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RNA-binding protein NONO promotes breast cancer proliferation by post-transcriptional regulation of *SKP2* and *E2F8*

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Abstract

The majority of breast cancers are primarily hormone-sensitive and can be managed by endocrine therapy, although therapy-resistant or hormone-refractory cancers need alternative treatments. Recently, increasing attention is being paid to RNA-binding proteins (RBP) in cancer pathophysiology. The precise role of RBP in breast cancer, however, remains to be clarified. We herein show that an RBP non-POU domain-containing octamer binding (NONO) plays a critical role in the pathophysiology of breast cancers regardless of their hormone dependency. Clinicopathological and immunohistochemical study of 127 breast cancer cases showed that NONO is a significant independent prognostic factor for breast cancer patients. Notably, siRNA-mediated NONO knockdown substantially repressed the proliferation of both hormone-sensitive MCF-7 and hormone-refractory MB-MDA-231 breast cancer cells. Integrative analysis combined with expression microarray and RIP-sequencing (RNA immunoprecipitation-sequencing) showed that NONO post-transcriptionally regulates the expression of cell proliferation-related genes by binding to their mRNAs, as exemplified by S-phase-associated kinase 2 and E2F transcription factor 8. Overall, these results suggest that NONO is a key regulator for breast cancer proliferation through the pre-mRNA splicing of cell proliferation-related genes and could be a potential new diagnostic and therapeutic target for advanced disease.

KEYWORDS

breast cancer, NONO, post-transcriptional regulation, RNA-binding protein, splicing

Abbreviations: CCNE2, cyclin E2; DAB, 3,3′-diaminobenzidine; DBHS, *Drosophila* behavior human splicing; E2F8, E2F transcription factor 8; ER, estrogen receptor; GEO, Gene Expression Omnibus; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; IDC, invasive ductal breast cancer; IHC, immunohistochemistry; IR, immunoreactivity; NONO, non-POU domain-containing octamer binding; NOPS, NONA/paraspeckle; PDCD4, programmed cell death 4; PgR, progesterone receptor; PI, propidium iodide; PSF, polypyrimidine tract-binding protein-associated splicing factor; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RBP, RNA-binding protein; RIP-seq, RNA immunoprecipitation-sequencing; RPKM, reads per kilobase of exon per million mapped reads; SKP2, S-phase-associated kinase 2; STR, short tandem repeat; TBST, Tris-buffered saline; TMPO, thymopoietin; TNBC, triple-negative breast cancer.

Iino and Mitobe have contributed equally to this manuscript.

Breast cancer is the malignancy with the highest incidence and the leading cause of cancer mortality among women worldwide.[1] The disease is categorized into intrinsic subtypes especially based on ER, PgR, and epidermal growth factor receptor 2 (ERBB2) status.[2] The majority of breast cancers are ER-positive and primarily treated with endocrine therapy, although long-term treatment often generates resistance.[3] ER-negative breast cancers are hormone-refractory and show poorer prognoses than ER-positive cancers.[1] Because alternative treatment for hormone-refractory cancers remains to be explored, it is requisite to identify new therapeutic targets.

Recent cancer research has shown that RBP, which regulate RNA modification and quality, are important factors for cancer development and progression.[4] RBP recognize particular sequences and/or structures of RNAs through protein-RNA binding,[5] thus RBP-binding RNAs could be predicted by in silico analysis.[6] Regarding the DBHS family RBP, attention is being paid to NONO in tumorigenesis.[7,8] DBHS proteins, consisting of tandem RNA-binding domains, NOPS domain and coiled-coil domain, are known as components of nuclear paraspeckles and are associated with transcriptional regulation, splicing and nuclear export.[9-13] As a splicing factor, NONO binds and regulates RNA expression of glucose transporter 2 and glucokinase through modulating RNA maturation.[14] NONO and another DBHS protein PSF bind to the C-terminal domain of RNA polymerase II and modulate transcription and splicing.[15] NONO promotes gastric cancer proliferation and invasion by activating ETS-1 transcription.[7] We previously showed that NONO contributes to the splicing of androgen receptor RNA in prostate cancer.[8] In breast cancer, functional and specific RNA targets of NONO remain unknown.

In the present study, we investigated the clinical relevance and molecular mechanisms of NONO in breast cancer. Our clinicopathological study showed that high expression of NONO independently correlates with poor prognosis of breast cancer patients. We showed that NONO contributes to breast cancer cell proliferation and cell cycle promotion. Our comprehensive study identified specific NONO target mRNAs, which are responsible for its oncogenic functions. Thus, our findings show that NONO and NONO-interacting RNAs are important for breast cancer progression and could be potential therapeutic targets for cancer.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and clinical data

Clinical samples were obtained from 127 Japanese female breast cancer patients who underwent surgical treatment from 2006 to 2013 at Toranomon Hospital (Tokyo, Japan), and patients' ages ranged from 31 to 81 years. Clinical samples excluded those who used preoperative chemotherapy or targeted therapies. Treatment was carried out according to the guidelines of the National Comprehensive Cancer Network.[16] Staging was determined by the TNM Classification of Malignant Tumours.[17] Clinical outcome was evaluated based on distant disease-free and overall survival, the former was defined as the time span from the date of surgery to the first distant recurrence or to the last follow up. Mean follow-up duration was 81 months (ranging from 8 to 118 months). This study abides by the Declaration of Helsinki principles and was approved by the ethical committee of Toranomon Hospital (approval no. 1327) and Saitama Medical University International Medical Center institutional review board (approval no. 17-024).

2.2 | Antibodies

Rabbit monoclonal antibodies to ERα (clone: SP1), PgR (clone: 1E2), and ERBB2 (clone: 4B5) were purchased from Roche. Mouse monoclonal antibodies to NONO (clone: aa.368-471) and β-actin (clone: AC-74) were from BD Biosciences and Sigma-Aldrich, respectively.

2.3 | Immunohistochemistry analysis

Immunohistochemical analysis for NONO was carried out using EnVision + visualization kit (Dako) as described previously.[18] Briefly, 4-μm tissue sections were deparaffinized with xylene and hydrophilized stepwise with ethanol, followed by washing using TBST with 0.05% of Tween-20, then heated at 115°C for 5 minutes in 10 mM sodium citrate buffer (pH 6.0) for antigenic activation. NONO antibody (1:10 000 dilution) was applied to the sections and incubated overnight at 4°C. Mouse IgG was used as a negative control for the first antibody. After rinsing in TBST, sections were incubated with EnVision + HRP-labeled polymerase (Dako) for 1 hour at room temperature and then washed with TBST. DAB substrate kits (Vector Laboratories) were used for visualization. Encapsulation was carried out by MountQuick. Immunostained slides were evaluated for intensity and proportional scores. Intensity scores were rated from 0 to 2 (0: negative, 1: weakly positive, 2: positive) and proportional scores were calculated as the percentage of stained tumor cells. IR was calculated by multiplying the intensity scores and proportional scores with a scale ranging from 0 to 200.[19] Strong NONO IR was defined at a threshold ≥120.

2.4 | Statistical analyses

Statistical analyses were conducted using JMP 11.0.2 (SAS Institute) or Excel Statistics 2016 (add-in software for Microsoft Excel) (SSRI). Correlation between NONO IR and clinicopathological factors was evaluated using Student's *t* test or Pearson's chi-squared (χ^2) test. Distant disease-free and overall survival curves were generated by the Kaplan-Meier method, and statistical significance was done by log-rank test. Univariate and multivariate analyses were carried out by a logistic regression model and Cox

proportional hazard model, respectively. In in vitro experiments, statistical analyses were carried out using Student's *t* test.

2.5 | Bioinformatics

Kaplan-Meier curves of distant disease-free and overall survival for breast cancer patients were analyzed by Kaplan-Meier plotter software (KM-plotter, http://kmplot.com/analysis/). *NONO*, *SKP2*, and *E2F8* expression in breast cancer and normal breast samples was retrieved from The Cancer Genome Atlas (TCGA, https://www.cancer.gov/tcga) Breast Statistics in the Oncomine Platform (https://www.oncomine.org/), including 532 invasive breast carcinoma, 61 paired normal breast cancer and three paired metastatic samples. Candidate upregulated genes (>1.5-fold, $P < .05$) in breast cancer versus normal breast tissues were screened by Oncomine. Coexpression analysis was carried out using cBioPortal for Cancer Genomics (http://www.cbioportal.org/),[21,22] with METABRIC (n = 1904)[23] and TCGA (n = 816)[24] datasets.

2.6 | Cell culture

MCF-7 and MDA-MB-231 cells were purchased from ATCC and cultured in DMEM with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO_2. Cell authentication was confirmed by STR profiling.

2.7 | siRNAs

Stealth RNAi siRNA (HSS143135 as siNONO #A) and Silencer Select siRNA targeting NONO (S9613 as siNONO #B) were from Invitrogen. siRNAs against *SKP2* and *E2F8* were designed using Enhanced siDirect siRNA design algorithm provided by RNAi Inc and synthesized by Sigma-Aldrich. Negative control siRNA (siControl) with no homology to known gene targets in mammalian cells was from RNAi Inc. Sequences of siRNAs are listed in Table S1.

2.8 | Quantitative reverse transcription polymerase chain reaction

RNA was extracted from cells using ISOGEN (Nippon Gene) and single-stranded cDNA was synthesized using Super Script III reverse transcriptase (Invitrogen) with oligo dT or random primers. qRT-PCR was carried out on StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using KAPA SYBR FAST qPCR Kit (KAPA Biosystems) and sets of gene-specific primers. RNA levels were analyzed by the ΔΔCt method according to the manufacturer's protocol and normalized to the values of *GAPDH*. Student's *t* test was used for statistical analysis and $P < .05$ was considered statistically significant. Sequences of primers used in this study are listed in Table S2.

2.9 | Western blotting

Cells were seeded in a six-well plate at a concentration of 350 000 cells/well and simultaneously transfected with siRNAs targeting NONO (#A or #B) or siControl at a final concentration of 10 nM with Lipofectamine RNAi Max (Thermo Fisher Scientific). Transfected cells were lysed in 2 × SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS and 5% 2-mercaptoethanol) at 72 hours after transfection. Extracted proteins were separated with SDS-PAGE and blotted on PVDF membrane, followed by antibody reactions.[25]

2.10 | Cell proliferation assay

Cells were seeded at 2000 cells/well in 96-well plates and simultaneously transfected with indicated siRNAs (10 nM) using Lipofectamine RNAiMAX. Cells were collected 1, 3, and 5 days after siRNA transfection. To evaluate cell proliferation ability, extracted DNA was stained with Hoechst 33 258 pentahydrate (Thermo Fisher Scientific) at a final concentration of 5 μg/mL. DNA content in each well was measured by 2030 ARVO X Multilabel Plate Reader (Perkin Elmer) at 355 nm for 0.1 second.

Cell proliferation based on cell viability was also assessed by using a kit containing the tetrazolium dye MTT (Promega). Cells were seeded on 96-well plates at a density of 2000 cells/well and transfected with siRNAs targeting NONO or siControl by using Lipofectamine RNAiMAX transfection reagent (Invitrogen). At the indicated time points after the transfection, 10 μL of a reagent solution containing MTT was added to each well and the cells were incubated for 2 hours at 37°C. Absorbance of the plates was read on a microplate reader at a wavelength of 492 nm.

2.11 | Cell cycle analysis

Cells were transfected with siRNA at a final concentration of 10 nM and collected at 48-72 hours after transfection, then fixed with 70% ethanol for 1 hour. Fixed cells were incubated with 5 μg/mL PI (Sigma-Aldrich), following RNase A treatment. DNA contents in PI-labeled cells were analyzed with BD FACSCalibur (BD Biosciences).

2.12 | Annexin V and PI staining

Cells were transfected with siRNA at a final concentration of 10 nM and collected at 72 hours after transfection. Apoptotic cells were stained with FITC Annexin V apoptosis detection kit (Becton Dickinson) following the manufacturer's instruction.[26] Annexin V- and PI-positive cells were analyzed with BD FACSCalibur.

2.13 | Microarray and pathway analysis

GeneChip Human Gene 1.0 ST Array from Affymetrix was used for expression microarray analysis according to the manufacturer's

protocol. Data were analyzed using Affymetrix Microarray Suite software. All microarray data are available in the GEO database with the accession numbers, GSE132742 and GSE132743. Pathway analysis was carried out using GSEA (http://software.broadinstitute.org/gsea) with hallmark gene sets and GO analysis with biological processes GO terms (https://david.ncifcrf.gov/).

2.14 | RNA immunoprecipitation-sequencing

RNA immunoprecipitation was carried out using EZ-magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's protocol. Cell nuclei were lysed using lysis buffer. Nuclear lysates were diluted and incubated with NONO antibody or IgG magnetic beads at 4°C for 3 hours. Library construction was carried out using SureSelect Strand-Specific RNA system from Agilent Technologies. Sequencing was done by HiSeq 2500 (Illumina). To remove ribosomal RNA sequence, we used Bowtie 2 version 2.2.6. Mapping to human genome (hg19) was done using TopHat 2.2.0 with Bowtie. Alignments were generated in SAM format from given single-end reads. Read tags were mapped to human RefSeq mRNA or to the GENECODE/NONCODE database. Expression levels of mapped transcripts were normalized into RPKM. Fisher's exact test was carried out to statistically determine the difference between NONO-immunoprecipitated and input samples. RIP-seq data were submitted to the GEO database with GEO accession number GSE133423.

2.15 | RNA immunoprecipitation assay

Cells were scraped from 10-cm dishes and collected into 1.5-mL tubes, and were then lysed with RIP buffer (150 mM KCl, 25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.5% NP40) followed by incubation with 1 μg NONO antibody for 3 hours at 4°C with rotation. NONO-RNA complexes were precipitated by Protein G Sepharose 4 Fast Flow beads (GE Healthcare). Beads were washed four times with RIP buffer and remaining RNAs were isolated with ISOGEN reagent.

3 | RESULTS

3.1 | NONO is a poor prognostic factor for breast cancer patients

To clarify the clinical significance of NONO in breast cancer, we carried out immunohistochemical analysis of NONO in 127 primary breast cancer tissue samples from patients. Among 127 cases, 31 and 96 patients had tumors with strong (Figure 1A) and weak NONO IR (Figure 1B), respectively. Normal breast ducts basically show weak NONO IR (Figure 1C). Student's *t* test or Pearson's χ^2 test analysis between NONO IR and clinicopathological factors showed that NONO IR status positively correlated with nuclear grade (P = .005) and negatively correlated with ER (P = .015) and PgR (P = .003) status (Table S3).

To examine whether NONO could be a prognostic factor for breast cancer patients, we next analyzed the relationship between NONO IR and patient survival. Kaplan-Meier method-based survival analyses showed that NONO IR was significantly associated with a poorer distant disease-free survival (Figure 1D) and a shorter overall survival of patients (Figure 1E). Furthermore, univariate and multivariate analyses of distant disease-free and overall survival showed that NONO IR was an independent prognostic factor for breast cancer patients (Table S4). Moreover, high *NONO* mRNA levels were associated with poorer distant disease-free survival of breast cancer patients compared with low *NONO* levels in whole subtypes (n = 3951) and in the ER-positive subtype (n = 2061), but not in the TNBC subtype (Figure S1A–C). TCGA breast cancer dataset in Oncomine Platform showed that *NONO* mRNA expression was increased in IDC samples when compared with normal breast samples (Breast) (Figure 1F).

3.2 | NONO knockdown represses breast cancer cell proliferation

As high *NONO* expression was significantly associated with poorer prognosis of breast cancer patients, we next assessed whether NONO contributes to breast cancer cell proliferation. Transfection of two distinct *NONO*-targeting siRNAs could significantly silence *NONO* levels in ER-positive MCF-7 cells (Figure 2A,B) and ER-negative MDA-MB-231 cells (Figure 2C,D) at both RNA and protein levels. NONO knockdown significantly impaired MCF-7 and MDA-MB-231 cell proliferation (Figure 2E,F, S2A,B). Cell cycle analysis showed that NONO knockdown decreased percentages of S-phase population in both cell types (Figure 2G,H). However, percentages of apoptotic cell populations in MCF-7 and MDA-MB-231 cells were not significantly altered by knockdown of NONO (Figure S3).

3.3 | NONO regulates cell proliferation-related pathways

To further clarify the biological function of NONO in breast cancer, we defined global gene expression alterations by NONO knockdown. Expression microarray analysis showed that 165 genes were downregulated (≤0.7-fold) by NONO knockdown in both MCF-7 and MDA-MB-231 cells, whereas 40 genes were upregulated (≥1.5-fold) in both cell types (Figure 3A,B). In pathway analysis by GSEA (Figure S4), the E2F pathway showed the strongest association with NONO knockdown in a highest normalized enrichment score (NES) value in both MCF-7 (Figure 3C,D) and MDA-MB-231 (Figure 3E,F) cells, and 121 and 68 core enrichment-associated E2F pathway genes were identified in MCF-7 and MDA-MB-231 cells, respectively (Table S5).

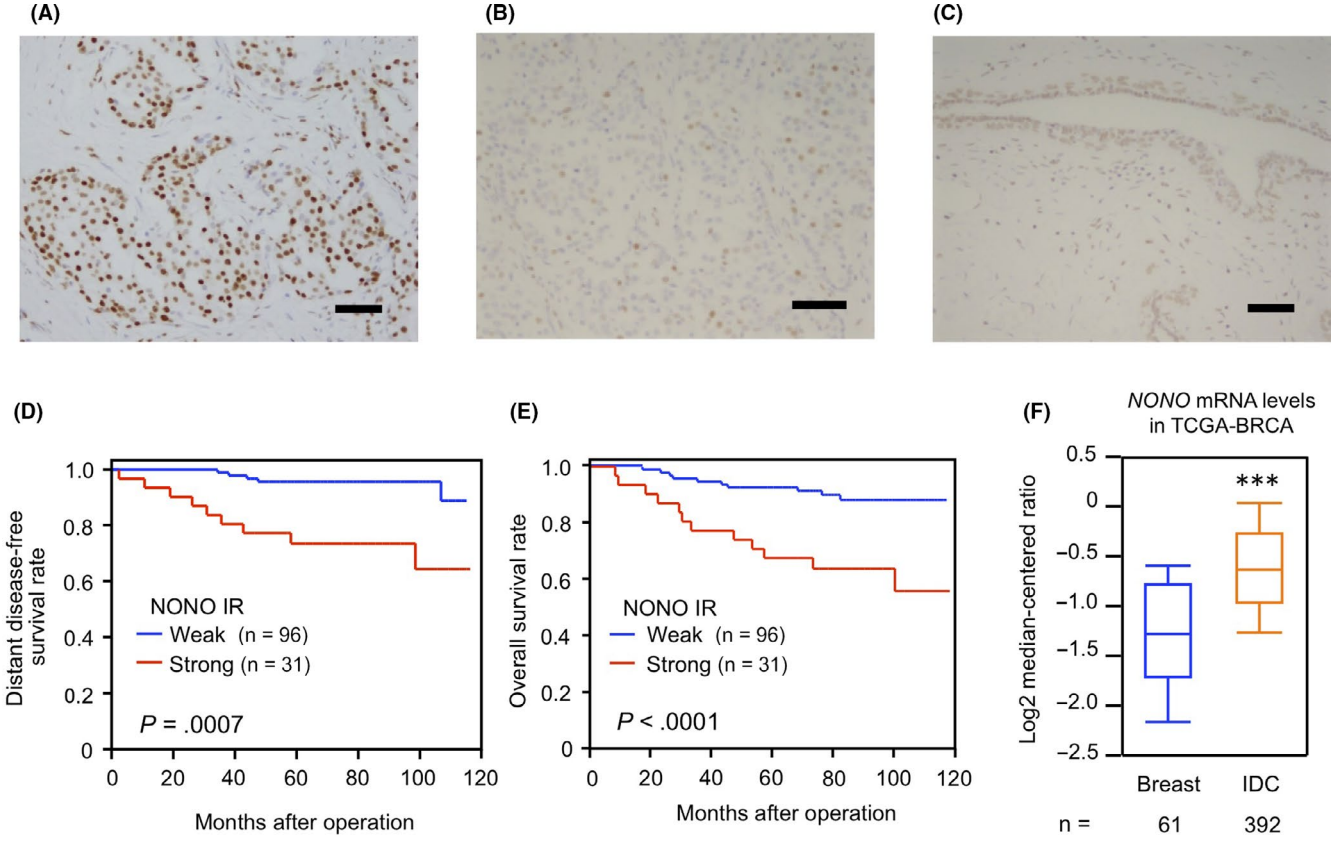

FIGURE 1 Non-POU domain-containing octamer binding (NONO) is a poor prognostic factor for breast cancer patients. A-C, Representative micrographs for breast cancer tissue sections with strong and weak NONO immunoreactivity (IR) (A and B, respectively) and benign breast ducts (C). Bar represents 100 μm. D, E, Distant disease-free survival (D) and overall survival (E) of breast cancer patients with strong or weak NONO IR were analyzed by Kaplan-Meier method. Red and blue lines represent strong (n = 31) and weak (n = 96) NONO IR cases, respectively. Statistical significance was evaluated by log-rank test. F, Comparison of *NONO* mRNA levels (Probe ID: A_24_P413437) in breast cancer and normal breast samples in The Cancer Genome Atlas (TCGA) breast cancer dataset based on Oncomine Platform. Breast, n = 61; invasive ductal breast cancer (IDC), n = 392. ***, $P < .001$

3.4 | NONO regulates cell proliferation-related genes by binding their RNAs

As NONO was reported to control splicing through binding target RNAs,[15] we carried out RIP-seq to identify NONO-associated RNAs in breast cancer cells (Figure 4A). RIP-seq identified 3698 and 3072 NONO-associated RNAs in MCF-7 and MDA-MB-231 cells, respectively, and 1444 RNAs were determined as common NONO-associated RNAs in both cell lines (fold enrichment >2, $P < 1e\text{-}5$) (Figure 4A).

Gene ontology-based pathway analysis showed that the GO term "G1/S transition of mitotic cell cycle" was the most significantly correlated pathway with 1444 common NONO-associated RNAs in both cells (Figure 4A). MCF-7 cell-specific NONO-associated RNAs (n = 2254) were predominantly correlated with the GO term "transcription" in addition to "cell cycle" (Figure S5A). In contrast, "phosphate metabolic process" was the most significantly correlated pathway among MDA-MB-231 cell-specific NONO-associated RNAs (n = 1628) (Figure S5B).

We further dissected candidate NONO target RNAs through integrated analysis of RIP-seq and microarray datasets. We dissected 452 and 348 NONO target RNAs, which are RIP-seq-determined NONO-associated RNAs that were shown as downregulated genes (<0.8-fold) by NONO knockdown in microarray analysis for MCF-7 (Figure 4B) and MDA-MB-231 cells (Figure 4C), respectively. GO-based pathway analysis showed that cell proliferation-related pathways were commonly enriched in the NONO-regulated RNAs in both cell lines (Figure 4B,C).

3.5 | NONO modulates SKP2 and E2F8 gene expression at post-transcriptional level

Our integrated analysis (Figure 4) suggested that NONO particularly regulates cell proliferation-regulated gene expression in ER-positive MCF-7 and TNBC MDA-MB-231 cells. To dissect common NONO targets in ER-positive and -negative breast cancer cells, we extracted 78 RNAs from NONO target RNAs in both MCF-7 and MDA-MB-231 cells (Figure S6A). GO-based pathway analyses showed that 78 candidate common NONO target RNAs were associated with cell proliferation-related pathways such as the GO term "DNA-dependent DNA replication" (Figure S6B). Similar to the 78 candidate common NONO

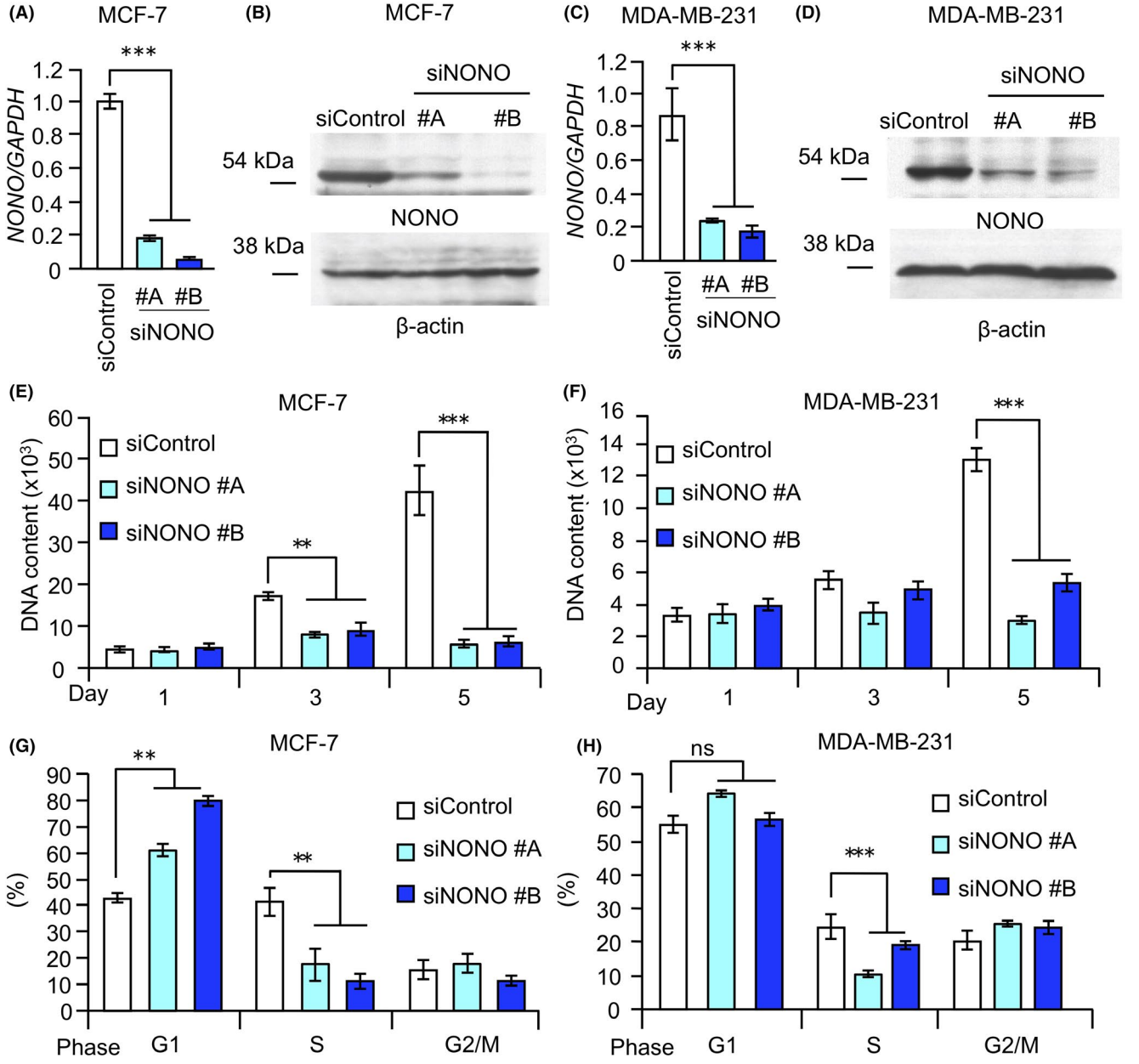

FIGURE 2 Non-POU domain-containing octamer binding (NONO) associates with breast cancer cell proliferation. A, C, Knockdown efficiency of siRNAs targeting NONO (siNONO #A and #B) in MCF-7 (A) and MDA-MB-231 cells (C) analyzed by qRT-PCR. Relative *NONO* mRNA levels were determined by normalizing to *GAPDH* mRNA levels and presented as mean fold change ± SD to siControl in each cell type (n = 3). B, D, Knockdown effects of siNONO in MCF-7 (B) and MDA-MB-231 cells (D) analyzed by western blotting. β-Actin was used as a loading control. E, F, Inhibitory effects of siNONO in MCF-7 (E) and MDA-MB-231 (F) cell proliferation analyzed by DNA assay. Results are shown as mean ± SD (n = 5). G, H, Cell cycle profiles of MCF-7 (G) and MDA-MB-231 (H) cells treated with indicated siRNAs analyzed by flow cytometry. Data indicate percentages of cell population in G1, S, G2/M phases (n = 3). **, $P < .01$; ***, $P < .001$; ns, not significant

target RNAs, 374 candidate MCF-7 cell-specific NONO target RNAs also correlated with cell proliferation-related pathways such as "cell cycle" (Figure S6C). Alternatively, "phosphorus metabolic process" and "negative regulation of apoptosis" pathways significantly correlated with 270 candidate MDA-MB-231 cell-specific NONO target RNAs (Figure S6D). Based on the statistical analysis by cBioPortal for Cancer Genomics,[21,22] we further selected 10 and 14 *NONO*-coexpressed RNAs in METABRIC (n = 1904)[23] and TCGA (n = 816)[24] breast cancer datasets, respectively (Spearman's correlation coefficient >0.2 and >0.3 for METABRIC and TCGA, respectively) (Figure S6A,E-H). We further focused on eight common *NONO*-coexpressed RNAs in both datasets. NONO knockdown-mediated mRNA downregulation was observed for four of eight genes in both MCF-7 and MDA-MB-231 cells, including *SKP2*, *E2F8*, *CCNE2*, and *TMPO* (Figure 5A,B, S7A,B). To analyze whether NONO contributes to post-transcriptional regulation of the four genes, we examined the expression of introns, which are involved in premature RNAs (pre-mRNAs). Intron levels of *SKP2* and *E2F8* were not significantly altered by NONO knockdown in both

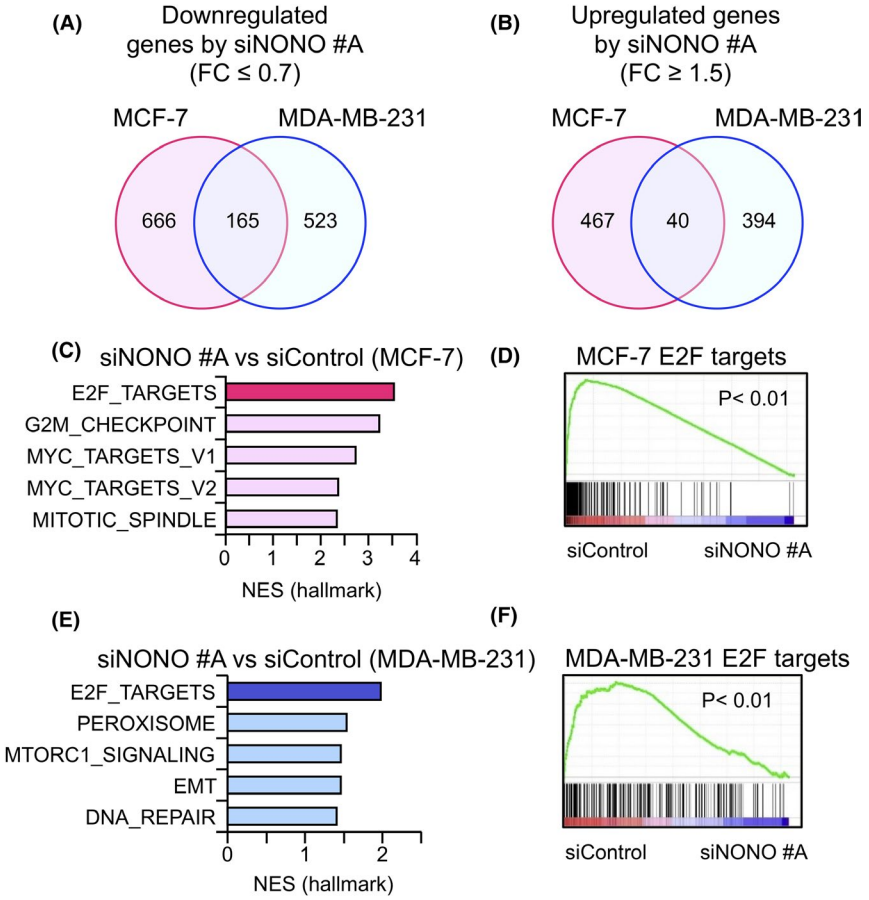

FIGURE 3 Non-POU domain-containing octamer binding (NONO) regulates cell proliferation-associated pathways. A, B, MCF-7 and MDA-MB-231 cells were transfected with NONO-targeting siRNA or control siRNA (siControl) for 48 h and subjected to expression microarray analysis. Venn diagrams indicate numbers of downregulated genes (A) by siNONO #A vs siControl (fold change [FC] ≤0.7) and upregulated genes (B) by siNONO #A vs siControl (FC ≥1.5) in each cell line. C, E, Top 5 NONO knockdown-associated signaling pathways analyzed by Gene Set Enrichment Analysis (GSEA) in MCF-7 cells (C) and MDA-MB-231 cells (E). EMT, epithelial-mesenchymal transformation; NES, normalized enrichment score. D, F, GSEA-determined enrichment plots for E2F target pathway in MCF-7 (D) and MDA-MB-231 (F) cells are shown

MCF-7 and MDA-MB-231 cells (Figure 5C,D), indicating that NONO interacts with *SKP2* and *E2F8* RNAs and regulates these expressions at post-transcriptional level. In contrast, NONO knockdown suppressed the expression of *CCNE2* (Figure S7C,D) and *TMPO* pre-mRNAs (Figure S7E,F), suggesting that NONO regulates the expression of *CCNE2* and *TMPO* at transcriptional level. RIP assay showed that NONO protein could significantly interact with *SKP2* and *E2F8* RNAs (Figure S7G-J).

3.6 | NONO-targeted SKP2 and E2F8 are key regulators for breast cancer cell proliferation

Finally, we assessed whether NONO/*SKP2* and NONO/*E2F8* axes contribute to breast cancer cell proliferation. Similar to *NONO* expression in breast cancers (Figure 1F), Oncomine-based expression analysis showed that *SKP2* and *E2F8* mRNAs were upregulated in IDC compared with normal breast tissue samples in TCGA breast cancer cohort (Figure 5E,F). KM-plotter breast cancer dataset showed that *SKP2* and *E2F8* were poorer prognostic factors for patients of all subtypes of breast cancer (Figure 5G,H) as well as ER-positive breast cancers (Figure S8A,B). *SKP2*- and *E2F8*-targeting siRNAs significantly suppressed RNA levels of *SKP2* and *E2F8*, respectively, in both MCF-7 (Figure 6A,B) and MDA-MB-231 (Figure 6C,D). Both *SKP2* and *E2F8* knockdown by siRNA significantly impaired MCF-7 (Figure 6E) and MDA-MB-231 (Figure 6F) cell proliferation.

Taken together, the results show that NONO plays a critical role in breast cancer cell proliferation by regulating cell proliferation-related genes *SKP2* and *E2F8* at the post-transcriptional level (Figure 6G).

4 | DISCUSSION

In the present study, immunohistochemistry-based prognostic analysis showed that strong NONO IR is a clinicopathological independent prognostic factor for breast cancer patients. NONO knockdown suppressed both ER-positive MCF-7 and ER-negative MDA-MB-231 cell proliferation. Microarray and pathway analyses suggested that NONO significantly associates with the cell proliferation-related pathway in both MCF-7 and MDA-MB-231 cells. RIP-seq showed that NONO interacts preferentially with cell proliferation-related RNAs and regulates their expression. Through integrated analysis of microarray and RIP-seq, together with the clinical database, we identified *SKP2* and *E2F8* as candidate NONO target RNAs. NONO suppression downregulated *SKP2* and *E2F8* mRNA levels but not their pre-mRNA levels. *SKP2* and *E2F8* are abundantly expressed in breast cancer, and their knockdown could attenuate MCF-7 and MDA-MB-231 cell proliferation. These results suggest that NONO modulates the expression of multiple cell proliferation-related genes at post-transcriptional level

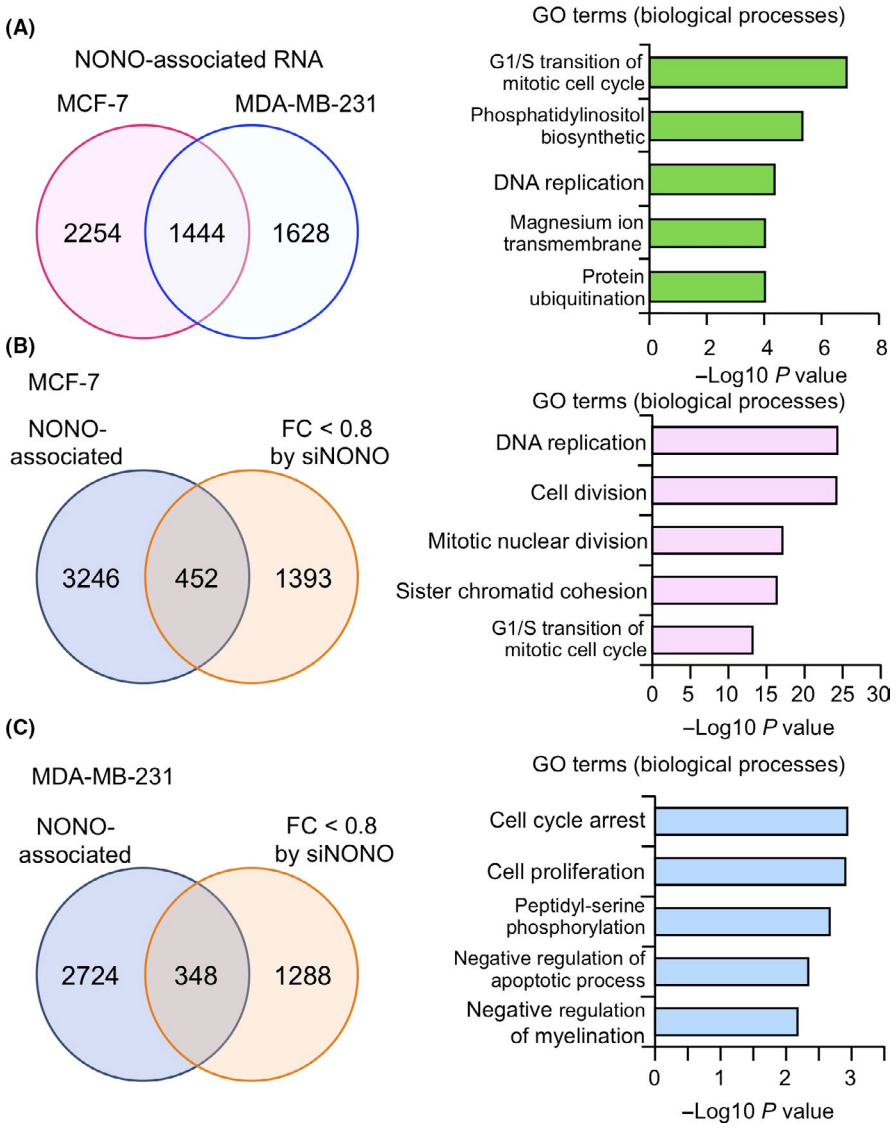

FIGURE 4 Non-POU domain-containing octamer binding (NONO) regulates cell proliferation-associated gene expression through RNA binding. A, Left: Summary of NONO-associated RNAs in MCF-7 and MDA-MB-231 cells. NONO-associated RNAs were determined by RNA immunoprecipitation-sequencing (RIP-seq) by precipitation with NONO antibody. Right: Pathway analysis of NONO-associated RNAs in MCF-7 and MDA-MB-231 cells was carried out based on Gene Ontology (GO). Top five associated GO terms and *P*-values are shown. B,C, Results of integrated analyses of microarray and RIP-seq in MCF-7 (B) and MDA-MB-231 (C) cells. Left: Left circle indicates NONO-associated RNAs from RIP-seq and right circle indicates downregulated genes by siNONO #A compared with siControl in microarray analysis. RNAs in merged fraction for right and left circles are designated as NONO target RNAs. Right: Top five pathways among NONO target RNAs were determined by GO analysis

and promotes breast cancer progression regardless of hormone dependency.

Our IHC-based prognostic analysis showed that high NONO protein expression was significantly associated with shorter overall and distant disease-free survival (Figure 1D,E) of breast cancer patients. Based on Kaplan-Meier plotter software-based analysis, a significant association was not shown between *NONO* high expression and poorer survival of TNBC patients (n = 255) in the dataset (Figure S1C). Nevertheless, it was observed that TNBC patients with *NONO* high expression have a lower survival rate compared to those with NONO low expression. Because the Kaplan-Meier plotter analysis was based on *NONO* mRNA expression, it still remains a possibility that NONO protein expression positively correlates with poor prognosis of TNBC patients. In terms of the interaction of NONO with other RBP, NONO exerts its function in TNBC cells by forming heterodimers with another DBHS family RBP PSF.[27] Therefore, future clinicopathological study will define the clinical relevance of NONO and its binding RBP partners in each breast cancer subtype.

NONO has been reported to interact with sterol regulatory element-binding protein-1A transcription factor, and to associate with MCF-7 cell proliferation,[28] although whether NONO contributes to ER-negative breast cancer progression remains to be clarified. In the present study, we showed that NONO knockdown impaired both MCF-7 and MDA-MB-231 cell proliferation, suggesting that NONO could be involved in common molecular mechanisms underlying the progression of both ER-positive and -negative breast cancers.

The present study showed that NONO interacts with its target RNAs and modulates their expression at post-transcriptional level, especially in the pre-mRNA splicing step. For instance, Benegiamo *et al*[14] reported that NONO interacts with metabolic enzyme RNAs in mouse liver cells. In human fetal adrenal H295R cells, NONO has been shown to regulate the splicing and degradation of phosphodiesterase mRNAs and to modulate cyclic AMP-dependent glucocorticoid biosynthesis.[29] In our RIP-seq analysis, we also observed significant interaction between NONO and phosphodiesterase *PDE3B* RNA in both MCF-7

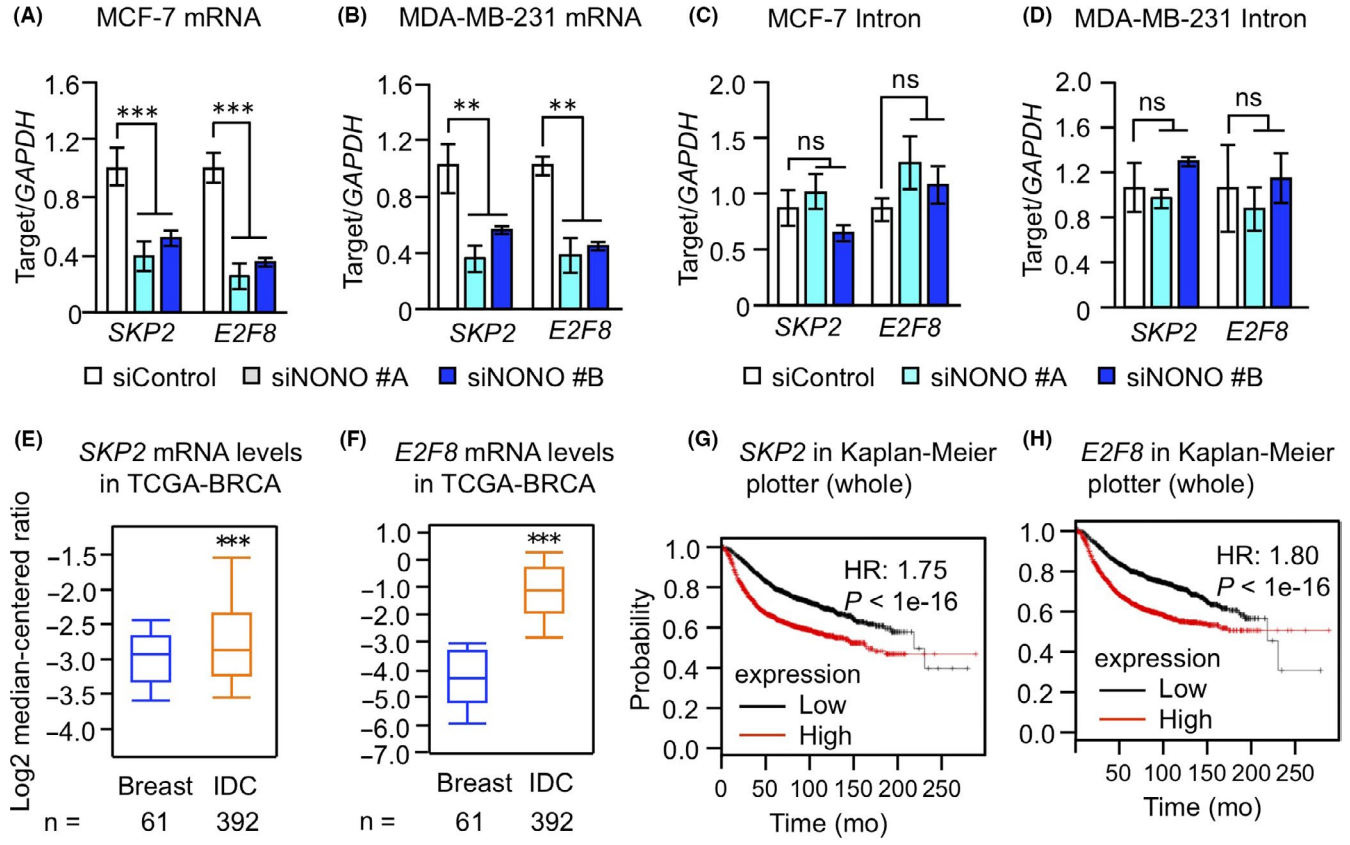

FIGURE 5 Non-POU domain-containing octamer binding (NONO) modulates *SKP2* and *E2F8* expression at the post-transcriptional level. A, B, Effects of NONO knockdown on *SKP2* and *E2F8* mRNA levels in MCF-7 (A) and MDA-MB-231 (B) cells. Data are normalized to *GAPDH* levels and presented as mean fold change ± SD vs siControl in each cell type (n = 3). C, D, Effects of NONO knockdown on *SKP2* and *E2F8* intron levels in MCF-7 (C) and MDA-MB-231 (D) cells. Data are normalized to *GAPDH* levels and presented as mean fold change ± SD vs siControl in each cell type (n = 3). E, F, Comparison of expression levels of *SKP2* (Probe ID: A_23_P156309) (E) and *E2F8* (Probe ID: A_23_P35871) (F) mRNAs in The Cancer Genome Atlas (TCGA) breast cancer and normal breast samples analyzed by Oncomine Platform software. Breast, n = 61; invasive ductal breast cancer (IDC), n = 392. G, H, Relapse-free survival curves analyzed by KM-plotter.[40] *SKP2* (Probe ID: 203625_x_at) (G) and *E2F8* (Probe ID:219990_at) (H) expression data are retrieved from 3951 breast cancer patients, respectively. *P*-value and hazard ratio (HR) are shown. **, $P < .01$; ***, $P < .001$; ns, not significant

and MDA-MB-231 cells (NONO-immunoprecipitated vs IgG-immunoprecipitated *PDE3B* expression was 11.31- and 2.81-fold in MCF-7 and MDA-MB-231 cells, respectively). In terms of some long non-coding RNAs (lncRNAs) previously identified as NONO interactors,[7,10,27,30] however, we have not observed substantial interactions in the present study. Presumably, the interactions between NONO and lncRNAs could be altered by individual cellular context, including species, tissue specificity, cellular conditions, and basal RNA expressions. In this study, we carried out microarray and RIP-seq analysis to dissect RNAs that could be targeted by NONO in breast cancer cells. Importantly, the pathway analysis based on both microarray and RIP-seq studies showed that cell proliferation-related pathways were particularly enriched in both MCF-7 and MDA-MB-231 cells. Although we assessed the effect of NONO knockdown on apoptotic cell death in breast cancer cells, siNONO did not substantially alter apoptotic cell profiles in both MCF-7 and MDA-MB-231 cells (Figure S3). In addition, pathway analysis based on microarray results in Figure 3 showed that NONO is well associated with cell proliferation pathways but not with apoptotic pathways, suggesting that NONO is a key modulator for breast cancer cell proliferation, and promotes cell proliferation mainly through accelerating cell cycle progression.

We also analyzed individual NONO targets in ER-positive MCF-7 and ER-negative MDA-MB-231 cells (Figure 4). MCF-7 cell-specific NONO target RNAs (Figure S6A) particularly associated with cell proliferation-related pathways (Figure S6C). Alternatively, MDA-MB-231 cell-specific target RNAs substantially associated with "phosphorus metabolic processes" (Figure S6D). Several genes, such as *thrombospondin 1*, *protein tyrosine phosphatases non-receptor type* and *protein kinase X-linked* in the "phosphorus metabolic processes" term have been reported to be involved in tumor progression of TNBC.[31-33] In addition, MDA-MB-231 cell-specific NONO target RNAs were also associated with apoptotic pathways (Figure S6D). Thus, we speculated that suppression of apoptosis may also contribute to the growth of ER-negative breast cancer cells. In the present study, we focused on NONO target RNAs in breast cancer cells regardless of ER status. Further

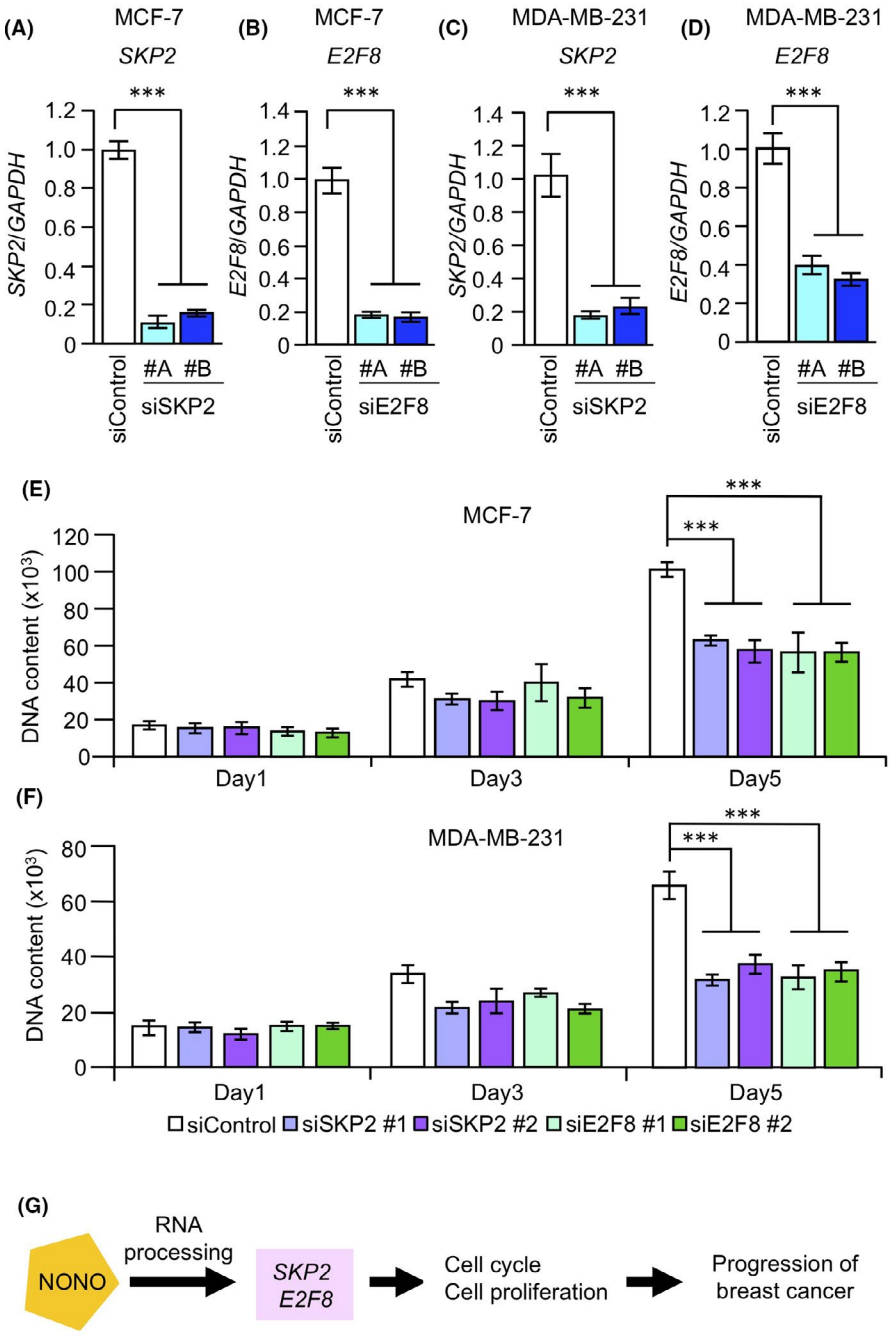

FIGURE 6 Non-POU domain-containing octamer binding (NONO)-targeted SKP2 and E2F8 are key regulators for breast cancer cell proliferation. A-D, Knockdown efficiency of siRNAs targeting SKP2 (A and C) and E2F8 (B and D) in MCF-7 (A and B) and MDA-MB-231 cells (C and D) analyzed by qRT-PCR. Relative *SKP2* or *E2F8* mRNA levels were normalized to *GAPDH* levels and presented as mean fold change ± SD to siControl in each cell type (n = 3). E, F, Inhibitory effects of indicated siRNAs on MCF-7 (E) and MDA-MB-231 (F) cell proliferation analyzed by DNA assay. Results are shown as mean ± SD (n = 5). (G) Working model of NONO in the proliferation and progression of breast cancer cells. ***, $P < .001$

studies will elucidate NONO-mediated post-transcriptional functions in TNBC cells.

Our integrated analysis identified *SKP2* and *E2F8* RNAs as prototypic NONO targets in breast cancer cells. SKP2 is an E3 ubiquitin ligase that promotes the degradation of tumor suppressor p27 and leads to cell proliferation.[34] SKP2 dysregulation was observed in several cancers including breast cancer. For instance, SKP2 is highly expressed in small cell and non-small cell lung cancers, and SKP2 suppression induces apoptosis and inhibits cell invasion.[35,36] SKP2 pharmacological inhibition also showed strong antitumor activity in lung and prostate cancers. In breast cancer, SKP2 promotes cell proliferation by suppressing the expression of PDCD4 protein, a tumor suppressor that plays a role in apoptosis and DNA damage response.[37] E2F8 belongs to the E2F family and is known as a DNA damage response factor.[38] In breast cancer cells, E2F8 overexpression is associated with chemotherapy resistance.[38] E2F8 was also reported to regulate *CCNE2* expression, leading to breast cancer proliferation.[39,40] We found that NONO knockdown suppresses *CCNE2* mRNA expression, suggesting that the NONO/E2F8 axis results in CCNE2 overexpression and breast cancer progression.

Taken together, our results show that NONO plays a critical role in breast cancer cell proliferation by regulating *SKP2* and *E2F8* RNA expression at the post-transcriptional level. NONO could be a new class of promising diagnostic and therapeutic targets for breast cancers, particularly for advanced tumors with high proliferative activity.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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