NONO Is a Negative Regulator of SOX2 Promoter

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Abstract. *Background/Aim:* Sex determining region Y (SRY)-box 2 (SOX2) is a transcription factor essential for the maintenance of proliferation and self-renewal of cancer stem cells and is associated with breast cancer initiation. Regulation of cancer stem cell plasticity by SOX2 requires both positive and negative SOX2 transcription factors, but the negative regulator is still largely unknown. *Materials and Methods:* SOX2 promoter-binding proteins were identified by liquid chromatography–mass spectrometry/mass spectrometry, luciferase assay, and chromatin immunoprecipitation. The effects of one such transcription factor on SOX2 expression was investigated by knockdown and overexpression experiments. *Results:* Non-POU domain-containing octamer-binding protein (NONO) (also known as 54-kDa nuclear RNA-binding protein, P54NRB) was identified as a SOX2 promoter-binding protein and a negative regulator of SOX2 expression. Its activity was controlled by its coiled-coil domain and the C-terminal domain. *Conclusion:* These results suggest that NONO acts as a key regulator of SOX2 transcription through the repression of SOX2 promoter activity in breast cancer cells.

Sex-determining region Y-box 2 (SOX2) is a transcription factor that is associated with tumor malignancy of glioma cells, ovarian cancer, head and neck cancer, and breast cancer (1). In breast cancer, the expression level of SOX2 is associated with tumor size and the number of metastatic lymph nodes (2). SOX2 overexpression also enhances tumor-initiating activity in breast cancer cell lines (3). These reports suggest that SOX2 is an important factor for maintenance of cancer stem cells (CSCs).

Recently, it was pointed out that CSCs have plasticity and may reversibly change their behavior between non-CSCs and CSCs (4). Therefore, if the properties of CSCs are controlled by SOX2, SOX2 expression must not only be induced but also suppressed. In many cancer types, SOX2 mRNA expression is mainly controlled by its promoter (5, 6), and nuclear transcription factor Y subunit alpha, transcription adaptor putative zinc finger, and glioma associated oncogene family zinc finger 1 have been reported as enhancers of the SOX2 promoter (7-9). However, the negative regulators of SOX2 transcriptional are largely unknown in breast cancer.

While non-pituitary-specific factor, octamer transcription factor, neural un-coordinated-86 (POU) domain-containing octamer-binding protein (NONO; previously known as 54-kDa nuclear RNA-binding protein/P54NRB) is an RNA splicing factor, it also binds to DNA using a POU-like element and regulates transcription through a coiled-coil domain (10). NONO interacts with SOX9 and induces transcription of collagen, type II, alpha 1 gene, which is a differentiation marker of chondrocytes, by binding to the promoter (11). NONO also induces transcription of sterol regulatory element-binding protein-1a in breast cancer (12). On the other hand, NONO acts as a transcriptional repressor

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for the connexin 43 gene (13) and cyclooxygenase-2 (COX2) (14). Characteristically, NONO is highly expressed in estrogen-receptor-positive breast cancer (15) but pathophysiological roles of NONO in human breast cancer are largely unknown. In this study, we investigated the molecular mechanisms regulating *SOX2* expression in breast cancer cell lines.

Materials and Methods

Cell lines. Human breast cancer cell lines, MCF-7 and T-47D were obtained from American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in Dulbecco's modified Eagle medium/Ham's Nutrient Mixture F-12 (DMEM/F-12) (Wako Pure Chemical, Osaka, Japan) supplemented with heat-inactivated 10% fetal bovine serum (Cambrex, East Rutherford, NJ, USA), 100 units/ml penicillin and 100 µg/ml streptomycin sulfate (both from Invitrogen, Carlsbad, CA, USA), and maintained at 37°C with 5% CO₂.

Identification of the region essential for transcriptional activity in the *SOX2* promoter. To identify *SOX2* promoter essential region, luciferase assay was used. The human *SOX2* promoter region was amplified from genomic DNA of MCF-7 cells (5). It was amplified using KOD-Plus-Neo DNA polymerase with proof reading activity (TOYOBO, Osaka, Japan) and 0.3 µM of specific primers. The sequences of the primers used were: P789 forward: 5'-GGTACCGGCCAAAGAGCTGAGTTGG-3', P629 forward: 5'-GGTACCAACTTCTAGTCGGGACTGTG-3', P467 forward: 5'-GGTACCCTGGCTGTTTCCAGAAATAC-3', P227 forward: 5'-GGTACCCTCAGTGGCTGGCAGGC-3', P68 forward: 5'-GGTACCGCTGATTGGTCGCTAGAAAC-3'; reverse: 5'-AAGCTTGAGGCAAACCTGGAATCAGGATC-3'. Thermal cycling procedure consisted of an initial denaturation for 2 min at 94°C. This was followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55-58°C for 30 s and extension at 68°C for 30 s. A-Tailing was then carried out by incubating 5.9 µl of the polymerase chain reaction (PCR) products with 1 µl of GoTaq polymerase (Promega, Madison, WI, USA), 2 µl of 5 × Go Taq reaction buffer, 0.6 µl of 2.5 mM MgCl₂, and 0.5 µl of 4 mM dATP for 30 min at 70°C. The A-tailed PCR products were then ligated into pGEM-T easy vectors (Promega), which were further digested with *KpnI* and *HindIII* (TAKARA Bio, Shiga, Japan) enzymes. Fragments obtained from the restriction digestion were inserted into pGL3-Basic luciferase vector (Promega). An electroporator was used to co-transfect 1 µg of generated vectors and 25 ng of the internal control, pRL-TK vector (Promega) into 5×10⁵ cells with or without 0.5 µg of complementary DNA (cDNA) expression vectors (CUY21SC; NepaGene, Ichikawa, Japan). Transfected cells were seeded into one well. Forty-eight hours after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with a luminometer MiniLumat (Berthold, Bad Wildbad, Germany). The results were normalized by dividing the luciferase reporter activity by that of the Renilla reporter, which were then statistically compared among the groups using Mann-Whitney *U*-test.

Biotin-streptavidin pulldown assay. To isolate *SOX2* promoter activity regulatory factors, biotin-streptavidin pulldown assay was performed. Nuclear fractions (100 µl) were prepared from 2×10⁶ MCF-7 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagent following the manufacturer's instructions (Thermo Fisher Scientific, Pittsburg, PA,

USA). Subsequently, 100 µl of the nuclear fraction was incubated with 17 µl of Streptavidin Mag Sepharose (GE Healthcare Bioscience, Pittsburgh, PA, USA) at 4°C for 2 h in 1 ml of binding buffer [20% glycerol, 20 mM Tris pH 7.5, 100 mM KCl, 1 mM dithiothreitol (DTT), 20 µg/ml bovine serum albumin (BSA), 2 mM EDTA] on a shaking incubator. After incubation, the mixtures were centrifuged at 5,000 × *g* for 1 min at 4°C. Supernatants were transferred to a fresh tube as the nuclear fractions were removed by avidin binding protein. Mag Sepharose-bound complex was washed twice with 100 µl of binding buffer without BSA and washed buffers were stored at -80°C. Human *SOX2* promoter-derived P227 and P68 regions were then amplified from MCF-7 genomic DNA using KOD Plus-Neo with biotin-labeled primers for P227 (forward, 5'-biotin-CTCAGTGGCTGGCAGGCTGG-3'), P68 (forward: 5'-biotin-GCTGATTGGTCGCTAGAAACC-3'), and reverse: 5'-GAGGCAAACCTGGAATCAGGATC-3'. The nuclear fractions bound to avidin binding protein were incubated with 50 µl of the biotin-labeled PCR products at 22°C for 4 h. The biotin-labeled DNA-nuclear protein complex was incubated with 17 µl of Streptavidin Mag Sepharose at 4°C for 1 hour and washed twice before it was re-suspended in Laemmli sample buffer with 5% (v/v) β-mercaptoethanol. To break the streptavidin-biotin interaction, 20 µl of the re-suspended protein complex was heated at 98°C before it was separated on Mini-PROTEAN® TGX™ Precast Gels (BIO-RAD, Hercules, CA, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were then stained using SilverQuest™ Silver Staining Kit (Invitrogen), and specific bands were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis.

In-gel digestion. To purify *SOX2* promoter-binding proteins from SDS-PAGE, in-gel digestion was performed. The SDS-PAGE gel was rinsed twice with 15 mM potassium ferricyanide and 50 mM sodium thiosulfate solution for 15 min to destain it. The gel was then rinsed twice with ultrapure water and the sample in the gel piece was reduced twice in a solution containing 50% acetonitrile (ACN), 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel piece was dehydrated in 100% ACN twice for 30 min each, and then rehydrated with an in-gel digestion reagent containing 10 µg/ml Sequencing-Grade Modified Trypsin (Promega) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. This procedure for in-gel digestion was performed overnight at 30°C. After digestion, the samples were lyophilized overnight using Labconco Lyph-Lock 1L Model 77400 (Labconco, Kansas, MO, USA). Lyophilized samples were then dissolved in 0.1% formic acid.

LC-MS/MS analysis. To identify *SOX2* promoter-binding proteins, LC-MS/MS analysis was performed. Twenty-five microliters of each sample were separated on a Zorbax 300SB-C18 column (75 µm × 150 mm; Agilent Technologies, Palo Alto, CA, USA). The capillary pump of Agilent 1100 LC/MSD Trap XCT (Agilent Technologies) was operated under the following conditions: solvent A: 0.1% formic acid, solvent B: ACN in 0.1% formic acid; column flow: 0.3 µl/min, primary flow: 300 µl/min; gradient: 0-5 min 2% solvent B, 60 min 60% solvent B; stop time: 60 min. Protein identification was performed in the Agilent Spectrum Mill MS proteomics workbench against the Swiss-Prot protein database search engine (<http://kr.expasy.org/sprot/>) and the MASCOT MS/MS Ions Search engine (http://www.matrixscience.com/search_form_select.html). The criteria for positive identification of proteins were set as follows: filter by protein score >10.0, and filter peptide by score >8, % scored peak intensity.

Quantitative reverse transcription-PCR (qRT-PCR). To analyze *SOX2* mRNA expression, qRT-PCR was performed on ABI Prism 7900HT (Applied Biosystems, Grand Island, NY, USA) using 2×QuantiFast SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA) with 0.5 μM primers. Specific primer sets used were as follows: human *SOX2* forward: 5'-TGGACAGTTACGCGCACAT-3', reverse: 5'-CGAGTAGGACATGCTGTAGGT-3'; and β-Actin forward: 5'-TTGCCGACAGGATGCAGAA-3' and reverse: 5'-GCCGATCCACACGGAGTACT-3'. The gene expression of *SOX2* and the endogenous control, β-Actin were analyzed in three independent qRT-PCR assays. The thermal cycling procedure included an initial denaturation step for 5 min at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Relative expression of the target gene was analyzed by the ΔΔCt method. Ratios of mRNA levels were expressed relative to those of the control group.

RNA repression by small-interfering RNA (siRNA). To repress *NONO* mRNA expression, *NONO*-specific siRNA was treated to MCF-7 or T-47D cells. BLOCK-iT RNAi Designer (Invitrogen) was used to design siRNAs with the following sequences were as follows: *NONO* sense, 5'-GCAUUCUGAAGUCUCUAATT-3'; antisense, 5'-UUAGAGACUUCAGGAAUGCTT-3'. AllStars Negative Control siRNA (Qiagen) was used as a negative control. Trypsinized MCF-7 cells (1×10⁶ cells) were re-suspended in 100 μl of Opti-Minimal Essential Medium (Invitrogen) followed by the addition of the siRNA such that the final concentration was 4 μM. Transfections were performed in an electroporation cuvette using a CUY21SC electroporator (NepaGene). The transfected cells were transferred to DMEM/F-12 containing 10% fetal bovine serum, incubated for 48 h in a humidified incubator maintained at 37°C and 5% CO₂. After 48 h, the cells were harvested for RNA or protein preparation.

Chromatin immunoprecipitation (ChIP) assay. To confirm *NONO* binding on *SOX2* promoter, ChIP assay was performed using anti-*NONO* antibody and qPCR for *SOX2* promoter region. Cells were seeded in 10-cm dishes and were harvested when it reached 80% confluency. Cross-linking was performed by incubating the cells in 500 μl of 1% formaldehyde prepared in phosphate-buffered saline (PBS) for 20 min at 20°C. After incubation, the cells were washed twice in ice-cold PBS, and lysed in 100 μl of ChIP lysis buffer (0.5% SDS, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA) for 30 min at 20°C. The cells lysates were then sonicated for 30×30 s at the maximum power setting on a Bioruptor Sonicator (Diagenode, Denville, NJ, USA) to generate DNA fragments of 150-500 bp in length. Sonicated samples were then centrifuged at 19,000 × g for 10 min at 4°C, and the supernatant was pre-cleared by incubating in 50% protein G agarose slurry (GE Healthcare) prepared in BSA/PBS for 2 h at 4°C. Pre-cleared samples were then incubated at 4°C overnight with 10 μg of normal mouse IgG (sc-2025, Santa Cruz Biotechnology, Dallas, TX, USA), anti-POL2 antibody (F-12, Santa Cruz Biotechnology), anti-*NONO* antibody (clone 3/p54nrb (RUO), BD Bioscience, Franklin Lakes, NJ, USA). The following day, 20 μl of protein G agarose was added and incubated with rotation for 2 h at 4°C, followed by a series of washes: twice with IP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100), twice with high-salt buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA, and 1% Triton X-100), once with LiCl buffer (250 mM LiCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% NP40, and 0.5% sodium deoxycholate), and twice with 10 mM Tris -1 mM EDTA (pH 8.0) buffer. Samples were eluted from the beads with 0.1 M Na₂CO₃ and 1% SDS by heating the mixture at 67°C for 30 min with intermittent vortexing at 10-min intervals. Samples were

reverse-crosslinked overnight at 65°C. Immunoprecipitated DNA and input DNA were incubated with RNase A and Proteinase K (Sigma, St Louis, MO, USA) at 45 °C for 2 h. DNA was purified using QIAquick PCR Purification Kit (Qiagen) and then analyzed using qPCR. Primer sequences were as follows: *SOX2* promoter -47 to -123, forward: 5'-CCTTTTCATGCAAAACCCG-3', reverse: 5'-GGGTTTCTAGCGA CCAATCA-3', and *SOX2* promoter -69 to +58, forward: 5'-CGCTGATTGGTCGCTAGAAAC-3', reverse: 5'-CCCCTTTTGC AAACACTCTC-3'. qPCR was performed on an ABI Prism 7900HT using previously described protocol for qRT-PCR. The relative gene expression was analyzed using ΔΔCt method and compared with normal mouse IgG.

Western blotting. To analyze protein expression level of *NONO* or *SOX2*, western blotting was performed. pcDNA3-NONO-1xFLAG (16, 17) or pCMV-3XFLAG (Mock, provided by Dr. Koji Nakagawa, Health Science University of Hokkaido School of Nursing & Social Services) were transfected into the cells by electroporation. Cells (1×10⁶) were lysed using 100 μl of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). Samples containing 20 μg of protein extracts were separated on a 14% polyacrylamide gels and transferred onto PVDF membranes (Immobilion P; Millipore, Billerica, MA, USA). Antibodies against *SOX2* (D6D9, Cell Signaling Technology, Danvers, MA, USA), *NONO* (clone 3/p54nrb (RUO), FLAG (M2: Sigma), β-Actin (clone C4: Millipore), anti-mouse IgG secondary antibodies, horseradish peroxidase (HRP)-linked F(ab')₂ fragment sheep or anti-rabbit IgG, and HRP-linked F(ab')₂ fragment donkey (GE Healthcare Bioscience) were used. Each protein band was detected using Western Chemiluminescent HRP Substrate (Immobilon, Millipore) and visualized by LAS1000mini (FUJIFILM Corp, Tokyo, Japan).

Immunocytochemistry. To analyze protein expression and localization of *NONO* or -*SOX2*, immunohistochemistry was performed using anti-*NONO* antibody or *SOX2* antibodies. MCF-7 cells were seeded on glass-based 24-well plates and fixed in 4% paraformaldehyde and 0.1% Triton X-100 at 20 °C for 30 min after they reached 80% confluence. They were washed twice with 1×PBS for 5 min each and incubated with two primary antibodies, anti-*SOX2* (D6D9) and anti-*NONO* (clone 3/p54nrb; RUO). The cells were then incubated with biotinylated sheep anti-rabbit IgG or Alexa 568 conjugated goat anti-mouse IgG (Invitrogen), and avidin-conjugated Alexa 488 (Invitrogen). All the antibodies were diluted in 1×PBS plus 3% BSA. 4,6-Diamidino-2-phenylindole (0.1 μg/ml) was used for nuclear staining in order to confirm cellular localization of *NONO* and *SOX2*.

Generation of POU binding element mutated promoter vector. To analyze the effect of mutation of POU binding element in luciferase vector, plasmid vectors were constructed. P227-pGEM T vector was used as a template for PCR. The DNA fragment was amplified by KOD-Plus-Neo using 0.3 μM of specific primers (P227 forward: 5'-GGTACCCTCAGTGGCTGGCAGGC-3', POU-M reverse: 5'-GGGTGGGTACAGCCCTGGGGCGGGGCCT-3', POU-M forward: 5'-AGGCCCGCCCCAGGGCTGTACCCACCC-3', reverse 5'-AAGCTTGAGGCAAACTGGAATCAGGATC-3') Mutated POU binding element is underlined in the above primer sequences. PCR products were extracted from agarose gel using QIAEXII (Qiagen), which served as templates for the second round of PCR using P227 forward and reverse primers. Thermal cycling procedure involved an initial denaturation step for 2 min at 94°C, followed by 20 cycles of 98°C for 10 s, 58°C 30 s, and 68°C for 30 s. A-tailing of the PCR

products was carried out, after which, they were ligated into pGEM-T vector. The pGEM-T was then digested with restriction enzymes, *Kpn* I and *Hind* III. The fragment obtained was ligated to pGL3-Basic luciferase vector. The reporter vector with mutation in the POU binding element was designated as P227-M.

Generation of *NONO* deletion mutants. To confirm SOX2 binding elements in *NONO* protein, plasmid vectors were constructed. pcDNA3-*NONO*-1xFLAG was used as a template for PCR. DNA fragment was amplified by KOD-Plus-Neo using 0.3 μ M of specific primers (Δ N forward: 5'-AGCCGTCTTTTGTGGGAAATC-3', reverse: 5'-CATGGTTCTATCTCCTTCGAAG-3', Δ R forward: 5'-TTAGATGATGAAGAGGGACT T-3', reverse: 5'-TCGTGGGTG AAGGTCTTC-3', Δ CC forward: 5'-ACCTTCCTGATGCGAGAG-3', reverse: 5'-ATATTCATACTCAAAGGAGC-3', Δ C forward: 5'-TATGACCCAGCCTTCTGTAC-3', reverse: 5'-TCCCTT GAATCCTTCCTGCTG-3'). Thermal cycling included an initial denaturation step for 2 min at 94°C, followed by 30 cycles at 98°C for 10 s, 58°C for 30 s, and 68°C for 3 min. PCR amplicons were digested with *Dpn* I. Digested PCR products were incubated with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA) for 30 min at 37°C, followed by overnight incubation with T4 DNA ligase (Promega) at 16°C. *NONO* coding regions of constructed vectors were sequenced, and expression of mutated *NONO* was confirmed by western blotting using FLAG antibody (M2: Sigma).

Flow cytometric (FACS) analysis. To confirm the SOX2-positive cell population in *NONO* siRNA-treated MCF-7 cells, FACS analysis was performed using antibody to SOX2. MCF-7 cells were fixed with 4% formaldehyde for 10 min at 37°C and permeabilized in ice-cold 100% methanol for 30 min. The cells were then treated with 3% BSA for 1 h and incubated with anti-SOX2 (D6D9) for 1 h at 20 °C. After washing with PBS, the cells were incubated with CF488A-conjugated goat anti-rabbit IgG antibody (Biotium, Hayward, CA, USA) for 1 h at 20°C. Immunostained cells were analyzed by FACS Aria III flow cytometer I (BD Biosciences, San Jose, CA, USA).

Sphere formation. To analyze cancer stemness in cells with repression of *NONO* expression, sphere formation assay was used. Control and *NONO*-specific siRNA transfected MCF-7 cells were seeded at 3,000/well on ultra-low-attachment 96-well plate (Sumitomo Bakelite Co., LTD, Tokyo, Japan) using FACS Aria III and cultured in a sphere-formation medium (DMEM/F-12 supplemented with 20 ng/ml of epidermal growth factor, 20 ng/ml of basic fibroblast growth factor, 100 units/ml penicillin, and 100 μ g/ml of streptomycin sulfate) for 7-10 days. Spheres formed were detected and counted under microscopic observation (IX73; Olympus, Tokyo, Japan).

Statistical analysis. Statistical analysis was performed to analyze whether or not there were any significant differences in the experimental results. Experiments were performed in triplicate or quadruplicate and conducted in a minimum of three independent trials. Data are presented as means $\pm$ standard deviations. Two-tailed *t*-tests were conducted in which the acceptable level of significance was *p*<0.05.

Results

To determine the transcriptional regulatory region of human SOX2 promoter (–789 to +253), deletion mutants were constructed. SOX2 promoter activity was reduced in all

deletion mutants, with –629 to –467 containing the SOX-binding site, and –227 to –68 containing two Sp1 and one POU binding sites in breast cancer cell MCF-7 (Figure 1A). The SOX2 promoter –227 to –68 has CpG island and a highly conserved Sp1- and POU-binding elements (Figure 1B). To identify SOX2 promoter-binding proteins, biotin-labeled promoter core region and MCF-7-derived nuclear fraction were used (Figure 1C). By LC-MS/MS analysis, *NONO*, eukaryotic translation elongation factor 1 α 2 (eEF1 α 2), and heterogeneous nuclear RNA binding protein-A1 (hnRNPA1) were identified as promoter-binding proteins (Figure 1C).

To confirm the effect of SOX2 promoter-binding proteins, promoter activity was examined with forced expression and knockdown experiments for these genes. Overexpression of *NONO* repressed P227 promoter activity compared with mock transfectant, but not that of P68 (Figure 2A). In addition, siRNA for *NONO* gene enhanced P227 promoter activity but not that of P68 (Figure 2B). The effect of *NONO* on P227 promoter was dependent on POU-like element (Figure 2C). Using ChIP assay, binding of *NONO* to SOX2 promoter at –227 to –69 was confirmed but not at –68 to +253 (Figure 2D). In addition, siRNA for *NONO* enhanced RNA polymerase II recruitment to SOX2 promoter (Figure 2E). SOX2 promoter activities were not affected by eEF1 α 2 and hnRNPA1 (data not shown). These results indicate that *NONO* acts as a negative regulator for SOX2 promoter.

NONO has several domain structures, such as RNA-binding domain and coiled-coil domain (18). To confirm SOX2 promoter regulatory domains in *NONO*, deletion mutants were constructed (Figure 3A). Deletion mutants of the coiled-coil domain and C-terminal region did not repress the activity of full length SOX2 promoter (–789 to +253) nor partial SOX2 promoter (–227 to +253) (Figure 3B). This indicates that *NONO* represses SOX2 promoter activities by coiled-coil domain and C-terminal region.

To confirm the role of *NONO* in SOX2 expression, *NONO* overexpression and knockdown experiments were performed. Overexpression of *NONO* reduced SOX2 protein expression (Figure 4A) while siRNA for *NONO* gene enhanced SOX2 protein expression (Figure 4B). siRNA for *NONO* induced SOX2 mRNA expression in breast cancer cell line MCF-7 and T-47D (Figure 4C) compared with siRNA control transfectant. siRNA for *NONO* enhanced the percentage of the SOX2-positive population in MCF-7 cells (Figure 4D). *NONO* was ubiquitously expressed in the nucleus and SOX2 was also expressed in the nucleus (Figure 4E). However, the expression of *NONO* was negatively correlated with that of SOX2, and *NONO*-positive, SOX2-negative cells existed in MCF-7 (Figure 4E, white arrow). It has been reported that SOX2 expression is associated with sphere-forming activity (3). *NONO* knockdown enhanced sphere formation of MCF-7 cells (Figure 4F). These results indicate that *NONO* is a repressor of SOX2 expression in breast cancer cell lines.

Figure 1. Identification of human sex-determining region Y-box 2 (SOX2) promoter-binding proteins in MCF-7 cells. A: SOX2 promoter deletion mutant activity in breast cancer cell lines. Light black bar: SOX-binding element, gray bar: Sp1-binding element, heavy black bar: pituitary-specific factor, octamer transcription factor, neural un-coordinated-86 (POU) l like-binding element, Sp: SOX2 promoter (–789 to +253). Renilla luciferase activity was used as an internal control. All promoter activities are shown relative to that of Sp promoter activity. B: Putative SOX2 promoter-binding factors and their binding elements in the region at positions –227 to +253. C: Identification of SOX2 promoter-binding proteins from nuclear extract of MCF-7 cells in the region at positions –227 to –69. NONO: non-POU domain-containing octamer-binding protein; eEF1α2: eukaryotic translation elongation factor 1 α 2; hnRNPA1: heterogeneous nuclear RNA binding protein-A1. *Significantly different at $p < 0.05$.

Discussion

In this study, we identified NONO as a SOX2 promoter-regulatory factor in human breast cancer cell lines. NONO regulated SOX2 promoter activity using the C-terminal region and coiled-coil domain, which were previously reported as a POU element-binding region (19). NONO has high homology with family gene splicing factor, proline- and glutamine-rich (SFPQ) and paraspeckle component 1 (PSPC1) in RNA binding domains (18). The complex of SFPQ and NONO binds retinoid X receptor and represses transcription using histone deacetylase recruitment (20). However, SFPQ and PSPC1 overexpression did not alter SOX2 protein expression (data not

shown). This is consistent with the low homology between the C-terminal regions of these two proteins and NONO.

Two patterns are known for transcriptional regulation by NONO (14, 21, 22). One is where a complex with a hormone receptor is formed to recognize a hormone receptor-binding sequence and to regulate transcription (21, 22). The other is where NONO directly binds to the POU element and regulates transcription, as seen in the transcriptional regulation of the COX2 gene (14). The present results indicate that the hormone receptor-binding element is absent, and that transcriptional activity was abolished by the POU element mutant, so that the transcriptional control of SOX2 by NONO is *via* the latter POU element-mediated pathway.

Figure 2. Non-POU domain-containing octamer-binding protein (NONO) is a negative regulator of sex-determining region Y-box 2 (SOX2) promoter activity in MCF-7 cells. A: Overexpression of NONO repressed SOX2 promoter activity. Mock: Mock transfectant, NONO: NONO-1xFLAG; P227: SOX2 promoter (–227 to +253); P68: SOX2 promoter (–68 to +253) B: siRNA treatment for NONO enhanced SOX2 promoter activity. Control: AllStars control siRNA, NONO: siRNA for NONO gene. C: NONO repressed SOX2 promoter activity via pituitary-specific factor, octamer transcription factor, neural un-coordinated-86 (POU) element. Mock: Mock transfectant, NONO: NONO-1xFLAG, P227-M: mutated P227 POU. D: Identification of NONO binding to the SOX2 promoter by chromatin immunoprecipitation (ChIP) assay. IgG was used as a negative control. Anti-polymerase II (POL2) was used as a positive control. Relative fold enrichment was determined by quantitative polymerase reaction. IgG: Mouse IgG, NONO: anti-NONO. E: NONO knockdown enhanced recruitment of POL2 to SOX2 promoter in MCF-7 cells (ChIP assay). In all luciferase assays, Firefly luciferase activity was normalized by that of Renilla luciferase. Significantly different at * $p < 0.05$ and ** $p < 0.005$.

Figure 3. Identification of the region of non-POU domain-containing octamer-binding protein (NONO) that is responsible for sex-determining region Y-box 2 (SOX2) promoter repression by use of deletion mutants. **A:** Expression of NONO-deletion mutants in a breast cancer cell line. These products were confirmed by western blotting using anti-FLAG antibody. Deletions of: ΔN : N-Terminal region, ΔR : RNA binding domain, ΔCC : coiled-coil domain, ΔC : C-Terminal region. WT: full length of wild-type protein. **B:** Deletion mutants of NONO (left panel) and their corresponding luciferase activity (right panel). Sp: SOX2 promoter (–789 to +253), P227: SOX2 promoter (–227 to +253), Firefly luciferase activity was normalized by that of Renilla luciferase. Significantly different at $*p < 0.05$.

NONO is an important factor for cell differentiation from stem cells. In mouse embryonic stem cells lacking *Nono* gene, differentiation is suppressed, and undifferentiated cells proliferate (23). In retinal epithelial cells that are important for the blood–retinal barrier, NONO plays an important role in inducing differentiation (24). NONO is highly expressed in various tumor cells and C-terminal deletions are frequently observed in tumor cells (25). Sphere-forming activity is a marker for cancer cell stemness (2). Our findings are consistent with these reports that wild-type NONO suppresses the expression of SOX2, an essential factor in stem cells. There were few reports on repressors of SOX2 transcription.

The interaction between epithelial–mesenchymal transition and mesenchymal–epithelial transition was reported to explain the plasticity of CSCs (4). Negative regulators of stem cell-related factors appear to be other players involved in the plasticity of CSCs. Our findings suggest that NONO regulates SOX2 expression in human breast cancer cells using reduction of promoter activity.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Designed research: S.L., J.H., and H.I.; performed research: S.L., H.T., and Y.K.; validation and formal analysis: T. H., Y.H., R.W., and S.H.; wrote the article: S.L., J.H., and H.I.

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Figure 4. Non-POU domain-containing octamer-binding protein (NONO) repressed sex-determining region Y-box 2 (SOX2) expression and enhanced sphere formation in breast cancer cell line. A: Overexpression of NONO repressed protein expression of SOX2 in MCF-7 cells. Mock: Mock transfectant, NONO: NONO-1xFLAG. B: siRNA treatment to silence NONO enhanced the protein expression of SOX2 in MCF-7 cells. Control: AllStars control siRNA, NONO: siRNA for NONO. β-Actin was used as an internal control. C: Silencing of NONO enhanced SOX2 mRNA expression. Control: AllStars control siRNA, NONO: siRNA for NONO. D: SOX2 protein-positive populations in NONO siRNA-treated MCF-7 cells. The SOX2-positive population was classified into three populations -/Low, +/Middle, and +/High according to the fluorescence intensity. Control: AllStars control siRNA. E: Immunostaining for NONO and SOX2 protein in the MCF-7 breast cancer cell line. Arrows: NONO-positive cells; DAPI: 4',6-diamidino-2-phenylindole. Bar: 50 μm. F: siRNA for NONO enhanced sphere formation in MCF-7 cell line. *Significantly different at $p < 0.05$.

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