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Differential Transactivation by Orphan Nuclear Receptor NOR1 and Its Fusion Gene Product EWS/NOR1: Possible Involvement of Poly(ADP-Ribose) Polymerase I, PARP-1

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ABSTRACT

In extraskeletal myxoid chondrosarcoma, a chromosomal translocation creates a gene fusion between EWS and an orphan nuclear receptor, NOR1. The resulting fusion protein EWS/NOR1 has been believed to lead to malignant transformation by functioning as a transactivator for NOR1-target genes. By comparing the gene expression profiles of NOR1- and EWS/NOR1-overexpressing cells, we found that they largely shared up-regulated genes, but no significant correlation was observed with respect to the transactivation levels of each gene. In addition, the proteins associated with NOR1 and EWS/NOR1 were mostly the same in these cells. The results suggest that these proteins differentially transactivate overlapping target genes through a similar transcriptional machinery. To clarify the mechanisms underlying the transcriptional divergence between NOR1 and EWS/NOR1, we searched for alternatively associated proteins, and identified poly(ADP-ribose) polymerase I (PARP-1) as an NOR1-specific binding protein. Consistent with its binding properties, PARP-1 acted as a transcriptional repressor of NOR1, but not EWS/NOR1, in a luciferase reporter assay employing PARP-1(−/−) fibroblasts. Interestingly, suppressive activity of PARP-1 was observed in a DNA response element-specific manner, and in a subtype-specific manner toward the NR4A family (Nur77, Nurr1, and NOR1), suggesting that PARP-1 plays a role in the diversity of transcriptional regulation mediated by the NR4A family in normal cells. Altogether, our findings suggest that NOR1 and EWS/NOR1 regulate overlapping target genes differently by utilizing associated proteins, including PARP-1; and that EWS/NOR1 may acquire oncogenic activities by avoiding (or gaining) transcription factor-specific modulation by the associated proteins. J. Cell. Biochem. 105: 785–800, 2008.

KEY WORDS: NOR1; NR4A FAMILY; EWS/NOR1; PARP-1; CHROMOSOMAL TRANSLOCATION

The EWS gene is involved in various human malignancies by way of chromosomal translocations [Delattre et al., 1992; Sorensen et al., 1994; Jeon et al., 1995]. It was first discovered in Ewing tumors with the translocation t(11;22), in which the EWS gene on chromosome 22 was fused to the gene for the transcription factor FLI-1 on chromosome 11, generating the fusion protein EWS/FLI-1. Subsequently, EWS was found to be fused to a variety of other transcription factors in other types of solid tumors, such as malignant melanomas of soft parts, and desmoplastic small round cell tumors. In extraskeletal myxoid chondrosarcoma, the EWS gene was found to be fused to the NOR1 gene on chromosome 9 [Labelle et al., 1995; Clark et al., 1996]. This t(9;22) chromosomal translocation has been reported in approximately 70% of cases [Stenman et al., 1995], and this gene fusion encodes a chimeric protein, called EWS/NOR1, containing the amino-terminal domain of EWS fused in-frame to the complete amino acid sequence of NOR1. We previously demonstrated that EWS/NOR1 interacts with the splicing factor U1C and affects pre-mRNA splicing [Ohkura et al., 2002]. In addition, Laflamme et al. [2003] reported that the homeotic transcription factor Six3 is expressed in extraskeletal myxoid chondrosarcoma and interacts directly with EWS/NOR1 to modulate its transcriptional activity.

EWS possesses a conserved RNA-recognition motif [Delattre et al., 1992] and can bind to RNA in vitro [Ohno et al., 1994], suggesting that it may be involved in RNA metabolism. On the other hand, NOR1 (also known as NR4A3) was originally identified in rat fetal forebrain undergoing apoptosis in our laboratory [Ohkura et al., 1994], and classified as a member of the NR4A subfamily (Nur77, Nurr1, and NOR1) within the nuclear receptor superfamily. NOR1 is an immediate-early gene product induced by mitogens, and is also involved in T-cell receptor-mediated apoptosis of immature thymocytes [Cheng et al., 1997]. NOR1 has been shown to

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transactivate target genes through both the monomeric DNA response element NBRE [Ohkura et al., 1999] and the palindromic response element NurRE [Drouin et al., 1998].

The EWS fusion proteins are believed to lead to malignant transformation by functioning as transcription factors, since the various EWS fusion proteins share a common structural pattern, in which the N-terminal domain of EWS, lacking the RNA-binding domain, is linked to the DNA-binding domain derived from one of a number of transcription factors, such as FLI-1, NOR1, and CHOP. The systematic presence of the DNA-binding domain strongly suggests that these fusion proteins exert their oncogenic potential, at least in part, by deregulating the expression of specific target genes. In addition, the EWS/NOR1 fusion protein binds to the same target DNA sequences of NOR1, and is about 270-fold more active than NOR1 in a reporter gene construct containing the NBRE response element [Labelle et al., 1999]. This suggests EWS/NOR1 to exert its oncogenic effects by acting as a transcriptional activator to regulate the expression of genes closely related to the NOR1-target genes.

Some observations, however, suggest that the transactivation of NOR1-target genes by EWS/NOR1 may not be suitable for accounting for the transforming activity of the fusion proteins; (1) The NOR1-target genes reported are not correlated with genes highly expressed in extraskeletal myxoid chondrosarcoma [Sjogren et al., 2003; Subramanian et al., 2005], and not directly associated with oncogenesis, (2) Overexpression of EWS/NOR1 in chondrocites induces cellular transformation [Filion and Labelle, 2004], but overexpression of NOR1 in cultured cells induces cell death (unpublished observation), (3) Overexpression of NOR1 in transgenic mice leads to a small body size and atrophy in the thymus and spleen, but no appearance of chondrosarcomas [Cheng et al., 1997; Kagaya et al., 2005], and (4) The abrogation of NOR1 and Nur77 in mice led to lethal acute myeloid leukemia, suggesting they may act as tumor suppressors [Mullican et al., 2007]. These observations give rise to the question of whether the EWS/NOR1 fusion protein is a simple transactivator that highly up-regulates NOR1-target genes.

To answer this question, we examined gene expression profiles of cells forced to overexpress NOR1 and EWS/NOR1 under genetically and epigenetically identical backgrounds, and searched for proteins specifically associated with NOR1 and EWS/NOR1. We show that whereas alternatively regulated genes are frequently observed in NOR1- and EWS/NOR1-expressing cells, the tendency for global regulation is similar in both. We also show that although the proteins associated with them are mostly the same, poly(ADP-ribose) polymerase I, PARP-1, prefers NOR1 over EWS/NOR1. PARP-1 is an eukaryotic nuclear protein, and multiple functions have been proposed for this protein, including involvement in DNA repair, a role in apoptosis, and roles in both transcriptional activation and repression [Rawling and Alvarez-Gonzalez, 1997; Oei et al., 1998; Cervellera and Sala, 2000; Hassa et al., 2003]. We demonstrate that PARP-1 functions as a transcriptional suppressor of NOR1 in a DNA response element-specific manner, and does not suppress EWS/NOR1-dependent transactivation. Thus, our work suggests that NOR1 and EWS/NOR1 transactivate common target genes via a common transcriptional machinery, and their transcriptional activities are modified by specifically associating proteins, including PARP-1.

MATERIALS AND METHODS

CELLS, PLASMIDS, AND ANTIBODIES

COS7, HeLa, 293, and 293T cells, and PARP-1(−/−) mouse embryonic fibroblasts, the latter kindly provided by Chugai Pharmaceutical Co. Ltd., were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Transient transfections were performed with Fugene 6 (Roche) according to the protocol supplied by the manufacturer. Various deletion constructs of NOR1 were generated by PCR amplification, and deletion constructs of PARP-1 were kindly provided by Dr. Mitsuko Masutani. Constructs with an amino acid replacement were generated using the QuickChange site-direct mutagenesis kit (Stratagene). These cDNA fragments were confirmed by DNA sequencing and restriction enzyme mapping. Expression plasmids for Flag- and myc-tagged proteins were constructed by inserting cDNA into the pCMV-Tag2 and Tag3 vector (Stratagene), respectively. The NBRE-Luc and NurRE-Luc reporter plasmids were kindly provided by Dr. Jeffrey Milbrandt and Dr. Jacques Drouin, respectively.

Mouse anti-NOR1 monoclonal antibody (H7833) was kindly provided by Dr. Toshiya Tanaka. All other antibodies were purchased commercially, including mouse anti-Flag (M2; Sigma), mouse anti-c-myc (9E10; Clontech), rabbit anti-PARP-1 (Santa Cruz Biotechnology), and rabbit anti-poly(ADP-ribose) (PAR; BD Biosciences) antibodies.

IMMUNOPRECIPITATION AND IMMUNOBLOT ANALYSIS

HEK293T Cells were transfected with the different constructs and lysed with a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and one Complete^{TM} protease inhibitor mixture tablet(Roche)/50 ml) and sonicated briefly. Anti-Flag agarose affinity gel (Sigma) was incubated with the lysates for 1 h at 4°C with gentle rocking. After four washes with PBS, SDS–PAGE sample buffer was added to the agarose beads. The immunoblot analysis, SDS–PAGE, and the transfer of proteins to PVDF membranes were performed according to standard procedures. The immunodetection of proteins was performed employing the ECL Plus Western blotting detection system (GE Healthcare Bio-Science).

ESTABLISHMENT OF THE INDUCIBLE NOR1- AND EWS/NOR1-EXPRESSING CELLS

HEK 293 tet-On advanced cells (Clontech) were transfected with the pTRE-tight vector containing full-length NOR1 or EWS/NOR1 cDNA, and selected in medium containing G418 (100 μg/ml) and hygromycin (100–150 μg/ml) for 2 weeks. Clones isolated were tested for the expression after the addition of doxycycline (1 μg/ml) for 24 h by Western blotting with anti-Flag antibody. Clones that showed positive expression with Dox and negative without Dox were selected, and then purified by limited dilution.

DNA MICROARRAY ANALYSIS

Tet-On-NOR1 (clone #69) and tet-On-EWS/NOR1 (clone #24) 293 cells were cultured with or without Dox (1 μg/ml) for 24 h. Total RNA was isolated from the cells using the RNeasy mini kit

(Qiagen). Biotinylated antisense cRNA was prepared by one cycle of in vitro amplification according to the protocol of One-Cycle Eukaryotic Target Labelling Assays (Affymetrix). Biotinylated cRNA was hybridized to an Affymetrix; GeneChip Human Genome U133 Plus 2.0 array at the Biomatrix Research Institute (Chiba, Japan). All data analyses were conducted with GeneSpring 7 (Silicon Genetics). The data from these experiments are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/) with accessions GSM281775, GSM281776, GSM281777, and GSM281778.

PURIFICATION OF NOR1 AND EWS/NOR1 COMPLEXES

The tet-On-NOR1 (clone #69) and tet-On-EWS/NOR1 (clone #24) cells were cultured with Dox (1 μg/ml) for 24 h. The cells were washed with PBS, and then nuclei were prepared by suspension in hypotonic buffer (10 mM Hepes-KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, and protease inhibitors) on ice for 10 min, followed by sedimentation at 1,500 rpm for 5 min. The nuclei were suspended in hypertonic lysis buffer (250 mM NaCl, 20 mM Hepes-KOH pH 7.9, 5 mM EDTA, 0.1% NP40, 10% glycerol, 1 mM DTT, 10 mM NaF and protease inhibitors), and the resulting extracts were clarified by centrifugation at 15,000 rpm for 1 h at 4°C. The clarified extracts were diluted, and incubated with anti-Flag M2 agarose for 2 h. The immunoprecipitated complexes were washed with wash buffer (20 mM Hepes pH 7.9, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.1% NP40, 100 mM KCl and protease inhibitors), and eluted by incubation with 3× Flag peptides (0.25 mg/ml in wash buffer). Eluted complexes were analyzed by 30–100% glycerol density gradient segregation (Beckman 46,000 rpm, 16 h at 4°C). Fractions from the density gradient were analyzed by SDS–PAGE followed by silver staining.

MASS SPECTROMETRY ANALYSIS

Bands visualized on the SDS–PAGE gel by silver staining were excised, destained, and incubated with 0.1 μg/μl of sequence grade modified trypsin (Promega) in digestion buffer (50 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$) overnight at 37°C. The digested samples were extracted with 5% trifluoroacetic acid and 50% acetonitrile, and analyzed by mass spectrometry as described previously [Yaguchi et al., 2004].

IMMUNOPRECIPITATION OF THE ENDOGENOUS NOR1 PROTEIN

For the induction of NOR1 with FCS stimulation, HeLa cells were plated 24 h prior to synchronization at G1 by serum deprivation. Cells were washed twice with PBS and incubated for 24 h in DMEM, after which the quiescent cells were stimulated to proliferate by incubation in DMEM containing 15% FCS. For TPA stimulation, HeLa cells were treated with TPA (20 nM) for 1 h. For cell stress, HeLa cells collected by trypsin treatment were sheared 30 times, and then harvested on dishes for 1 h. They were lysed with a hypotonic buffer (20 mM Hepes-KOH, pH 7.9, 5 mM KCl, 0.5 mM $MgCl_2$, and protease inhibitors), and then centrifuged at 2,000 rpm for 10 min. The precipitates, as a nuclear fraction, were lysed with a lysis buffer, and then immunoprecipitaed with anti-NOR1 monoclonal antibody, and protein G-sepharose (GE Helthcare Bio-Science).

CHROMATIN IMMUNOPRECIPITATION ASSAYS

Chromatin immunoprecipitation assays were performed using a ChIP assay kit (Upstate Biotechnology) with modifications. Tet-On 293 cells cultured with or without Dox for 24 h were cross-linked with 1% formaldehyde for 10 min at 37°C. Cells were washed with PBS and resuspended in lysis buffer (50 mM Tris–HCl pH 8.1, 10 mM EDTA, 1% sodium dodecyl sulfate and protease inhibitors). Lysates were sonicated, and diluted with ChIP dilution buffer. The diluted lysates were pre-cleaned with protein A agarose with sonicated sarmon sperm DNA, and then incubated with anti-Flag M2 agarose at 4°C overnight. Antibody/protein/DNA complexes were washed, eluted, and incubated at 65°C for 8 h to reverse formaldehyde cross-links. DNA recovered by phenol/chloroform extraction and ethanol precipitation was amplified by real-time PCR using an SYBR Green PCR kit (Qiagen).

IMMUNOCYTOCHEMISTRY

COS7 cells grown on coverslips were transfected with the GFP-fused NOR1 expression vector (pEGFP-NOR1), fixed with 4% formaldehyde in PBS for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Immunostaining with anti-PARP-1 antibody was performed as described previously [Yaguchi et al., 2004].

LUCIFERASE REPORTER ASSAY

The transfection of 293 cells and PARP-1(−/−) fibroblasts was performed using Fugene 6 according to the manufacturer's instructions. All transfections involved the luciferase-reporter plasmid and Renilla luciferase plasmid phRG-B (Promega) to normalize for transfection efficiency. Cells were transfected in 24-well plates and harvested 48 h after transfection. All transfections were carried out in triplicate. Promoter activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations.

GLUTATHIONE *S*-TRANSFERASE PULL-DOWN ASSAYS

For the preparation of glutathione *S*-transferase (GST)-fusion proteins, pGEX-6P vectors (GE healthcare Bio-Science) were used. The fusion proteins were affinity-purified from the soluble fraction of cell extract with glutathione-sepharose beads (GE healthcare Bio-Science) according to the manufacture's instructions. In vitro binding assays were performed by incubating GST fusion protein or an equal amount of GST resin and [^{35}S]methionine-labeled, in vitro transcribed/translated proteins produced in rabbit reticulocyte lysate (Promega). Proteins were incubated at 4°C for 2 h in the binding buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$). Bound proteins were washed four times with binding buffer and subjected to SDS–PAGE and autoradiography.

RESULTS

GENERATION OF INDUCIBLE 293 CELLS EXPRESSING NOR1 AND EWS/NOR1

To examine the differences between NOR1 and EWS/NOR1 against the same cellular background, we generated NOR1- and EWS/NOR1-overexpressing cells derived from the same cell line. We used a tetracycline-controlled transactivator system to generate

293 cells that inducibly overexpress Flag-tagged NOR1 or EWS/NOR1 (Fig. 1A) under the control of the CMV promoter. In the presence of the tetracycline derivative doxycycline (Dox), expression of NOR1 or EWS/NOR1 is induced. All cells in this study were cultured in the absence of Dox before induction of the proteins to prevent any potential physiological consequences from the expression of the proteins.

In the established clones, NOR1 or EWS/NOR1 expression was clearly detected 24 h after the treatment with Dox (Fig. 1B). To explore the effect of NOR1 or EWS/NOR1 expression in the cells, we first examined the cell growth rate and morphology. The NOR1-expressing cells (clones #54 and #69; cultured in the presence of Dox) had lower growth rates than the corresponding control cells (identical clones cultured in the absence of Dox) (Fig. 1C). In contrast, EWS/NOR1-expressing cells (clones #24 and #111 with Dox) had similar growth curves to the corresponding control cells. Next, we examined morphological changes of these cells after the induction using a phase-contrast microscope (Fig. 1D). In the NOR1-expressing cells, a round shape and detachment from the substratum were observed 72 h after the treatment, suggesting apoptosis or cell cycle arrest. To test this, we tested for apoptosis using a cell death detection ELISA kit (Roche), and for cell cycle arrest by flow cytometry. The NOR1-expressing cells had increased numbers of apoptotic cells compared to the corresponding control 48 h after the induction of NOR1 expression (Fig. 1E). Apoptosis of the EWS/NOR1-expressing cells did not differ significantly between the control and the cells treated with Dox. In addition, NOR1-expressing cells had smaller populations in G2/M and S than the corresponding control cells 12 h after the induction (Fig. 1F). Collectively, these results suggest that the lowered cell growth rate observed in NOR1-expressing cells was due, at least in part, to an increase of apoptotic cell death and inhibition of cell cycle progression. Thus, differences in phenotype between NOR1- and EWS/NOR1-expressing cells could be recapitulated partially in our systems.

COMPARISON OF GENE EXPRESSION PROFILES BETWEEN NOR1- AND EWS/NOR1-EXPRESSING CELLS

The observed differences between NOR1 and EWS/NOR1-expressing cells may reflect differences in their transcriptional regulation. To analyze the altered gene expression induced by NOR1 or EWS/NOR1 expression, we exploited the Affymetrix GeneChip DNA microarray. Total RNA was obtained from the tet-On-NOR1 (clone #69) or tet-On-EWS/NOR1 (clone #24) cells cultured with or without Dox for 24 h, and subjected to analysis. Expression profiles in each clone were generated by comparing the gene expression between the cells treated with and without Dox (Y and X axes, respectively; Fig. 2). In the two clones cultured with Dox, up-regulated genes were more prominently detected than down-regulated genes. This is consistent with previous observations that the two proteins act as transcriptional activators. Moreover, the up-regulated genes in NOR1-overexpressing cells ($>\times 2.0$) showed a tendency to be up-regulated ($>\times 1.0$) also in EWS/NOR1-overexpressing cells, and vice versa. However, a battery of genes showed selective up-regulation in either NOR1- or EWS/NOR1-overexpressing cells, even if classified as up-regulated ($>\times 1.0$) in both cells (Fig. 3A). Among the up-regulated genes in NOR1- (1,318 genes) and EWS/NOR1-expressing cells (1,190 genes), only 130 were up-regulated in both (Fig. 3B). The up-regulated genes in each clone mainly encoded cell cycle regulators, apoptosis-related factors, and differentiation-related factors. Similarly, only 117 genes were down-regulated in both NOR1-expressing cells and EWS/NOR1-expressing cells, whereas 969 were down-regulated in NOR1-expressing cells and 1,039 in EWS/NOR1-expressing cells (Fig. 3C). These results suggest that while the two proteins recognize common target genes in the genome, they differ in transactivation activity.

Within the up-regulated genes shared in both clones, Eno3 contains an NBRE in just upstream of the 5′-flanking region of the transcription start site. Using reporter gene constructs containing the approximately 1 kb promoter region of Eno3, we observed typical responses to NOR1 and EWS/NOR1: namely, both NOR1 and EWS/NOR1 transactivated the reporter construct (Fig. 4A); EWS/NOR1 showed stronger transcriptional activity than NOR1; and those transactivation were dependent on the NBRE (Fig. 4C). Moreover, chromatin immunoprecipitation showed that NOR1 and EWS/NOR1 directly bound to the endogenous Eno3 promoter region in the cells (Fig. 4B). These results fit the established concept that EWS/NOR1 highly transactivates NOR1-target genes. However, a majority of the up-regulated genes in either NOR1- or EWS/NOR1-expressing cells do not contain an NBRE or NurRE within 2.0 kb upstream of the transcription start site and intron 1, both of which are frequently identified as regions responsible for coregulator-mediated transactivation. Moreover, even in the genes that possess an NBRE in their promoter region, the regions (approximately 1 kb) were not sufficient to recapitulate the differential induction by NOR1 and EWS/NOR1 in the reporter assay (data not shown). These results suggest a possibility that other mechanisms are required for the differential expression by NOR1 and EWS/NOR1, in addition to the NBRE-dependent transactivation. Since nuclear receptors have been shown to regulate gene expression via cross-talk with other transcription factors [Lin et al., 2004; Mix et al., 2007; Shatnawi et al., 2007], transcription factors that cross-talk with NOR1 and EWS/NOR1 might be important in modulating their activities. Therefore, we next examined differences in associated proteins between NOR1 and EWS/NOR1.

IDENTIFICATION OF PROTEINS ASSOCIATED WITH NOR1 AND EWS/NOR1

To identify the proteins specifically associated with NOR1 and EWS/NOR1, we employed a purification process using anti-Flag antibody-conjugated beads. NOR1 and EWS/NOR1 complexes were immunoprecipitated from the nuclear extracts of the cells cultured in the presence or absence of Dox for 24 h. The immunoprecipitants were eluted from the beads by incubating with synthetic Flag peptides, and then resolved by SDS–PAGE. Multiple bands ranging from 30 to 150 kDa were observed in each fraction (Fig. 5, upper panel). Most bands in the silver-stained gels were recognized in the NOR1- and EWS/NOR1-expressing cells equally, suggesting that the basic transcriptional machinery was the same for the NOR1 and EWS/NOR1-dependent regulation. To analyze the complexes precisely, we then subjected the eluted NOR1- and EWS/NOR1-containing complexes to glycerol density gradient segregation, and found that several bands differed in the two gels (Fig. 5, lower

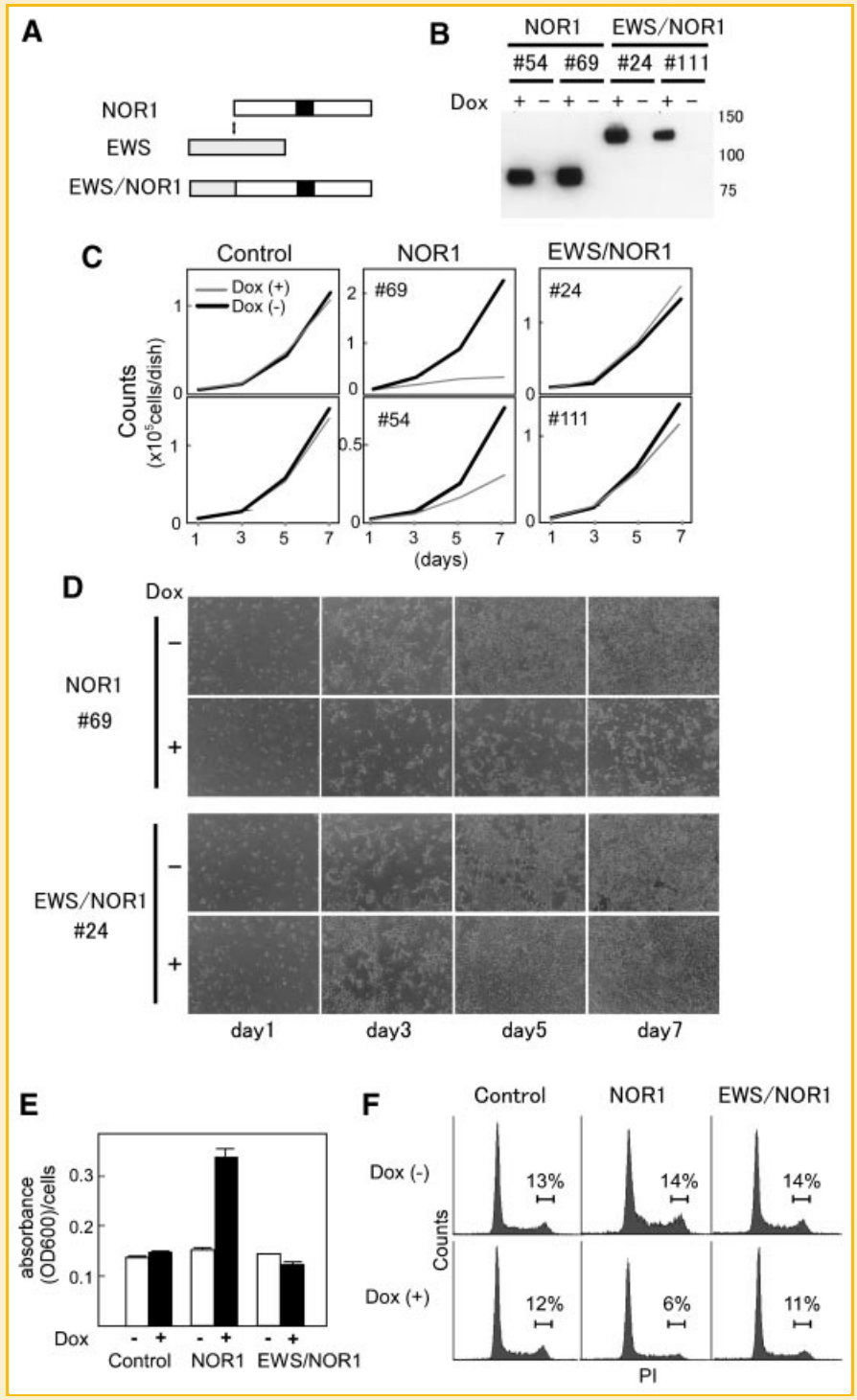

Fig. 1. Differences between NOR1- and EWS/NOR1-expressing cells. A: Schematic representation of EWS, NOR1, and EWS/NOR1 proteins. An arrow and closed boxes indicate a break point of the fusion, and the DNA binding domain of NOR1, respectively. B: Inducible expression of NOR1 and EWS/NOR1. 293 cells stably transfected with pTet-On advanced and pTRE-tight-Flag-tagged NOR1 or EWS/NOR1 vectors, were treated with (+) or without (−) Dox for 24 h. Cell lysates were subjected to Western blotting with anti-Flag antibody. C: Effect of the NOR1 and EWS/NOR1 expression on cell growth. Tet-On-NOR1 (clone #54 and #69), tet-On-EWS/NOR1 (clones #24 and #111), and control 293 cells were treated with/without Dox for 7 days. Cell growth was examined by using a Coulter counter at indicated time points. D: Morphological changes after the induction of NOR1 or EWS/NOR1 expression. Tet-On-NOR1 (clone #69) and tet-On-EWS/NOR1 (clone #24) 293 cells treated with/without Dox were monitored by phase-contrast microscopy. E: Apoptotic cell death was increased in NOR1-expressing cells. Apoptotic cell death of tet-On-NOR1, tet-On-EWS/NOR1, and control cells treated with/without Dox for 48 h was examined by using a cell death detection ELISA kit. F: Cell cycle progression was inhibited in NOR1-expressing cells. Tet-On-NOR1, tet-On-EWS/NOR1, and control cells treated with/without Dox for 12 h were fixed, stained with PI, and then analyzed by flow cytometry. Populations in G2/M are indicated in each panel.

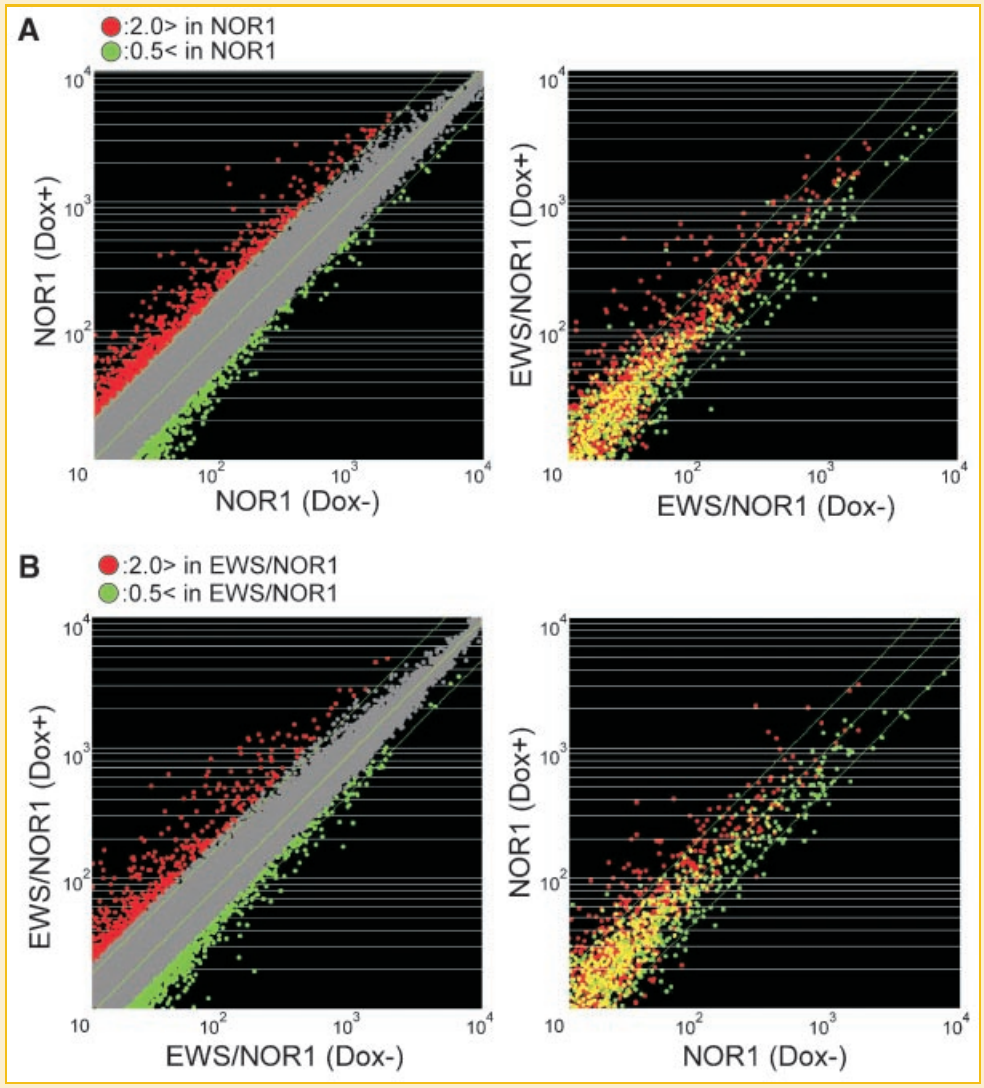

Fig. 2. Changes in global gene expression after the NOR1 and EWS/NOR1 induction. Tet-On-NOR1 (clone #69) and tet-On-EWS/NOR1 (clone #24) cells were cultured for 24 h with/without Dox. Total RNA isolated from the cells was subjected to the Affymetrix GeneChip analysis. The diagrams indicate scattered plots of genes positioned by the expression levels in the cells. Expression levels in each gene in the cells treated with Dox and without Dox are represented by Y and X axes, respectively (log scale). A: Up- ($>\times 2.0$) and down-regulated ($<\times 0.5$) genes in tet-On-NOR1 cells after the treatment are indicated by red and green, respectively (left panel). In the diagram of tet-On-EWS/NOR1 cells (right panel), only the up- and down-regulated genes in NOR1-expressing cells are shown in the same colors as on the left. B: Up-regulated and down-regulated genes in tet-On-EWS/NOR1 cells are also indicated by red and green, respectively (left panel). In NOR1 tet-On cells (right panel), up- and down-regulated genes in tet-On-EWS/NOR1 cells are shown as on the left.

panels). Only the predominant bands that differed between the NOR1 and EWS/NOR1 immunoprecipitants were isolated, and subjected to mass spectrometry for identification. Among the candidate proteins that differed in their affinity for NOR1 and EWS/NOR1, we identified one at 120 KDa, which was pre-dominantly detected in the NOR1-immunoprecipitants, as poly(ADP-ribose) polymerase I, PARP-1. Unfortunately, the other proteins were not identified by mass spectrometry due to the insufficiency of the proteins.

CONFIRMATION OF THE INTERACTION BETWEEN NOR1 AND PARP-1

The interaction of PARP-1 with Flag-tagged NOR1 was examined by immunoprecipitation with anti-Flag followed by immunoblotting with anti-PARP-1 antibody (Fig. 6A). PARP-1 was clearly associated with NOR1, but little associated with EWS/NOR1. Interestingly, another member of the NR4A family, Nurr1 but not Nur77, associated with PARP-1. To examine the intracellular distribution of NOR1 and PARP-1, we performed an immunocytochemical analysis with anti-PARP-1 antibody in COS7 cells transfected with the GFP-fused NOR1 expression plasmid. The GFP-NOR1 fusion protein was located in the nucleus with a granular distribution, and partially co-localized with PARP-1 (Fig. 6B). To further confirm the endogenous interaction, we examined the association between endogenous NOR1 and PARP-1 under conditions where NOR1 expression is induced. While the endogenous NOR1 expression was barely

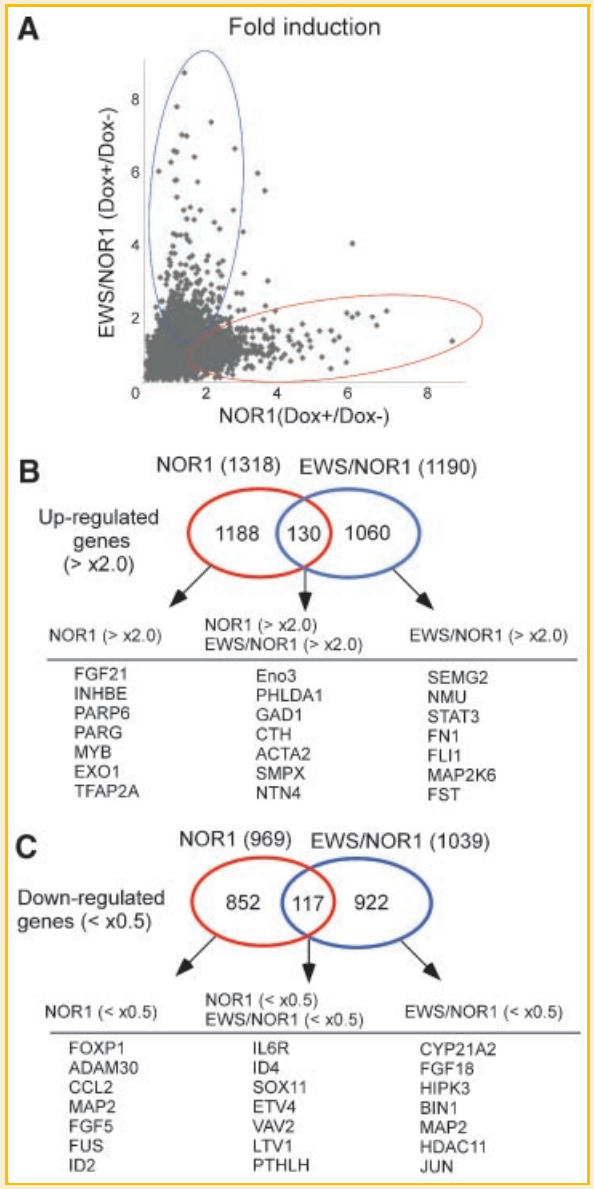

Fig. 3. Sets of genes are differentially regulated in cells expressing NOR1 and EWS/NOR1. A: Fold induction of each gene, represented as a ratio between expression levels in Dox-treated and un-treated cells, compared between NOR1- and EWS/NOR-expressing cells. X and Y axes indicate fold induction observed in NOR1- and EWS/NOR1-expressing cells, respectively. Genes induced preferentially in NOR1- and EWS/NOR1-expressing cells are indicated by red and blue circles, respectively. B: A Venn diagram of sets of up-regulated genes in NOR1- and EWS/NOR1-expressing cells. C: A Venn diagram of sets of down-regulated genes in NOR1- and EWS/NOR1-expressing cells. Figures indicate the number of genes categorized in each subset. Some typical genes in each subset are also listed below.

detectable in normally growing and resting cells, its expression was rapidly induced by extracellular stimuli such as fetal calf serum (FCS), 12-*o*-tetradecanoylphorbol 13-acetate (TPA) and cell stress. For the FCS stimulation, HeLa cells were deprived of FCS for 24 h then stimulated with it for 1, 2, and 4 h. Cells were subsequently lysed, fractionated into cytosolic and nuclear fractions, and the nuclear fractions were immunoprecipitated with the anti-NOR1 monoclonal antibody. The association of NOR1 with PARP-1 was observed in the starved condition (time 0) (Fig. 6C). After the FCS treatment, endogenous NOR1 expression was enhanced rapidly, and the bands immunoreactive with anti-NOR1 antibody shifted to a high molecular weight. This shift supposes phosphorylation of the NOR1 protein, since NOR1 is highly phosphorylated by FCS (data not shown). Although the production of NOR1 protein was up-regulated by FCS in a time-dependent manner, the interaction between endogenous NOR1 and PARP-1 was attenuated by the stimulation. NOR1 expression was also induced by cell shearing stress [Bandoh et al., 1997]. HeLa cells were collected by trypsin treatment, sheared, and then harvested in complete medium. After 1 h, the expression of NOR1 was slightly increased by shearing stress, and interaction between NOR1 and PARP-1 was observed (Fig. 6D). In contrast, on treatment with TPA for 1 h and FCS for 4 h, the expression of NOR1 was highly induced, but the association was weak. These results indicate that the association was influenced, at least in part, by posttranslational modifications including phosphorylation. We also confirmed that NOR1 physically interacted with PARP-1 by conducting GST pull-down assays (Fig. 7A), and that the interaction was not bridged by DNA by using DNase I treatment (Fig. 7B). Furthermore, we verified that NOR1 was not a substrate for poly(ADP-ribosyl)ation by PARP-1 by examining the poly(ADP-ribosyl)ation of NOR1 in cells treated with the DNA-damaging agent bleomycin (Fig. 7C).

NOR1–PARP-1 INTERACTION REQUIRES THE DNA-BINDING DOMAINS OF BOTH PROTEINS

Although EWS/NOR1 contains a full-length NOR1, little association between EWS/NOR1 and PARP-1 was detectable. Therefore, we next examined the regions of the proteins responsible for the interaction. NOR1 consists of an N-terminal transactivating domain, a central DNA-binding domain (DBD), and a C-terminal putative ligand-binding domain. To map the interaction domain in NOR1, we performed protein-binding experiments using truncated mutants lacking the N-terminal or C-terminal domain of the protein. Flag-tagged NOR1 expression vectors or truncated mutants were transfected into HEK293T cells. Immunoblot analysis of the products with antibodies to Flag confirmed the presence of the mutants. As shown in Figure 8A, the N-terminal or C-terminal-lacking mutants (ΔC1, ΔC2, ΔN1, and ΔN2) retained the ability to associate, but further deleted constructs (ΔC3 and ΔN3) lost this ability. These results indicate that domains required for the association with PARP-1 involve a central region of NOR1, which corresponds to the DBD. We further observed that the association between NOR1 and PARP-1 was disrupted by a single point mutation, Cys to Tyr at codon 293 (C293Y), of NOR1, which corresponds to the first Cys constituting the zinc finger motif.

Full-length PARP-1 encompasses three functional domains, the N-terminal DBD, the central automodification domain, and the C-terminal catalytic domain. To map the interaction domain in PARP-1 also, myc-tagged truncated mutants of PARP-1 along with Flag-tagged NOR1 expression vectors were transfected into HEK293T cells. As shown in Figure 8B, deletion of the N-terminal DBD (TPH), or zinc finger motif (TPK) impaired and weakened the

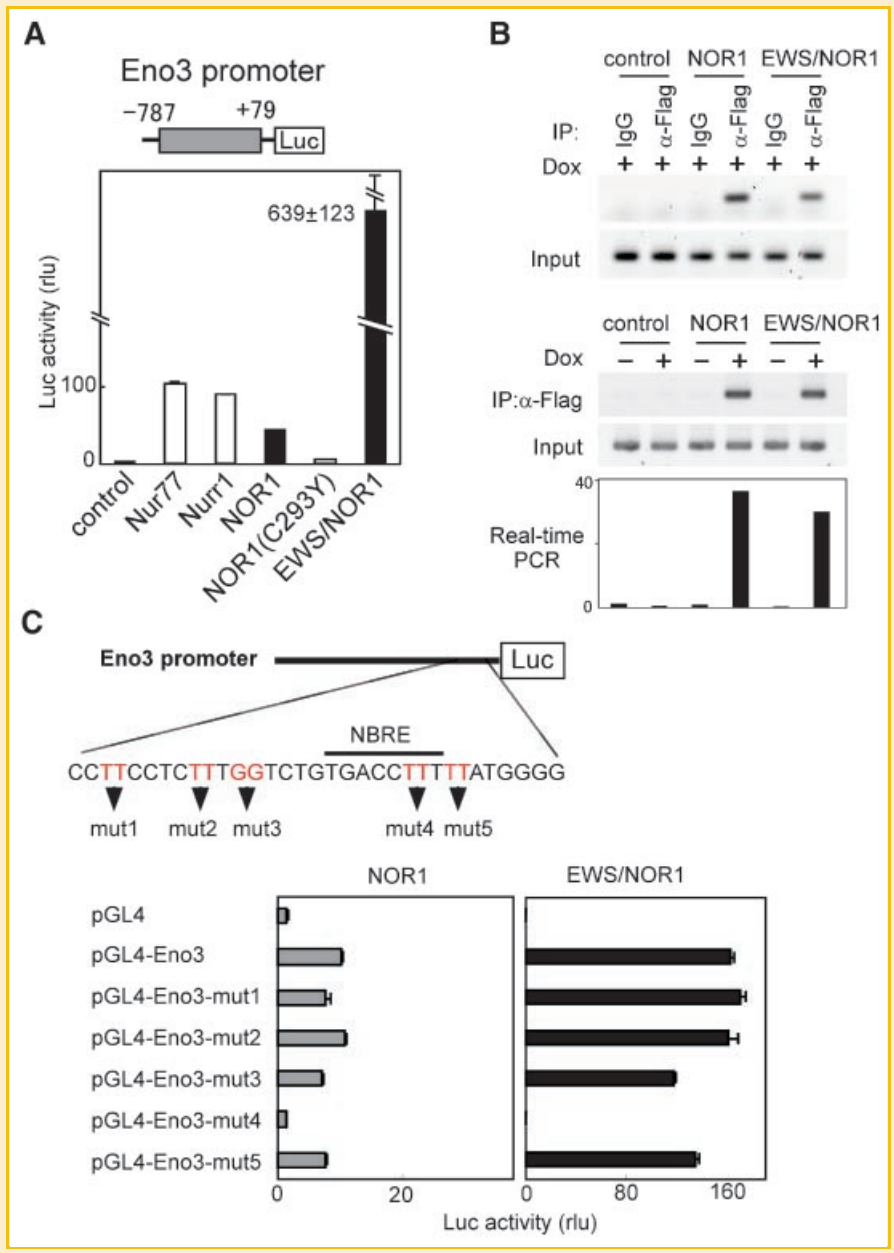

Fig. 4. Eno3 shows typical induction patterns by NOR1 and EWS/NOR1. A: The Eno3 promoter was transactivated by the Nur77 family (Nur77, Nurr1, and NOR1) and EWS/NOR1 in the reporter assay. NOR1(C293Y) indicates a mutant that has one amino acid substitution Cys to Tyr at codon 293 of NOR1, which corresponds to the first Cys constituting the zinc finger motif. 293T cells were transfected with the expression vector containing the listed genes along with the Eno3 reporter construct. Twenty-four hours after the transfection, cells were lysed and subjected to the luciferase assay. A renilla luciferase expression plasmid was cotransfected as an internal control for transfection efficiency. Luciferase activity normalized to renilla luciferase activity is shown (mean ± SE, n = 3). B: NOR1 and EWS/NOR1 can bind to the promoter region of Eno3. Chromatin immunoprecipitaion with the anti-Flag antibody or mouse IgG was preformed in tet-On-NOR1, tet-On-EWS/NOR1 and control cells treated with/without Dox. Immunoprecipitants and inputs were subjected to PCR amplification to detect the promoter region of Eno3 (upper and middle panels). The amount of immunoprecipitaed DNA of the region was also compared among the cells treated with/without Dox using the real-time PCR (lower panel). C: Eno3′s activation by NOR1 and EWS/NOR1 was dependent on the response element NBRE. 293T cells were transfected with the NOR1 or EWS/NOR1 expression vector along with the mutated Eno3 promoter-reporter constructs (mut1 to mut5), which have two nucleotides replacements in the vicinity of NBRE. The positions of the replacement are indicated schematically.

association, respectively. Moreover, amino acid replacements in the zinc finger motifs (TPZ1 and TPZ2) also led to less association. These results indicate that the association between NOR1 and PARP-1 requires zinc finger motifs of both proteins.

THE N-TERMINAL REGION OF NOR1 IS IMPORTANT FOR THE INTERACTION

The region of NOR1 associated with PARP-1 was located in the DBD, and the association was effectively abolished by a single point

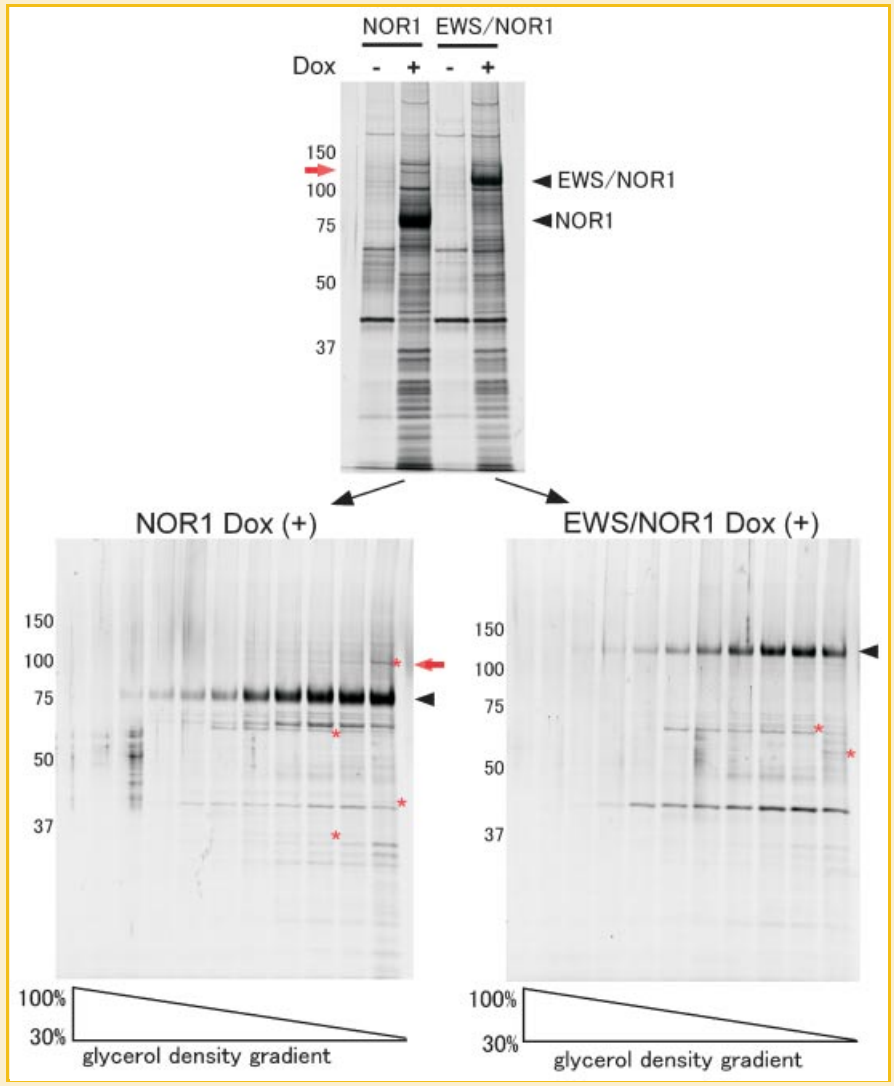

Fig. 5. Identification of proteins alternatively interacting with NOR1 and EWS/NOR1. Complexes containing Flag-tagged NOR1 and EWS/NOR1 were immunoprecipitated with the anti-Flag antibody from tet-On-NOR1 (clone #69) and tet-On-EWS/NOR1 (clone #24) cells with/without Dox. The complexes were eluted by incubation with Flag peptides, and separated by 10% SDS-PAGE (upper panel). Immunoprecipitants derived from the Dox-treated tet-On-NOR1 and tet-On-EWS/NOR1 cells were further subjected to glycerol density gradient segregation (lower panels). A photographic image of gels stained with silver is shown. The arrowheads and stars indicate immunoprecipitated NOR1 and EWS/NOR1 proteins, and candidate proteins showing alternative binding with NOR1 and EWS/NOR1, respectively. Arrows indicate the position of PARP-1, which was determined as an NOR1-binding protein by mass spectrometric analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mutation here, C293Y. However, DBD is also conserved in EWS/NOR1, which showed an attenuated association with PARP-1. Moreover, one of the Nur77 family members, Nur77, could not associate with PARP-1 (Fig. 6A), even in the presence of a DBD sharing approximately 98% sequence similarity with that of NOR1. Thus, to further clarify the requirement of the interaction, we used genetic approaches with chimeric constructs, which were generated by exchanging domains between Nur77 and NOR1. Deleted and chimeric constructs of Nur77 or NOR1 were transfected into the HEK293T cells, immunoprecipitated with anti-Flag antibody, and subjected to immunoblotting with anti-PARP-1 antibody. Coimmunoprecipitation of PARP-1 was detected with the N-terminal-deleted constructs of Nur77 (Nur77ΔN) and NOR1 (NOR1ΔN), and C-terminal-deleted construct of NOR1 (NOR1ΔC), but not with the C-terminal-deleted construct of Nur77 (Fig. 9). A chimeric construct composed of the N-terminal region of NOR1 and Nur77ΔN associated with PARP-1. In contrast, a construct composed of the N-terminal region of Nur77 and NOR1ΔN barely associated with PARP-1. These results indicate that the N-terminal region of Nur77 interfered with the association between PARP-1 and Nur77, and that subtype specificity for the binding of PARP-1 was reliant on the difference in the N-terminal regions of the NR4A family. Therefore, the attenuated association between PARP-1 and EWS/NOR1 might depend on the structural alteration of the N-terminal region of NOR1, since the gene fusion caused by the chromosomal translocation fused EWS to the N-terminus of NOR1.

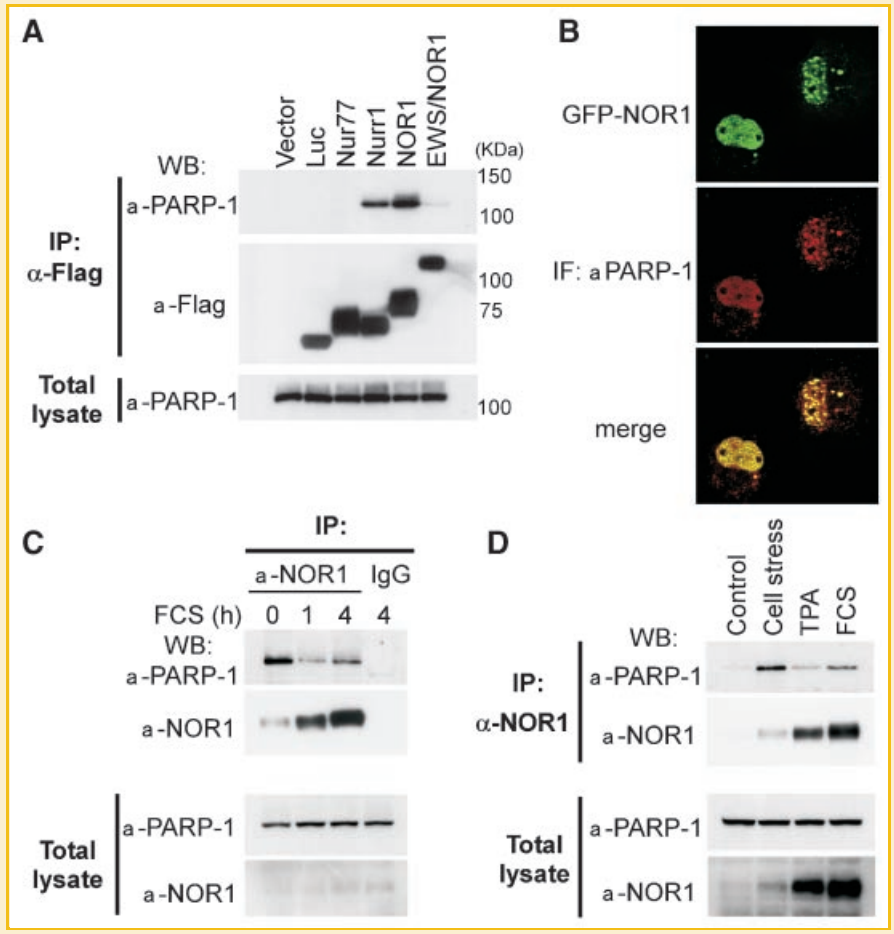

Fig. 6. PARP-1 associates with NOR1, but barely with EWS/NOR1. A: Flag-tagged Nur77, Nurr1, NOR1, EWS/NOR1 and luciferase (Luc; control) were immunoprecipitated from transfected HEK293T cells using anti-Flag antibody. Immunoprecipitated proteins (IP) were detected by Western blotting (WB) with anti-Flag or anti-PARP-1 antibodies. B: Intracellular colocalization of NOR1 and PARP-1. COS7 cells transfected with the GFP-fused NOR1 expression vector were subjected to immunofluorescence staining (IF) with anti PARP-1 antibody. C: Endogenous interaction between NOR1 and PARP-1. HeLa cells starved for 24 h were stimulated with FCS for the period indicated. Nuclei prepared from the cells were lysed and immunoprecipitated with anti-NOR1 monoclonal antibody. Mouse IgG was used as a control. Immunoprecipitated proteins were detected by Western blotting with anti-NOR1 monoclonal antibody and anti PARP-1 antibody. D: NOR1 induced by stimuli associates with PARP-1. NOR1 induced by cell stress, TPA, and FCS in HeLa cells was immunoprecipitated with anti-NOR1 monoclonal antibody, and then subjected to Western blotting.

PARP-1 SUPPRESSES NOR1-, BUT NOT EWS/NOR1-, DEPENDENT TRANSACTIVATION

NOR1 is thought to exert its biological function as a transcription factor by binding to specific consensus DNA sequences, NBRE and NurRE, within the promoter region of target genes. NBRE was isolated by genetic screening in yeast, and recognized by the NR4A receptors as a monomer [Wilson et al., 1991]. NurRE was identified as a responsive element of Nur77 in the 5′ upstream region of pro-opiomeranocortin (POMC), and recognized as a homodimer [Philips et al., 1997; Maira et al., 2003]. To study the role of PARP-1 in a physiological context, we first examined the effect of PARP-1 on the transcriptional activity of the NR4A receptors through these response elements. NOR1, Nurr1, or Nur77, together with the NBRE-Luc or NurRE-Luc reporter plasmid, was transfected into the PARP-1(−/−) mouse embryonic fibroblasts, a cell line lacking PARP-1 derived from a PARP-1 knockout mouse. The cells were harvested 48 h after the transfection, and the luciferase levels were quantified. NOR1, Nurr1, and Nur77 transactivated both the NurRE-Luc and NBRE-Luc reporter constructs, as reported previously [Wilson et al., 1991; Philips et al., 1997] (Fig. 10A). An increased amount of PARP-1 caused a substantial decrease in NOR1 and Nurr1, but not Nur77, -dependent transcriptional activity through the palindromic DNA response element NurRE. This result is consistent with the PARP-1-binding property. On the other hand, through the monomeric DNA response element NBRE, PARP-1 did not have any effect on the Nur77-, Nurr1-, and NOR1-dependent transcriptional activity. To examine the involvement of poly(ADP-ribose) polymerase activity in the suppression, we further examined the suppressive activity of a enzymatically inactive mutant of PARP-1. An increased amount of PARP(K893I) mutant, which lacks poly(ADP-ribose) polymerase activity [Simonin et al., 1993], also caused a substantial decrease in the NOR1-dependent transcriptional activation (Fig. 10B), indicating that the suppressive effect of PARP-1 was independent of its poly(ADP-ribose) polymerase activity.

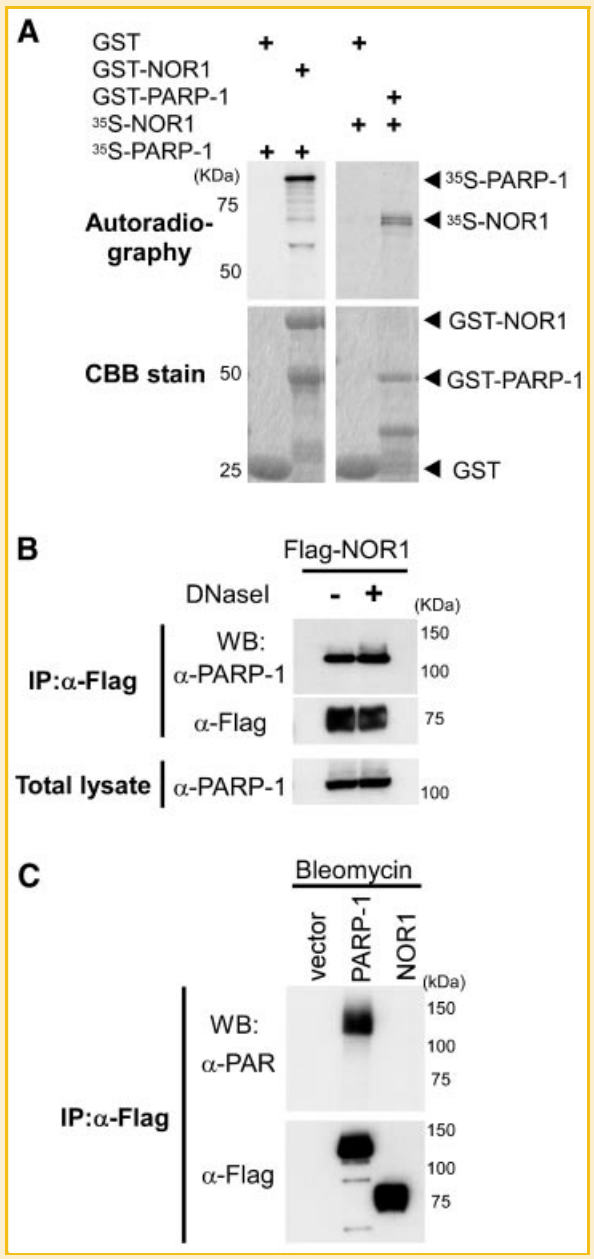

Fig. 7. NOR1 interacts physically with PARP-1. A: GST-fused NOR1 (amino acids 231–626) or PARP-1 (7–232) was incubated with in vitro transcribed/translated [^{35}S]-labeled PARP-1 or NOR1, respectively. Bound proteins were subjected to SDS–PAGE and autoradiography. B: Interaction between NOR1 and PARP-1 is not impaired by DNaseI treatment. Flag-tagged NOR1 was immunoprecipitated from transfected HEK293T cells using anti-Flag antibody. Immunoprecipitaed proteins were treated with DNaseI for 1 h at 37°C in DNaseI buffer, washed with lysis buffer, and subjected to Western blotting with anti-Flag or anti-PARP-1 antibodies. C: NOR1 is not poly(ADP-ribosyl)ated by PARP-1. HEK293T cells were transfected with Flag-tagged PARP-1 or NOR1. Twenty-four hours after the transfection, the cells were treated with bleomycin (0.4 mg/ml) for 1 h, and then lysed with lysis buffer. Cell extracts were immunoprecipitated with anti-Flag antibody, and subjected to Western blotting using anti-poly(ADP-ribose) (PAR) or anti-Flag antibodies.

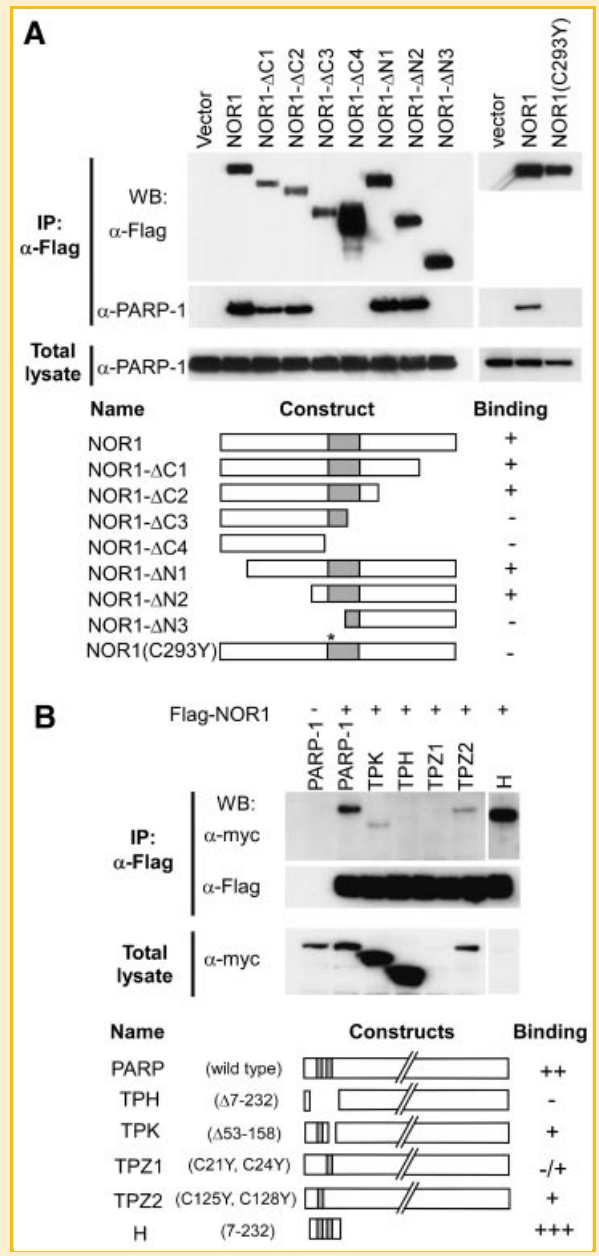

Fig. 8. Interacting domains of NOR1 and PARP-1 for their interaction. A: The central DNA binding domain of NOR1 is required for the association with PARP-1. HEK293T cells were transfected with Flag-tagged NOR1 deletion constructs. Cell extracts were immunoprecipitated with anti-Flag antibody. Immunoprecipitated proteins were detected by Western blotting with anti-Flag or anti-PARP-1 antibodies. The lower panel indicates schematic representations of the NOR1 deletion constructs. Gray boxes indicate the DNA binding domain. NOR1(C293Y) represents an NOR1 mutant containing a single Cys replacement to Tyr at codon 293. B: The N-terminal DNA binding domain of PARP-1 interacts with NOR1. HEK293T cells were co-transfected with Flag-tagged NOR1 and myc-tagged PARP-1 mutants. Immunoprecipitated proteins with anti-Flag antibody were detected by Western blotting with anti-Flag or anti-myc antibodies. The lower panel indicates schematic representations of the PARP-1 deletion constructs. Gray boxes indicate zinc finger motifs.

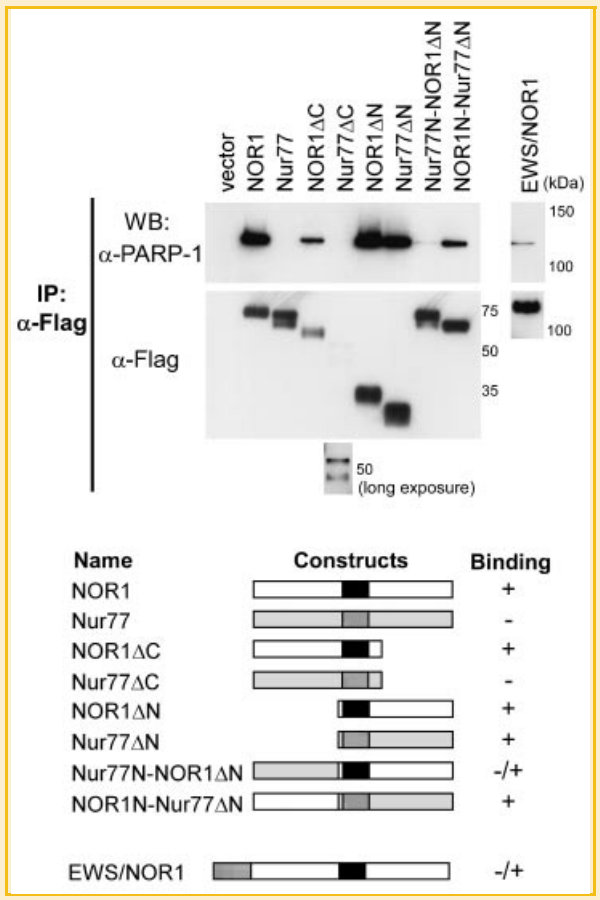

Fig. 9. The N-terminal region of NOR1 is important for the PARP-1-interaction. Flag-tagged deleted and chimeric constructs of NOR1 and Nur77 were immunoprecipitated from transfected cells using anti-Flag antibody, and immunoprecipitated proteins were detected by Western blotting with anti-Flag or anti-PARP-1 antibodies. Flag-tagged EWS/NOR1 was also immunoprecipitated and subjected to Western blotting with anti-PARP-1. The lower panel indicates schematic representations of the deleted and chimeric constructs of NOR1 and Nur77, and EWS/NOR1. Dotted, closed, and hatched boxes indicate the N-terminal region of EWS, the DNA binding domains of NOR1 and Nur77, respectively.

We next examined the effect of PARP-1 on the EWS/NOR1-dependent transcriptional activity. As shown in Figure 10C, PARP-1 had no effect on the transcriptional activity through the palindromic NurRE or monomeric NBRE response element, consistent with the binding property. PARP-1 also did not show the significant effect on the NOR1- and EWS/NOR1-dependent transcriptional enhancement through the Eno3 promoter, which contains a single copy of NBRE. These results suggest that EWS/NOR1 is not involved in the PARP-1-mediated transcriptional attenuation, and thus, PARP-1 might be one of the factors that govern the differences in control mechanisms between NOR1 and EWS/NOR1.

DISCUSSION

NOR1 is a member of the NR4A subfamily within the nuclear receptor superfamily. The NR4A family has been characterized as immediate-early gene products produced in response to a variety of mitogenic stimuli such as growth factors and liver regeneration, and appear to be involved in T-cell receptor-mediated apoptosis of immature thymocytes, and vascular smooth muscle cell proliferation [Cheng et al., 1997; Nomiyama et al., 2006]. On the other hand, the EWS/NOR1 fusion gene, which is created by the chromosomal translocation t(9;22) in extraskeletal myxoid chondrosarcoma, has been classified as an oncogene, since its translocation was observed in extraskeletal myxoid chondrosarcomas recurrently, and forced expression of EWS/NOR1 in the fetal rat chondrogenic cell line CFK2 resulted in cellular transformation [Filion and Labelle, 2004]. Since EWS/NOR1 has been shown to possess strong transcriptional activation activity compared to NOR1, and to bind to the NOR1 target DNA sequences equally in vitro, it has been believed that EWS/NOR1 causes malignant transformation by functioning as a transcription factor activating NOR1-target genes.

To address the functional differences between NOR1 and EWS/NOR1, we established NOR1- and EWS/NOR1-expressing clones from the same cell line to avoid the influences derived from differences of intracellular components, extracellular environment, and genetic background. We observed that the majority of genes are differentially regulated in NOR1- and EWS/NOR1-expressing cells, as expected, even under conditions where the two proteins were expressed constitutively at similar levels for identical periods in the same cell line. Although differences in cell type, clonal variation, and induction (i.e., immediate-early response vs. constitutive expression), as well as other oncogenic mutations in skeletal myxoid chondrosarcomas, may be responsible, at least in part, for the differences between NOR1- and EWS/NOR1-induced phenotypes, this result confirmed the existence of a functional difference in their transcriptional activities. Subramanian et al. [2005] found that some sets of genes are commonly up-regulated in extraskeletal myxoid chondrosarcomas by examining the

Fig. 10. PARP-1 suppresses transcriptional activities of NOR1, but not EWS/NOR1. A: PARP-1 suppresses NOR1- and Nurr1-dependent transactivation in a DNA response element-specific manner. PARP-1(−/−) fibroblasts were transfected with the NurRE-Luc or NBRE-Luc reporter construct, and an expression vector encoding Nur77, Nurr1, or NOR1, along with an increased amount of the PARP-1 expression vector. Transfected cells were grown for 48 h, and extracts of the harvested cells were tested for luciferase activity. A renilla luciferase expression plasmid was cotransfected as an internal control for transfection efficiency (mean ± SE, n = 3). Data are also shown as percent changes from NR4A-transfected samples without PARP-1 expression vector (100%) in lower panels. B: Poly(ADP-ribose) polymerase activity of PARP-1 is not required for the transcriptional suppression. PARP-1(−/−) fibroblasts were transfected with the NurRE-Luc and NOR1 expression vectors along with an increased amount of the PARP-1 or PARP(K893I) expression vectors. PARP(K893I), which contains one amino acid replacement Lys to Ile at codon 893, has been shown to lack the poly(ADP-ribose) polymerase activity. C: Transactivation activity of EWS/NOR1 is independent of the PARP-1-mediated suppression. PARP-1(−/−) fibroblasts were transfected with NBRE-Luc or NurRE-Luc and EWS/NOR1 expression vector along with an increased amount of PARP-1 expression vector. D: PARP-1 has no effect on the NOR1- and EWS/NOR-dependent transcriptional enhancement through the Eno3 promoter. PARP-1(−/−) fibroblasts were transfected with NOR1, EWS/NOR1, and PARP-1 expression vectors, as indicated, along with the Eno3 promoter-Luc construct (pGL4-Eno3).

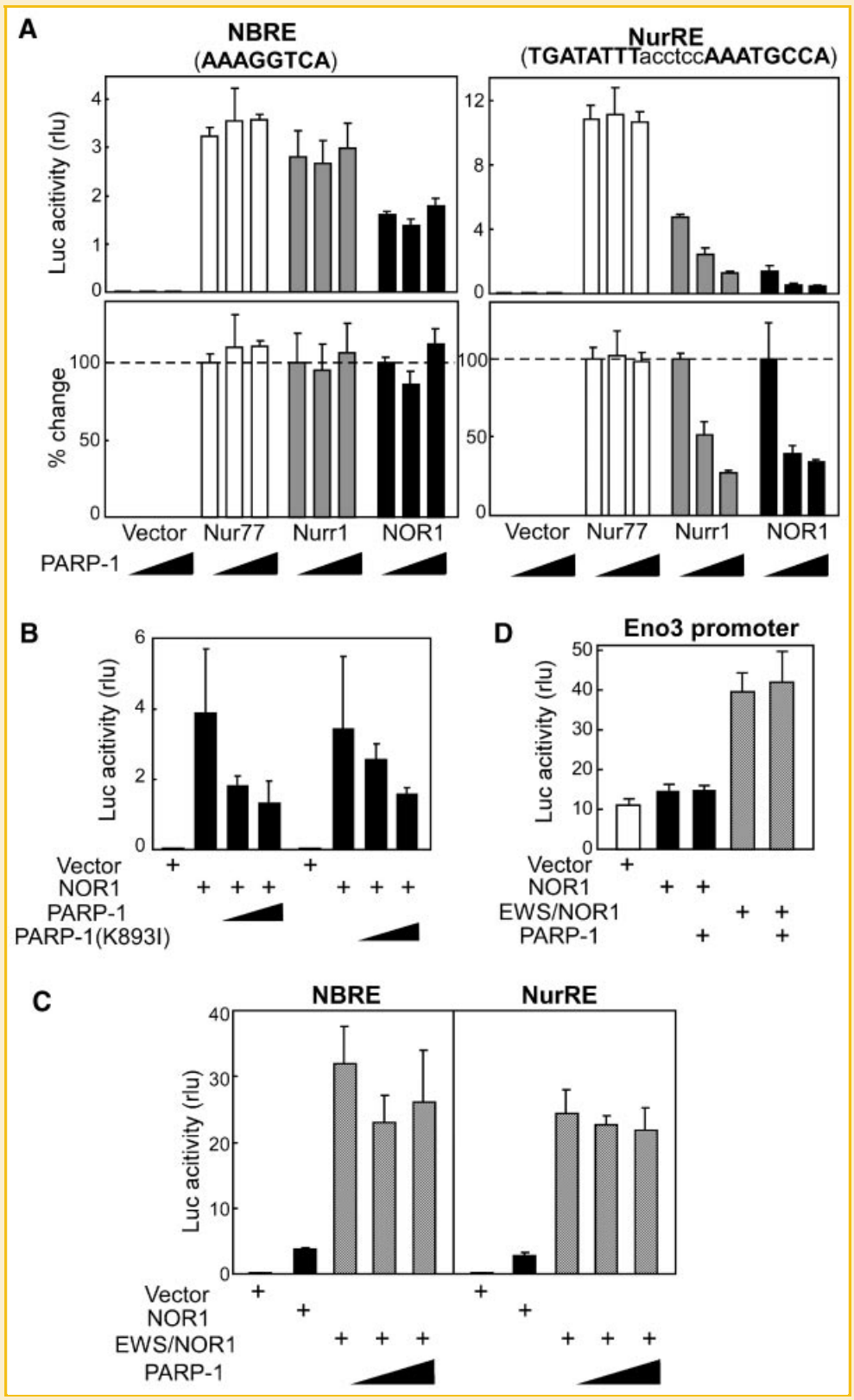
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Fig. 10.

expression profiles of sarcomas and carcinomas. Most of them, however, differed from the NOR1-target genes reported, also suggesting a difference between NOR1- and EWS/NOR1-dependent transcriptional control. Therefore, it is unlikely that the simple overexpression of NOR1 is sufficient to induce the sets of genes that are required for the pathogenesis of extraskeletal myxoid chondrosarcoma. On the other hand, we observed that a limited number of genes, such as the Eno3 gene, were up-regulated by both NOR1 and EWS/NOR1. In the case of the Eno3 promoter, the induction by NOR1 and EWS/NOR1 was clearly dependent on the NBRE located just upstream of the transcriptional initiation site. These results are consistent with the established idea that EWS/NOR1 is a strong transcriptional activator of NOR1-target genes. In addition, we observed that up-regulated genes in NOR1-overexpressing cells tended to be up-regulated also in EWS/NOR1-overexpressing cells, and vice versa; and that the proteins associated with NOR1 were mostly the same as those associated with EWS/NOR1. Altogether, these results suggest that, whereas NOR1 and EWS/NOR1 transactivate closely overlapping target genes via a common transcriptional machinery, the two proteins utilize a mechanism that confers differential transcriptional activity on the machinery to achieve their specific biological roles.

Nuclear receptors, including NOR1, transactivate their target genes basically through specific DNA response elements, which are similar to, but distinct from each other within the nuclear receptor superfamily. We observed that the majority of the genes up-regulated by NOR1 or EWS/NOR1 did not contain an NBRE or NurRE within 2 kb upstream of the transcription start site and intron 1. Moreover, even in the genes possessing an NBRE in their promoter regions, their alternative induction by NOR1 or EWS/NOR1 was not recapitulated by the reporter gene constructs containing those promoter regions. Although the observed differences in the expression profiles might involve secondary effects of NOR1- and EWS/NOR1-induced genes, they suggest the existence of additional regulatory mechanisms in addition to the NBRE-dependent transactivation. Given that some nuclear receptors achieve transactivation via interaction with coregulators [Lin et al., 2004; Mix et al., 2007; Shatnawi et al., 2007], one possible explanation is that NOR1 as well as EWS/NOR1 transactivates target genes through not only direct DNA binding but also interaction with transcription factors and co-regulators. However, we cannot rule out the possibilities that EWS/NOR1 utilizes completely different DNA response elements from NOR1, and that posttranslational modifications of NOR1 and EWS/NOR1 change their DNA sequence specificity and/or transactivation ability.

Important to elucidating the functional differences between NOR1 and EWS/NOR1 is the identification of interacting proteins. Using transcriptional complex purification and density gradient segregation, followed by MS, we identified one protein, PARP-1, as interacting with NOR1. PARP-1 is a nuclear enzyme that catalyzes the transfer of ADP-ribose to a specific subset of nuclear substrates, such as histones and transcription factors. Although PARP-1 has been studied for its role in DNA repair, there have been several reports demonstrating its role in both the activation and repression of transcription. For example, PARP-1 has been shown to bind the oncogenic protein B-Myb to enhance its transactivating property [Cervellera and Sala, 2000], and to be necessary for retinoic acid-dependent transcription [Pavri et al., 2005]. We also observed that the transactivation activity of NOR1 was attenuated by coexpression of PARP-1 through NurRE. Consistent with the binding property, this suppressive activity of PARP-1 was not observed in the transactivation by EWS/NOR1. Therefore, we speculate that aberrant expression of the EWS/NOR1 fusion protein in myxoid chondrosarcoma may favor disease progression by escaping from the transcriptional suppression of PARP-1. Moreover, we also detected other proteins that differed in their binding with NOR1 versus EWS/NOR1 (shown in Fig. 5), suggesting that EWS/NOR1 activates specific sets of genes in concert with interacting proteins that differ from those of NOR1. Further experiments are required to clarify the involvement of EWS/NOR1- or NOR1-interacting proteins in their specific transcriptional activities.

In addition to EWS/NOR1, other types of EWS-fused gene products have been reported in tumors. EWS/FLI-1 is the most common fusion construct in Ewing tumors, occurring in 85% of reported cases, while EWS/ERG, EWS/FEV, EWS/ETV1 and TLS/ERG occur at much lower frequencies [Janknecht, 2005]. EWS/FLI-1 induced the rapid onset of myeloid/erythroid leukemia in mice [Torchia et al., 2007]. However, transgenic mice made to overexpress FLI-1 do not develop leukemia, although activation of FLI-1 is required for the development of erythroleukemia [Zhang et al., 1995]. EWS/FLI-1 also showed stronger transcriptional activity than FLI-1 [Oikawa, 2004], but this observation suggests the existence of differences between FLI-1 and EWS/FLI-1 in transcriptional control. Whereas EWS/FLI-1 and EWS/NOR1 showed stronger transcriptional activation than FLI-1 and NOR1, respectively, the EWS-related fusion gene products may have qualitatively different transactivation activities conferred by specifically associated proteins, in addition to strong transcriptional activation, for their transforming activity.

PARP-1 has been shown to affect transcriptional activity through the ADP-ribosylation of transcription factors, such as TBP, TFIIF, SP-1 and CREB [Rawling and Alvarez-Gonzalez, 1997; Oei et al., 1998]. These proteins are highly specific substrates for poly(ADP-ribosyl)ation, and modifications prevent them from binding to their respective DNA consensus sequences. Our work demonstrated that PARP-1′s function as a transcriptional corepressor modulating NOR1-dependent transcription did not require the PARP-1 polymerase activity. This suggests that events other than poly(ADP-ribosyl)ation are the basis of the suppression. Similarly, the interaction between NF-kB p65 and PARP-1 has been shown not to require the enzymatic activity of PARP-1 as a coactivator [Hassa et al., 2003]. PARP-1 also enhances the transactivation of B-Myb, independently of PARP-1′s enzymatic activity [Cervellera and Sala, 2000]. These observations indicate PARP-1-mediated transcriptional suppression to depend on direct interaction independent of enzymatic activities. In addition, repression by PARP-1 was detected in experiments using the reporter construct containing the dimeric response element NurRE, but not the monomeric NBRE. Since the NurRE was recognized through the homodimer of NR4As [Maira et al., 2003], the specificity might be dependent on the conformational accessibility caused by NOR1 and PARP-1, and/or the involvement of PARP-1-mediated DNA sequence recognition. These

results further suggest PARP-1 to play a role in modifying the DNA sequence specificity of NOR1, in addition to the suppressive activity.

Our results also indicate a subtype-specific interaction between PARP-1 and the NR4A receptors. The association was observed in NOR1, weakly in Nurr1, but not at all in Nur77, although the three have almost the same amino acid sequence in the DNA-binding domain, which corresponds to the domain interacting with PARP-1. We found that the central DNA-binding domain of Nur77 was capable of binding PARP-1, whereas the N-terminal region of Nur77 disrupted the interaction. Since the homology in the N-terminal transactivation domain among the NR4A family is low, one possibility is that differences in the N-terminal sequence cause conformational changes, resulting in masking of the PARP-1-binding domain and thereby preventing the interaction. In addition, EWS/NOR1, which consists of the N-terminal half of EWS fused to the N-terminus of the full-length NOR1, was unable to bind PARP-1 either, supporting the hypothesis that the conformational change in the N-terminus of NOR1 affects its binding with PARP-1.

We showed that PARP-1 was a negative regulator of NOR1 and Nurr1, but not Nur77, through the palindromic response element NurRE, but not the monomeric response element NBRE. Members of the NR4A family have been shown to be expressed in various tissues including developmental brain and endocrine tissues, with partially overlapping expression profiles [Zetterstrom et al., 1996]. Thus, our finding that PARP-1 suppressed the NR4A-dependent transcriptional activity in a subtype- and DNA response element-specific manner, suggests that PARP-1 plays a role in the target gene-specific and tissue/developmental stage-selective regulation of the NR4A receptors. PARP-1 was reported to function as a coregulator of retinoic acid receptor (RAR)-mediated gene expression acting in concert with the mediator complex [Pavri et al., 2005]. Moreover, PARP-1-association was reported in other nuclear receptors, such as the 9-*cis* retinoic acid receptor (RXR) [Miyamoto et al., 1999] and progesterone receptor (PR) [Ghabreau et al., 2004]. Therefore, PARP-1 may be involved in the mechanisms by which a limited number of nuclear receptors acquire diversity of transcriptional regulation in normal cells.

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