NLRR2 is transcriptionally regulated through JNK/c-Jun pathway and enhances cell survival in neuroblastoma

(NLRR2 は JNK/c-Jun 経路により転写制御され、神経芽腫の細胞生存を高める)

千葉大学大学院医学薬学府

先端医学薬学専攻

（主任：田川雅敏 教授）

Afzal Sheikh

Abstract

The novel human gene family encoding Neuronal Leucine Rich Repeat (NLRR) proteins were identified as prognostic markers from our previous screening of primary neuroblastoma (NB) cDNA libraries. Of the NLRR gene family members, NLRR1 and NLRR3 are associated with the regulation of cellular proliferation and differentiation, respectively. However, the functional regulation and clinical significance of NLRR2 in NB remain unclear . We evaluated the expression of *NLRR2* in 78 NBs by quantitative real time PCR. High levels of *NLRR2* expression were significantly associated with a poor prognosis of NB (p=0.0009). *In vitro* experiments showed that the exogenous expression of *NLRR2* enhanced cellular proliferation and induced resistance to retinoic acid (RA)-mediated cell growth inhibition in NB cells. Knock-down of *NLRR2* exhibited growth inhibition effects in NB cells and enhanced RA-induced cell differentiation. In NB cells treated with RA, NLRR2 expression was increased and was correlated with the upregulation of c-Jun, a member of the activator protein-1 (AP-1) family. The expressions of NLRR2 and c-Jun were suppressed by treatment with a JNK inhibitor, which ameliorated the promoter activity of the *NLRR2* gene. An AP-1 binding consensus was identified in the *NLRR2* promoter region to which c-Jun was recruited. Moreover, knock-down of c-Jun reduced *NLRR2* expression, suggesting that *NLRR2* is an inducible gene regulated by the JNK pathway to enhance cell survival and inhibit NB cell differentiation. Therefore, NLRR2 might have an important role in NB aggressiveness and might be a potential therapeutic target for the treatment of RA resistant and aggressive NBs as well as other cancers.

Introduction

Neuroblastoma (NB) is the most common form of malignancy in early childhood and is derived from the sympathoadrenal lineage of neural crest progenitor cells.[1] NB exerts heterogeneous clinical behavior. Some NBs regress spontaneously while others progress to highly metastatic tumors with a poor prognosis despite intensive multimodal therapy.[2] However, the pathogenesis of NB is poorly understood as very few gene defects have been identified in this often-lethal tumor. Frequently detected genetic alterations are limited to MYCN amplification and ALK activation.[3-5] Aberrant gene expression patterns have been shown to be important in the clinical outcome of NB. For example, high expression of the neurotrophic receptor TrkA has been identified as a prognostic factor for a favorable outcome because it inhibits tumor growth and angiogenesis, whereas TrkB and its ligand, brain-derived neurotrophic factor, are associated with aggressive NB with MYCN amplification that enhances tumor growth and angiogenesis.[4,6-8] Some factors related to NB pathogenesis are involved in proliferation, differentiation, and metastasis. However, little is known about their biological function with regards to tumor growth aggressiveness and this has negatively impacted on the development of therapeutic drugs. Therefore, studies on the molecular mechanisms of NB are required to develop new therapeutic strategies to treat aggressive NB.

Retinoic acid is an important metabolite that functions as a morphogen in the developing nervous system *in vivo*, and which induces the differentiation of neuronal cells *in vitro*.[9] It has been implicated in human NB because RA treatment inhibits NB proliferation, differentiates into neuronal-like cells, reduces cell migration and invasiveness.[10-13] These properties have been used as a basis for the clinical application of RA, one of the limited drugs currently used to treat cancers.[10-14] It has become standard practice to treat high-risk NB patients after marrow or stem cell transplantation.[15] Despite multiple clinical efforts, the prognosis remains poor for this enigmatic disease because of the high rate of resistance and metastasis in acute promyelocytic leukemia as well as human NB cell lines that express multidrug resistance genes during differentiation by RA.[16,17] Therefore, to overcome drug resistance in the aggressive NBs, it is important to identify factors that contribute to RA resistant and RA-mediated NB cell differentiation provides a good model for this study. RA binds to RAR and activates a signal transduction pathway that involves multiple cytoplasmic signaling molecules such as JNK and ERK. The JNK pathway has been proposed to induce a signaling cascade during RA-mediated differentiation of NB as well as non–NB.[18-20] The downstream component of JNK/SAP MAPK is AP-1, which is a homo- or heterodimeric complex consisting of c-Jun, c-fos, Maf and ATF2. Based on the cellular context, the composition of the AP-1 dimeric complexe determines its functions in the regulation of differentiation, proliferation, and apoptosis.[19-23] However, the direct molecular mediator in transmitting RA-signal to regulate neuritis extension in NB has remained unknown. Here, we report that NLRR2 acts as an inhibitor for the RA-mediated differentiation of NB, therefore contribute to NB aggressiveness.

NLRR2 is a member of the NLRR genes family that encode a glycosylated transmembrane protein with a leucine rich repeat (LRR) domain containing 11 or 12 LRR, an immunoglobulin c2-type domain, and a type III fibronectin domain in its extracellular domain. Like other NLRR family proteins, NLRR2 has highly conserved amino acid sequence in the extracellular domain which has substrate or chemical binding sites and four potential glycosylation sites. NLRR2 also possesses ww interacting domains in the short intracellular region, which mediate protein-protein interactions and might provide a basis of signaling events for *NLRR* function in tumorigenesis.[24-26] We previously reported that NLRR1 enhances epidermal growth factor (EGF)-mediated MYCN induction in NB resulting in the acceleration of tumor growth *in vivo* and that a high expression of NLRR1 mRNA levels is associated with a poor prognosis of NB. In contrast, low NLRR3 expression levels were correlated with a poor prognosis in NB patients.[27-29] However, very little is known about the role of *NLRR2* in tumor progression except it has been reported to be amplified and overexpressed in malignant gliomas.[30] The current study reveals that RA functions as a negative feedback regulator through the upregulation of NLRR2 during RA-mediated differentiation in NBs. NLRR2 might be a useful pharmacological indicator to predict RA efficiency in NB treatment.

Results

Expression of *NLRR2* is associated with the poor prognosis of neuroblastoma and enhances oncogenic transformation *in vitro* and *in vivo*.

Previously, we identified NLRR family genes which are differentially expressed between favorable and unfavorable NBs from the screening of nearly 2000 novel genes using our unique NB cDNA libraries. Human *NLRR2* is a highly expressed gene in NB-derived cell lines[26] and may be used to define clinical relevance between favorable and unfavorable NBs. We performed gene expression analysis by real-time PCR using NB cDNA from 78 patients. Kaplan-Meier survival curves indicated that NBs with high *NLRR2* expression significantly associated with a poor clinical prognosis (P<0.001) (Figure 1a). To investigate the oncogenic effect of NLRR2 in NBs, we stably expressed *NLRR2* in SK-N-BE cells that resulted in significant increas of proliferation (P<0.001) compared with the mock stable cells (Figure 1b). However, these findings by real-time PCR data do not demonstrate why NLRR2 is a poor prognosis factor for NB patients. Therefore, we investigated the biological function of NLRR2 by overexpressing or knocking down *NLRR2* and measuring cell growth. Overexpression of *NLRR2* resulted in a significant (P<0.01) increase in NB cell proliferation (Figures 1c and d). In addition, the down regulation of *NLRR2* by siRNA-mediated knock-down significantly (P<0.01) reduced SK-N-BE cell growth (Figure 1e). To confirm the function of NLRR2 *in vivo*, we locally treated mice bearing tumors derived from SK-N-BE cells with atelocollagen complexed with si*NLRR2* . Compared with the control siRNA group, siNLRR2 treatment significantly reduced the tumor growth of SK-N-BE xenograft tumors (Figures 1f, g and h). These data suggest that the function of *NLRR2* is involved in tumorigenesis and is associated with a poor prognosis of NB.

NLRR2 inhibits differentiation in NB cells

To confirm whether NLRR2 has a function in cell growth and differentiation processes of NB, we investigated morphological changes in cells upon the modulation of *NLRR2* expression. After transfection of siNLRR2, the growth of SK-N-BE cells was significantly repressed by RA treatment (Figures 2a and b). Interestingly, the differentiation data revealed that *NLRR2*-knockdown cells were more susceptible to RA-mediated differentiation (Figures 2c and d). The enhanced cell differentiation by *NLRR2* knock-down was confirmed by a higher level of GAP43, a neuronal differentiation marker expression (Figure 2e). GAP43 is also associated with neuritis extension.[31-33] These data suggest that *NLRR2* knock-down retards cell growth and enhances cell differentiation induced by RA treatment.

c-Jun is important for the regulation of NLRR2 expression

We next examined NLRR2 expression during RA-induced differentiation (Supplementary figure S2) of NB cells. Interestingly, mRNA and protein levels of NLRR2 expression were elevated in RA-treated NB cells (Figures 3a and b). Consistent with previous reports showing the RA-mediated induction of c-Jun[18,19,34] c-Jun expression in NB cells was also increased upon RA treatment. To investigate whether c-Jun was important for the regulation of NLRR2 expression, NB cells were transiently transfected with *c-Jun* siRNA, resulting in reduced expressions of NLRR2 mRNA and protein (Figures 3c and d). Because RA treatment failed to rescue the NLRR2 expression in *c-Jun* knock-down cells, these data demonstrated that c-Jun functions by regulating RA-induced NLRR2 expression.

RA enhances the recruitment of c-Jun to the *NLRR2* promoter

To clarify the transcriptional regulation of the *NLRR2* gene, we generated luciferase reporter constructs containing −790 bp to +110 bp fragments of the *NLRR2* gene where +1 represents the transcriptional initiation site. The promoter activity determined by luciferase reporter analysis was increased in response to RA treatment (Figure 4a). The study using a series of deletion mutants of the promoter constructs showed that the luciferase activity was highest in between the −790 and −560 region, which contains a c-Jun binding site of TPA-responsive element (TRE), TGACAAA. We performed a promoter assay in HeLa cells because they have a high transfection efficiency. RA-treatment induced *NLRR2* and *c-Jun* expression (Supplemental figure S3). We also confirmed similar luciferase activity data in SK-N-BE cells (Figure 4b) using a −790 to +110 promoter construct including AP-1 binding site. Next, we performed a chromatin immunoprecipitation (ChIP) assay to determine whether c-Jun directly binds to the promoter region of *NLRR2*. As shown in Figure 4c, the recruitment of c-Jun to the *NLRR2* promoter was increased by RA treatment, indicating that *NLRR2* is transcriptionally regulated by c-Jun.

NLRR2 expression is regulated by the JNK pathway

NLRR2 promoter activity was suppressed in SK-N-BE cells following treatment with a JNK inhibitor (Figure 5a). To confirm that NLRR2 is regulated through the JNK pathway, we treated TGW and SK-N-BE cells with a JNK inhibitor to examine the expression of NLRR2. As expected, JNK inhibition reduced the expression of NLRR2 and c-Jun in both the mRNA and protein levels (Figures 5b and c, respectively). Furthermore, the RA-mediated increased expression of NLRR2 was reduced by JNK inhibition (Supplemental figure S4). To confirm the function of the JNK pathway in the regulation of NLRR2 expression, we used sorbitol, which induces JNK activation.[35,36] The phosphorylation of JNK and c-Jun as well as the expression of NLRR2 were induced by sorbitol treatment in SK-N-BE cells and co-treatment with a JNK inhibitor ameliorated the induction of NLRR2 expression (Figures 5d and e). Thus, the JNK pathway is particularly important for regulating NLRR2 expression in NB cells.

NLRR2 exhibits survival to RA and other stress-mediating agents in NB cells.

Because the JNK pathway was induced by RA and sorbitol was involved in the regulation of NLRR2 expression, we next treated NB cells with other agents that cause cellular stress. Treatment with tunicamycin (TM), an endoplasmic reticulum stress agent and cisplatin (CDDP), a DNA-damaging agent, induced *NLRR2* expression in NB cells (Figures 6a and b). To examine whether the induced NLRR2 was associated with cell survival against cellular stress, NLRR2-stably expressing cells were treated with RA and TM and cell viability was examined. As shown in Figure 6c and 6d, NLRR2-stable cells were significantly resistant to RA and TM treatment compared with control cells. We also checked the morphology of the NLRR2 stable and mock cells upon RA-treatment to study the differentiation function of NLRR2. Data showed that the stable expression of NLRR2 significantly (*P*<0.01) inhibited RA-induced differentiation compared with controls (Supplemental figure S5). Therefore, after the induction of NLRR2 by cellular stress-mediating agents, NLRR2 in turn reduce their cytotoxicity of NB cells.

Discussion

Our results clearly demonstrate the clinical relevance of NLRR2 expression and its function in RA-induced cell differentiation. NLRR2 enhances the cell survival of RA-treated NB cells, while other family members, NLRR1 and NLRR3, are associated with cell proliferation and differentiation.[28,29] In addition, mouse NLRR3 was reported to modulate MAPK signaling through the EGFR pathway,[35] indicating that NLRR family proteins might have functional relevance in cell signaling events. The present study suggests that the high expression of *NLRR2* is significantly associated with the poor prognosis of NB, consistent with data analyzed using an R2 platform. Of note, a similar observation was reported for malignant gliomas,[30] Local treatment of siNLRR2 in a mouse xenograft model of NB significantly reduced tumor growth, suggesting that the low expression of NLRR2 solely restricts tumor progression and serves as a prognostic marker of NB. Further study may clarify the clinical significance of NLRR2 expression in tumorigenesis.

Our *in vitro* experiments that modulated the expression of NLRR2 in NB cells indicated the potential molecular mechanisms of how *NLRR2* might be involved in the poor clinical outcome of NB. NLRR2-overexpressing cells showed enhanced cell proliferation and a significant resistance phenotype to RA treatment. In contrast, *NLRR2* knock down cells were more susceptible to RA-mediated cell growth inhibition and exhibited significantly (P<0.01) increased numbers of differentiated cells compared with control cells. These data suggest that the function of NLRR2 is to inhibit the differentiation process and support the cell survival of RA-treated cells.

RA is a potent differentiation inducer of NB cells and causes a marked suppression of MYCN expression.[29,36,37] However, our previous observation revealed that NLRR2 expression was not correlated with MYCN expression (data not shown). In the present study, we found that c-Jun plays a key role in regulating *NLRR2* gene expression in RA-treated NB cells. RA-treated NB cells exhibited the co-induction of NLRR2 and c-Jun, whereas expressions of other AP-1 members such as c-fos and ATF-2 were reduced (Supplemental figure S6). The activity of JNK appears to be important for the induction of NLRR2, which is consistent with previous reports regarding the induction and activation of c-Jun in a downstream of the JNK pathway after RA treatment.[19,20] It is noteworthy that other stress-inducing agents such as TM and CDDP also induced NLRR2 in NB cells. The significant survival difference observed in the clinical subsets with low and high expressions of NLRR2 may be explained by the stress-induced up-regulation of NLRR2, which in turn contributes to the drug resistance of NB cells. An anti-apoptotic role of activated c-Jun/AP-1 transcription factor was reported to protect human tumor cells from DNA-damaging agents including TM and CDDP (38). Thus, the effects observed in this study suggest that activated AP-1 complexes exert a favorable influence on cell survival that counters diverse external stresses induced by drugs.

Although the JNK and p38MAPK pathways can potentially synergize to induce AP-1 transcriptional activity and share c-Jun as a common substrate (39), we found a limited effect of p38MAPK inhibition on RA-induced differentiation as well as NLRR2 expression in NB cells (data not shown). There is considerable evidence that neurite outgrowth stimulated by RA is partially dependent on JNK activity (18). c-Jun/AP-1 was reported to be involved in neuronal differentiation and protection from apoptosis in NB cells.[40] However, the relevant functions and target genes of AP-1 in neuronal differentiation have not been elucidated yet. In particular, it is unclear whether AP-1 in NB cells facilitates cell death or, conversely, is a part of a protective response against differentiation or apoptosis. Our experimental evidence suggests that c-Jun/AP-1 in RA-treated NB cells plays a protective role by up-regulating the expression of NLRR2. Future studies might explain why the prognosis of NB treated with RA remains poor, with a high rate of resistance and metastasis.[16,17]

The present study indicates the clinical relevance of NLRR2 and a regulatory mechanism of *NLRR2* transcription in RA-treated NB cells. The expression of *NLRR2* is transcriptionally regulated by the JNK-c-Jun axis and enhances cell survival in drug-treated NB cells. Therefore, NLRR2 might be a key target molecule for the development of new therapeutic strategies to overcome RA resistance in aggressive NLRR2-expressing NBs.

Materials and Methods

Cell culture

Human NB-derived TGW, SMS-SAN and non-NB HeLa cells were collected from The Children's Hospital of Philadelphia cell line bank (Philadelphia, PA, USA). SK-N-BE and NB cells were collected from the European Collection of Cell Cultures (Wiltshire, UK) cell bank. NB cells were maintained in RPMI 1640 medium (Wako, Osaka, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, CA, USA), 50 μg/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). HeLa cells were maintained in DMEM medium (Wako) with the same supplements. All cells were cultured in a humidified chamber provided with 5% CO_2 at 37°C.

siRNA-mediated knock-down

A mixture of two sets of siRNA sense and antisense sequences was used: siNLRR2-1: 5′-CUACAGGAACUCUAUCUCATT-3′ (sense); 5′-UGAGAUAGAGUUCCUGUAGTT-3′ (antisense); siNLRR2-2: 5′-CCAACUUGGAGAUACUCAUTT-3′ (sense); 5′-AUGAGUAUCUCCAAGUUGGTT-3′ (antisense), which were designed to target human *NLRR2* (Takara, Shiga, Japan). c-Jun siRNA was purchased from Cell Signaling Technology (Boston, MA, USA). Control non-targeting siRNA was purchased from Thermo Fisher Scientific (Waltham, MA, USA). NB cells were transfected with siRNA by forward-transfection according to the manufacturer's protocol using Lipofectamine RNAiMAX reagent (Invitrogen). We used siRNA (concentration 50 nM) for siNLRR2 and 200 nM for si-c-Jun because these concentrations were determined to work well in a preliminary study (Supplemental figure S1). Forty-eight hours after incubation at 37°C in a CO_2 incubator, gene knock-down was evaluated by quantitative real-time RT-PCR.

Cell growth assays

SK-N-BE and SMS-SAN cells were transfected with an *NLRR2* expression vector using Lipofectamine 2000 in Opti-MEM medium, which was replaced after 12 hours of transfection with RPMI medium supplemented with heat-inactivated 10% FBS for 24 hours. Cells were then plated into 96-well plates at a cell density of 500 cells/well and their proliferation was monitored by a live cell imaging system (IncuCyte, Essen Bioscience, Morgan Rd, MI,USA).

RT-PCR and quantitative real-time PCR (qPCR)

RT-PCR was performed to check the expression of *NLRR2*, *c-Jun*, *GAP43* and *GAPDH*. To perform RT-PCR, total RNA was extracted from cells by RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and reverse transcription was performed with SuperScript II reverse transcriptase (Invitrogen). The specific primers used were as follows: *NLRR2*, 5′-cccgagactgtgattccat-3′ and 5′-aagttgctgaggccagaaaa-3′; *c-Jun*, 5′-agcggaccttatggctacag-3′ and 5′-ccgttgctggactggattat-3′; *GAP43*, 5′-gagagcagttcgacctagtcc-3′ and 5′-tgcggccttaatgagctttat-3′; and *GAPDH*, 5′-acctgacctgccgtctagaa-3′ and 5′-tccaccaccctgttgctgta-3′. cDNAs prepared from primary NB tissue samples and cultured cells were used to quantify β-actin using a β-actin control reagent kit purchased from Applied Biosystem (Drive Foster City, CA, USA). *NLRR2* and *c-Jun* mRNA expressions were measured by the SYBR green real-time system using the following primers: *NLRR2*, 5′-ctcctgagggccattgaca-3′ and 5′-cgccaatcatgagtatctccaa-3′; and *c-Jun*, 5′-cggagaggaagcgcatga-3′ and 5′-ttcctttttcggcacttgga-3′. All these real-time quantifications were performed using a 7500 Real Time PCR System (Applied Biosystems).

Immunoblotting

Whole cell lysates were prepared using ice-cold RIPA buffer containing proteinase and phosphatase inhibitors. Protein concentration was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were then blocked with 5% skim milk at room temperature for 1 hour. After blocking, the membranes were incubated at 4°C overnight with anti-NLRR2 (MBL, Nagoya, Japan), anti-c-Jun, anti-JNK, anti-phospho-JNK, anti-phospho-cJun, anti-GAP43 (Cell Signaling Technology) and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) antibodies.

RA treatment

To study the effect of RA (Sigma-Aldrich) on *NLRR2* expression, TGW cells (1 µM) and SK-N-BE, SMS-SAN and HeLa cells (2.5 µM) were treated with RA or dimethyl sulfoxide. After the indicated time period, NLRR2 expression was measured by RT-PCR, qPCR and western blot analysis. To investigate the effect of RA on NB cell growth and differentiation, *NLRR2* was stably expressed or knocked down using an *NLRR2* expression vector or siNLRR2, respectively. After 36 hours of transfection, the cells were seeded at 5 × 10^3 (stably expressing cells) or 1 × 10^3 (knock-down cells) in 96-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) and allowed to adhere overnight followed by RA treatment. At the indicated time points, cell survival was determined using IncuCyte (Essen Bioscience).

Luciferase assay

An *NLRR2* promoter region (−790 to +110) was generated by PCR amplification using human placental genomic DNA and cloned into a pGL3 basic vector (Promega, Fitchburg,WI, USA). Cells were plated in a 96-well plate at a density of 2 × 10^3/well and transiently transfected with the indicated luciferase reporter constructs (200 ng). At 48 hours after transfection, cell lysates were prepared using passive lysis buffer (PLB) and their luciferase activity was measured by dual luciferase assay kit according to the manufacturer's protocol (Promega). *Renilla* luciferase was used as an internal control.

ChIP assay

A ChIP assay was performed using a ChIP assay kit according to the protocol provided by Millipore (Billercia, MA, USA). Precipitated DNA and control input DNA were purified using a QIAquick PCR purification kit (Qiagen). Purified DNA was amplified by PCR using the following set of primers: 5′-gttcctgactgaaattgacc-3′ and 5′-ttgcaaaggccaggagacgaatg-3′ targeting the *NLRR2* core promoter region (−790 to +110). A 5′-tcaaggagcgcctggctct-3′ and 5′-atggcagaatcccaatccg-3′ primer set was used for the region that does not contain any AP-1 consensus sequence.

In vivo tumorigenicity assays

SK-N-BE cells at a density of 1×10^7 were subcutaneously inoculated into 7-week-old female SCID mice. One week after inoculation, when the tumors had an average volume of 70 ± 30 mm^3, a mixture of 1 nmol of control or a mixture of two sets of *NLRR2* siRNA and 200 µl of atelocollagen (Koken, Tokyo, Japan) was injected to the site of the tumor to evaluate the growth inhibition effect. Injections were administered twice at 7-day intervals. Tumor size was measured every week after they were visible. The volume of each tumor was calculated using the formula: Tumor volume=(length × width^2)/2. Animal experiments were performed in compliance with the regulations for animal experiments of IACUC.

Statistical analysis

Results were shown as the mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences of means between two groups. $P < 0.05$ was considered statistically significant.

References

1 Chang HH, Lee H, Hu MK, Tsao PN, Juan HF, Liao YF *et al*. Notch1 expression predicts an unfavorable prognosis and serves as a therapeutic target of patients with Neuroblastoma, *Clin Cancer Res* 2010; **16**: 4411-20.

2 Brodeur GM, Nakagawara A. Molecular basis of clinical heterogeneity in Neuroblastoma. *Am J Pediatr Hematol Oncol* 1992; **14**: 111-6.

3 John M, Maris MD. Recent Advances in Neuroblastoma. *N Engl J Med* 2010; **362**: 2202-2211.

4 Nakagawara A, Arima M, Azar CG, Scavarda NJ, Brodeur GM. Inverse relationship between trk expression and N-myc amplification in human neuroblastomas. *Cancer Res* 1992; **52**: 1364–68.

5 Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M *et al*. Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 2008; **16**: 971-4.

6 Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human Neuroblastoma. *N Engl J Med* 1993; **328**: 847-54.

7 Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-B and BDNF in human neuroblastomas. *Mol Cell Biol* 1994; **14**: 759–767.

8 Nakagawara A, Arima-Nakagawara M, Azar CG, Scavarda NJ, Brodeur GM. Clinical significance of expression of neurotrophic factors and their receptors in neuroblastoma. *Prog Clin Biol Res* 1994; **385**: 155–61.

9 Duester G. Retinoic acid synthesis and signaling during early embryogenesis. *Cell* 2008; **134**: 921-931.

10 Miloso M, Villa D, Crimi M, Galbiati S, Donzelli E, Nicolini G *et al.* Retinoic acid-induced neuritogenesis of human neuroblastoma SH-SY5Y cells is ERK independent and PKC dependent. *J Neurosci Res* 2004; **75**: 241-52.

11 SideII N, Altman A, Haussler MR, Seeger RC. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human Neuroblastoma cell lines. Exp Cell Res 1983; 148: 21-30.

12 Messi E, Florian MC, Caccia C, Zanisi M, Maggi R. Retinoic acid reduces human neuroblastoma cell migration and invasiveness: effects on DCX, LIS1, neurofilaments-68 and vimentin expression. *BMC Cancer* 2008; **8**: 30.

13 McBurney MW, Jones-Villeneuve EM, Edwards MK, Anderson PJ. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* 1982; **299**: 165-7.

14 Tang XH, Gudas LJ. Retinoids, retinoic acid receptors, and cancer. *Annu Rev Pathol* 2011; **6**: 345-64.

15 Huang S, Laoukili J, Epping MT, Koster J, Hölzel M, Westerman BA, *et al.* ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome. *Cancer Cell* 2009; **15**: 328-40.

16. Tallman MS, Andersen JW, Schiffer CA *et al.* All-trans retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol. *Blood* 2002; **100**: 4298-302.

17 Bates SE, Mickley LA, Chen YN, Richert N, Rudick J, Biedler JL *et al.* Expression of a drug resistance gene in human neuroblastoma cell lines: modulation by retinoic acid-induced differentiation. *Mol Cell Biol* 1989; **9**: 4337-44.

18 Yu YM, Han PL, Lee JK. JNK pathway is required for retinoic acid-induced neurite outgrowth of human Neuroblastoma, SH-SY5Y. *Neuroreport* 2003; **14**: 941-945.

19 Kanungo J, Potapova I, Malbon CC, Wang HY. MEKK4 mediates differentiation in response to retinoic acid via activation of c-Jun N-terminal kinase in rat embryonal carcinoma P19 cells. *J Biol Chem* 2000; **275**: 24032-9.

20 Leppä S, Saffrich R, Ansorge W, Bohmann D. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. *EMBO J* 1998; **17**: 4404-13.

21 Karin M, The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 1995; **270**: 16483-6.

22 Ameyar M, Wisniewska M, Weitzman JB. A role for AP-1 in apoptosis: the case for and against. *Biochimie* 2003; **85**: 747–52.

23 Raivich G, Behrens A. Role of the AP-1 transcription factor c-Jun in developing, adult and injured brain. *Prog Neurobiol* 2006; **78**; 347-63.

24 Ohira M, Morohashi A, Inuzuka H, Shishikura T, Kawamoto T, Kageyama H, et al. Expression profiling and characterization of 4200 genes cloned from primary neuroblastomas: identification of 305 genes differentially expressed between favorable and unfavorable subsets. *Oncogene* 2003; **22**: 5525–5536.

25 Haines BP, Gupta R, Jones CM, Summerbell D, Rigby PW *et al*. The *NLRR* gene family and mouse development: Modified differential display PCR identifies *NLRR-1* as a gene expressed in early somitic myoblasts. *Dev Biol* 2005; **281**: 145-59.

26 Hamano S, Ohira M, Isogai E, Nakada K, Nakagawara A. Identification of novel human neuronal leucine-rich repeat (hNLRR) family genes and inverse association of

expression of Nbla10449/hNLRR-1 and Nbla10677/hNLRR-3 with the prognosis of primary neuroblastomas. *Int J Oncol* 2004; **24**: 1457-66.

27 Hossain MS, Ozaki T, Wang H, Nakagawa A, Takenobu H, Ohira M, Kamijo T *et al*. N-MYC promotes cell proliferation through a direct transactivation of neuronal leucine-rich repeat protein-1 (*NLRR1*) gene in Neuroblastoma. *Oncogene* 2008; **27**: 6075-6082.

28 Hossain S, Takatori A, Nakamura Y, Suenaga Y, Kamijo T, Nakagawara A. NLRR1 enhances EGF-mediated MYCN induction in neuroblastoma and accelerates tumor growth in vivo. *Cancer Res* 2012; **72**: 4587-96.

29 Akter J, Takatori A, Hossain MS, Ozaki T, Nakazawa A, Ohira M, *et al.*, NLRR3 Orphan Receptor Gene is Negatively Regulated by MYCN and Miz-1, and Its Downregulation is Associated with Unfavorable Outcome in Neuroblastoma. *Clin Can Res* 2011; **17**: 6681-92.

30 Almeida A, Zhu XX, Vogt N, Tyagi R, Muleris M, Dutrillaux AM, *et al*. GAC1, a new member of the leucine-rich repeat superfamily on chromosome band 1q32.1, is amplified and overexpressed in malignant gliomas. *Oncogene* 1998; **16**: 2997-3002

31 Zhao JC, Zhang LX, Zhang Y, Shen YF. The differential regulation of Gap43 gene in the neuronal differentiation of P19 cells. *J Cell Physiol* 2012; **227**: 2645-53.

32 Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci* 1997; **20**: 84–91.

33 Aarts LH, Schotman P, Verhaagen J, Schrama LH, Gispen WH. The role of the neural growth associated protein B-50/GAP-43 in morphogenesis. *Adv Exp Med Biol* 1998; **446**: 85–106.

34 Kitabayashi I, Kawakami Z, Chiu R, Ozawa K, Matsuoka T, Toyoshima S *et al*. Transcriptional regulation of the c-jun gene by retinoic acid and E1A during differentiation of F9 cells. *EMBO* J 1992; **11**: 167-75.

35 Fukamachi K, Matsuoka Y, Ohno H, Hamaguchi T, Tsuda H. Neuronal leucine-rich repeat protein-3 amplifies MAPK activation by epidermal growth factor through a carboxyl-terminal region containing endocytosis motifs. *J Biol Chem* 2002; **277**: 43549-52.

36 Thiele C.J., Reynolds C.P., Israel M.A., Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. *Nature* 1985; **313**: 404–406.

37 Reynolds C.P., Kane D.J., Einhorn P.A., Matthay K.K., Crouse V.L., Wilbur J.R., *et* al. Response of neuroblastoma to retinoic acid in vitro and in vivo. *Prog Clin Biol Res* 1991; **366**: 203–211.

38. Potapova O, Basu S, Mercola D, and Nikki J. Holbrook N.J.Protective Role for c-Jun in the Cellular Response to DNA Damage. *The Journal of Biological Chemistry* 2001, **276**: 28546-28553.

39 Erwin F, Wagner and Angel R.N. Signal integration by JNK and p38MAPK pathways in cancer development. *Nature Reviews* 2009; **9**: 537-549.

40 Feng Z, Li L, Ng PY, Porter AG. Neuronal differentiation and protection from nitric oxide-induced apoptosis require c-Jun-dependent expression of NCAM140. *Mol Cell Biol* 2002; **22** :5357-66.

Figure legends

Figure 1. NLRR2 is correlated with poor survival outcome in human NB and regulates tumor cell growth *in vitro* and *in vivo*. (a) Kaplan-Meier survival curves. Expression levels of *NLRR2* were designated as high (n=23, red line) and low (n=55, blue line). High *NLRR2* mRNA expression was significantly correlated with poor survival outcome (p=0.001). (b) Mock and NLRR2 bulk stable cells were generated in SK-N-BE cells and NLRR2 expressions were confirmed by western blotting (left panel). Stable expression of *NLRR2* exhibited significant (p=0.001) enhancement of cell growth (right panel). (c, d) *NLRR2* was transiently overexpressed in SK-N-BE and SMS-SAN cells and confirmed by western blotting. Over-expression of *NLRR2* significantly (p<0.05) enhanced cell growth in SK-N-BE (c) and SMS-SAN (d) cells. (e) *In vitro* knock-down of *NLRR2* reduced NB cell growth. (f) SCID mice were subcutaneously inoculated with 1×10^7 SK-N-BE cells. Two weeks after inoculation, when the tumors had an average volume of 70 ± 30 mm^3, 1 nmol of control siRNA or a mixture of two siNLRR2 siRNA with 200 µl of atelocollagen (Koken, Tokyo, Japan) was injected to the tumor mass to evaluate the growth inhibition effect. siNLRR2 treatment significantly (p<0.01) reduced the tumor growth compared with the control siRNA-treated group. (g, h). Four weeks after treatment, the tumors were removed (g). The tumor weight was significantly (p<0.01) reduced in the siNLRR2-treated group compared with the control siRNA group (h).

Figure 2. NLRR2 knock down cells are susceptible to RA-mediated inhibition of cell survival and differentiation. (a, b) SK-N-BE cells were transiently transfected with control siRNA or siNLRR2 and cell growth was measured using a real-time cell imaging

system. Knock-down of *NLRR2* expression was confirmed by quantitative real time PCR (a). The inhibition of cell growth by RA treatment was significantly increased in *NLRR2* knock down cells (p<0.01). (c, d). SK-N-BE cells were transiently transfected with control siRNA or siNLRR2 followed by RA (2.5 μM) treatment for three days. The percentage of differentiated cells per field was counted (5 fields per well, 5 wells in each group). Data are presented as the mean ± SD. (e) SK-N-BE cells were transiently transfected with control siRNA or siNLRR2 followed by RA treatment for the indicated time period. GAP43, a marker for the neuronal differentiation, and NLRR2 expressions were examined by western blot analysis. *NLRR2* knock down cells with RA treatment showed a higher expression of GAP43.

Figure 3. c-Jun is important for regulating *NLRR2* expression induced by RA. (a, b) NLRR2 and c-Jun were upregulated during RA-mediated differentiation at the mRNA (a) and protein (b) levels in SK-N-BE, TGW and SMS-SAN cells. NB cells were treated with RA (1.0 μM for TGW cells, 2.5 μM for SK-N-BE and SMS-SAN cells) for the indicated time periods. NLRR2 and c-Jun expressions were determined by RT-PCR and western blot analysis. (c, d). SK-N-BE cells were transfected with control or c-Jun siRNA. Forty-eight hours after transfection, the expressions of c-Jun and NLRR2 were measured by quantitative real time PCR (c). c-Jun knock-down cells treated with RA showed a reduced expression of NLRR2 (d).

Figure 4. *NLRR2* transcription is enhanced by RA through the recruitment of c-Jun onto the promoter of *NLRR2* while JNK inhibition suppresses the promoter activity. **(**a) Luciferase reporter constructs −790, +110; −560, +110; −315, +110; −147, +110 and −47,

+110 (left panel) with renilla luciferase vector were introduced to HeLa cells and the promoter activity was measured by dual luciferase assay. RA treatment enhanced the *NLRR2* promoter activity (−790, +110) (right panel). (b) RA treatment enhanced *NLRR2* promoter activity in SK-N-BE cells. SK-N-BE cells were transfected with *NLRR2* core luciferase reporter constructs (−790, +110) with renilla luciferase vector followed by RA treatment for 36 hours and promoter activity was measured. (c) RA-mediated c-Jun recruitment to the *NLRR2* promoter region containing AP-1 consensus sequence was confirmed by ChIP assays.

Figure 5. The JNK pathway is important for regulating NLRR2 expression. (a) JNK inhibition reduced *NLRR2* promoter activity in SK-N-BE cells. SK-N-BE cells were transfected with the (−790, +110) promoter construct and a Renilla luciferase vector. At 24 hours after the transfection, cells were treated with SP600125 (50 μM) for 24 hours and luciferase assay was performed. (b, c) TGW and SK-N-BE cells were treated with SP600125 for 24 hours and the expression of c-Jun and NLRR2 were analyzed by western blotting and RT-PCR analyses. JNK inhibition by SP600125 suppressed the expression of NLRR2 and c-Jun at mRNA (b) and protein levels (c) in TGW and SK-N-BE cells. (d, e) JNK activation by sorbitol treatment (300 μM) in SK-N-BE cells induced NLRR2 expression which was blocked by SP600125 pretreatment. Total JNK, p-JNK, total c-Jun and p-c-Jun were examined by western blot analysis (d) and *NLRR2* mRNA expression was determined by RT-PCR (e).

Figure 6. *NLRR2* expression is induced by stress signals and contributes to drug resistance in neuroblastoma cells. (a) TGW, SK-N-BE and SMS-SAN cells were treated

with TM for the indicated time period. The expressions of NLRR2 mRNA were measured by quantitative real time PCR. TM induced the expression of *NLRR2*. (b) SK-N-BE, SMS-SAN cells were treated with CDDP for the indicated time period. The expressions of *NLRR2* mRNA were measured by quantitative real time PCR. *NLRR2* expression was induced by CDDP. (c d) SK-N-BE mock and *NLRR2* bulk stable cells were treated with RA and TM. Cell survival was analyzed using a real-time cell imaging system. *NLRR2* bulk stable cells showed significant resistance to RA (c) and TM-mediated (d) cell growth inhibition.

Supplementary Information

Supplemental figure 1. siNLRR2 (50 nM) and si-c-Jun (200 nM) reduced the expression of NLRR2 and c-Jun in SK-N-BE cells, respectively. SK-N-BE cells were transfected with siRNA according to the manufacturer's protocol. Expression of *NLRR2* and *c-Jun* was detected by real time PCR and RT-PCR, respectively

Supplemental figure 2. Retinoic acid (RA) induced TGW cell differentiation in a dose dependent manner. TGW cells were treated with RA (0, 0.5, 1.0, 2.5, 2.5 or 5.0 μM) for 24 hours and the percentage of differentiated cells was counted (left and right panel).

Supplemental figure 3. RA induced *NLRR2* and *c-Jun* expression in HeLa cells. HeLa cells were stimulated with RA for the indicated time period. *NLRR2* levels were determined by quantitative RT-PCR.

Supplemental figure 4. RA-mediated upregulation of *NLRR2* was suppressed by JNK inhibition. TGW cells were stimulated with RA in the presence or absence of SP600125 for 24 hours. *NLRR2* levels were determined by quantitative RT-PCR.

Supplemental figure 5. Exogenous expression of NLRR2 inhibited RA-induced differentiation of NB cells. SK-N-BE mock and *NLRR2* bulk stable cells were treated with RA for 4 days. Cell images were taken from a real-time cell imaging system. The percentage of differentiated cells per field was counted (3 fields per well, 5 wells in each group). Data are presented as the mean ± SD.

Supplemental figure 6. c-Fos and ATF-2 expression was suppressed by RA in NB cells. NB cells were treated with RA (1.0 μM for TGW cells, 2.5 μM for SK-N-BE and SMS-SAN cells) for the indicated time periods. c-Fos and ATF-2 expressions were determined by western blotting.

Figures

Figure 1. NLRR2 is correlated with poor survival outcome in human NB and regulates tumor cell growth *in vitro* and *in vivo*.

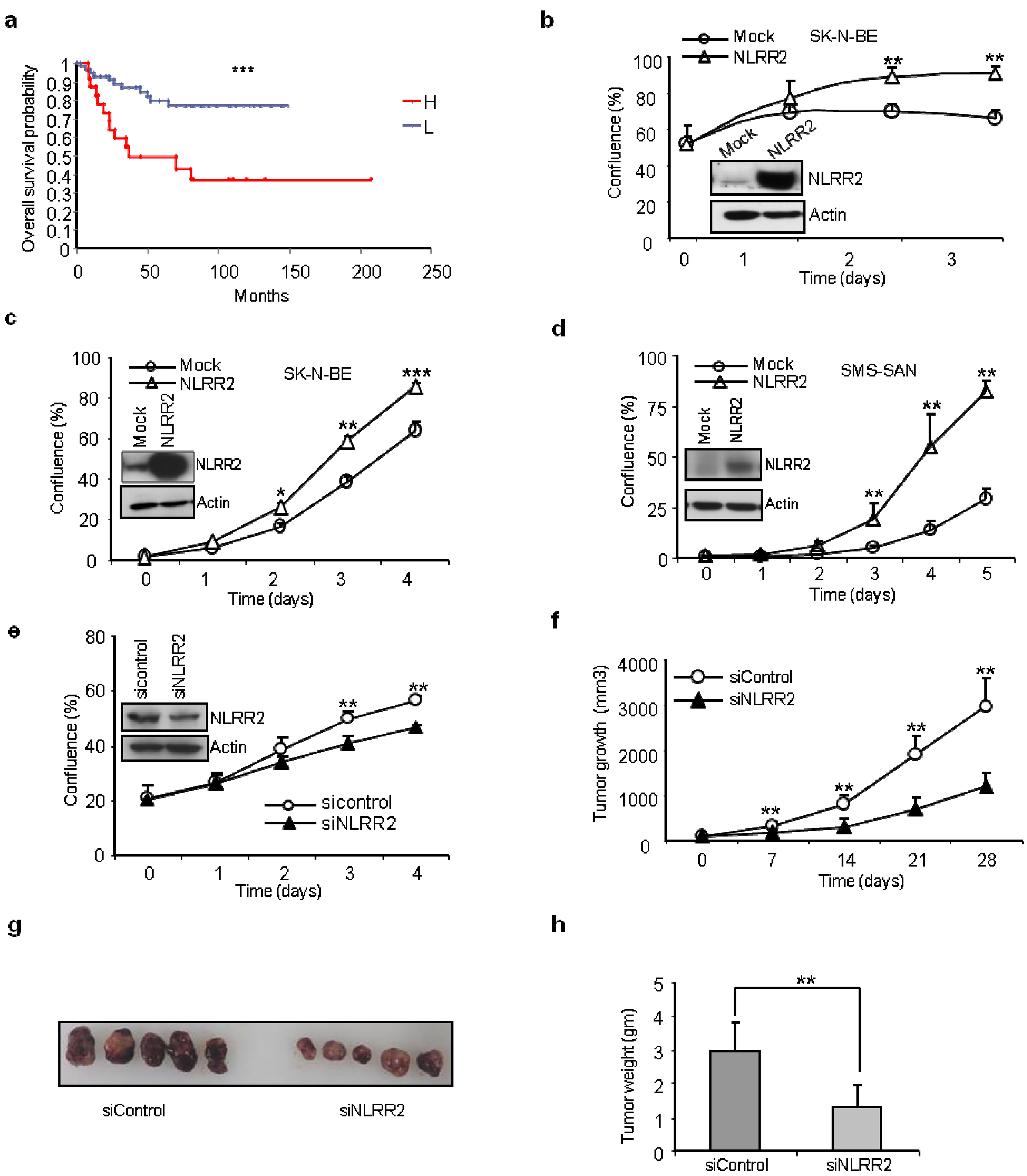


Figure 2. NLRR2 knock down cells are susceptible to RA-mediated inhibition of cell survival and differentiation.

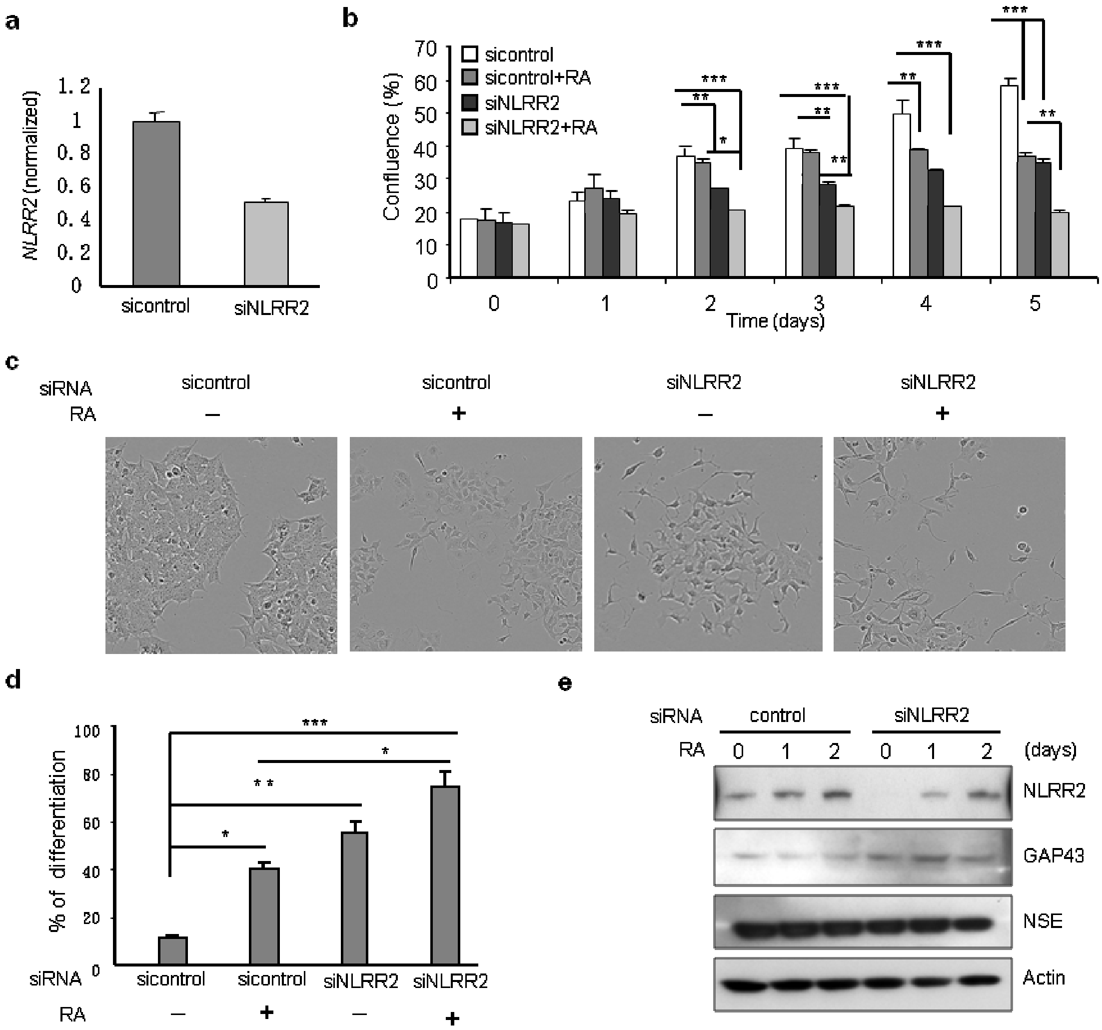


Figure 3. c-Jun is important for regulating *NLRR2* expression induced by RA.

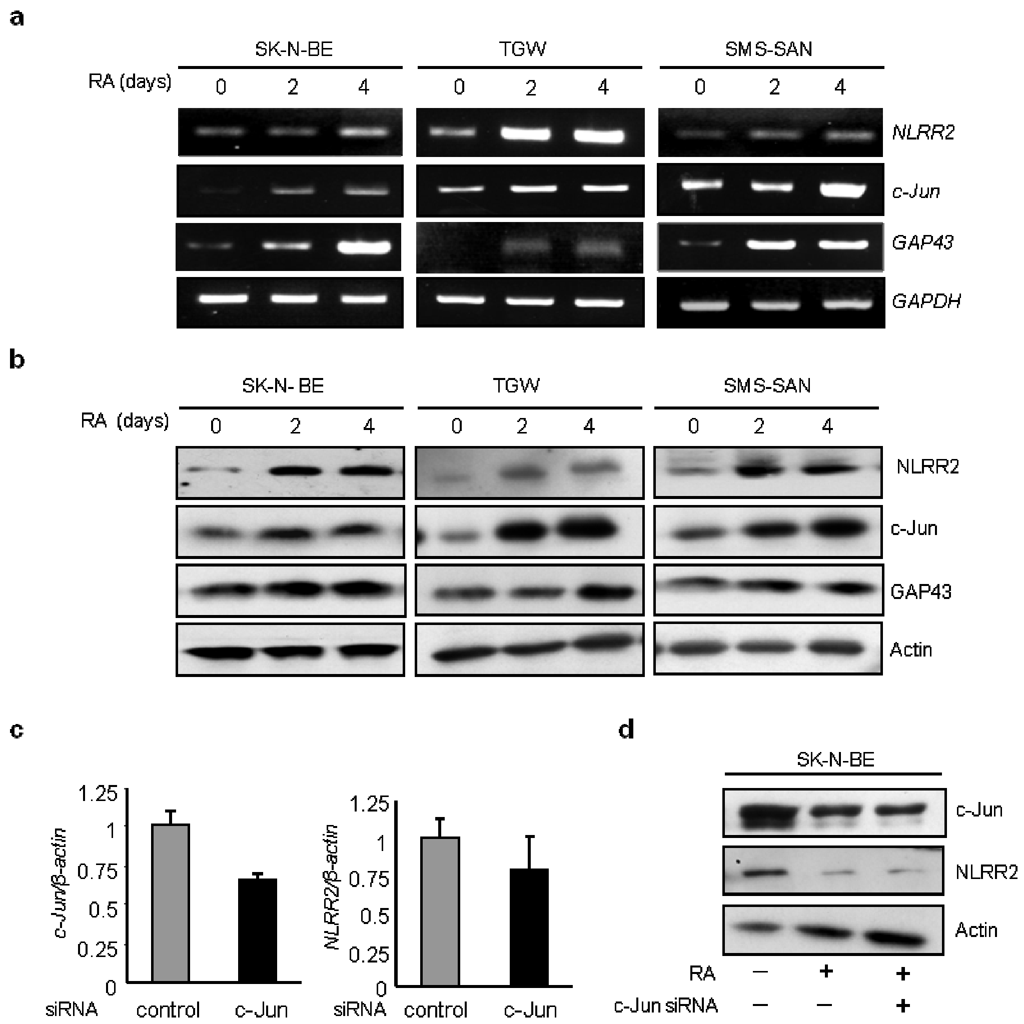


Figure 4. *NLRR2* transcription is enhanced by RA through the recruitment of c-Jun onto the promoter of *NLRR2* while JNK inhibition suppresses the promoter activity.

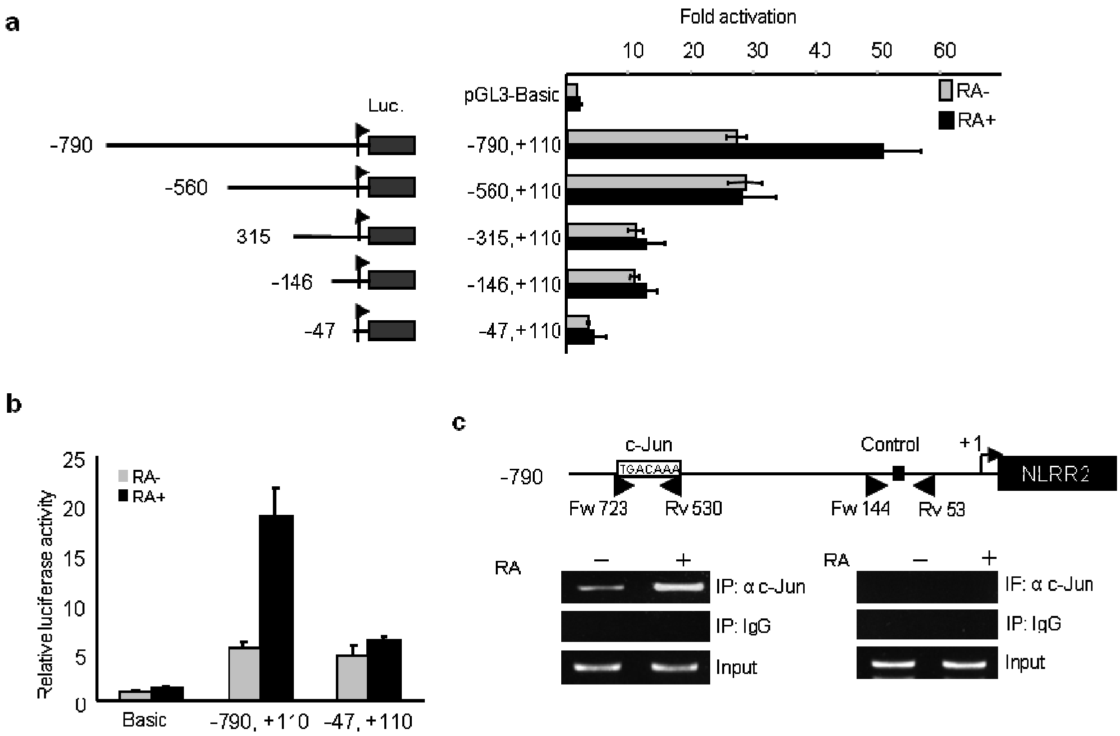


Figure 5. The JNK pathway is important for regulating NLRR2 expression.

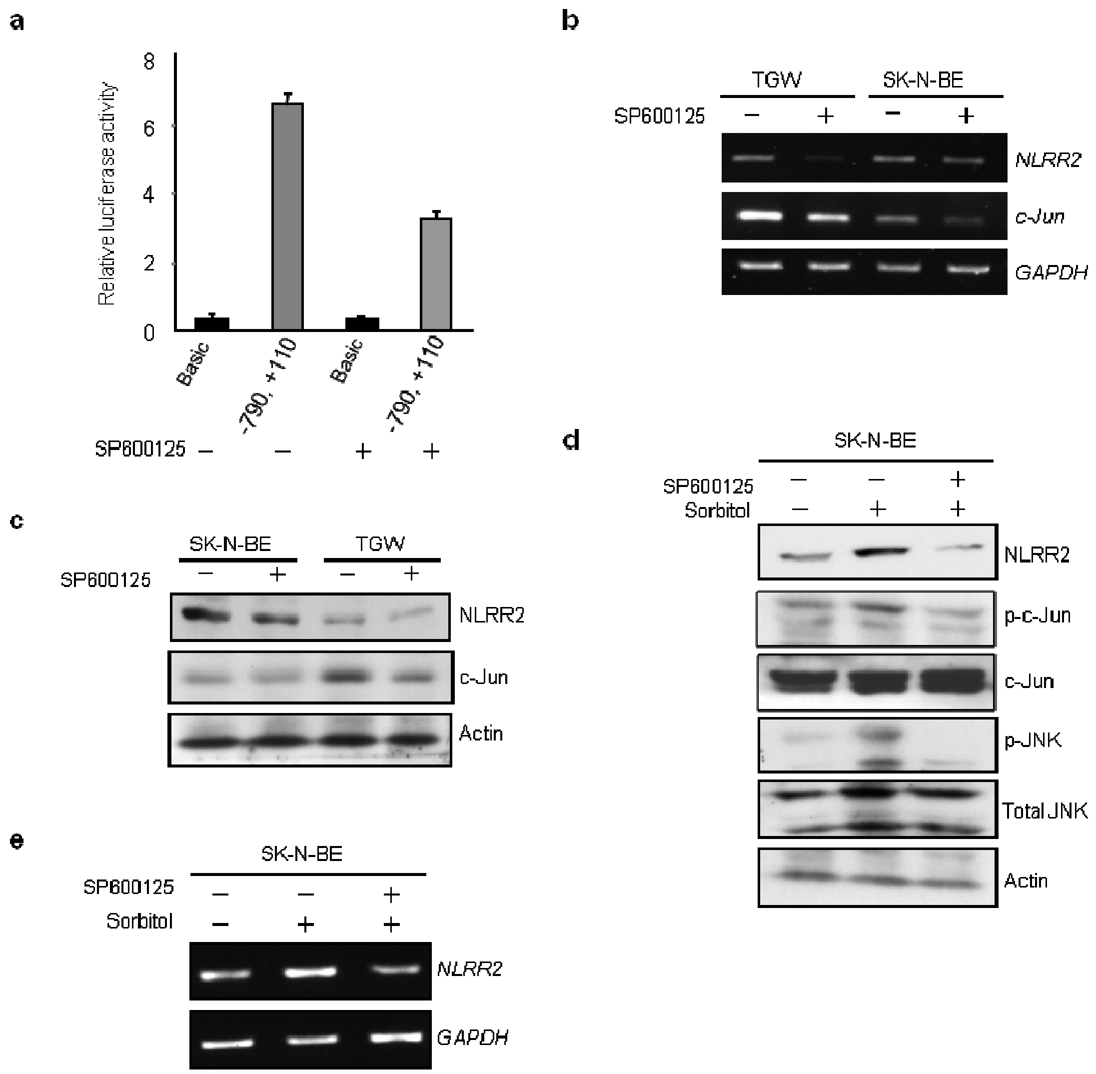
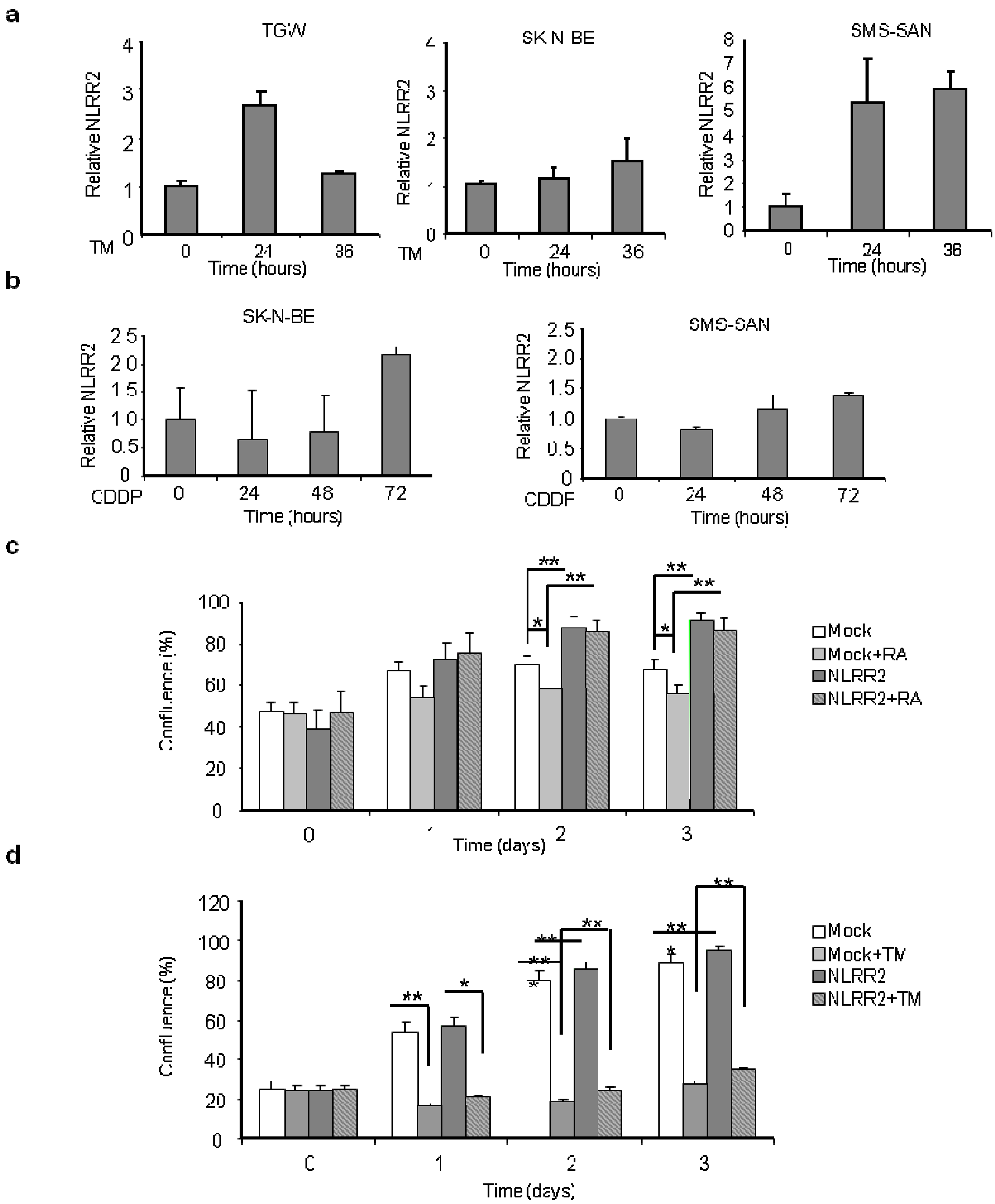
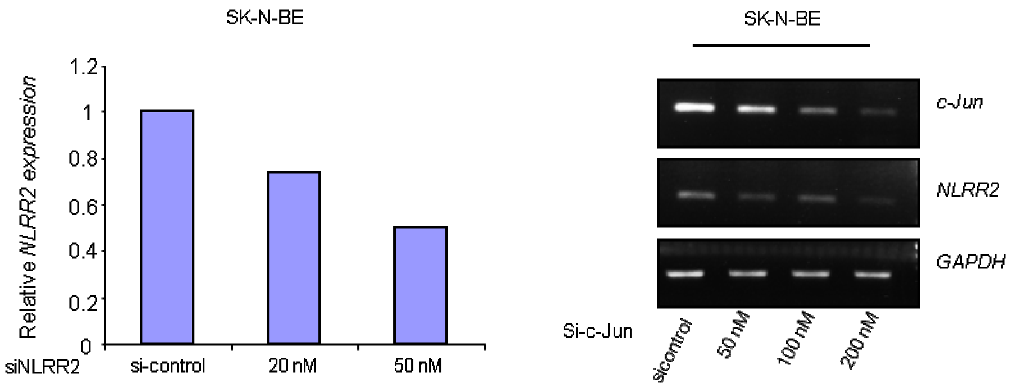


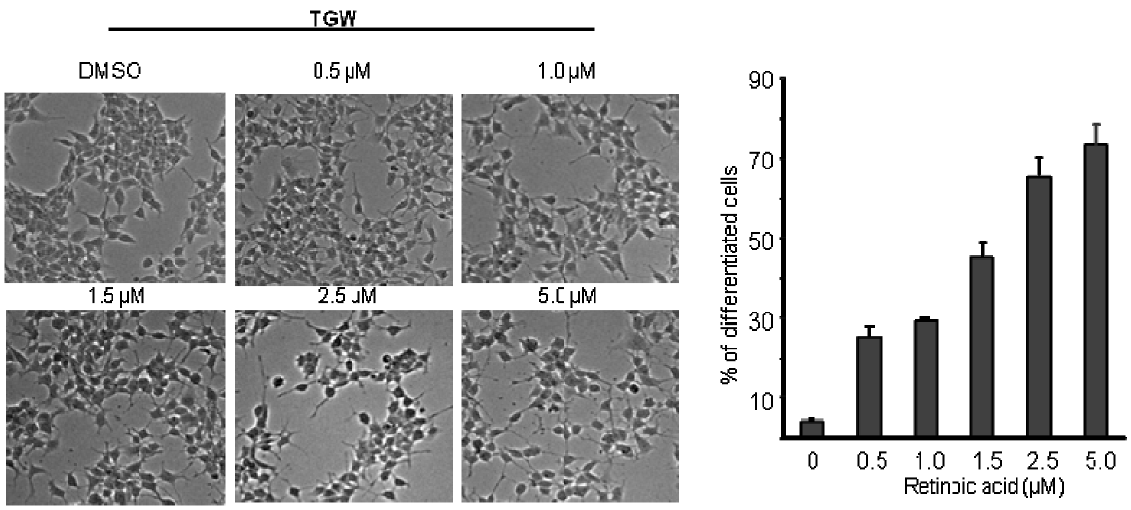
Figure 6. *NLRR2* expression is induced by stress signals and contributes to drug resistance in neuroblastoma cells.



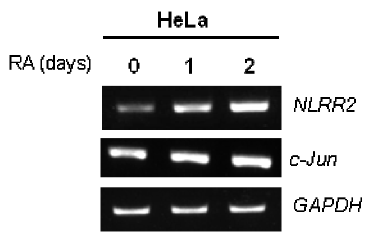
Supplemental figure 1. siNLRR2 (50 nM) and si-c-Jun (200 nM) reduced the expression of NLRR2 and c-Jun in SK-N-BE cells, respectively.



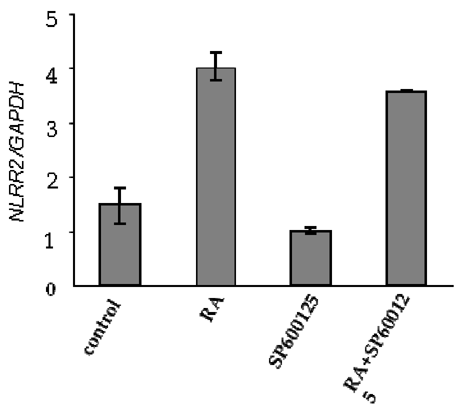
Supplemental figure S2. Retinoic acid (RA) induced TGW cell differentiation in a dose dependent manner.



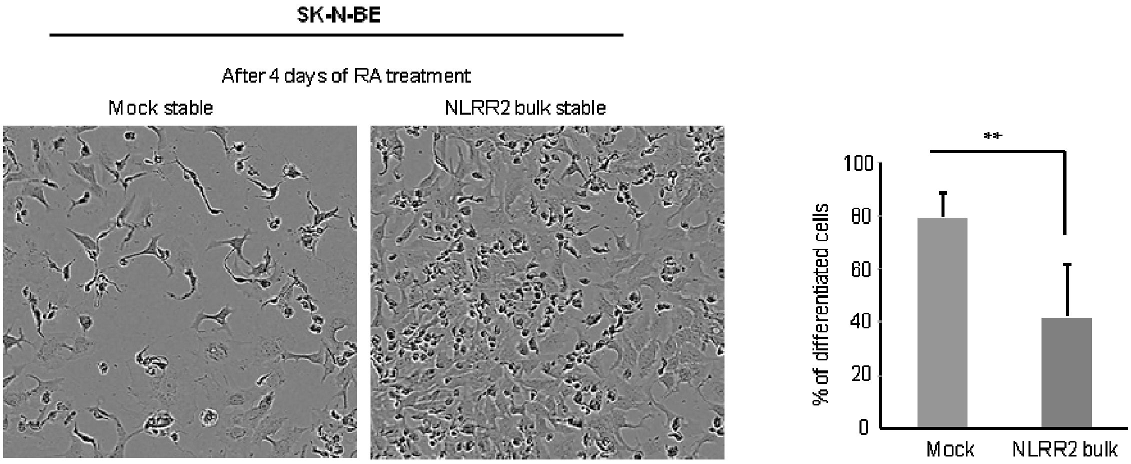
Supplemental figure S3. RA induced *NLRR2* and *c-Jun* expression in HeLa cells.



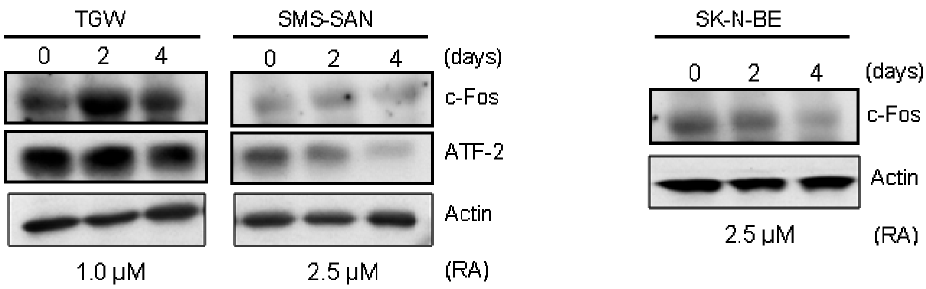
Supplemental figure S4. RA-mediated upregulation of *NLRR2* was suppressed by JNK inhibition.



Supplemental figure S5. Exogenous expression of NLRR2 inhibited RA-induced differentiation of NB cells.



Supplemental figure S6. c-Fos and ATF-2 expression was suppressed by RA in NB cells.



Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 19890276 to A.T., Ministry of Education, Culture, Sports, Science and Technology KAKENHI Grant Number 22791016, 25830092 to A.T. The authors would like to thank, Drs. Y. Nakamura and N. Koshikawa for their excellent advice and to Drs. T. Ozaki, M. Ohira, Y. Suenaga, J. Akter, and R. Islam, (Chiba Cancer Center Research Institute) for their fruitful scientific discussions.