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Functional interplay between MYCN, NCYM, and OCT4 promotes aggressiveness of human neuroblastomas

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Key words

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Neuroblastoma is a pediatric solid tumor that originates from embryonic neural crest cells. The *MYCN* gene locus is frequently amplified in unfavorable neuroblastomas, and the gene product promotes the progression of neuroblastomas. However, the molecular mechanisms by which *MYCN* amplification contributes to stem cell-like states of neuroblastoma remain elusive. In this study, we show that *MYCN* and its *cis*-antisense gene, *NCYM*, form a positive feedback loop with *OCT4*, a core regulatory gene maintaining a multipotent state of neural stem cells. We previously reported that *NCYM* is co-amplified with the *MYCN* gene in primary human neuroblastomas and that the gene product promotes aggressiveness of neuroblastoma by stabilization of MYCN. In 36 *MYCN*-amplified primary human neuroblastomas, *OCT4* mRNA expression was associated with unfavorable prognosis and was correlated with that of *NCYM*. The OCT4 protein induced both *NCYM* and *MYCN* in human neuroblastoma cells, whereas NCYM stabilized MYCN to induce *OCT4* and stem cell-related genes, including *NANOG*, *SOX2*, and *LIN28*. In sharp contrast to MYCN, enforced expression of c-MYC did not enhance *OCT4* expression in human neuroblastoma cells. All-*trans* retinoic acid treatment reduced MYCN, NCYM, and OCT4 expression, accompanied by the decreased amount of OCT4 recruited onto the intron 1 region of *MYCN*. Knockdown of NCYM or OCT4 inhibited formation of spheres of neuroblastoma cells and promoted asymmetric cell division in *MYCN*-amplified human neuroblastoma cells. These results suggest that the functional interplay between MYCN, NCYM, and OCT4 contributes to aggressiveness of *MYCN*-amplified human neuroblastomas.

Neuroblastoma is a pediatric solid tumor that arises in sympatho-adrenal tissues.[1] Amplification of the *MYCN* oncogene is frequently observed in unfavorable neuroblastomas,[2] and aberrant expression of *MYCN* contributes to neuroblastoma progression.[3] The transcription factor MYCN regulates a wide variety of biological phenomena, including cell-cycle progression, apoptosis, differentiation, and stemness.[4,5] *MYCN* transgenic mice spontaneously develop neuroblastomas,[3] but unlike human *MYCN*-amplified neuroblastomas, the mice rarely have metastatic tumors. Recently, we reported that *NCYM*, a *MYCN cis*-antisense gene, encodes a protein that functions as an onco-promoting factor.[6] The coding sequence of *NCYM* is not evolutionally conserved in mice, and the *NCYM* gene is co-amplified with *MYCN* in human primary neuroblastomas.[6] The MYCN protein directly targets *NCYM* for transcriptional activation, whereas NCYM stabilizes MYCN protein, forming a positive autoregulatory loop.[6,7] Expression of NCYM caused metastatic tumors in *MYCN/NCYM* double transgenic mice and inhibited apoptotic cell death.[6] However, these results do not rule out the possibility that NCYM is involved in other cellular phenotypes to promote the aggressiveness of neuroblastoma.

Neuroblastomas originate from neural crest cells that differentiate into multiple cell lineages.[8] Some neuroblastoma cells retain multipotency and highly express stem cell-related genes, such as *OCT4*[9] and *LIN28*.[10] Intermediate (I)-type neuroblastoma cells highly express OCT4 and differentiate into neuroblastic (N)-type or substrate adherent (S)-type cells in response to retinoic acid or BrdU treatment, respectively.[11] $OCT4^+$/Tenascin C^+ neuroblastoma cells were reported to

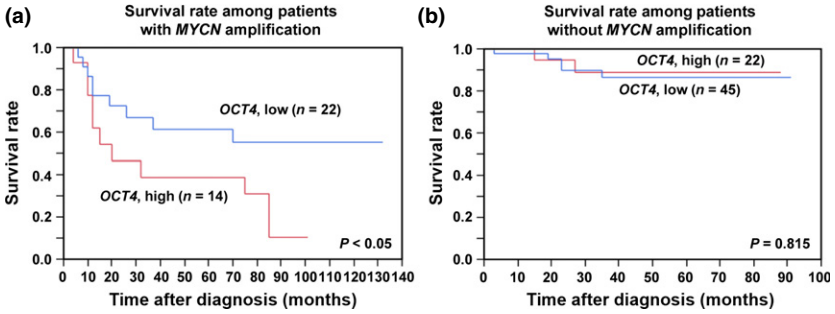

Fig. 1. High *OCT4* expression correlates with unfavorable prognosis in human *MYCN*-amplified neuroblastomas. (a) Overall survival of patients with *MYCN*-amplified neuroblastomas according to relative *OCT4* expression levels (n = 36; high, n = 14; low, n = 22). *P*-value by log–rank test. (b) Overall survival of patients with *MYCN*-non-amplified neuroblastomas according to relative *OCT4* expression levels (n = 67; high, n = 22; low, n = 45). *P*-value by log–rank test. *OCT4* mRNA expression designated high or low according to the average value.

serve as progenitors of tumor-derived endothelial cells, promoting neovascularization of the tumors.[9] Furthermore, OCT4 is expressed in side-population cells of neuroblastoma.[12] Despite these correlations between OCT4 expression and the stem cell-like state of neuroblastomas, the functional roles of OCT4 in neuroblastoma pathogenesis remain unclear. In this study, we investigated the biological and clinical significance of OCT4 in neuroblastomas and found that the newly evolved network between MYCN, NCYM, and OCT4 regulates aggressiveness of human neuroblastomas.

Materials and Methods

Immunofluorescence analysis. BE(2)-C and SK-N-AS cells were grown on coverslips and transfected with indicated shRNAs. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, blocked in 3% BSA, stained with the indicated antibodies, and examined with a laser scanning confocal microscope (DMI 4000B; Leica, Wetzlar, Germany).

Asymmetric cell division assay. We tested whether neuroblastoma cells showed asymmetric distribution of nuclear mitotic apparatus protein (NuMA). Asymmetric distributions of NuMA to one side of the cell were counted during mitotic stages. The spindle apparatus were also stained with anti-tubulin-α antibody to avoid false results caused by uneven dyeing. The antibodies used were anti-NuMA (Novus Biologicals, Littleton, CO, USA), and anti-tubulin-α (Thermo Fisher Scientific, Wilmington, DE, USA).

Statistical analysis. All data were presented as the mean ± standard deviation and were obtained from three independent experiments. Statistical significance in the clinical data was calculated using the log–rank test, χ^2-test, and Student's *t*-test. Hazard ratios were calculated using univariate and multivariate Cox regression analysis. Statistical analyses were undertaken using JMP 9 (SAS Institute Japan, Tokyo, Japan). Statistical significance was set at $P < 0.05$.

More detailed descriptions of the Material and Methods are described in Document S1.

Results

High expression of *OCT4* associated with poor prognoses in *MYCN*-amplified human neuroblastomas. To examine the prognostic significance of *OCT4* mRNA expression in human neuroblastoma, total RNA was extracted from 36 *MYCN*-amplified and 67 *MYCN*-non-amplified primary neuroblastomas and subjected to quantitative real-time RT-PCR. *MYCN* amplification was examined as previously described [13]. Kaplan–Meier analysis showed that high levels of *OCT4* mRNA expression were significantly associated with poor outcomes in *MYCN*-amplified human neuroblastomas (Fig. 1a), but not in *MYCN*-non-amplified human neuroblastomas (Fig. 1b).

Expression levels of *OCT4* correlated with prognostic factors. We next checked the relationship between the expression of *OCT4* and prognostic factors. The expression levels of *OCT4* were significantly correlated with International Neuroblastoma Staging System (INSS) stage, Shimada pathology, and expression of *NCYM* and *MYCN* in *MYCN*-amplified primary neuroblastomas (Table 1). In addition, univariate Cox regression analysis of 36 *MYCN*-amplified primary neuroblastomas indicated that high levels of *OCT4* mRNA expression tended to correlate with poor prognosis (Table S1). Multivariate Cox regression analysis also revealed that *OCT4* mRNA expression was not independent of *NCYM* and *MYCN* mRNA expression in *MYCN*-amplified primary neuroblastomas (Table S2).

NCYM induced *OCT4* via induction of MYCN. We next examined the factors that predict *OCT4* expression in primary neuroblastomas by multiple regression analysis (Table S3). The expression levels of *NCYM*, *NANOG*, *KLF4*, and c-*MYC* and *MYCN* amplification significantly contributed to the prediction of *OCT4* expression in primary neuroblastomas (Table S3). Furthermore, the expression levels of *NCYM* mRNA were positively correlated with those of *OCT4* and *NANOG*, whereas

Table 1. Prognostic significance of *OCT4* expression and other clinical factors in *MYCN*-amplified neuroblastomas (χ^2-test)

Factor		*OCT4* mRNA expression		*P*-value
		Low (n = 22)	High (n = 14)	
Age, months	<18 (n = 22)	11	11	0.079
	≥18 (n = 14)	11	3	
INSS stage (3 or 4)	3 (n = 9)	8	1	<0.05
	4 (n = 27)	14	13	
Tumor origin	Adrenal gland (n = 32)	19	13	0.534
	Others (n = 4)	3	1	
Shimada classification	Favorable (n = 6)	6	0	<0.001
	Unfavorable (n = 30)	16	14	
MYCN mRNA expression	Low (n = 25)	18	7	<0.05
	High (n = 11)	4	7	
NCYM mRNA expression	Low (n = 26)	21	5	<0.001
	High (n = 10)	1	9	

INSS, International Neuroblastoma Staging System.

KLF4 expression was inversely correlated with that of *MYCN* and *NCYM* (Table S4).

These results prompted us to assess whether NCYM regulates OCT4 as well as stem cell-related genes in human neuroblastoma cells. Overexpression of NCYM or MYCN, but not c-MYC, induced *OCT4* mRNA expression (Figs S1,S2) as well as *NANOG*, *LIN28*, and *SOX2*, whereas neither NCYM nor MYCN enhanced c-*MYC* or *KLF4* (Fig. S1). Knockdown of NCYM decreased OCT4 and MYCN expression at both mRNA and protein levels (Fig. 2a,b), and suppressed their promoter activities (Fig. 2c). In addition, the expression levels of a stem cell-related protein CD133 were also downregulated by NCYM knockdown (Fig. S3A). A previous report suggested that MYCN is directly recruited onto the distal enhancer of human *OCT4*.[14] We thus checked the recruitment of MYCN onto putative E-box sites found in human OCT4 enhancer regions (Fig. 2d). Endogenous MYCN protein was recruited onto the distal enhancer region (Fig. 2d,e, #1), but not in the proximal enhancer region (Fig. 2d,e, #2). Knockdown of NCYM diminished MYCN binding to the distal enhancer of OCT4 (Fig. 2e). Together, these results suggest that NCYM regulates *OCT4* transcription by induction of MYCN.

In sharp contrast to human neuroblastoma cells, overexpression of NCYM in mice did not induce stem cell-related genes either *in vitro* (Fig. S4) or *in vivo* (Fig. S5). Furthermore, the E-box at the distal enhancer region of OCT4 is not evolutionally conserved among species (Fig. S6A).

Transcription of *MYCN* in human neuroblastoma directly stimulated by OCT4. OCT4, SOX2, and NANOG form core networks in embryonic stem (ES) cells by their mutual transcriptional regulations.[15] We thus examined whether OCT4 regulates *MYCN/NCYM* transcription in human neuroblastoma cells. In BE(2)-C *MYCN*-amplified neuroblastoma cells, shRNA-mediated knockdown of OCT4 downregulated MYCN at both the mRNA and protein levels (Fig. 3a,b). CD133 was also suppressed by OCT4 knockdown (Fig. S3B). Although OCT4 knockdown decreased *NCYM* mRNA expression, it showed marginal effects on the expression of NCYM protein (Fig. 3a, b). In *MYCN* non-amplified SK-N-AS cells, overexpression of OCT4 induced the expression and promoter activities of *MYCN* and *NCYM* (Fig. S7), as well as the expression of *NANOG* and *SOX2* (Fig. S8A). In BE(2)-C cells, OCT4 knockdown suppressed the promoter activities of *OCT4* and *MYCN*, whereas it did not affect *NCYM* promoter activity (Fig. 3c). These results suggest that OCT4 may not directly affect *NCYM* transcription at the endogenous expression level. Overexpression of OCT4 enhanced activities of MYCN reporter constructs containing the intron 1 region of *MYCN* (Fig. 3d). We found two putative OCT4 binding sites within the intron 1

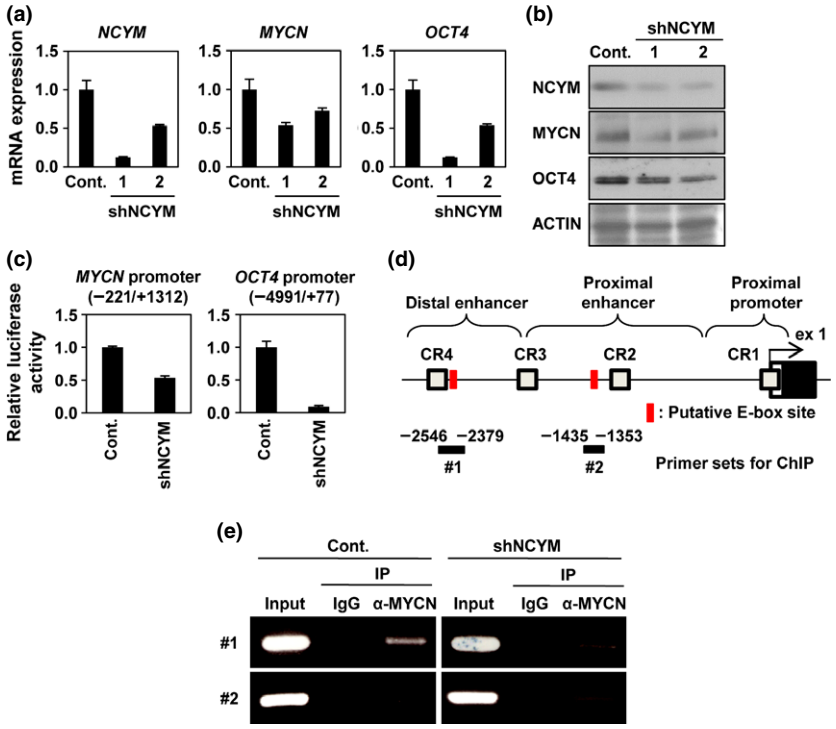

Fig. 2. NCYM regulates OCT4 through the recruitment of MYCN onto the *OCT4* promoter region in neuroblastoma cells. (a) Quantitative real-time RT-PCR analyses of *NCYM*, *MYCN*, and *OCT4* in NCYM shRNA-transfected BE(2)-C intermediate (I)-type neuroblastoma cells. Seventy-two hours after infection, mRNA expression levels were measured by real-time RT-PCR with β-*actin* as an internal control (Cont.). (b) Western blot analyses of NCYM, MYCN, and OCT4 proteins in NCYM shRNA-transfected BE(2)-C I-type neuroblastoma cells. Seventy-two hours after infection, cells were subjected to Western blot analyses. ACTIN was used as loading control. (c) Luciferase activity of *MYCN* and *OCT4* reporters in NCYM shRNA-transfected BE(2)-C I-type neuroblastoma cells. Seventy-two hours after infection, cells were subjected to luciferase reporter assay. Data are shown as the fold change in the luciferase activity. The activities were standardized by control cells. (d) Schematic depiction of the *OCT4* promoter region. The *OCT4* promoter is divided into three regions (distal enhancer, distal promoter, and proximal promoter). Each conserved region (CR1–4) and exon 1 of human OCT4 (ex1) are boxed. The gray, white, and black boxes indicate the conserved region, 5′-UTR, and coding region, respectively. The locations of the ChIP primers are indicated by bold lines. The putative E-box sites are shown in red boxes. (e) Identification of the MYCN binding region in the *OCT4* promoter by ChIP assays. BE(2)-C I-type neuroblastoma cells were transfected with control shRNA or NCYM sh-1. Seventy-two hours after infection, cells were subjected to ChIP assay. Genomic DNA was amplified by PCR by specific primer sets as shown by bold black lines #1 and #2 in panel (d). The PCR bands indicated in panel #1 indicate amplification of the distal enhancer region; PCR bands indicated in panel #2 indicate amplification of the proximal enhancer region. IP, Immunoprecipitation.

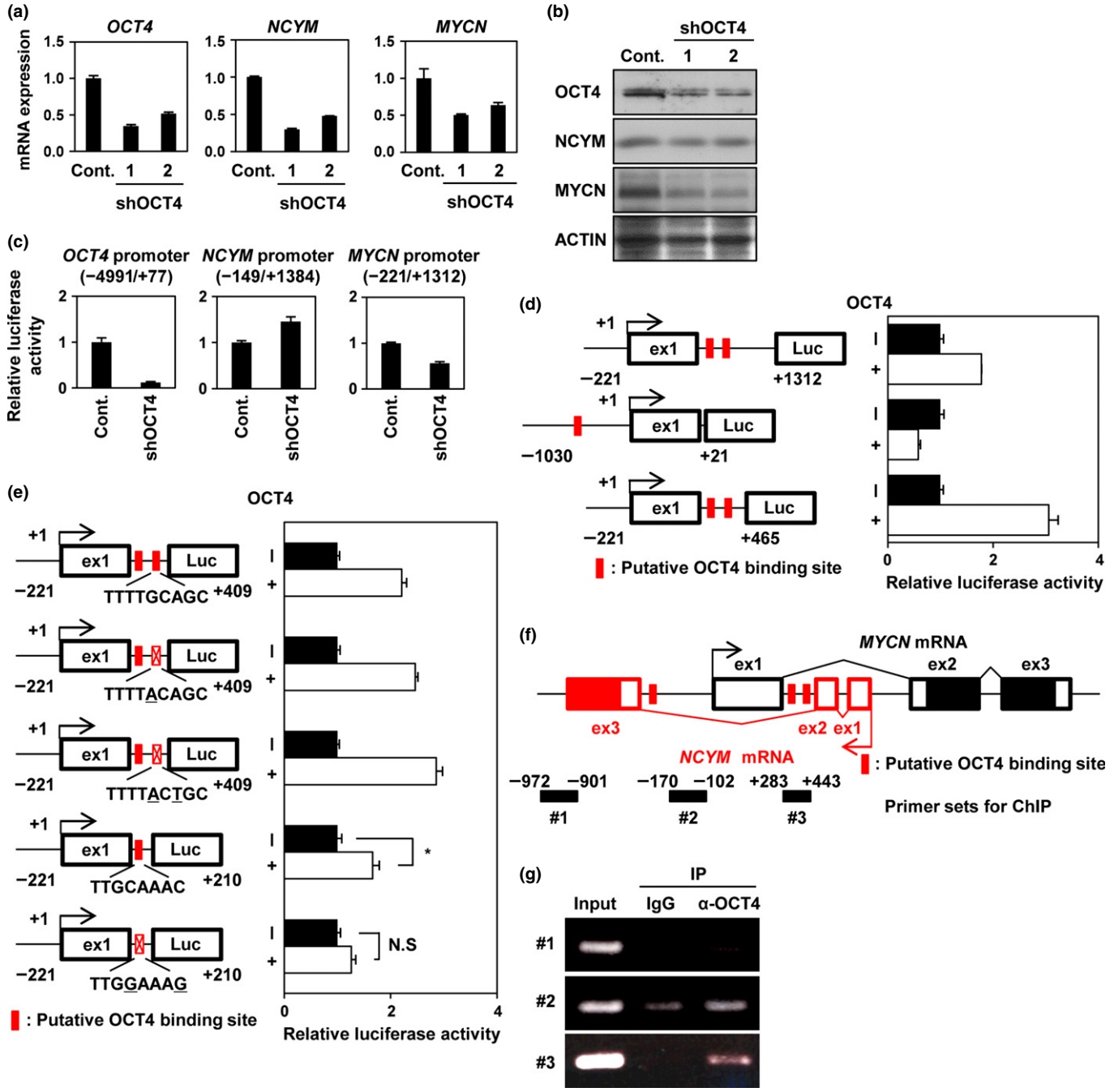

Fig. 3. OCT4 induces transcription of *MYCN* in neuroblastoma cells. (a) Quantitative real-time RT-PCR analysis of *NCYM*, *MYCN*, and *OCT4* in OCT4 shRNA-transfected BE(2)-C intermediate (I)-type neuroblastoma cells. Seventy-two hours after infection, mRNA expression levels were measured by real-time RT-PCR with β-*actin* as an internal control (Cont.). (b) Western blot analyses of NCYM, MYCN, and OCT4 proteins in OCT4 shRNA-transfected BE(2)-C I-type neuroblastoma cells. Seventy-two hours after infection, cells were subjected to Western blot analyses. ACTIN was used as loading control. (c) Luciferase activity of *OCT4*, *NCYM*, and *MYCN* reporters after OCT4 shRNA-transfected BE(2)-C I-type neuroblastoma cells. Seventy-two hours after infection, cells were subjected to luciferase reporter assay. Data are shown as the fold change in the luciferase activity. The activities were standardized by control cells. (d) Luciferase activity of *MYCN* (−221/+1312, −1030/+21, and −221/+465) reporters after OCT4 transfection of SK-N-AS neuroblastoma cells. Forty-eight hours after transfection, cells were subjected to luciferase reporter assay. Data are shown as the fold change in luciferase activity. The activities were standardized by control cells. (e) Luciferase activity of *MYCN* reporters (−221/+409, −221/+409 mutant 1, −221/+409 mutant 2, −221/+210 mutant, and −221/+409 mutant) after OCT4 transfection of SK-N-AS neuroblastoma cells. Forty-eight hours after transfection, cells were subjected to luciferase reporter assay. Data are shown as the fold change in the luciferase activity. The activities were standardized by control cells. The putative OCT4 binding sites are indicated in red boxes. Statistical significance determined by the Student's *t*-test, $*P < 0.05$. (f) Schematic of the *MYCN*/*NCYM* promoter and coding region, divided into three exons (ex 1–3). Each translated region is boxed. The red and black boxes indicate NCYM and MYCN regions, respectively. Locations of the ChIP primers are indicated by the bold line. Putative OCT4 binding sites are indicated by red boxes. (g) Identification of the OCT4 binding region in the *MYCN*/*NCYM* region by ChIP assays in BE(2)-C I-type neuroblastoma cells. Seventy-two hours after infection, cells were subjected to ChIP assay. Genomic DNA was amplified by PCR using the primer sets shown in panel (f). IP, Immunoprecipitation.

region, and generated luciferase reporter constructs harboring mutations in the OCT4 binding sites (Fig. 3e). Mutations in the upstream OCT4 binding sequence diminished OCT4-mediated enhancement of *MYCN* promoter activity, whereas *MYCN* promoter constructs containing the WT upstream OCT4 site sustained the response to OCT4 overexpression (Fig. 3e). Chromatin immunoprecipitation assay showed that OCT4 was directly recruited onto the intron 1 region of *MYCN* (Fig. 3f,g, #3). We also observed the recruitment of OCT4 to the promoter of *MYCN* (Fig. 3f,g, #2), although the OCT4 site in the *MYCN* promoter was not responsible for OCT4-mediated enhancement of *MYCN* promoter activity (Fig. 3e). We next checked the conservation of the OCT4 binding site within intron 1 among species, and found that it is mostly conserved among primates, but not in mice (Fig. S6B).

OCT4 is downregulated on differentiation of neuroblastoma cells. BE(2)-C I-type neuroblastoma cells are stem cell-like cells that show the ability to differentiate into N-type cells in response to retinoic acid treatment[11] and MYCN expression is downregulated during the differentiation.[11] We assessed the expression of NCYM and OCT4 in BE(2)-C I-type cells treated with all-*trans* retinoic acid (ATRA) (Fig. 4a,b). As reported previously,[11] BE(2)-C I-type cells differentiated into N-type cells with marked neurite extensions (Fig. 4a,b), accompanied by a rapid decrease of MYCN expression (Fig. 4c,d). The decrease of MYCN was followed by the downregulation of NCYM, OCT4, NANOG, and SOX2 (Figs 4c,d,S8B,C), whereas no significant changes were observed in the expression levels of *NANOG* mRNA (Fig. S8B). A neural maker GAP43 was induced in the ATRA-treated neuroblastomas cells (Fig. S8C). In good accordance with the strong correlation in primary tumors (Table S4), *NCYM* and *OCT4* expression showed similar expression patterns in ATRA-treated BE(2)-C cells (Fig. 4c,d). Furthermore, ATRA treatment decreased MYCN binding to the distal enhancer of *OCT4* (Fig. 4e) and OCT4 binding to the intron 1 region of *MYCN* (Fig. 4f). Therefore, retinoic acid-induced neuronal differentiation abrogated the positive autoregulatory loops formed by MYCN, NCYM, and OCT4 through the simultaneous downregulation of their expression.

Self-renewal of neuroblastoma cells maintained by OCT4 and NCYM. We next examined whether OCT4 and NCYM contributes to self-renewal of neuroblastoma cells. Knockdown of NCYM or OCT4 in BE(2)-C cells inhibited formation of spheres of neuroblastoma cells and cellular invasion, whereas the cell proliferation was not significantly changed within 3 days after shRNA transduction (Fig. 5). Izumi *et al.*[16] reported that neuroblastoma cells have stem cell-like characteristics showing both asymmetric and symmetric cell divisions *in vitro* and that MYCN suppresses the asymmetric cell division (ACD). Consistent with the previous report,[16] immunocytochemistry analyses showed a high percentage of cells exhibiting ACD in SK-N-AS *MYCN*-non-amplified cells compared with BE(2)-C *MYCN*-amplified cells (Fig. S9). The shRNA-mediated knockdown of NCYM or OCT4 significantly increased the number of cells exhibiting ACD in BE(2)-C cells

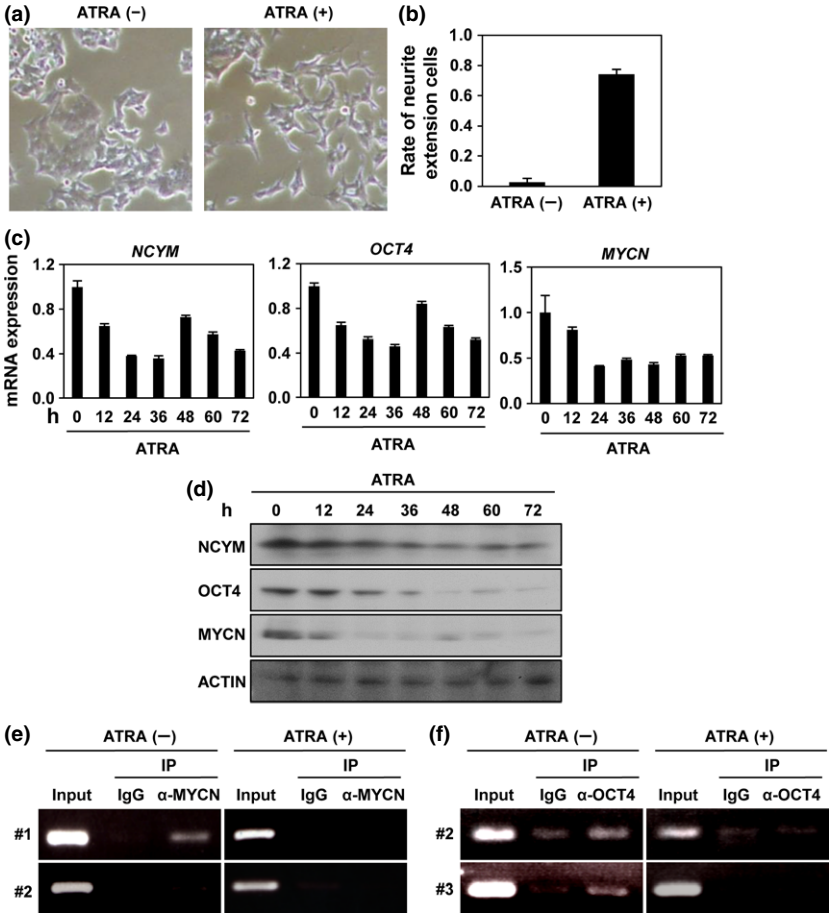

Fig. 4. All-*trans* retinoic acid (ATRA)-induced neuronal differentiation abrogates the positive autoregulatory loops formed by MYCN, NCYM, and OCT4. (a) Morphology of BE(2)-C intermediate (I)-type neuroblastoma cells treated with or without ATRA. (b) Percentage of BE(2)-C I-type neuroblastoma cells with marked neurite extensions relative to control with or without ATRA. Error bars represent SEM from three independent experiments. (c) Quantitative real-time RT-PCR analysis of *NCYM*, *MYCN* and stem cell-related genes in ATRA-treated BE(2)-C I-type neuroblastoma cells. mRNA expression levels were measured by real-time RT-PCR with β-*actin* as an internal control. (d) Western blot analyses of NCYM, MYCN, and OCT4 proteins in ATRA-treated BE(2)-C I-type neuroblastoma cells. ACTIN was used as loading control. (e) Identification of the MYCN-binding region in the *OCT4* promoter by ChIP assays. BE(2)-C I-type neuroblastoma cells were treated with or without ATRA. (f) Identification of the OCT4 binding region in the *MYCN/NCYM* promoter by ChIP assays. BE(2)-C I-type neuroblastoma cells were treated with or without ATRA.

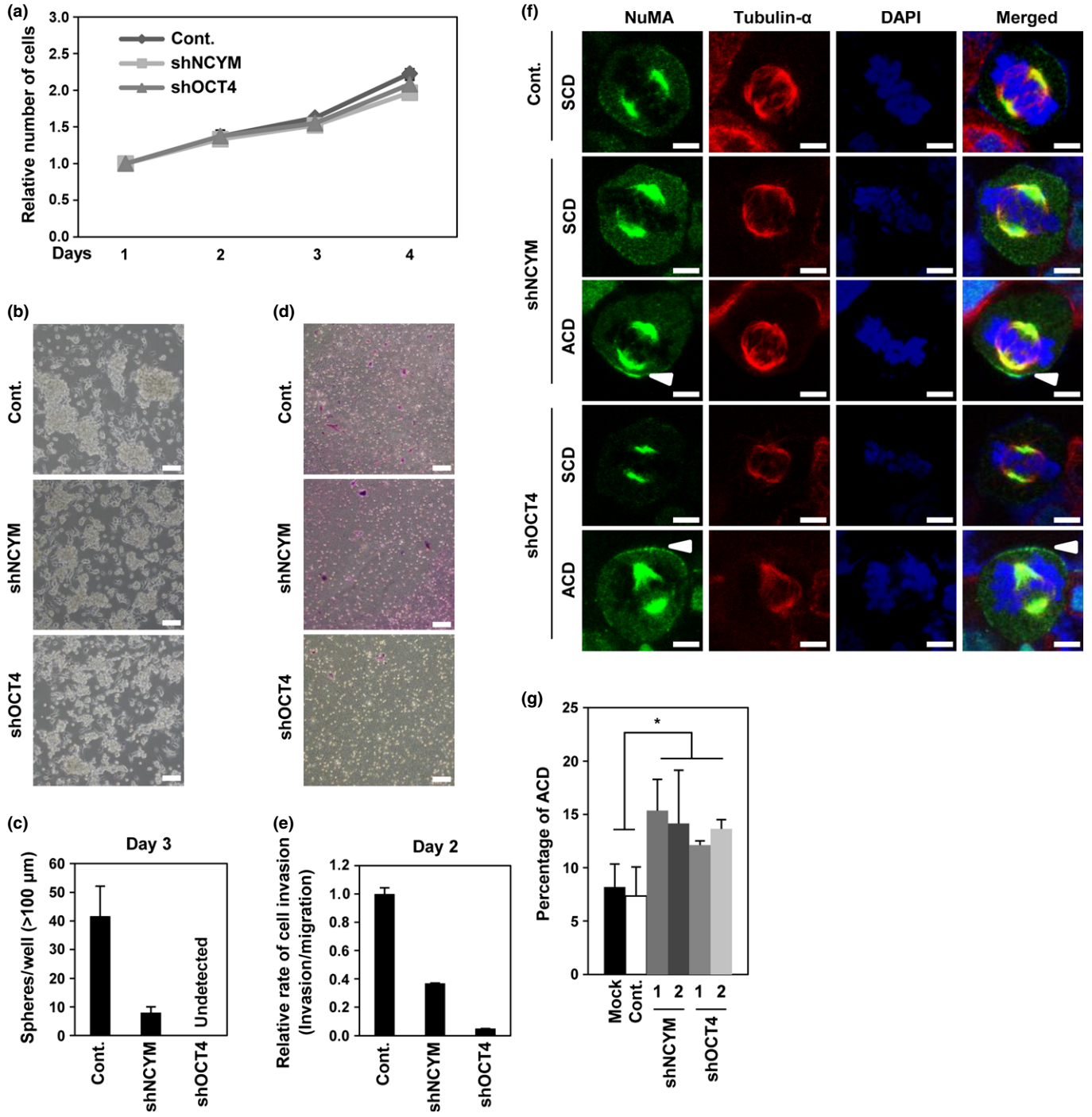

Fig. 5. NCYM and OCT4 control self-renewal of neuroblastoma cells. (a) Cell viability assay of BE(2)-C intermediate (I)-type neuroblastoma cells with NCYM or OCT4 shRNA-mediated knockdown. Cell proliferation was examined by WST assays at the indicated time points. (b) Sphere formation assay of BE(2)-C I-type neuroblastoma cells. Representative images show induction of sphere-forming activity after knockdown of NCYM or OCT4. Scale bar = 100 μm. (c) Quantification of sphere numbers from panel (b). The numbers of spheres were counted 72 h after infection. Error bars represent SEM from three independent experiments. (d) Invasion assay of BE(2)-C I-type neuroblastoma cells. Representative images show invasion activity after knockdown of NCYM or OCT4. Scale bar = 100 μm. (e) Quantification of BE(2)-C I-type neuroblastoma cells invading Matrigel relative to control (Cont.) migration after NCYM or OCT4 shRNA-mediated knockdown from panel (d). The numbers of spheres were counted 48 h after infection. Error bars represent SEM from three independent experiments. (f) Representative images of symmetric distribution of nuclear mitotic apparatus protein (NuMA) during the late stage of mitosis in shRNA-treated neuroblastoma cells. Tubulin-α is indicated in red, NuMA is green, and DNA is blue. Arrows show the distribution of NuMA on the cell cortex. Scale bar = 5 μm. (g) Quantification of cells with asymmetric cell division (ACD) in shRNA-transfected human neuroblastoma cells during late metaphase and anaphase. Error bars represent SEM from three experiments. Statistical significance determined by the Student's *t*-test, $*P < 0.05$. SCD, Symmetric cell division.

(Fig. 5f,g). Collectively, these results suggest that NCYM and OCT4 maintain self-renewal of human neuroblastoma cells.

Discussion

Here, we found that OCT4 promotes aggressiveness of *MYCN*-amplified neuroblastoma cells by forming a positive regulatory loop with MYCN/NCYM (Fig. 6). Despite a correlation between OCT4 expression and a stem cell-like state of neuroblastomas, the clinical significance of OCT4 in neuroblastomas has remained elusive. In this study, we found that *OCT4* was correlated with *NCYM* expression and undifferentiated pathological characteristics in Shimada pathology. Furthermore, the expression levels of *OCT4* were associated with unfavorable outcomes in *MYCN*-amplified tumors, but not in *MYCN*-non-amplified tumors. Previous studies showed that *MYCN* expression was inversely correlated with c-*MYC* in neuroblastoma[17] and that low expression levels of *KLF4* mRNA were associated with poor neuroblastoma outcome.[18] Our results showed that *NCYM* was positively correlated with *NANOG* expression and was inversely correlated with *KLF4* and c-*MYC*. As overexpression of OCT4 induced *NANOG* mRNAs, the correlation between *NANOG* and *MYCN/NCYM* in neuroblastomas may be explained by their common upstream regulator, OCT4. *In vitro* experiments showed that overexpression of NCYM induced *OCT4*, *SOX2*, and *NANOG*, but not c-*MYC* or *KLF4*. Therefore, among stem cell-related genes, *NCYM* mainly regulated the transcription of genes related to maintenance of pluripotency of ES cells[19–21] in human neuroblastoma cells. The NCYM protein stabilized MYCN to stimulate *OCT4* transcription, whereas OCT4 induced *NCYM* and *MYCN* through direct transcriptional activation of *MYCN*. Therefore, MYCN, NCYM, and OCT4 cooperate to induce each other, resulting in keeping their own expression at high levels and maintaining self-renewal of cells in *MYCN*-amplified neuroblastomas. Differentiation-inducing therapy by retinoic acid treatment has improved the overall survival of patients with *MYCN*-amplified neuroblastomas,[22] and ATRA treatment abrogated the mutual transcriptional regulations between MYCN, NCYM, and OCT4, inducing neuroblastoma cell differentiation. The ATRA treatment rapidly decreased *NCYM* mRNA within 24 hours, but the protein levels of NCYM were hardly downregulated compared with those of MYCN or

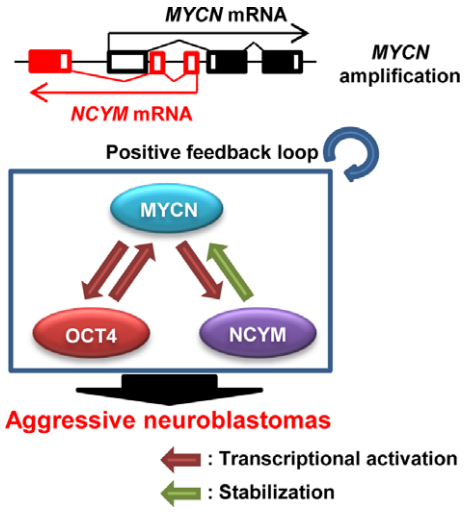

Fig. 6. Schematic model of MYCN/NCYM–OCT4 networks in *MYCN*-amplified human neuroblastomas.

OCT4. Therefore, the NCYM protein may be relatively more stable than MYCN or OCT4 proteins. Previous studies have shown that OCT4-positive neuroblastoma cells have resistant potency to conventional therapy[12] and multipotency to differentiation.[9] Thus, the functional interplay between MYCN/NCYM and OCT4 may contribute to maintenance of the multipotent status of OCT4-positive cells and the disruption of the MYCN/NCYM–OCT4 network could be a good therapeutic strategy for aggressive tumors.

Pezzolo *et al.*[9] reported that 2–30% of OCT4-positive cells were detected in approximately 90% of neuroblastoma samples (21 of 23). Thus, in contrast to other cancer stem cells,[23] the stem cell-like populations of neuroblastomas may not be small. In addition, high OCT4 expression was correlated with poor prognoses in patients with *MYCN*-amplified neuroblastomas, but not *MYCN*-non-amplified tumors, although the expression levels of OCT4 in *MYCN*-non-amplified tumors were comparable to those in *MYCN*-amplified tumors. These results indicate that *OCT4* requires *MYCN* amplification to promote aggressiveness of neuroblastomas. As NCYM inhibits apoptosis in *MYCN*-amplified tumors,[6] NCYM may be required for efficient proliferation of multipotent OCT4-positive cells. Therefore, variable amounts of OCT4-positive cells in *MYCN*-amplified tumors may reflect the different percentages of proliferative stem cell-like cells, influencing the prognoses of patients.

Previous reports have shown the physiological roles of OCT4 in the transcriptional regulation of *MYC* family members in various species.[24–26] OCT4 stimulates *MYC* transcription for cell proliferation in human and mouse ES cells[24,25] and activates myc transcription for cell survival during zebrafish gastrulation.[26] In the present study, we found the pathological significance of OCT4 for *MYCN* transcription in human neuroblastoma cells. The OCT4 binding sequence in *MYCN* intron 1 is not present in mice, but it is mostly conserved in other mammals. Although the E-box responsible for MYCN-mediated *MYCN/NCYM* transcription is highly constrained in mammals (Fig. S10), NCYM coding sequences are conserved only in humans and monkeys.[6] Therefore, the transcriptional regulation of *MYCN* by OCT4 and the positive autoregulation of MYCN may have evolved before the emergence of the *NCYM* gene, and NCYM strengthens the MYCN–OCT4 network by stabilizing MYCN, thereby inducing *OCT4* transcription. *NCYM* is positively selected during evolution;[6] however, its physiological roles in normal stem cells have remained unknown. Because new genes have been reported to rapidly become essential after emergence,[27,28] future studies will need to examine the physiological roles of the MYCN/NCYM–OCT4 networks in the maintenance of human normal stem cells.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

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

Fig. S1. NCYM and MYCN induce expression of embryonic stem cell-related genes.

Fig. S2. Overexpression of c-MYC does not enhance expression of *OCT4*, *NCYM*, or *MYCN* in SK-N-AS human neuroblastoma cells**.**

Fig. S3. Knockdown of NCYM or OCT4 decreases expression of CD133 in neuroblastoma cells.

Fig. S4. NCYM and MYCN do not induce mouse *Oct4* in mouse neuroblastoma cells.

Fig. S5. Expression of *OCT4* mRNA in tumors developed from *MYCN* and *MYCN/NCYM* transgenic mice.

Fig. S6. Conservation of OCT4 binding and E-box site in *OCT4* promoter and *MYCN/NCYM* regions.

Fig. S7. OCT4 induces the expression of *NCYM and MYCN* in neuroblastoma cells.

Fig. S8. All-*trans* retinoic acid (ATRA) suppresses stem cell-related genes.

Fig. S9. Asymmetric cell division in human neuroblastoma cells.

Fig. S10. Conservation of the E-box site at the *MYCN/NCYM* region.

Table S1. Univariate Cox regression analysis using *OCT4* mRNA expression level and clinical prognosis factors in *MYCN*-amplified neuroblastomas.

Table S2. Multivariate Cox regression analysis using *OCT4* mRNA expression level and clinical prognosis factors in *MYCN*-amplified neuroblastomas.

Table S3. Multiple regression analysis for factors associated with *OCT4* mRNA expression in neuroblastomas.

Table S4. Correlation between *NCYM* and *MYCN* mRNA expression and embryonic stem cell-related genes in neuroblastomas.

Doc. S1. Detailed descriptions of Materials and Methods.