, and mitotic cell cycle process. Moreover, GSEA showed that the gene sets of E2F targets, G2M checkpoint and MYC target were regulated by EHMT2 knockdown in both cell lines (Fig. 2C). We performed further analysis of the 557 genes to assess the overlapping functions in both cell lines. EHMT2 knockdown analysis showed that 254 genes were upregulated and 303 genes were downregulated. In GO term analysis, the 303 down-regulated genes presented chromosome-related terms (homologous chromosome segregation, centrosome location, mitotic sister chromatid segregation, mitotic nuclear division and kinetochore) and microtubule-related terms (microtubule bundle formation and microtubule-based movement). In addition, the 254 up-regulated genes enriched cellular response-related terms. Overall, we suggest that EHMT2 is involved in cell cycle-related processes in both cell lines (Fig. 2D).

3.4. EHMT2 knockdown regulated DNA repair related genes in the MB231 cell line

Compared to that in MCF7 cells (siCont vs siEHMT2), we identified 1208 DEGs for EHMT2-regulated genes in MB231 cells only. In GO analysis, the response of cell death, cellular response to stress, chromosome organization, DNA conformation change, and sister chromatid segregation were enriched by EHMT2 knockdown in MB231 cells (Fig. 3A). In detail, among the 1208 genes, 604 down-regulated genes represented cell cycle-related terms and DNA repair-related terms. The 604 up-regulated genes by EHMT2 knockdown in MB231 cells were involved in negative regulation of cell proliferation, regulation of apoptotic process, positive regulation of apoptotic process (Fig. 3B). Moreover, in the KEGG pathway analysis, we also found that EHMT2 knockdown affected DNA replication, the cell cycle, nucleotide excision repair, ECM-receptor

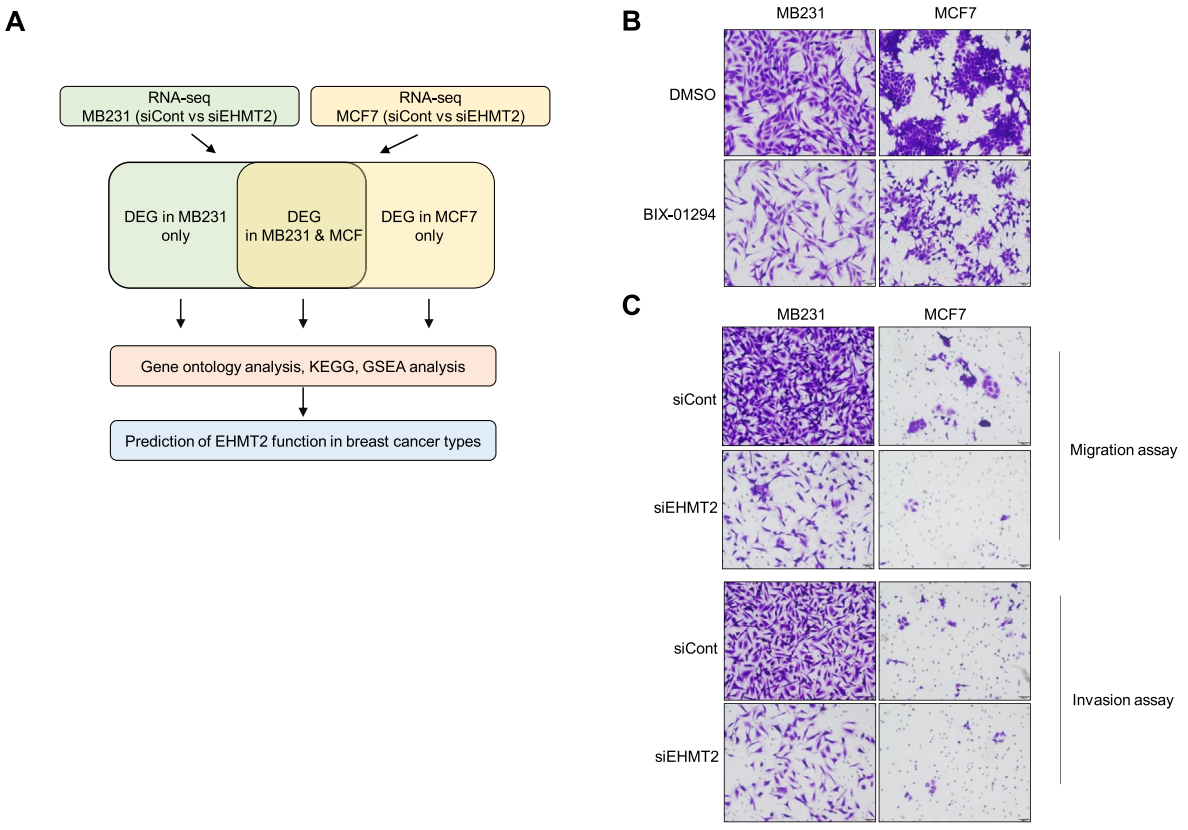

Fig. 1. EHMT2 suppressed cell growth and migration/invasion in MB231 and MCF7 cells.
A. Research strategy in this study. **B.** Cell growth assay after BIX-01294 treatment (2 μM). After MB231 cells were treated with BIX-01294 for 24 h, the cells were fixed with 100% methanol and stained with crystal violet. **C.** Migration and invasion assays after EHMT2 knockdown. Cell migration and invasion assays were performed over 48 h. Migrated and invaded cells were stained with crystal violet.. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

interaction, and focal adhesion (Fig. 3C). Therefore, we suggest that EHMT2 knockdown up-regulated the apoptosis process and down-regulated the DNA conformation related genes of MB231 cells.

3.5. *EHMT2 knockdown induced cell apoptosis in MCF7 cells*

In GO analysis using 1055 genes (MCF7 only), several cellular processes, such as cotranslational protein targeting to membrane, defense response, innate immune response and regulation of neuron death (Fig. 4A). Unlike MB231 cells, 363 downregulated genes represented the translational-related terms (ribosomal large subunit biogenesis, tRNA processing, translational initiation, translational elongation, and ribosomal large subunit assembly). Interestingly, upregulated genes showed involvement in several signaling pathway terms (MAPK cascade, response to interferon-gamma, immune response, positive regulation of I-kappaB signaling) and apoptosis terms (apoptotic process, apoptotic signaling pathway) (Fig. 4B). Additionally, in the KEGG pathway analysis, EHMT2 knockdown involved several signaling pathways as well as apoptosis in MCF7 cells (Fig. 4C). Therefore, we suggest that EHMT2 knockdown induced cell apoptosis via the dysregulation of the translational process in MCF7 cells.

4. Discussion

Transcriptome analysis using RNA-seq to study the role of EHMT2 in breast cancer is a useful analysis pipeline because there are many analytical methods, such as GO analysis (biological processes), GSEA, and KEGG pathway analysis. Using DEG analysis, we can easily obtain data, especially on cell proliferation, apoptosis, migration/invasion, and cell maintenance-related pathways or genes, and compare the properties of each cell line. Finally, we can predict and suggest the functions of candidate genes in several types of cancers. Thus, this analytical pipeline can be applied to all genes and all cancers to identify candidate genes for cancer therapy.

In this study, to assess the function of EHMT2 in different types of breast cancer cell lines, we used an RNA-seq analytical pipeline after the knockdown of EHMT2 and performed GO analysis, KEGG analysis, and GSEA. In both the MB231 and MCF7 cells, EHMT2 knockdown induced cell apoptosis via the regulation of cell cycle-related genes (GO analysis), E2F targets, the G2M checkpoint, and MYC target-related genes (GSEA). Moreover, GO analysis using the DEGs specific to each of the breast cancer cell line subtypes showed that EHMT2 upregulated apoptosis related genes and GO terms in MB231 and MCF7 cells, respectively. However, in MB231 cells, EHMT2 knockdown mainly down-regulated cell cycle related terms, but in MCF7 cells, EHMT2 knockdown affected to translational regulation genes and GO terms (Figs. 3B and 4B). Based on this result, in each cell lines, EHMT2 knockdown induced cell apoptosis via up-regulation of apoptosis related genes, and down-regulation of cell cycle, translational related genes. Thus, we suggest that EHMT2 is an important regulator of breast cancer proliferation and migration/invasion.

In summary, EHMT2 showed cell types specific functions in MB231 and MCF7. In both of cell lines, EHMT2 related to the cell cycle process, in MB231 only, EHMT2 associated the DNA replication and repair process. In addition, in MCF7 only, EHMT2 regulated the translational process (Fig. 4D). Based on the role of EHMT2 in

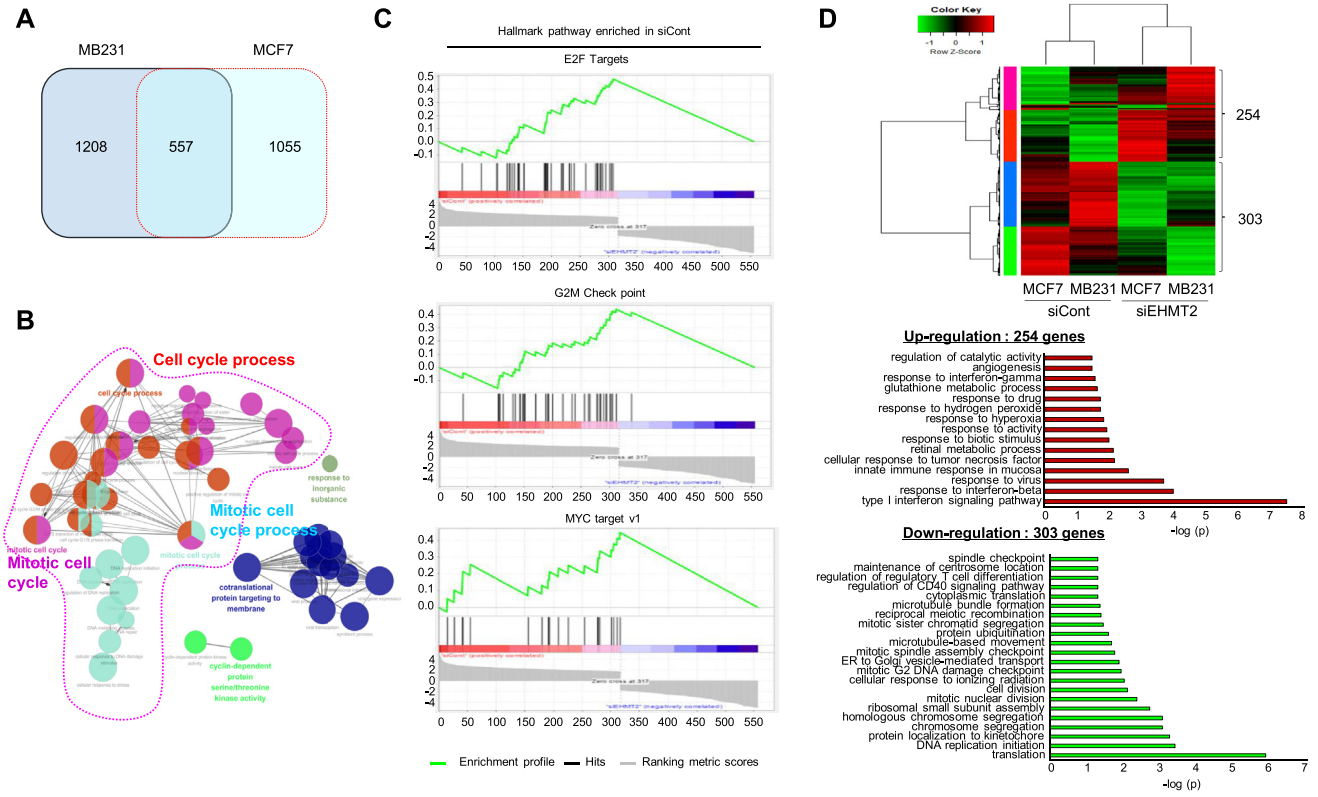

Fig. 2. Prediction of overlapping EHMT2 functions in MB231 and MCF7 cells.
A. The Venn diagram of DEGs after EHMT2 knockdown in MB231 and MCF7 cells. **B.** GO pathway term enrichment networks. GO pathway term networks with 557 genes functionally grouped by ClueGO. **C.** GSEA analysis using 557 genes. **D.** Heatmap of 557 genes (left). DAVID-based GO analysis of RNA-seq results with the up- and downregulated genes of the 557 genes. The enriched terms are shown.

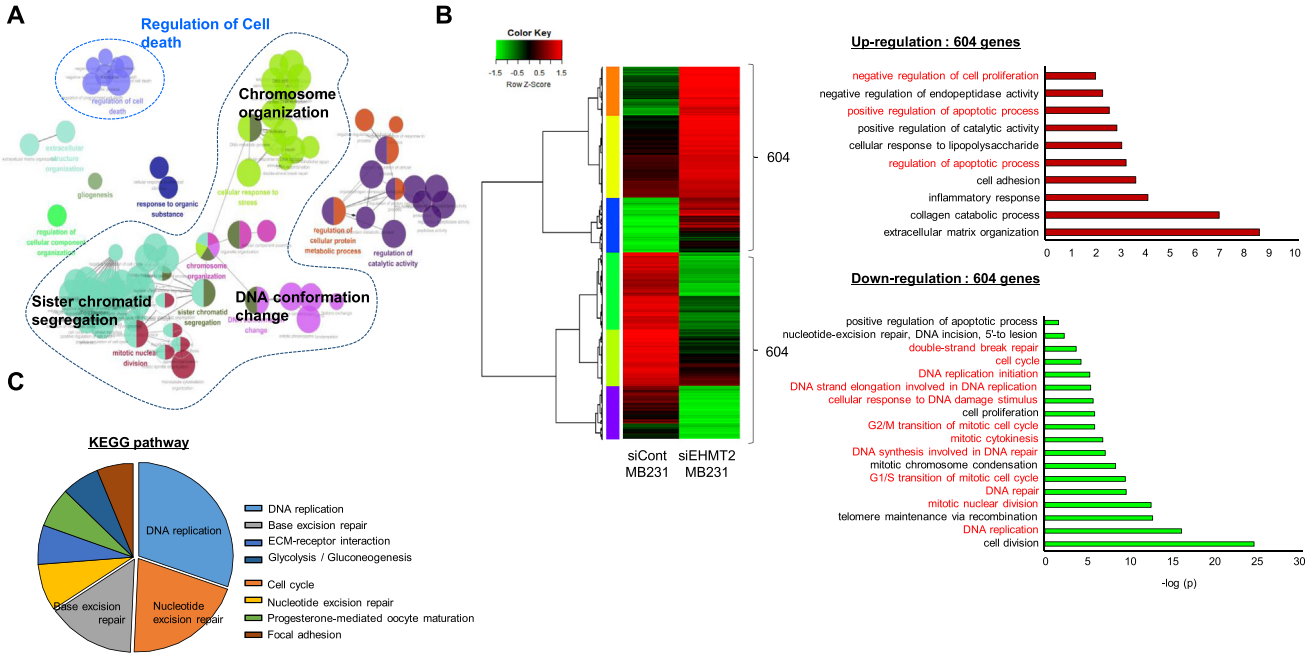

Fig. 3. EHMT2 regulated DNA repair related terms in MB231 cells.
A. GO analysis (biological processes) of the DEGs after EHMT2 knockdown in MB231 cells only, grouped by ClueGO. **B.** Heatmap of 1208 genes (left). DAVID-based GO analysis of RNA-seq results with the up- and downregulated genes of the 1208 genes. The enriched terms are shown. **C.** DAVID-based KEGG pathway analysis of 1208 genes.

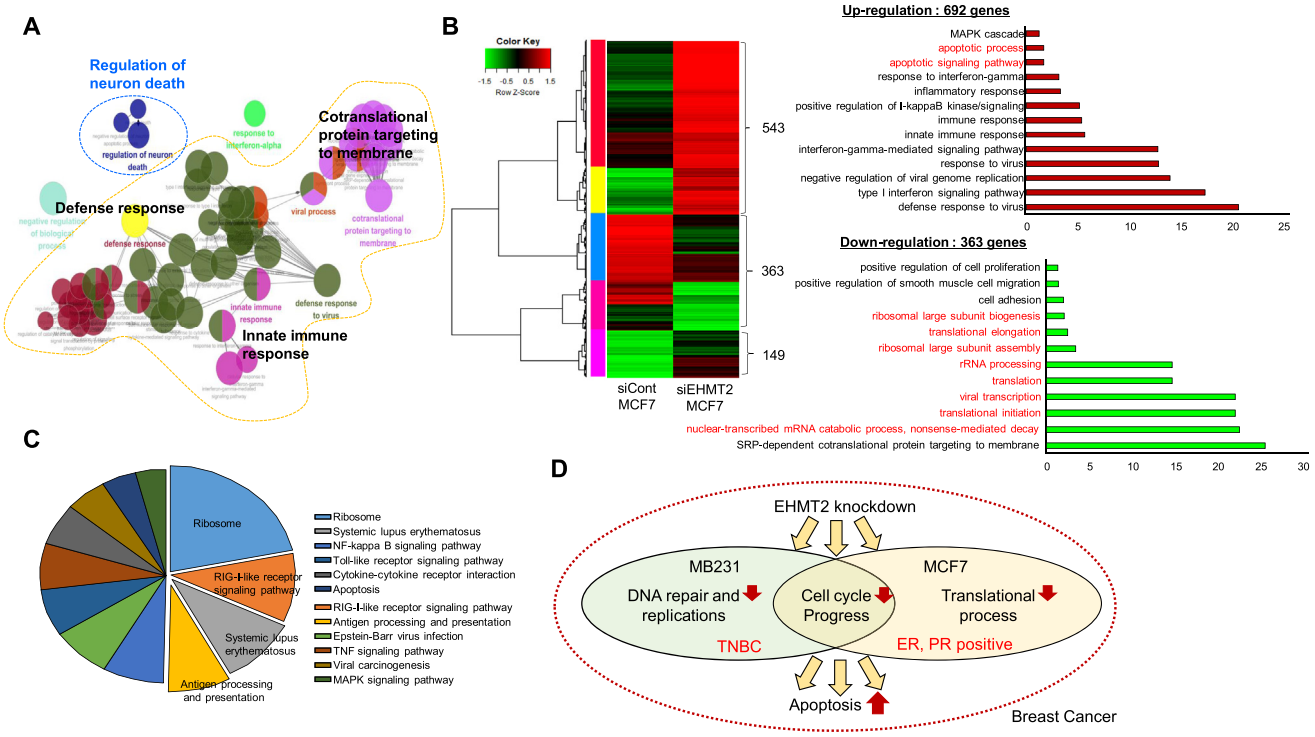

Fig. 4. EHMT2 induced cell apoptosis related terms in MCF7 cells.
A. GO analysis of the DEGs after EHMT2 knockdown in MCF7 cells only, grouped by ClueGO. **B.** Heatmap of 1055 genes (left). DAVID-based GO analysis of RNA-seq results with the up- and downregulated genes of the 1055 genes. The enriched terms are shown. **C.** DAVID-based KEGG pathway analysis of 1055 genes. **D.** Schematic summary of EHMT2 functions in breast cancer.

several types of breast cancer cell lines, in further studies, EHMT2-specific inhibitors must be developed for breast cancer treatment. Currently, although there is no clinical study on EHMT2 inhibitors, EHMT2 is an attractive therapeutic target for treating all types of breast cancer.

Declaration of competing interest

The authors declare no conflicts of interest.

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