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Nucleolar protein PES1 is a marker of neuroblastoma outcome and is associated with neuroblastoma differentiation

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Key words

Differentiation, neuroblastoma, nucleolus, PES1, tumorigenesis

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Neuroblastoma (NB) is a childhood malignant tumor that arises from precursor cells of the sympathetic nervous system. Spontaneous regression is a phenomenon unique to NBs and is caused by differentiation of tumor cells. PES1 is a multifunctional protein with roles in both neural development and ribosome biogenesis. Various kinds of models have revealed the significance of PES1 in neurodevelopment. However, the roles of PES1 in NB tumorigenesis and differentiation have remained unknown. Here we show that NB cases with *MYCN* amplification and clinically unfavorable stage (INSS stage 4) express higher levels of *PES1*. High *PES1* expression was associated with worse overall and relapse-free survival. In NB cell lines, *PES1* knockdown suppressed tumor cell growth and induced apoptosis. This growth inhibition was associated with the expression of NB differentiation markers. However, when the differentiation of NB cell lines was induced by the use of all-trans retinoic acid, there was a corresponding decrease in *PES1* expression. *Pes1* expression of tumorspheres originated from *MYCN* transgenic mice also diminished after the induction of differentiation with growth factors. We also reanalyzed the distribution of PES1 in the nucleolus. PES1 was localized in the dense fibrillar component, but not in the granular component of nucleoli. After treatment with the DNA-damaging agent camptothecin, this distribution was dramatically changed to diffuse nucleoplasmic. These data suggest that PES1 is a marker of NB outcome, that it regulates NB cell proliferation, and is associated with NB differentiation.

Neuroblastoma (NB) is the most common extracranial solid malignant tumor of childhood and is derived from precursor cells of the sympathetic nervous system.[1] Unlike in the case of other malignant tumors, a subset of patients with NBs show spontaneous regression without any treatment. This phenomenon is caused by differentiation and programmed cell death of tumor cells.[2] Differentiation induction and proliferation inhibition with 13-cis-retinoic acid is thus one of the effective therapies for high-risk NBs.[3] *MYCN* is a well-known driver gene of NBs. Twenty to thirty percent of NB cases show *MYCN* amplification and poor prognosis.[4] Although MYCN, which has a helix–loop–helix domain and belongs to the MYC family of transcription factors, activates the transcription of various kinds of growth signals, recent data show that MYCN is also required for the differentiation program.[5]

The gene *PES1* (and its zebrafish ortholog pes) is highly conserved from yeast to humans. *PES1* was originally identified in zebrafish in an insertional mutagenesis screen. *PES1* mutant embryos exhibited brains with reduced volumes and other developmental abnormalities.[6] *PES1* mutant overexpression disrupted oligodendrocyte development, oligodendrocyte migration, and axon extension.[7] In the developing *Xenopus laevis*, *pes1* is expressed in the migrating cranial neural crest cells. Inhibition of *pes1* using morpholino oligonucleotide caused neural crest migration impairment.[8] In developing mice, *Pes1* displayed a distinct spatial and temporal pattern of gene expression with high levels in the germinal zone and other specific brain regions that contain neural progenitor cells and postmitotic neurons.[9] In addition to these neurodevelopmental roles, PES1 plays a crucial part in ribosome neogenesis,[10,11] particularly in pre-ribosomal RNA (pre-rRNA) processing. In interphase cells, PES1 is localized in the nucleolus, an intranuclear body where ribosome neogenesis takes place.[12,13] Previous reports revealed that *PES1* was upregulated in glioblastoma, breast cancer, and head and neck squamous cell carcinoma.[9,14,15] The close relationship between ribosome biogenesis and carcinogenesis could be the reason for these upregulations. *PES1* knockdown has been shown to inhibit the proliferation and tumorigenicity of breast cancer cell lines.[16]

As described above, PES1 has two important roles, namely neural development and oncogenesis. These two key processes are themselves characteristics of NBs. Therefore, in the current study we investigated the relationship between PES1 and NBs. We show here that *PES1* is a prognostic marker of NBs and

regulates NB proliferation and apoptosis. Reduced *PES1* expression is associated with NB differentiation in NB cell lines and tumorsphere models. In addition, our analysis with confocal microscopy showed that PES1 is distributed in the dense fibrillar component (DFC) in the nucleolus, and its distribution is radically changed after treatment with the DNA-damaging agent camptothecin (CPT).

Materials and Methods

Mice. The *MYCN* transgenic (Tg) mice[17] were maintained in the animal facility at Nagoya University Graduate School of Medicine (Nagoya, Japan), where they were housed in a controlled environment and provided with standard nourishment and water. Normal ganglia and precancerous and tumor tissues from WT, hemizygous, and homozygous *MYCN* Tg mice were dissected and minced, and then total RNA was extracted. This study was approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine.

Gene expression profiling. Gene expression profiling was carried out as previously described.[18] Samples were analyzed with a GeneChip Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA, USA). The data have been deposited in the Gene Expression Omnibus database of NCBI (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE43419.

Human neuroblastoma tumor profile. Kaplan–Meier analysis of the prognosis of NB patients was performed on the R2 website (http://r2.amc.nl). The dataset was from the AMC cohort study of 88 NB patients (GSE16476). This dataset was profiled on the Affymetrix HGU133 plus2.0 platform and normalized using the MASS5.0 algorithm.

Cell culture. The human neuroblastoma cell lines NB39 and TNB1 were obtained from Riken Cell Bank (Tsukuba, Japan). SH-EP and *MYCN*-inducible SH-EP cell line (SH-EP^{MYCN}) cells were gifts from Dr. Schwab of the Division of Tumor Genetics, German Cancer Research Center (Heidelberg, Germany).[19] They were cultured with DMEM (D5796; Sigma-Aldrich, St. Louis, MO, USA) or RPMI-1640 (R8758; Sigma-Aldrich) or a mixture of some other media following the manufacturer's instructions. Induction of *MYCN* was regulated by removal of tetracycline (1 μg/mL). The media were supplied with 10% heat-inactivated FBS in an incubator with humidified air at 37°C with 5% CO_2. They were routinely authenticated on the basis of their viability, growth rate, and morphology by microscopic examination.

RNA interference. To deliver shRNA expression construct into NB cell lines, commercially available lentiviral shRNA vectors were used. Non-targeting shRNA (Sigma-Aldrich) and specific shRNAs for *PES1* (Open Biosystems, Little Chalfont, UK) were used. The shRNA sequences are listed in Table S1.

Plasmids and their transfection. Human *MYCN* construct was generated by PCR using the Gateway recombination system. *MYCN* construct was cloned into CSII-CMV-RfA-IRES-venus plasmid. This plasmid and CSII-CMV-venus control plasmid were kindly provided by Dr. Miyoshi of Riken. For producing lentiviral particles, HEK293T cells were co-transfected with shRNA plasmids or overexpression plasmid in addition to psPAX2 (Addgene, Cambridge, MA, USA) and pMD2.G (Addgene) plasmids using FuGENE HD reagent (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. The neuroblastoma cell lines were infected in the presence of 8 μg/mL polybrene (Sigma-Aldrich).

Quantitative RT-PCR. RNA extraction from cultured cells and tumorspheres, and quantitative RT-PCR were carried out as previously described.[20] Quantitative PCR analyses were carried out using an MX3000P or MX3005P real-time QPCR System (Agilent, Santa Clara, CA, USA). Quantitative PCR was carried out with ReverTra Ace (Toyobo, Osaka, Japan) and Thunderbird SYBR qPCR Mix (Toyobo). The relative expression levels were acquired according to the manufacturer's instructions. The primers used are listed in Table S2.

Immunocytochemistry. Immunocytochemistry was carried out as previously described using the following primary antibodies: rabbit anti-pescadillo (A300-902A; Bethyl Laboratories, Montgomery, TX, USA), mouse anti-pescadillo (#ab88543; Abcam, Cambridge, UK), rabbit anti-nucleolin (#ab22758; Abcam), mouse anti-phospho-histone H2AX (Ser139) (#05-636; Millipore, Billerica, MA, USA), and mouse anti-neuronal class III β-tubulin (Tuj1) (MMS-435p; Covance, Princeton, NJ, USA). Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG were used as secondary antibodies. Hoechst 33258 was used to visualize nuclei.

Primary culture of tumorspheres from primary tumors of *MYCN* transgenic mice. The procedure was carried out as previously described.[21] Briefly, dissected tumor tissue was minced and digested with 0.25% trypsin (Sigma-Aldrich). To obtain tumorspheres from the primary tumors, the cells were suspended in DMEM/F12ham (Sigma-Aldrich) plus 10 ng/mL epidermal growth factor, 15 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA), 2% B27 supplement, 1% penicillin/streptomycin (PS; Gibco, Waltham, MA, USA), 15% FBS (HyClone, Waltham, MA, USA), 1% non-essential amino acid, 1% sodium pyruvate, and 55 μM β-mercaptoethanol. Cells were cultured in a non-treated Petri dish in a 37°C, 5% CO_2 tissue culture incubator. To induce differentiation, spheres were collected and resuspended with medium containing DMEM/F12ham, 1% FBS, 2% B27 supplement, 1% sodium pyruvate, 10^3 U/mL leukemia inhibitory factor (LIF), and 1% PS. The spheres were seeded into a poly-D-lysine/laminin/fibronectin pre-coated dish. After 12 h the medium was changed to differentiation medium containing DMEM/F12 Ham, 1% FBS, 2% B27 supplement, 1% N2 supplement, 50 ng/mL nerve growth factor (NGF), 50 ng/mL neurotrophin-3 (NT3), and 1% PS.

TUNEL assay. The TUNEL assay was carried out using the APO-DIRECT kit (556381; BD Pharmingen, San Jose, CA, USA). Cell fixation and staining was carried out following the manufacturer's instructions. The cells were analyzed with BD FACSCalibur (BD Pharmingen) and BD CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

Data analysis. Results are expressed as the mean ± SD. Their homoscedasticities were checked by the *f*-test. Statistical significance was evaluated with a two-tailed, unpaired Student's *t*-test.

Results

***PES1* expression increased with NB tumor development in *MYCN* Tg mouse model.** To examine the relationship between NB formation and *PES1* expression, we used a *MYCN* Tg mouse model.[17] In this model, human *MYCN* is overexpressed under the tyrosine hydroxylase promoter, which is active in migrating neural crest cells. At 2 weeks of age, the superior mesenteric ganglion (SMG) of WT mice and hemizygous or homozygous *MYCN* Tg mice are similar in size. However, compared to those of 2-week-old WT mice, the SMGs of 2-week-old homozygote *MYCN* Tg mice show accumulation of immature neuroblasts microscopically. These SMGs can be

regarded as "precancerous" or "hyperplastic" lesions.[18,20,22,23] Finally, at age 6–14 weeks, 100% (for homozygotes) or approximately 70% (hemizygotes) of Tg mice develop NB and die. Histology of tumors of both hemizygous and homozygous *MYCN* Tg mice are similar and equivalent to human neuroblastomas.[23] We first examined the expression profiles of the SMGs of 2-week-old WT mice, the SMGs of 2-week-old homozygous *MYCN* Tg mice, and the terminal tumors of 6-week-old hemizygous or homozygous *MYCN* Tg mice (GSE43419). The precancerous lesions (2-week-old homozygous MYCN Tg mice SMG) showed higher *Pes1* expression than the normal ganglia, and the terminal tumors of hemizygous or homozygous *MYCN* Tg mice showed even higher *Pes1* expression than the precancerous ganglia or WT ganglia (Fig. 1). This suggests that *Pes1* expression increased as the NBs grew in the *MYCN* Tg mouse model.

***PES1* expression associated with poor prognosis of NB.** We then investigated *PES1* expression in human NB cases. For this analysis we used the database from an AMC cohort study of 88 NB patients (GSE16476) and the R2 genomics analysis and visualization platform. Expression of *PES1* was significantly higher in the *MYCN*-amplified cases ($P = 6.9 \times 10^{-10}$; Fig. 2a). *MYCN* amplification has been associated with rapid disease progression and unfavorable prognosis.[4] As the *PES1* promotor region has an E-box, the MYCN binding sequence, we examined whether *MYCN* induction enhances *PES1* expression. *MYCN* induction in SH-EP^{MYCN},[19] SY5Y and SH-EP cell lines significantly increased *PES1* expression (Fig. S1). This result was consistent with Figure 2(a). Tumor stage is also an important prognostic factor of NBs. Neuroblastomas were previously classified based on the International Neuroblastoma Staging System (INSS).[24] Although this classification is widely accepted, in 2009, the International Neuroblastoma Risk Group proposed a new staging system.[25] Both classifications include the designations 4S or MS, in addition to 4 and M, which indicate metastatic diseases. The S is short for "special," and indeed the 4S and MS stages are unique to NBs. The 4S (or MS) cases show favorable prognosis and spontaneous regression in spite of multiple metastases of the skin, liver, or bone marrow. We investigated the *PES1* expression in each of the INSS stages using the same cohort. Stage 4 cases showed higher levels of *PES1* expression than stage 1–3 cases ($P = 0.008$; Fig. 2b). In addition, compared to stage 4S cases, stage four cases also showed significantly higher *PES1* expression ($P = 0.016$; Fig. 2b). The correlation of *PES1* expression with *MYCN* status or clinical stage raised the possibility that *PES1* might be a good prognostic marker. We therefore analyzed the prognostic value of *PES1* expression using Kaplan–Meier survival curves. High *PES1* expression was significantly associated with poorer overall survival ($P = 8.4 \times 10^{-5}$ after Bonferroni correction; Fig. 2c) and relapse-free survival ($P = 5.4 \times 10^{-3}$ after Bonferroni correction; Fig. 2d). Previous reports have indicated that malignant tumors express higher *PES1* levels than normal tissues or benign tumors in other types of cancers.[9,14–16,26] In line with previous findings, these results indicate that *PES1* expression is correlated with *MYCN* status and clinical stage, and that *PES1* is a prognostic marker of NBs.

***PES1* knockdown suppressed NB cell growth and induced apoptosis.** As NB cases with high *PES1* expression are associated with unfavorable prognosis, we next investigated the role of PES1 in NB cell lines. We knocked down *PES1* expression using two different shRNAs against human *PES1*. The shRNAs efficiently knocked down *PES1* expression at both the mRNA and protein level (Fig. 3a,b). Short hairpin RNAs suppressed the growth of both a *MYCN*-amplified cell line (NB39) and a *MYCN* non-amplified cell line (SH-EP) (Fig. 3c,d). We next carried out a TUNEL assay to examine whether these growth suppressions were caused by apoptosis. Compared to non-targeting shRNA, shRNAs against *PES1* dramatically increased the percentage of TUNEL-positive cells (Fig. 3e,f). These data suggest that *PES1* knockdown inhibited NB cell growth and this inhibition was caused by apoptosis.

As already described, tumor cell differentiation is both a characteristic and an important treatment strategy of NB.[3] Accordingly, we next investigated whether *PES1* knockdown causes the differentiation of NB cells. Differentiated cells possess more and longer neurites and express higher levels of differentiation markers (GAP43, RET).[27] Although *PES1* knockdown did not induce clear increase in neurite outgrowth

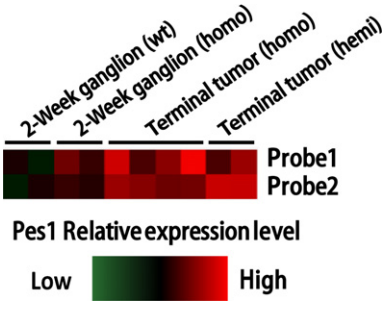

Fig. 1. *Pes1* expression in a neuroblastoma mice model. Results of a microarray analysis of the relative expression levels of *Pes1* in superior mesenteric ganglia of wild-type mice (wt), 2-week homozygous *MYCN* transgenic (Tg) mice (precancerous lesions) and terminal tumors (hemizygous and homozygous *MYCN* Tg mice). hemi, hemizygous *MYCN* Tg mice; homo, homozygous *MYCN* Tg mice.

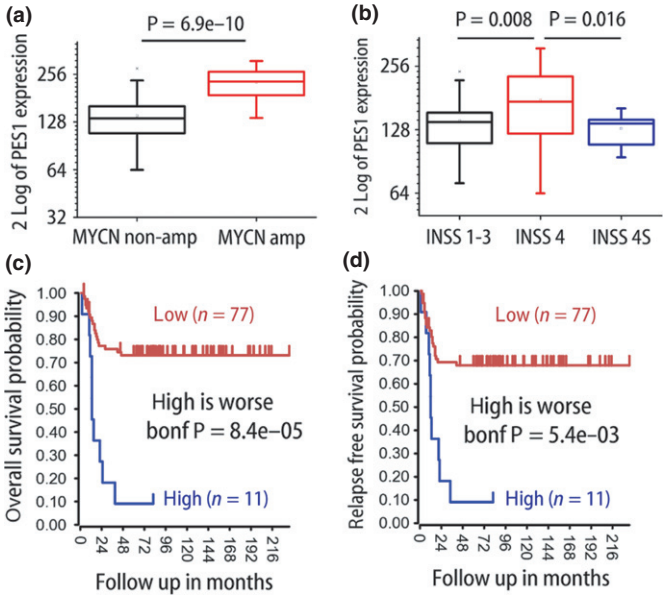

Fig. 2. *PES1* expression in human neuroblastoma cases and patient prognosis (AMC cohort, GSE16476). (a) *PES1* expression in *MYCN* non-amplified/amplified cases ($n = 72$, 16 each). (b) *PES1* expression in each International Neuroblastoma Staging System (INSS) stage (INSS 1–3, $n = 36$; INSS 4, $n = 40$; INSS 4S, $n = 12$). (c,d) Kaplan–Meier curves and log–rank test for overall survival (c) and relapse-free survival (d). Bonf, after Bonferroni correction.

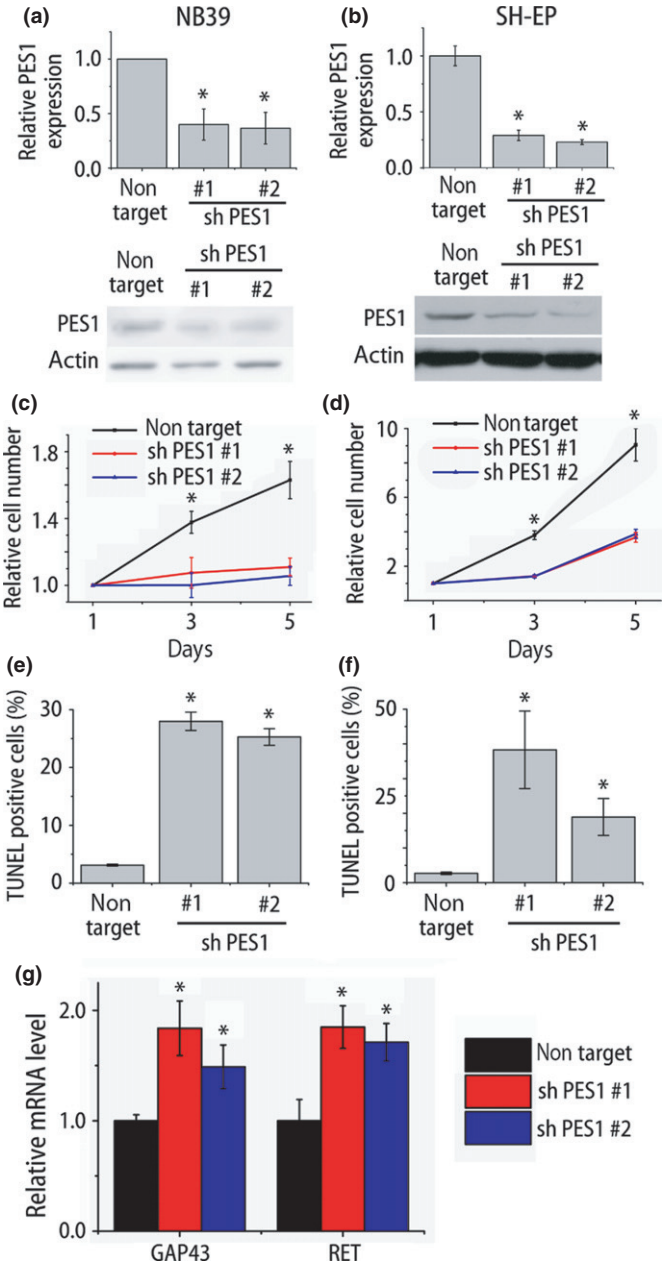

Fig. 3. *PES1* knockdown inhibited neuroblastoma (NB) cell growth and induced apoptosis. (a,b) Quantitative RT-PCR analysis and Western blot analysis of PES1 expression in NB cell lines (NB39 and SH-EP) after shRNA treatments ($n = 3$). (c,d) Cell proliferation analysis of NB cell lines after treatment of non-target shRNA or shRNA against *PES1* (NB39, $n = 4$; SH-EP, $n = 3$). (e,f) Quantification of TUNEL-positive apoptotic cells ($n = 3$). (g) Quantitative RT-PCR analysis of differentiation-related gene expressions of NB39 ($n = 4$). $^*P < 0.05$ non-target versus shRNA against *PES1* (Student's *t*-test).

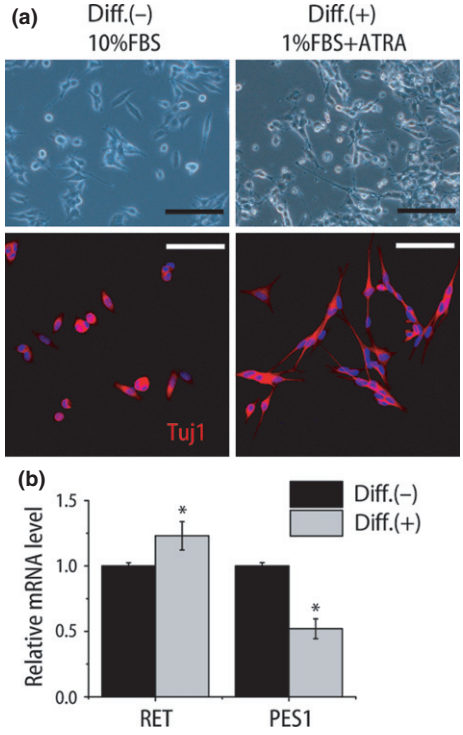

Fig. 4. Induced differentiation attenuated *PES1* expression in neuroblastoma cell line. (a) Phase contrast images (top panels) and confocal image of fluorescence immunostaining for β-tubulin (Tuj1) (red, bottom panels) of NB39 cells under normal culture conditions (10% FBS; Diff.(–)) and differentiation conditions (1% FBS + 1 μM all-trans retinoic acid [ATRA]; Diff.(+)). Hoechst 33258 was used to visualize nuclei. Scale bar = 100 μm. (b) Quantitative RT-PCR analysis of differentiation-related gene (*RET*) and *PES1* expressions ($n = 4$). $^*P < 0.05$ normal culture conditions versus differentiation conditions (Student's *t*-test).

(data not shown), the results of quantitative PCR showed an increase in the expression of differentiation-related genes (Fig. 3g). As the cell shape was not remarkably changed, we could not conclude that *PES1* knockdown caused the differentiation. But this result raised the possibility that *PES1* expression is associated with differentiation status.

Differentiation induction in NB cell lines decreased *PES1* expression. We then examined whether the induction of differentiation can change the *PES1* expression. Historically, various methods have been used to induce NB differentiation, including serum starvation and treatment with all-trans retinoic acid (ATRA).[28–31] A derivative of vitamin A, ATRA is essential for development and differentiation. All-trans retinoic acid-induced differentiation of leukemic cells has been shown to dramatically improve the prognosis of patients with acute promyelocytic leukemia.[32] In addition, ATRA can differentiate NB cells. We induced differentiation in NB cell lines with serum reduction (from 10% FBS in original culture to 1%) and ATRA (1 μM). This treatment increased the number and length of the neuritic extensions of NB39 and TNB1 cells (Fig. 4a, Fig. S2), and these neurites were positive for Tuj1, a neuronal marker. This differentiation induction was further verified by the increased expression of a differentiation-related gene (Fig. 4b, Fig. S2).[33] Interestingly, the differentiation induction decreased *PES1* expression (Fig. 4b, Fig. S2). This result suggests the possibility that ATRA-induced differentiation modulates *PES1* expression.

Differentiation induction in mouse NB model also decreased *PES1* expression. To further verify the association of *PES1* with tumor cell differentiation, we tried another NB model. We used tumorspheres derived from an *MYCN* Tg mouse primary tumor.[21] Differentiation induction with NGF, NT3, and N2 supplement caused radial neurite outgrowth (Fig. 5a). Concurrently, the expressions of differentiation marker (Tuj1) increased and stemness markers (Snail and Musashi) decreased (Fig. 5b). In line with the results in the NB cell lines, *Pes1* expression was reduced with the differentiation induction

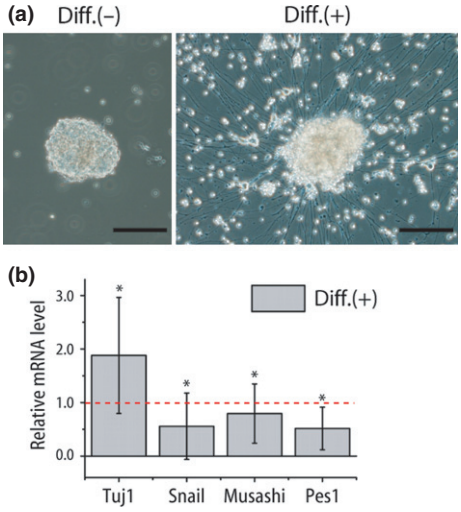

Fig. 5. Induced differentiation decreased *Pes1* expression in *MYCN* transgenic (Tg) mice model tumorsphere. (a) Phase contrast images of tumorspheres established from *MYCN* Tg mice primary tumor under normal culture conditions and differentiation conditions (NGF, NT3 addition). Scale bar = 100 μm. (b) Quantitative RT-PCR analysis of differentiation (β-tubulin, Tuj1) or stemness-related gene (*Snail* and *Musashi*) and *Pes1* expressions ($n = 4$). Only the values of differentiation conditions are shown (normal culture condition = 1.0). The result is the average of four independently established tumorsphere samples. *$P < 0.05$ normal culture conditions versus differentiation conditions (Student's *t*-test).

(Fig. 5b). These results suggest that *Pes1* expression was negatively correlated with the differentiation conditions in two kinds of different NB models.

PES1 localized in DFC in nucleolus and DNA damage induced redistribution. As previously reported, PES1 was localized in the nucleolus within the nucleus, where ribosome neogenesis takes place.[9,11] As one of the major roles of PES1 is 32S pre-rRNA processing,[10,34] this distribution is reasonable. The nucleolus consists of three distinct subnucleolar compartments called the fibrillar center, the DFC, and the granular component (GC) from inside to outside,[35] and ribosome maturation also proceeds from inside to outside. A previous analysis using tagged PES1 showed its GC distribution.[11] However, we found that endogenous PES1 is surrounded by nucleolin, a major nucleolar protein that is found in DFC and GC,[36] with nucleolin showing "donut-like" fluorescence (Fig. 6a). Considering the fact that pre-rRNA resides in the DFC, this result suggest that PES1 is mainly localized in DFC of the nucleolus, not in the GC.

In addition to its principal role, ribosome neogenesis, the nucleolus also works as a stress sensor of DNA damage.[37,38] A previous report found that *PES1* knockdown induced the expression of phosphorylated H2AX (γH2AX), a DNA damage marker, and delayed the recovery from DNA damage.[39] The authors indicated some association between PES1 and DNA damage response, but the relationship is still unclear. Therefore, we next investigated the dynamics of PES1 under DNA damage. Camptothecin is a topoisomerase-1 inhibitor that causes DNA damage and is used to treat cancers.[37,40] In the interphase TNB1 cells, PES1 is located in the nucleolus. After treatment with CPT (1 μM) for 5 h, γH2AX was expressed strongly in the nucleus and formed γH2AX foci (Fig. 6b).[41] PES1, together with nucleolin, showed a radical change in distribution to a diffuse nucleoplasmic distribution (Fig. 6b,c). As DNA-damaging agents are known to cause nucleolar disruption,[42] this result was not surprising. The reason for this distribution change and its biological significance remains to be investigated. This result suggests that DNA damage can redistribute PES1 as well as nucleolin. PES1 expression partially, but not completely, overlapped with γH2AX (Fig. 6d).

Discussion

The present study addressed the roles of PES1 in NB tumorigenesis by using a clinical NB database of mRNA expression and survival, human NB cell lines, an NB model in MYCN Tg mice, and tumorspheres derived from *MYCN* Tg mice. *MYCN*

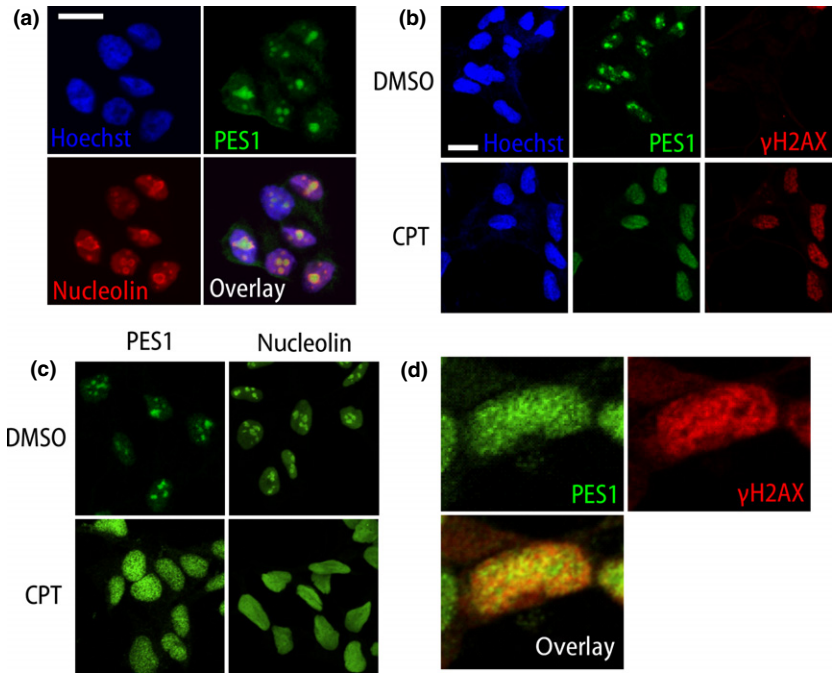

Fig. 6. Endogenous PES1 localization and its redistribution after DNA damage. (a) Confocal images of TNB1 neuroblastoma cells immunostained for PES1 (green) and nucleolin (red) with nuclear stain (Hoechst33258, blue). PES1 is encircled by nucleolin, which shows "donut-like" staining. (b,c) Confocal images of TNB1 cells after DMSO or camptothecin (CPT; 1 μM) treatment. Scale bar = 15 μm. (d) Enlarged confocal images of TNB1 cells after CPT (1 μM) treatment. One nucleus was enlarged.

Tg mice are a reliable NB model that recapitulates the clinical features of NB, histology, and molecular changes.[17,43] Our study revealed that PES1 is involved in NB cell growth, survival, and differentiation. Its high expression is associated with poor prognosis. *PES1* knockdown suppresses NB tumor cell growth and induces apoptosis. In their differentiated state, the NB cell lines and tumorspheres show lower *PES1* expression. In contrast, *PES1* knockdown induces differentiation features. In addition, we found PES1 distribution in DFC in the nucleolus and diffuse redistribution into the nucleoplasm after CPT treatment.

Our study showed that *PES1* is a prognostic marker of NBs. Neuroblastoma cases with unfavorable prognosis (*MYCN* amplified or INSS 4) show high *PES1* expression. Previous reports indicated that cancerous tissue expressed more *PES1* than normal tissue or benign tumors.[9,14–16,26] However, these results were based on the results of tissue microarray or cell lines. We showed the relationship between *PES1* expression and prognosis in NBs in this study for the first time. As previously shown in colon cancer cell lines,[26] PES1 knockdown strikingly suppressed NB cell growth in our study.

We showed that *PES1* knockdown upregulated some differentiation-related genes. To further elucidate the relationship between *PES1* expression and differentiation, we used two different kinds of models to induce NB differentiation. One is ATRA treatment of NB cell lines, and the other is tumorsphere differentiation with growth factors. We previously reported a new tumorsphere culture condition,[21] and found that this condition worked well in the present experimental scheme. In both models, we successfully induced differentiation as demonstrated by morphology change (neurite growth) and differentiation marker expression. Under these conditions, *PES1* was downregulated. However, we could not successfully rescue these phenomena by *PES1* overexpression (data not shown). These results suggest that PES1 is required but not sufficient to suppress NB cell differentiation.

We also reanalyzed PES1 distribution in the nucleoli using confocal microscopy. In our analysis, PES1 was encircled by nucleolin. Nucleolin and nucleophosmin are the two major protein components of the nucleolus. Previous reports have described multiple functions of nucleolin from rRNA splicing to ribosome assembly. Our results indicate that PES1 is mainly distributed in DFC, but not GC, whereas nucleolin is localized in DFC and GC. As DFC is an rRNA-rich lesion in nucleoli, the DFC distribution of PES1 makes sense. We also observed a dynamic change of PES1 distribution after treatment with the DNA-damaging agent CPT. The relationship between PES1 and DNA damage response is still unclear. One previous study indicated that PES1 works protectively against DNA damage and PES1 knockdown induces γH2AX expression.[39] From our results and previous knowledge, we can hypothesize that *PES1* knockdown causes DNA damage and accumulated DNA damage drives cells to apoptosis.

Another interesting fact is that PES1 has been known to have a BRCA1 C-terminal domain (BRCT domain).[12] The BRCT domain is a phosphoprotein binding domain and an integral signaling module in the DNA damage response.[44] As the role or binding partners of the BRCT domain of PES1 is not known, we investigated the binding partners of PES1 using liquid chromatography–mass spectrometry with peptide mass fingerprinting (LC-MS/MS). At this stage, NONO and SFPQ are among the list of identified proteins as binding partners (data not shown). These proteins form heterodimers and enhance DNA strand break rejoining.[45,46] These results also suggested the possible close association of PES1 with DNA damage. But we need further study to elucidate the role of PES1 in DNA damage response.

In this article, we described multiple functions of the PES1 protein. The classical and best-known role of PES1 is ribosome genesis.[10,11]. The ribosome is an essential intracellular organelle to protein synthesis, and its dysfunction cause severe cellular disturbance. The growth inhibitions and apoptosis observed in our study (Fig. 3) might be caused by this ribosomal dysfunction. In addition, as mentioned above, DNA damage caused by *PES1* knockdown could also be the cause of apoptosis and growth inhibitions. Arrest of cell division is a prerequisite to enter a program of differentiation.[47] Therefore, the phenotypes related to differentiation (Figs. 3–5) are closely linked to growth inhibition.

In summary, we showed that PES1 is a good prognostic marker of NBs. As a multifunctional protein, PES1 is involved in NB cell survival and differentiation. DNA damage might contribute to apoptosis regulation in NB cells.

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Disclosure Statement

The authors have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. MYCN induction in MYCN single-copy cell lines.

Fig. S2. Induced differentiation in TNB1 neuroblastoma cells.

Table S1. Short hairpin RNA target sequences (PES1).

Table S2. Primer sequences for PCR experiments.