DOWNREGULATION OF MYCN ONCOGENE BY RETINOIC ACID IN NEUROBLASTOMA

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ABSTRACT

Neuroblastoma is the most common extracranial solid tumor for children. Up to 30% of neuroblastoma tumors show the gene amplification of MYCN oncogene. Because MYCN plays a critical role, MYCN gene amplification is considered an important risk factor of neuroblastoma. Retinoic acid (RA) is one of the few agents which can improve the survival rate of patients in the high risk group. RA induces the growth arrest and cell differentiation of neuroblastoma cells. RA represses MYCN expression in neuroblastoma and it is the first molecular event which occurs prior to the cell differentiation. Therefore, it is believed that the downregulation of MYCN is the key step for cell differentiation of neuroblastoma by RA and its clinical benefit. However, the molecular mechanism of how RA downregulates MYCN is still unknown. A recent study revealed that the binding site of E2F transcription factor within the MYCN promoter is the key element for the repression of MYCN by RA in neuroblastoma.

In this study, we figured out that MYCN downregulation starts before or around 6 hours after RA treatment, and the bindings of E2F proteins and Rb family proteins onto the MYCN promoter did not change during RA treatment. However, reporter assays with RNA interference revealed that Rb is essential for the repression of MYCN by RA, even though its binding onto the MYCN promoter does not change. Our findings indicate that Rb is involved in MYCN repression, acting as the connector for the real repressor, and RA treatment activates the real repressor at the MYCN promoter.

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LIST OF ABBREVIATIONS

ALK	**anaplastic lymphoma kinase**
APL	**acute promyelocyte leukemia**
BDNF	**brain-derived neurotrophic factor**
BRG1	**human brahma related gene**
CCG	**Children Cancer Group**
CDK	**cyclin-dependent kinase**
ChIP	**chromatin immunoprecipitation**
COG	**Children's Oncology Group**
DP	**dimerization partner proteins**
DM	**double minute**
EFS	**event-free survival**
FCS	**fetal calf serum**
HAT	**histone acetyltransferase**
HDAC	**histone deacetylase**
HMT	**histone methyltransferase**
HSR	**homogenous staining region**
INRG	**International Neuroblastoma Risk Group**
INRGSS	**International neuroblastoma risk group staging system**
INSS	**International Neuroblastoma Staging System**
IP	**immunoprecipitation**
LOH	**loss of heterozygosity**
MKI	**mitosis-karyorrhexis index**
MRD	**minimal residual disease**
NB	**neuroblastoma**

POG	**Pediatric Oncology Group**
RA	**retinoic acid**
RAR	**retinoic acid receptor**
RARE	**retinoic acid response elements**
RARR	**retinoic acid responsive region**
ROS	**reactive oxygen species**
RXR	**retinoid X receptor**
TSA	**tricostatin A**
VDR	**vitamin D receptor**

SPECIFIC AIMS AND HYPOTHESES

Specific Aim 1: Investigate the bindings of E2F proteins onto the binding site within the MYCN promoter in neuroblastoma during RA treatment.

Hypothesis: The bindings of E2F proteins onto the MYCN promoter change when neuroblastoma cell is treated with RA and this event leads to the downregulation of MYCN oncogene.

Specific Aim 2: Investigate the interaction between Rb tumor suppressor proteins and the complex of E2F proteins and DNA sequence within the MYCN promoter in neuroblastoma during RA treatment.

Hypothesis: The binding of Rb tumor suppressor proteins to the complex of E2F proteins and DNA sequence within the MYCN promoter increases in neuroblastoma during RA treatment.

Specific Aim 3: Determine whether Rb is essential key factor for the downregulation of MYCN oncogene and the induction of cell differentiation by RA in neuroblastoma.

Hypothesis: Tumor suppressor gene, Rb is the essential key molecule which is involved in the transcriptional repression of MYCN oncogene and cell differentiation in neuroblastoma during RA treatment.

CHAPTER 1. BACKGROUND

1.1 Neuroblastoma

1.1.1 Epidemiology

Neuroblastoma (NB) is the second most common solid tumor in children following brain tumor and the most common cancer in infants, accounting for 8-10% of all childhood cancers [1]. In the United States, approximately 800 cases of neuroblastoma are diagnosed each year, which corresponds to roughly 1 case per 7000 live births [2, 3]. The prevalence of neuroblastoma is slightly higher in boys than girls and male-to-female sex ratio is 1.1 to 1. In the cooperative group studies at POG (Pediatric Oncology Group) and CCG (Children Cancer Group) institutions from 1986 to 2001, approximately 90% of neuroblastoma patients were younger than 5 years old and 98% were younger than 10 years old [1].

1.1.2 Clinical presentation

The anatomic distribution of neuroblastoma primary tumors is well-established in the neuroblastoma research community, and tumors can arise anywhere along the sympathetic nerve system is located. The most common location of primary tumors is the abdomen, especially from the adrenal medulla. Primary tumors can also be found in the chest, pelvis, cervical region, and other regions [4, 5]. Neuroblastoma can metastasize to lymph nodes, bone marrow, cortical bone, liver, skin, and other parenchymal organs by hematogenous and

lymphatic pathways [4, 6-8]. The tumor also can spread to the lungs and the central nervous system, but it is rare and more commonly found in the recurrent disease or the end-stage disease [9].

There is one distinct stage, 4S (S=special) which is about 5% of all neuroblastoma cases. Infants in this stage have small primary tumors with metastases in liver, skin, or bone marrow. However, these tumors almost always regress spontaneously [5].

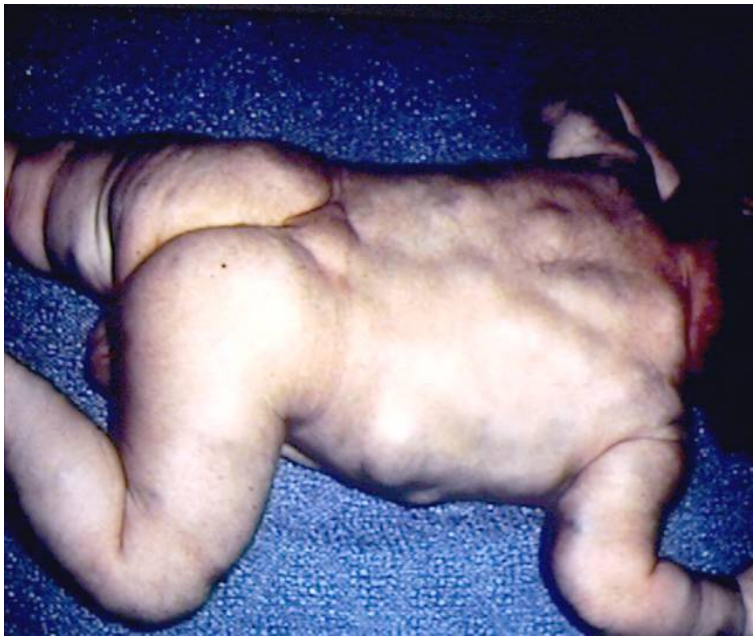


Figure 1. Neuroblastoma patient with 4S disease.
Picture provided by Dr. Wada.

1.1.3 Pathology

Neuroblastoma is thought to originate in the pluripotent nerve cell of the neural crest and can arise from anywhere the sympathetic nerve is found [10]. But the etiology of neuroblastoma is unknown. However, many genetic aberrations which might associate with neuroblastoma were reported.

The most important genetic aberration in neuroblastoma is the gene amplification of MYCN oncogene. MYCN is located on the distal short arm of chromosome 2 (2p24), but some neuroblastoma tumors exhibit extra copies of MYCN which reside extra-chromosomally within double minutes (DMs) or intra-chromosomally within the homogenous staining region (HSRs) [10]. The gene amplification of MYCN in DMs are more common in primary neuroblastoma tumors and in HSRs are more common in neuroblastoma cell lines [1].

Copy number of amplified-MYCN gene in tumor cells varies and can be more than 500-fold, and most cooperative groups define genomic amplification as 4 times the normal number of MYCN copies [1, 10].

MYCN rules the biological feature of neuroblastoma and the amplification of MYCN is associated with advanced stages, unfavorable biological features, and a poor outcomes [11-13]. Thus, MYCN is considered the most important risk factor in neuroblastoma patients.

1.1.4 Staging of neuroblastoma

Staging neuroblastoma patients is very important, because disease stage is correlated with patient outcome and, therefore appropriate therapy needs to be stratified. Consequently staging systems for neuroblastoma patients have been developed in the United States [14], Japan [15], and other countries since Evans et al. introduced the first neuroblastoma-specific staging system in 1971 [16]. However, there were differences between those staging systems, especially for intermediate-stages, and their results could not be compared directly each other. Therefore, representatives from major cooperative groups and countries met in 1987 and in 1991 to establish the International Neuroblastoma Staging System (INSS) [17, 18]. In this system, a localized tumor which is surgically resectable is categorized as stage 1. Localized tumors with gross residual disease and localized tumors with ipsilateral lymph node involvement are classified into Stage 2A and 2B, respectively. A tumor with continuous infiltration across the vertebral column is stage 3. Any primary tumor with invasion upon distant lymph nodes, liver, bone marrow, skin and/or other organs except as defined for stage 4S. Stage 4S is a localized primary tumor with limited invasion only upon bone marrow, skin or liver in children under 1 year of age. Stage 4S is a distinct stage, distinguished from stage 4, because of higher event-free survival, and unique, favorable pattern, such as spontaneous regression [1, 5].

Although currently INSS is utilized by all major cooperative groups and countries, new staging system, International neuroblastoma risk group staging system (INRGSS) [19] is likely replacing the INSS. It is because staging in INSS

can vary depending on the skill and aggressiveness of the surgeon, therefore, alternative presurgical staging system, INRGSS which is based on clinical assessment and biological characteristic of neuroblastoma tumor was developed in order to make therapeutic decisions [5].

Stage	Definition
Stage 1	Localized tumor with complete gross excision, with or without microscopicresidual disease; reresentative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with the primary tumor may be positive).
Stage 2A	Localized tumor with incomplete gross excision; representative ipsilateral lymph nodes negative for tumor microscopically.
Stage 2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically.
Stage 3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement.
Stage 4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined by 4S).
Stage 4S	Localized primary tumor (as defined for stage stage 1, 2A or 2B), with dissemination limited to skin, liver and /or bone marrow (limited to infants < 1 year of age).

Table 1. International Neuroblastoma Staging System.
Table adapted from Brodeur et al. 1993.

1.1.5 Risk groups of neuroblastoma

As just previously mentioned, in order to determine patient therapy, it may be beneficial to consider the biological characteristics of neuroblastoma tumors besides disease stage, because staging system is just for classification of the disease extent at the time of diagnosis and cannot predict disease progression accurately. Therefore, in order to classify patients into each risk group, risk stratification systems have been developed considering several risk factors, such as age [20], DNA ploidy [21-23], MYCN amplification [12, 13], histological feature [24-27], disease stage [17, 18], and allelic aberration [28-31]. Also, other markers have been proposed, such as serum ferritin, NSK, LDH, and circulating GD2 [32-35]. For example, COG (Children's Oncology Group) has three categories of risk group (low, intermediate, and high [36].

MYCN gene amplification status is the most important risk factor which places the patients, such as infants in stage 4, into high-risk group [12, 13, 37]. The MYCN expression affects the biological feature of neuroblastoma tumors, such as aggressive malignant phenotype, the relapse of disease, and tumors in multiple sites [12, 13]. This genomic aberration is highly correlated with other risk factors, such as advanced age and unfavorable histology, therefore, poor outcomes [12, 13].

Age at diagnosis is another important risk factor which correlates to patient outcomes [20]. In general, younger age is favorable and correlates to good outcomes and 1 year is considered a cutoff point in many groups, such as COG,

but in International Neuroblastoma Risk Group (INRG) consensus pretreatment classification schema, 18 months is considered the border-line that determines the outcome of patients [38].

“The Shimada index” which is a histopathologic classification system for neuroblastoma, is used to group patients into a favorable group and unfavorable group focusing on the amount of Schwannian cell stroma, the degree of cell-differentiation, and mitosis-karyorrhexis index (MKI) of the neuroblastic cells [24, 39]. This Shimada system was developed by Joshi and is widely used for neuroblastoma patients [27].

Favorable: (1) stroma rich, all ages, no nodular pattern, (2) Stroma poor, age 1.5-5 years old, differentiated, MKI < 100, (3) Stroma poor, age < 1.5, MKI < 200.

Unfavorable: (1) stroma rich, all ages, nodular pattern, (2) stroma poor, age > 5 years old, (3) stroma poor, age 1.5-5 years old, undifferentiated, (4) stroma poor, age < 1.5 years old, MKI > 200.

INSS stage	Age (years)	MYCN status	Other
Low-risk neuroblastoma			
1	0-21	Any	
2	<1	Any	
	1-21	=	
	1-21	↑	Shimada histology favorable
4S	<1	=	Shimada histology favorable, DNA index hyperdiploid
I=ntermediate-risk neuroblastoma			
3	<1	=	
	1-21	=	Shimada histology favorable
4	<1	=	
4S	<1	=	Shimada histology unfavorable or DNA index diploid
High-risk neuroblastoma			
2	1-21	↑	Shimada histology unfavorable
3	Any	↑	
	1-21	=	Shimada histology unfavorable
4	<1	↑	
	1-21	Any	
4S	<1	↑	

Table 2. Children's Oncology Group risk stratification system.
Arrows indicate the *MYCN* gene amplification, equal signs indicate nonamplified *MYCN*, "Any" indicates any *MYCN* status. Table adapted from Cheung and Cohn, 2005.

1.1.6 DNA ploidy

Determination of tumor cell DNA ploidy has prognostic value to stratify therapy for neuroblastoma patients under 18 months old with stage 4, and 4S disease, but not in older patients [21, 22, 40]. Hyperdiploidy is a form of genetic instability which is frequently observed in neuroblastomas and is associated with favorable outcomes. On the other hand, near-diploid and near tetraploid which are detected in patients older than 12 months, are related to structural abnormalities with allelic loss of chromosome 1p and amplification of MYCN gene and related to aggressive tumors and poor outcome [10]. Usually hyperdiploid and near triploid are detected in patients younger than 12 months or with low risk tumors with few or no structural chromosome abnormalities [10]. The mechanisms of these DNA aneuploidies in neuroblastomas are still unclear.

1.1.7 Other DNA aberrations

Besides MYCN amplification, there are other DNA aberrations which are often found in neuroblastoma and correlate with neuroblastoma characteristics.

Extra copies of 17q are found in neuroblastoma and they associate with aggressive tumors and poor outcomes. Studies suggest that unbalanced gain of 17q may occur in more than 50% of all neuroblastomas [41, 42]. The genes which are responsible for aggressive features are unknown yet, but BIRC5 (survivin, apoptosis inhibitor), PPM1D, and NME1 (NM23) are possible targets of the gain of 17q [43-45].

Allelic loss at 1p is found is 30-35% tumors. This loss correlates with MYCN amplification and advanced stage [46]. Even though it is still controversial whether the 1p LOH (loss of heterozygosity) is an independent indicator of prognosis, several studies suggest that allelic loss at 1p36 can be predictor for the increased risk of relapse of localize tumors and tumors without MYCN amplification [1, 28, 29, 47]. Deletions of tumor suppressor genes which are involved in the 1p36 deletion, such as CHD5, KIF1BB, and miR-34a might be the key molecular events [48-52].

Allelic loss of 11q is found in 35-45% of primary neuroblastoma tumors [30, 53]. This aberration associates with high-risk features which contribute to bad outcomes [31]. Therefore, it is a candidate of prognostic marker and recent data suggest that it predicts disease relapse [31, 54]. Even though it is not certain, one tumor suppressor gene, CADM1 at 11q23 is one of the candidates that are involved in 11q deletion [55].

The missense or nonsense mutations in homeobox gene PHOX2B are often found in children with sporadic or familial neuroblastoma and congenital central hypoventilation syndrome and/or Hirschsprung's disease [56, 57].

The gain of 17q, ALK (anaplastic lymphoma kinase) has been attracting the attention of many researchers in the field. In 2008, Chen et al. reported that the mutations or amplification of ALK was found in 18 (8.4%) out of 215 fresh tumors from neuroblastoma patients [58]. Also, in 2012, Schonferr et al. reported that 49 cases of ALK mutation out of 709 neuroblastoma cases (6.9%) were found and

observed in 8.9% of MYCN amplified tumors [59]. Schonferr et al. also reported gain-of function mutants in ALK stimulate the MYCN exspression in neuronal and neuroblastoma cell lines and, furthermore, ALK inhibitors abrogate the MYCN expression [59]. Also, activating mutations in the tyrosine kinase domain are found in most cases of hereditary neuroblastoma. These mutations in the germ-line are caused by single-base substitutions in the key regions of the kinase domain, and result in constitutive activation of the kinase and a premalignant state.

Another observation which currently is getting examined is CD133 (prominin-1). CD133 is a transmembrane glycoprotein which is expressed on the surface of stem cells and cancer stem cells [60, 61]. Takenobu et al. reported that several neuroblastoma cell lines and neuroblastoma tumor samples express CD133. They also reported that CD133 accelerates cell proliferation and represses cell differentiation of neuroblastoma. Furthermore, they found out that CD133 suppresses the transcription of RET tyrosine kinase, and overexpression of RET rescues cell differentiation of neuroblastoma from inhibition by expression of CD133 [62].

1.1.8 Treatment of neuroblastoma

The traditional treatment modalities for neuroblastoma are, (1) surgery, (2) chemotherapy, (3) radiotherapy. Immunotherapy has been established as a new therapy for advanced disease.

For low-risk neuroblastoma, surgical removal of the primary tumor is considered the main treatment option [63-65]. For stage 1 neuroblastoma, surgery alone is effective as initial therapy. And the second surgery can manage the local recurrences, and even metastatic recurrences can be managed with chemotherapy [66]. Even for stage 2 of the disease, it can be managed with only surgery. However, historically, many of stage 2 patients also receive chemotherapy, and their survival results were very high [67]. But it is still controversial whether chemotherapy/radiation therapy should be recommended, because tumors can regress spontaneously [1].

Treatment for intermediate-risk neuroblastoma varies, because this is a heterogeneous group consisting mainly of very young patients with metastatic disease, or patients, regardless of age, with unresectable primary tumors. Age and the MYCN amplification are important factors for strategies. But, most patients are treated with moderate dose-intensive or intensive chemotherapy including cyclophosphamide, doxorubicin, cisplatin, and etoposide, and radiation [1]. Schmidt et al. reviewed that the CCG reported that while 3-years EFS (event-free survival) rate for infants with MYCN single-copy neuroblastomas treated with moderately intensive chemotherapy was 93%, for infants with MYCN-amplified tumors treated with much more intensive therapy was 10% [37].

The therapies for high-risk neuroblastoma have three phases, (1) intensive induction chemotherapy, (2) myeloablative consolidation therapy with stem cell rescue, and (3) targeted therapy for minimal residual disease maintenance. Survival rate of high-risk neuroblastoma has been improving (about 30%, 1986-

1995), but further research should be conducted [1]. Intensive induction chemotherapy is to induce maximum reduction of tumor at primary and metastatic sites. In this therapy, patients are treated with several cycles with drugs such as cisplatin, cyclophosphamide, doxorubicin, teniposide, etoposide, pirarubicin, and vincristine. Even now, the doses of those drugs, length of treatment period, number of cycles to be repeated, the combinations of these drugs are tested for better therapy [68-72]. The consolidation therapy comes following the intensive induction chemotherapy. The goal of this therapy is to eliminate clones of tumor which are resistant and survive induction chemotherapy. This therapy consists of myeloablative chemotherapy and autologous bone marrow infusion. Melphalan, cisplatin, teniposide, doxorubicin, etoposide, carboplatin, and radiation are used for myeloablative therapy [73-77]. For stem cell rescue, even though autologous bone marrow transplant works better, allogeneic transplant also works and it may be investigated more [78-80].

Minimal residual disease (MRD) maintenance therapy is used to eradicate any residual tumor cells with agents which are theoretically active against highly chemoresistant MRD after consolidation therapy. In fact, in spite of the improvements in induction chemotherapy and consolidation therapy, lots of high-risk neuroblastoma patients experience disease relapse, even though no disease is detectable after consolidation therapy [1]. Therefore, it is assumed that microscopic residual disease is often present even after consolidation therapy and relapsed diseases are highly chemoresistant [81]. For this purpose, several agents are used to eliminate MRD which is chemoresistant. 13-cis retinoic acid is

one of those agents which can increase the survival rate of high risk neuroblastoma patient [5, 73]. Retinoic acid induces the growth arrest and neuronal differentiation of neuroblastoma tumor cell [82, 83]. In randomized trial, the cohort of patients who received 13-cis RA had a significantly improved EFS probability (46% vs. 29%) [73]. In addition to 13-cis retinoic acid, the human-mouse chimeric monoclonal antibody, ch14.18 is used [84]. This antibody is specific to the cell surface ganglioside GD2 and shows the effect even in refractory neuroblastoma patients [85, 86]. As immunotherapy agents, GM-CSF and IL-2 are used besides ch14.18 and 13-cis RA [1, 87].

1.1.9 Current issue of clinical therapy of neuroblastoma

A large number of high-risk neuroblastoma patients experience disease relapse, despite of therapeutic improvements. Recurrent or refractory diseases are chemoresistant [81] and currently there are no known curative salvage regiments for them. Therefore, more understanding of molecular basis of high-risk neuroblastoma and active agents are needed. And several highly active agents, such as new chemotherapeutic agents, target-delivered radionuclides, new retinoids, kinase inhibitors, antiangiogenesis agents, and immunotherapy agents have been identified and tested in clinical trials [5].

1.2 MYCN oncogene

1.2.1 MYCN and the Myc family

As mentioned above, MYCN is the most important factor that determines the biological features of neuroblastoma tumors and correlates with disease aggressiveness.

MYCN is a member of the Myc family. Myc genes, which include b-Myc, c-Myc, N-myc, L-myc and s-Myc, encode transcription factors that play a key role in cell proliferation, differentiation, and death through gene-specific transcriptional activation or repression. Myc genes contain several domains such as Myc Box 1, Myc Box 2 and basic-helix-loop-helix-leucine zipper (bHLHZip) motif which work for DNA binding and protein-protein interaction [88-91].

Researchers revealed that MYCN functions are very similar to c-Myc. Also the fact that knock-in mice in which c-Myc gene was replaced with MYCN are viable and appear normal, suggests that MYCN can replace c-Myc functionally [92]. However, unlike c-Myc which is expressed extensively, gene expression of MYCN is restricted. MYCN is expressed in a transient and organ-specific fashion during fetal and post fetal development and normally they are not detectable in mature tissue, except in B-cell in early stages [92, 93]. In the brain, MYCN is expressed in neuroblasts up to the onset of differentiation [94]. MYCN is also expressed at high levels in neuroblasts migrating from the neural crest through the adrenal cortex, while neuroblasts forming the adrenal medulla express low levels of MYCN mRNA. This implies that migrating cells receive signal upon

entering the adrenal cortex that downregulates MYCN transcription and allows the cells to begin the process of differentiation [95].

1.2.2 MYCN and neuroblastoma

Again, in 20-40% of neuroblastoma and neuroblastoma cell lines, the amplification and/or overexpression of MYCN oncogene is observed and its ampilification can be more than 300-fold or even 500-fold [92]. In neuroblastoma, MYCN abnormalities are present as (1) extrachromosomal double minutes (DMs), (2) homogeneously staining regions (HSR), and (3) partial monosomy for short arm of chromosome 1 and 17 [96, 97].

Even though it is well-known that MYCN expression in neuroblastoma correlates with its characteristics, malignancy and prognosis [12], surprisingly it is still unclear how this molecule functions. Nara et al. reported that the knockdown of MYCN by RNA interference (RNAi) induced the growth arrest, cell differentiation, and apoptotic activity in MYCN-amplified-neuroblastoma cell line and these results suggested that MYCN may be a key factor for tumorgenicity of neuroblastoma [98]. In fact, Weiss et al. reported that the MYCN overexpression causes neuroblastoma in transgenic mice [99]. Also, Goodman et al. reported that MYCN associates with tumor invasiveness [100], and Tanaka et al. found out that MYCN promotes the invasion of neuroblastoma cells by downregulating integrin [101]. Also, in 2011, Fletcher et al. revealed that MYCN upregulates

GSTP1 along with ABC transporters and causes multidrug resistance in neuroblastoma [102].

On the other hand, it is still controversial whether the MYCN expression in neuroblastoma is associated with only tumor malignancy. Some studies have reported that moderate MYCN function gain might favor spontaneous regression [103]. Moreover, there are several studies which report that MYCN associates with apoptosis in neuroblastoma cell. These data mention that MYCN expression sensitizes neuroblastoma cells for apoptosis by inducing proteins which lead cell to apoptosis, such as p53, Bak, Bax, and CD95 [104-108].

Other findings about MYCN in neuroblastoma are, the one important signal which induces MYCN expression in neuroblastoma is IGF [92], MYCN shows positive auto-regulation in neuroblastoma by binding its own intron 1 [109], Aurora A stabilizes and protects MYCN protein [110].

1.3 Retinoic acid

1.3.1 Retinoic acid

There are several treatments for neuroblastoma patients, such as surgery, chemotherapy, radiotherapy, and immunotherapy. Retinoic acid (RA) is one of the few agents which are considered the standard care for neuroblastoma patients in the high risk groups [73, 82].

Retinoic acid is one of the retinoids. Retinoid is the collective term of these compounds and it consists of vitamin A, and its derivatives and analogues. Vitamin A is a lipophilic vitamin and consists of vitamin A_1 and vitamin A_2. Vitamin A_1 consists of retinol which has hydroxyl radical (-CH_2OH) on the end of its carbon chain, retinal which has aldehyde radical (-CHO) as well, and retinoic acid which has a carboxy radical (-COOH), respectively. Vitamin A_2 consists of analogues of vitamin A1, 3-dehydro-retinol, 3-dehydroretinal, and 3-dehydroretinoic acid. Vitamin A has multiple physiological functions such as development, reproduction, vision, and immunity [111].

Retinoids are important regulator of cellular proliferation and differentiation in embryonic development, reproduction, morphogenesis, homeostasis, and diseases. Also, it is known that retinoic acid inhibits cell proliferation and/or induces cell differentiation in many cancer cells, such as lymphoma, leukemia, prostate cancer, hepatocellular carcinoma, kidney cancer, lung cancer, and neuroblastoma [112-119].

Within an organism, retinoids function by binding nuclear receptors which are retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Both of RARs and RXRs have subtypes, α, β, and γ, and they belong to the superfamily of the steroid/thyroid hormone nuclear receptors [120-124]. Those receptors work as ligand-induced transcription factors and consist of three functional domains; ligand-binding domain [125-127], dimerization domain, and transactivation domain. The DNA binding domain contains two zinc finger motifs which interact with DNA. RARs bind DNA as heterodimer with RXRs, and RXRs interact with

DNA as homodimers and heterodimers with other members of the family, such as RARs, vitamin D receptors.

RARs and RXRs interact with specific DNA sequences, called retinoic acid response elements (RAREs). RAREs consist of direct repeats of the sequence, A/GGGTCA and are found in promoters of many genes [128]. These genes play important roles for cellular proliferation, differentiation, and apoptosis, such as Myc family, Cyclin D1, TCF-1, PPAR-γ, MMP-7, Axin-2, and CD44, therefore, it is presumed that abnormality of RARs and RXRs correlates with canceration of cell. Abnormality of RARα in 100% of acute promyelocyte leukemia (APL) and functional aberration of RXRα by abnormal phosphorylation by Ras/MAPK/ERK in canceration mechanism of liver cancer, are examples [11].

1.3.2 Retinoic acid and neuroblastoma

In 1982, Sidell et al. found that all-trans-retinoic acid induced the growth inhibition and cell differentiation in a neuroblastoma cell line [129, 130]. Subsequently, between the early 1980's and 1990's, researchers revealed that retinoic acid inhibits the cell growth and induces cell differentiation and/or apoptosis in neuroblastomas [130-136]. However, the mechanisms of RA-induced cell differentiation are not clear yet, despite significant research in this area.

Again, the mechanisms through which RA inhibits neuroblastoma growth and induces cell differentiation/apoptosis are not clear. However, because

neuroblastoma tumors express RARs and RXRs [137], and there is a correlation between expression of these receptors and prognosis [138], it is likely that these receptors are involved in the pathogenesis of neuroblastoma [11], even though the relations between retinoid receptors and canceration in neuroblastoma are not clear.

Researchers have investigated the interactions between retinoic acids and RARs/RXRs, and revealed that all-trans RA binds only RARs, but 9-cis RA binds both RARs and RXRs, and 13-cis RA has low affinities for both RARs and RXRs [136]. Also retinoic acids regulate the expressions of these receptors and other genes in neuroblastoma [136]. All three RAs upregulate RARα, RARβ, RARγ, RXRα, and RXRγ, but not RXRβ, and all-trans RA and 9-cis RA downregulate RXRβ, but 13-cis RA does not [136, 139, 140]. These findings suggest that RAs, RARs, and RXRs are involved in different mechanisms in growth inhibition of neuroblastoma. However, there is much research that reports on the mechanisms/models/pathways which are independent from RARs and RXRs. In 1993, Kaplan et al. reported that RA induces cell differentiation of neuroblastoma through the upregulation of TrkB which responds to brain-derived neurotrophic factor (BDNF) [141]. Also, Holzel et al. reported that tumor suppressor NF1 is a key factor which responds to RA treatment and represses RAS-MEK signaling which represses ZNF423, a transcriptional coactivator of RARs [142].

In 1985, Thiele et al. reported a major breakthrough. Thiele stated from her research that prior to cell differentiation of neuroblastoma, RA induces the

downregulation of MYCN whose expression has a strong correlation with aggressive tumors and the poor outcome of neuroblastoma patients [131].

1.3.3 Retinoic acid and MYCN

Following Thiele's finding, researchers investigated the effect of retinoic acid in MYCN gene expression of neuroblastoma. There are many reports that MYCN correlates with neuroblastoma characteristics, such as growth speed, tumor aggressiveness, drug resistance, and the poor outcome of patients. Therefore, it is felt that MYCN downregulation is the key step for cellular-differentiation of tumors and tumor remission. Currently, it is well-known that retinoic acid downregulates gene expression of MYCN oncogene in neuroblastomas [95, 132, 143-145]. Thiele et al. also reported that RA downregulates other proto-oncogenes, such as c-myb, c-Ha-ras, and c-efb-B in neuroblastoma during RA-induced differentiation [95, 132, 146].

1.3.4 Limitation of RA treatment

Although retinoic acid has unparalleled effects on neuroblastoma patients, there are some issues and limitation of its usage. The most important issue of RA usage is that RA doesn't work in all cases of neuroblastoma. It is also known that neuroblastoma can acquire resistance to certain drugs, and also can become resistant to RA [81, 147].

Another issue is there are several side effects of retinoic acid. It is known that RA causes “retinoic acid syndrome”. Symptoms are pyrexia, dyspnea, pleural effusion, interstitial pneumonia, lung congestion, and anoxemia [148]. Other side effects of retinoic acid are hyperleukocytosis, infectious diseases such as pneumonia and septicemia, delirium, thrombosis, and angitis [148]. Other common side effects of retinoids are cheilitis, liver toxicity, conjunctivitis, hypertriglyceridemia, and mucocutaneous dryness [149].

Besides these side effects, hypercalcemia has been reported in patients who receive therapy with retinoic acid [149-151]. Studies reported that this is the dose-limiting-toxicity and its symptoms are anorexia, asthemia, vomiting, headache, hypertension, convulsion, arthralgia, and myalgias [149]. Osteoblastic lesions are often reported with hypercalcemia. Teratogenicity is another important issue of retinoic acid [148].

1.4 Other therapeutic reagents and new drugs for neuroblastoma

There are other new reagents which have the potential for neuroblastoma therapy. Other vitamins such as vitamin D which shows anti-proliferative effects [152-155], vitamin E which has growth-arresting propaties and differentiating effects [156-158], and vitamin K which induces apoptosis [159], micro-RNAs, DMFO, and DMSO are some examples which have been investigated [160-162].

Fenretinide (N-(4-hydroxyphenyl) retinamide) is a promising new synthetic retinoic acid. The same as all-trans-retinoic acid and 13-cis-retinoc acid,

fenretinide demonstrates cytotoxicity and growth inhibition of neuroblastoma in vitro and in vivo [163-168]. Additionally, it is particularly worth noting that fenretinide inhibits cell proliferation in RA-resistant neuroblastoma cell lines [169]. The studies of fenretinide have reported that this new retinoid causes apoptosis and/or necrosis in neuroblastoma cells by inducing caspase-8 activation in neuroblastoma cells [170], inducing mitochondrial ROS (reactive oxygen species) [171], increasing ceramide levels and inducing cell death in a p53- and caspase-independent manner [169, 172], inducing Bak protein which belongs to a family of Bcl-2 [173], and raising endoplasmic reticulum stress and activating JNK pathway [174].

After the success of the phase I trial [175, 176], fenretinide has been studied more [177, 178] and now it is in the phase II trial [179]. And even the metabolites of fenretinide such as 4-oxo-4-HPR are currently under investigation for a new treatment of high-risk neuroblastoma [180].

Other promossing reagents are microRNAs which suppress MYCN expression such as microRNA-34a, let-7, and mir-101. These microRNAs have anti-proliferation effects in vitro and in vivo [52, 181-183]. MicroRNA-34a even induces apoptosis in neuroblastoma cells [52].

Other reagents and molecules which have the potential for neuroblastoma therapy have been investigated, such as interferon gamma [184, 185], Akt inhibitor [186], vasoactive intestine peptide [187, 188], histone deacetylase

inhibitors [189-192], phenylacetate [193, 194], and shRNA targeting MYCN virus vectors [195, 196].

1.5 Previous research on MYCN regulation and RA

Even though it is well-known that RA inhibits neuroblastoma and is clinically very important, the molecular mechanism through which RA works against neuroblastoma is unclear even after decades of research.

Once Thiele et al. found that RA downregulates the gene expression of MYCN oncogene before cells start differentiating [95, 131, 132], researchers started trying to reveal the molecular mechanism of this event, assuming MYCN downregulation is the key event for the induction of cell differentiation in neuroblastoma by RA. However, it is still unclear. And one important point is that RARE and RXRE are not found within the MYCN gene.

In 1997, Wada et al. figured out that a certain sequence within the MYCN promoter, just upstream of exon1 (-186 to -160) is essential to respond to the RA signal in neuroblastoma cells and this sequence was named “retinoic acid responsive region” (RARR) (figure 1) [184]. In 2002, Inge et al. discovered that Sp1 is important for MYCN expression [197]. In 2003, Tuthill et al. figured out that, in neuroblstoma, MYCN expression is driven by Sp1 and Sp3 on promoter [198], and Kramps et al. also showed E2F and Sp proteins activate MYCN expression in neuroblastoma [199]. In 2008, however, Kanemaru et al. found that Sp1 and Sp3 are not key factors which are modified by RA for MYCN downregulation in

neuroblastoma, because there is not any significant difference with RA treatment and without RA treatment [200].

Lastly, Takeuchi reported that the E2F binding site within the MYCN promoter is the key element for the downregulation of MYCN by RA (figure 2). He reported that the mutation or deletion within the binding site of E2F transcription factors impaired the response to RA treatment by reporter assay with various mutants of the MYCN promoter (figure 3 and 4) [201].

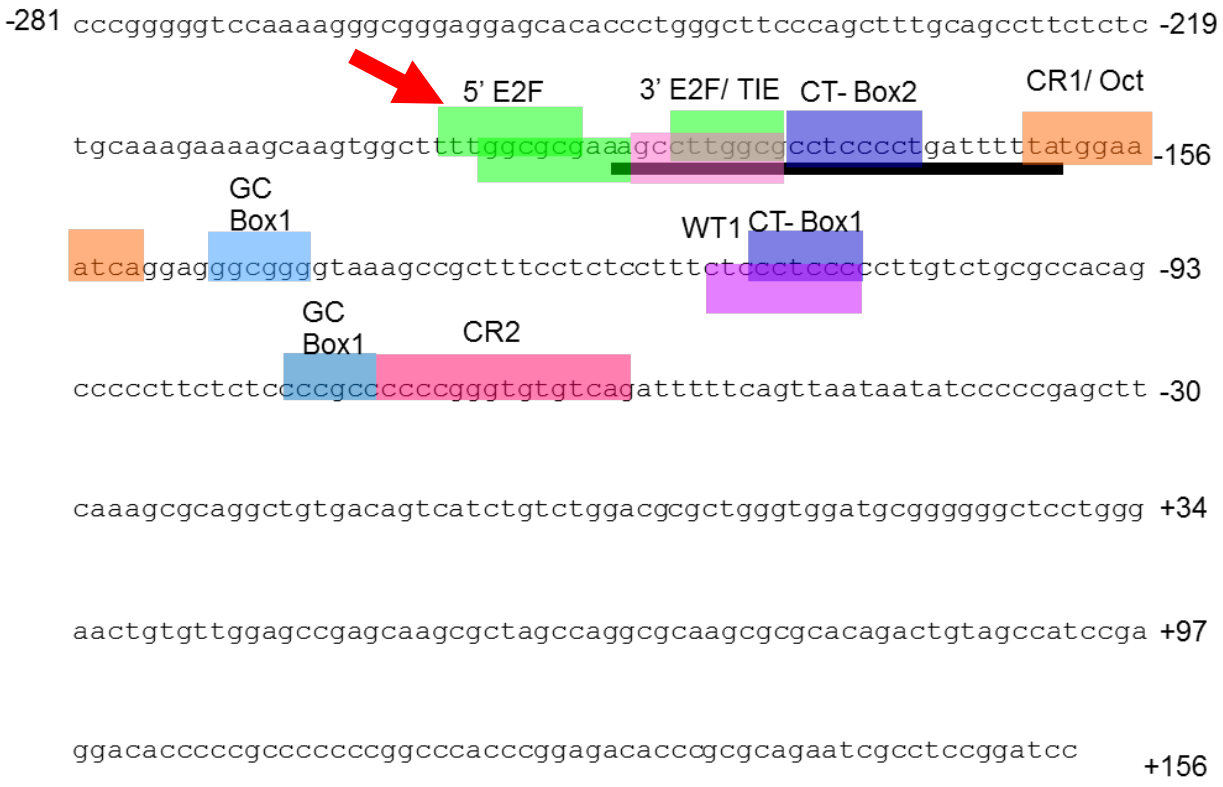


Figure 2. MYCN basal promoter.
Black underline indicates RARR. Red arrow indicates the key element which Takeuchi found out. Figure referred from Takeuchi, 2009.

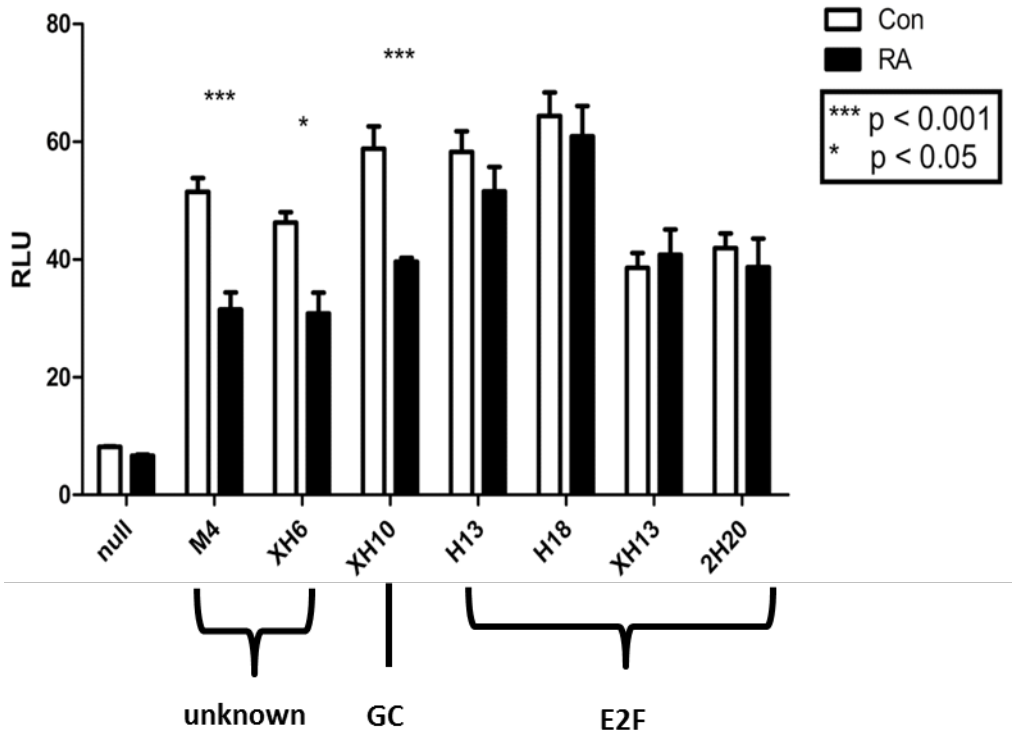


Figure 3. Randam mutant promoter analysis.
A promoterless luciferase construct (null) or luciferase constructs containing various random murtants of the MYCN promoter were transiently contransfected with a SV40 Renilla luciferase construct (pGL4.73). Following transfection, SH-SY5Y cells were treated with either DMSO, solvent alone or 5 µM RA for 48 hours. The relative light units (RLU) are expressed as a ratio of luciferase: Renilla luciferase. Each bar represents the average of three replicates. Figure adapted from Takeuchi, 2009.

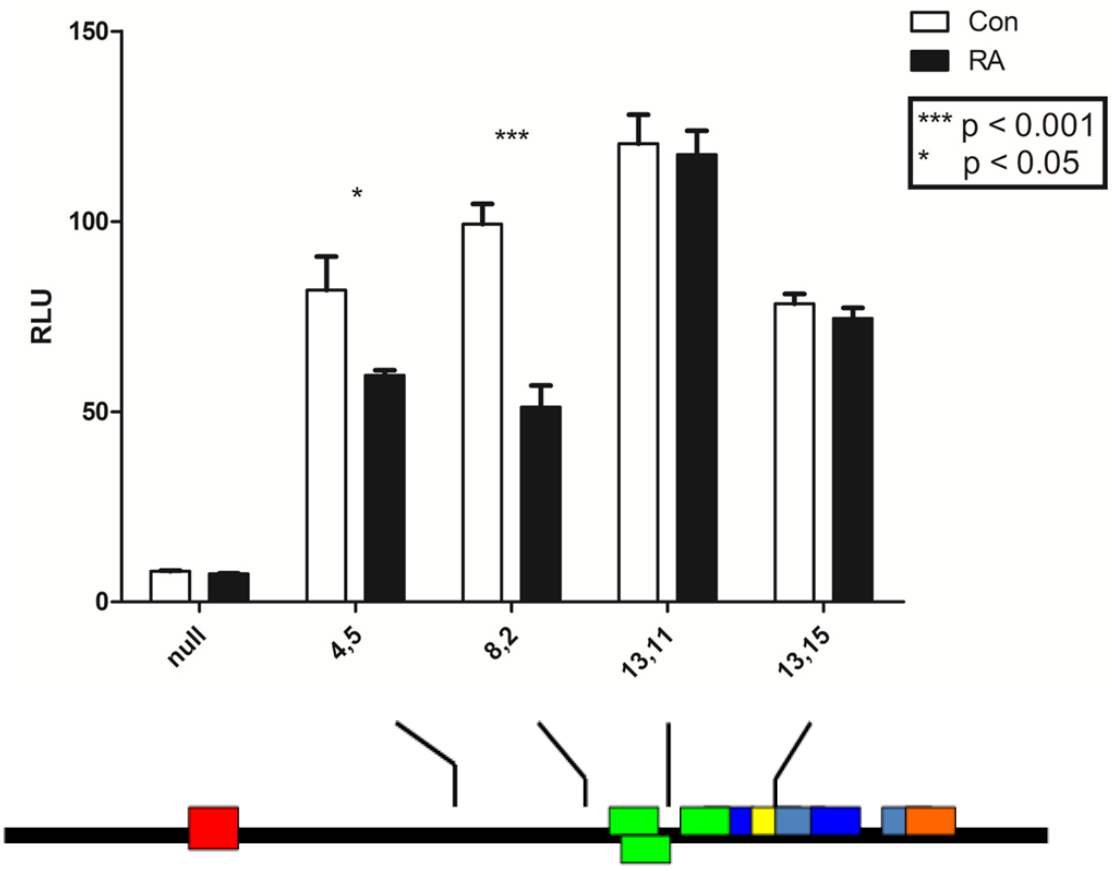


Figure 4. 5' deletion mutant promoter analysis.
A promoterless luciferase construct (null) or luciferase constructs containing various 5' mutants of the MYCN promoter, were transiently cotransfected with a SV40 Renilla luciferase construct (pGL4.73). Following transfection, SH-SY5Y cells were treated with either DMSO, solvent alone or 5 μM RA for 48 hours. The relative light units (RLU) are expressed as a ratio of luciferase:Renilla luciferase. Each bar represents the average of three replicates. Each 3' deletion mutant is associated with a colored dot. These dots correspond to the position of the deletion mutant on the diagram below the graph. E2F = green box; CTboxes = dark blue box; GCboxes = light blue box; CR1/Oct binding motif = yellow box; CR2 = orange box. Figure adapted from Takeuchi, 2009.

1.6 E2F transcription factors and cell cycle

E2F transcription factors are a family of at least 8 DNA binding proteins that govern the expression of various genes, which are important for cell cycle progression, such as cyclin E, cyclin A, CDK2, c-Myb, and MYCN[202, 203]. About 40 binding sites were characterized in promoters [204]. E2F1 regulates around 1000 genes [205].

In order to bind to DNA, E2Fs form heterodimer with dimerization partner proteins (DP) [206, 207]. To activate target genes, E2Fs recruit histone acetyltransferase (HAT), such as CBP, p300, Tip60, and P/CAF [206, 207]. These enzymes acetylate histone. The acetylation of histone tail loosens the DNA-histone contacts. Therefore, histone acetylation results in the increase of the interaction between transcription factors and promoters [202].

E2F proteins have the binding site for the Rb family which is known as tumor suppressor genes. The activity of E2F proteins are controlled by the Rb family [204, 208].

New studies propose that E2F1, E2F2, and E2F3 are activator E2Fs which promote the expression of their target genes, and E2F4, E2F5, E2F6, E2F7, and E2F8 are repressor E2Fs which negatively regulate gene expression [206, 207, 209]. But, still new studies are ongoing to uncover the functions of E2Fs. Recent studies reported that E2F7 and E2F8 have activator role [210-212].

1.7 Rb tumor suppressor gene and Rb family

The retinoblastoma protein (Rb or Rb105) is a well-known tumor suppressor protein and a member of the Rb protein family that also includes p107 and p130 [213, 214]. They also are mentioned as a pocket protein family [213, 215]. These three proteins have overlapping functions in the cell cycle control, mainly anti-proliferative control by interacting with E2F transcription factors. The pocket proteins repress the E2F activity by binding them and inhibit the cell cycle progression at G1/S phase transition [215, 216]. These ability to repress E2F are regulated by posttranslational modification, phosphorylation and it is mediated by cyclin/CDK (cyclin-dependent kinase) complexes [217, 218]. Cyclin/CDK complexes inactivate Rb through phosphorylation and promote the cell cycle progression. In hypophosphorylated form, Rb is able to bind to E2F proteins resulting in the inhibition of E2F transcriptional activity. However, when cyclin/CDK complexes phosphorylate Rb, hyperphosphorylated Rb releases E2F resulting in the expression of target genes for cell cycle progression [208, 213-216, 219, 220]. There are 16 potential cyclin/CDK phosphorylation sites on Rb and many of them were detected in vivo [208, 219, 221, 222].

Rb protein forms the complex by recruiting other co-repressors which modify chromatin structure for cell cycle inhibition, such as histone deacetylase enzymes (HDACs), the histone methyltransferase (HMTs), DNA methyltransferase, and heterochromatin protein, HP1 [203, 223-231]. Histone deacetylase decetylates the histone tail and facilitates nucleosome packing which results in the reduction of transcription factor access to promoter [202, 203]. Histone methyltransferase

such as SUV39H1, SUV39H2, methylate lysine 9 and create the binding site for HP1 within histone H3 to repress transcription [207, 230].

Interestingly, functions of Rb, especially with posttranslational modification such as phosphorylation, acetylation, methylation, SUMOylation, and ubiquitinylation, are not well-understood and new studies are ongoing to uncover its functions and mechanisms [221, 232-234].

1.8 Interaction of E2F and Rb

The mechanism of G_1/S cell cycle transition that CDK/cyclin complexes inactivate Rb and release E2F through phosphorylation is widely accepted. However, the model of Rb-E2F interaction is still controversial. The classical model proposes that Rb binds to E2F transcription factors and sequesters them from their target genes. For cell cycle progression, CDK/cyclin complexes inactivate Rb through phosphorylation and hyperphosphorylated Rb releases E2F (figure 5) [203]. Another alternate model proposes that Rb interacts with E2Fs on promoter and represses their transcriptional activities. Phosphorylation by CDK/cyclin complexes impairs the affinity of Rb to E2Fs (figure 6) [235].

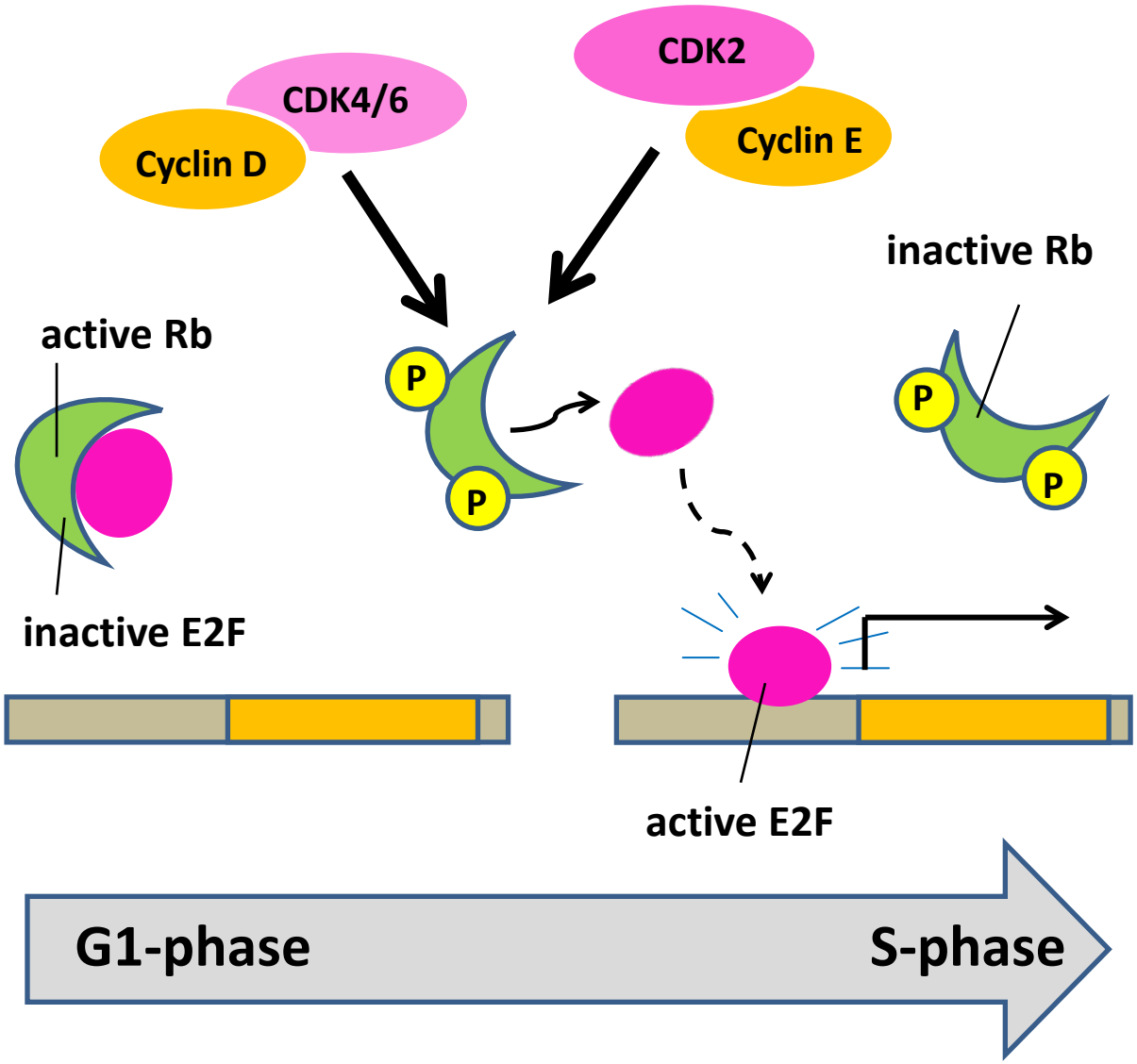


Figure 5. Classic model of the interaction between E2F and Rb.
The Rb protein inhibits the E2F activity and the progress of the cell cycle when it is unphosphorylated. The complexes of CDK/cyclin phosphorylate Rb and free E2F for the progression of the cell cycle. Figure referred from Trimarchi, 2002.

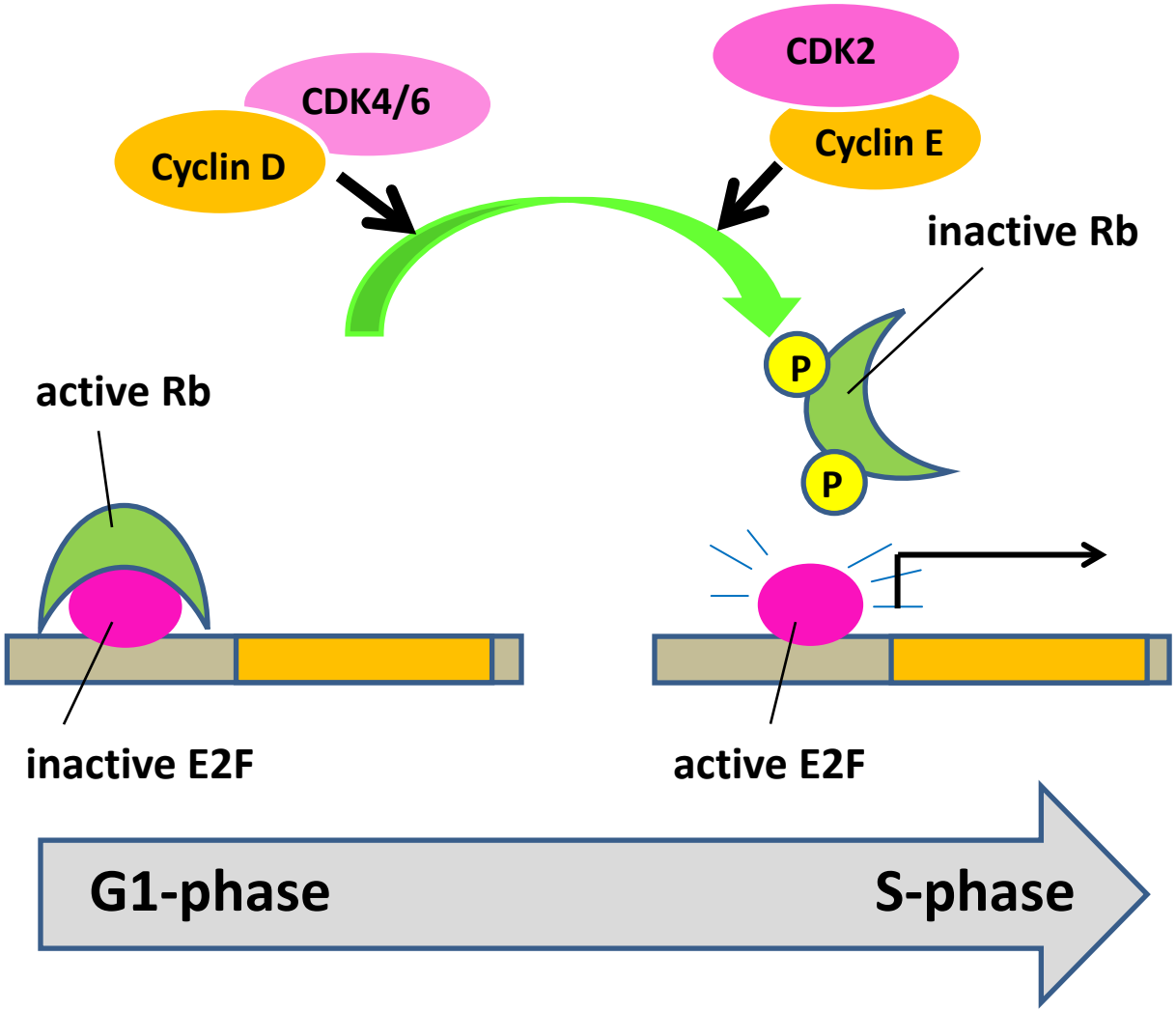


Figure 6. Alternate model of the interaction between E2F and Rb. The Rb protein inhibits the E2F and suppresses cell cycle progression. CDK/cyclin complexes inactivate Rb through phosphorylation and stimulate the progression of the cell cycle. Figure referred from Ajioka, 2014.

CHAPTER 2. SIGNIFICANCE AND OBJECTIVE

2.1 Significance of this project

Because MYCN is strongly associated with poor outcomes of neuroblastoma patients, regulating this oncogene may be beneficial to the therapy of neuroblastoma patients.

For the last three decades, scientists made lots progress and have revealed that RA represses neuroblastoma proliferation and downregulates gene expression of MYCN oncogene in neuroblastoma. At the hand of their tireless efforts, RA is now clinically utilized as one of the few agents which increase the survival rate of neuroblastoma patients in the high-risk group. However, there are limitations of therapeutic usage of RA, because of its side effects and inadequate understanding of how this drug works, even though RA has maintained unparalleled benefits and possesses further potential for new and better therapy for neuroblastoma patients

By investigating molecular interactions in neuroblastoma cells during RA treatment, this research will provide further insight and better understanding how RA downregulates the expression of MYCN oncogene. In conclusion, our study will lead us to better clinical applications.

2.2 Objective

MYCN oncogene is strongly associated with biological features of the neuroblastoma tumor and, therefore, the outcome of patients. Inducing the downregulation of MYCN is thus considered very beneficial for treatment of neuroblastoma patients.

Accordingly, revealing and understanding the molecular mechanism and/or molecular pathway through which RA exerts its effects on MYCN promoter activity potentially provides us new knowledge, which guides us to better usage of RA and the discovery of new drugs for neuroblastoma patients, such as the new combinations of RA and additional drugs that inhibit molecules which, in turn, inhibit RA activity; and also which enhance the molecules that promote RA signal. Additionally, such information might also suggest that avoiding RA treatment will help us circumvent futile attempts at treatment, as well as those subsequent side effects, when the key molecules that respond to RA are not functional in neuroblastoma patients, as a result of mutation.

Since Thiele et al. found that RA downregulates MYCN expression, researchers have been trying to reveal its molecular mechanism, and now we know that the binding site of E2F transcription factor within MYCN promoter is the key element for MYCN downregulation by RA in neuroblastoma.

Here, the objective of this research is to reveal the molecular events for the downregulation of MYCN oncogene on the E2F binding site within the MYCN promoter by investigating the interactions between the binding site of E2F within the MYCN promoter and proteins, such as E2F and Rb.

CHAPTER 3. RESULTS

3.1 Specific Aim 1. Investigate the bindings of E2F proteins onto the binding site within the MYCN promoter in neuroblastoma during RA treatment.

3.1.1 Hypothesis

The bindings of E2F proteins onto the MYCN promoter change when neuroblastoma cell is treated with RA and this event leads to the downregulation of MYCN oncogene.

3.1.2 Rationale

The molecular mechanism through which RA downregulates MYCN oncogene is still unclear. Previously, Takeuchi figured out that the binding site of E2F transcription factors within the MYCN promoter is a key element for the downregulation of MYCN by RA [201]. E2F transcription factors play a critical role in cell cycle progression in response to mitogenic stimulation [236]. The next logical step is to examine the molecular events which repress the MYCN transcription within this E2F binding site during MYCN downregulation by use of RA. The classic model of E2F-Rb interaction (figure 5) predicts us that RA regulates the interaction of E2F and MYCN promoter. Accordingly we will

investigate the molecular interactions, mainly between the E2F protein and DNA within the E2F binding site on the MYCN promoter.

3.1.3 Examine RA effect on cell morphology

RA effect on cell morphology in neuroblastoma cell was examined with MYCN-amplified cell line, LA-N-5 (shown in figure 7). Cells were treated with 5 μM RA or control solvent. The morphological changes of LA-N-5 cells that occurred during RA treatment were depicted in figure 8. RA treatment caused a dramatic increase in a morphologic differentiation as evidenced by the formation of long neurites. Compared with control cells, RA treated cells had thin elongated and branched neurite extensions. Within the first 4 days after RA treatment, many LA-N-5 cells began to extend the neuritis. After 10 days of RA treatment, the neurite processes began to form the neuritic bundles. Also, the growth inhibition of LA-N-5 cells by RA was apparent by 4 days.

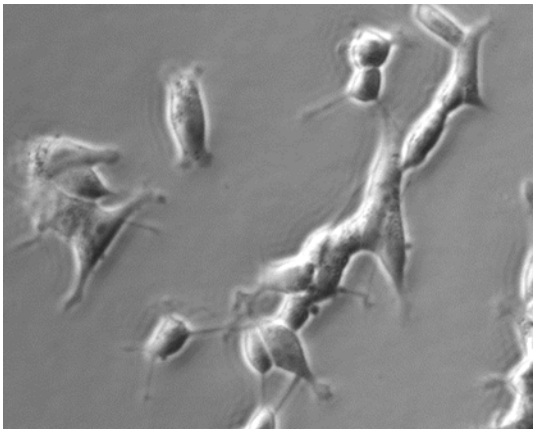


Figure 7. Neuroblastoma cell line, LA-N-5 without any treatment.

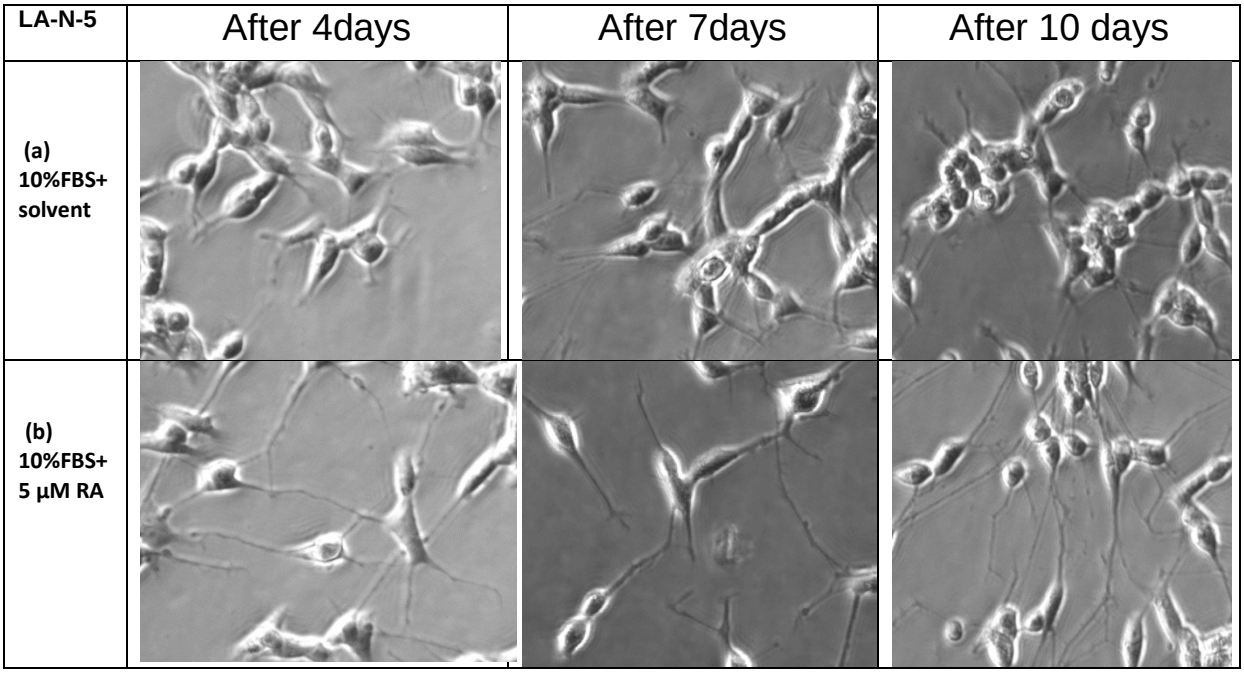


Figure 8. Morphological differentiation of RA-treated LA-N-5 neuroblastoma cells.
Cells were treated with (a) control solvent or (b) 5 µM RA. RA treatment caused a dramatic increase in a morphologic differentiation as evidenced by the elongated and branched neurite extensions.

3.1.4 Analysis of MYCN expression at protein level during RA treatment by Western blot

To study RA effect on MYCN expression at protein level in neuroblastoma LA-N-5 cells, Western blot was performed. Cells were cultured with control solvent or 5 µM RA for indicated periods and protein was extracted. As shown in figure 9, MYCN expression was decreased dramatically with 5 µM RA treatment. Downregulation of MYCN was detected after 24 hours of RA treatment.

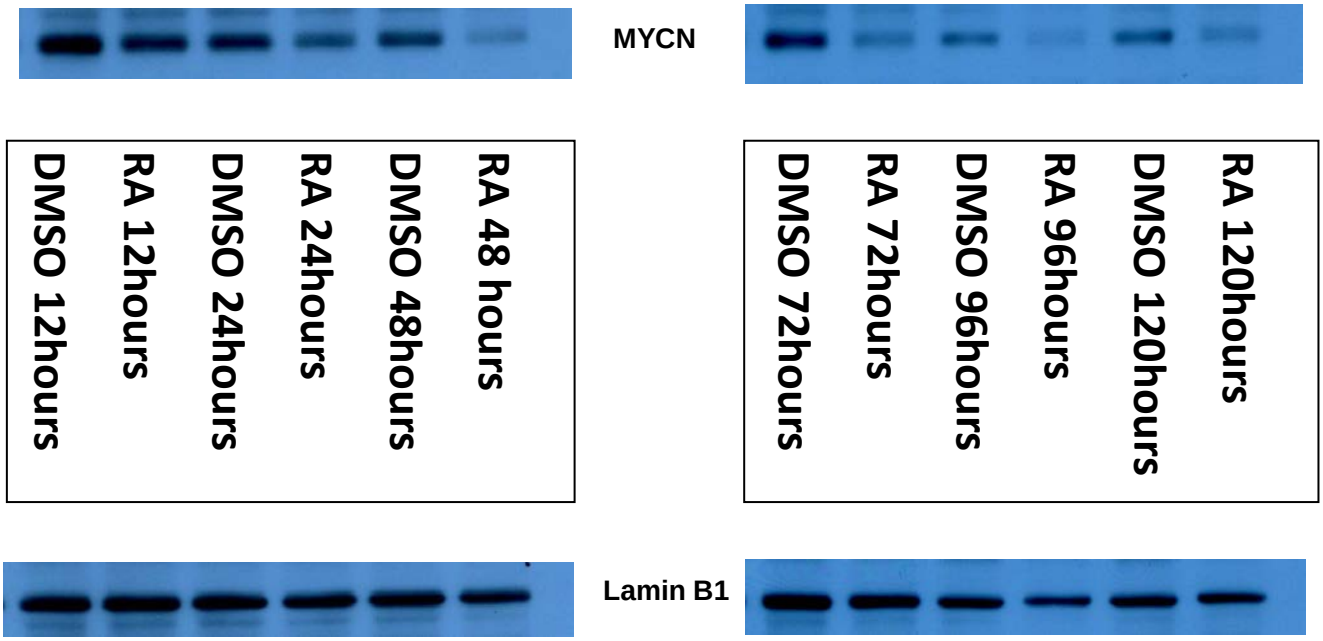


Figure 9. Western blot results with LA-N-5.
Total cellular protein was extracted from LA-N-5 cells treated with control solvent or 5 µM RA for 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours. MYCN expression was decreased dramatically with 5 µM RA treatment within 24 hours.

3.1.5 Analysis of MYCN expression at mRNA level during RA treatment by RT-qPCR

Gene expression of MYCN at mRNA level in neuroblastoma LA-N-5 cells during RA treatment was examined by RT-qPCR. Cells were cultured with control solvent or 5 µM RA for indicated periods. After mRNA extraction, RT-qPCR was performed. As shown in figure 10, MYCN expression was decreased with RA treatment. Downregulation was detected from 6 hours after RA treatment. This result suggests that the molecular event on the E2F binding site within the MYCN promoter occurs around, or even before 6 hours of RA treatment.

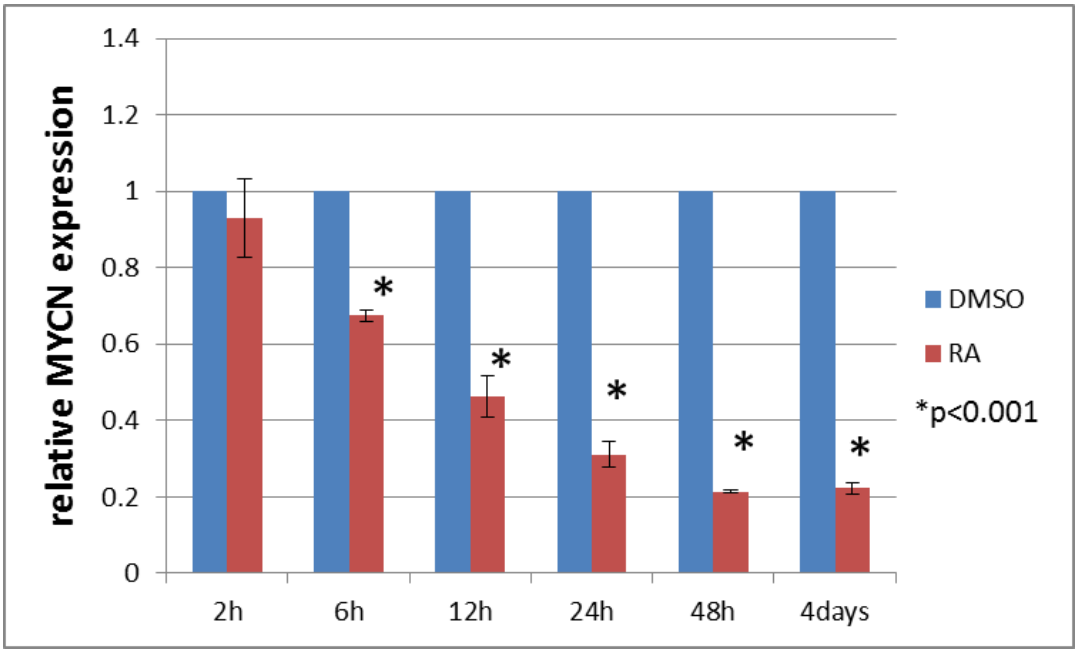
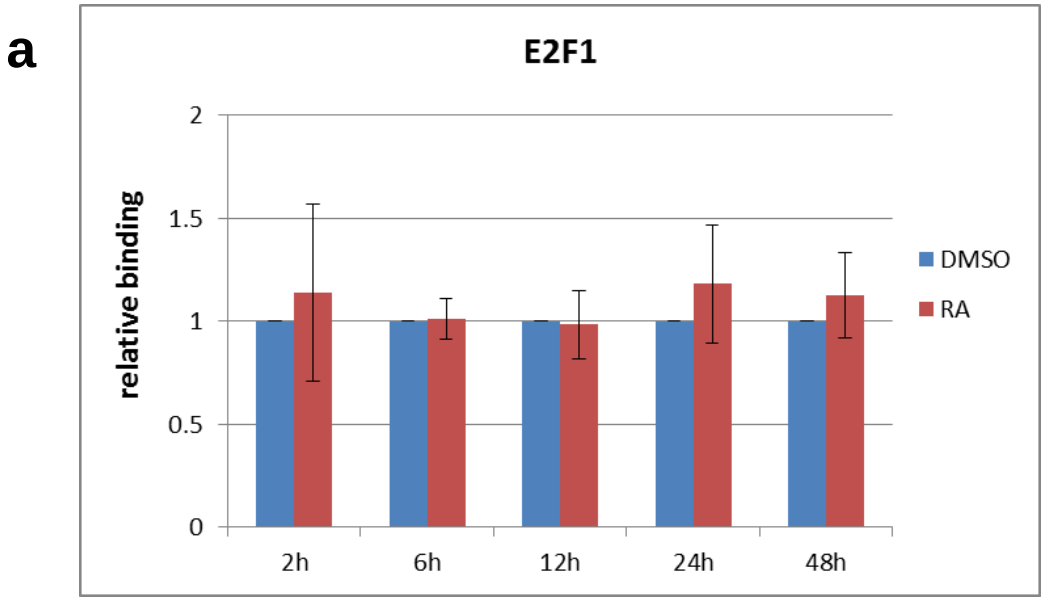


Figure 10. MYCN mRNA downregulation by RA.
Total cellular RNA was extracted from LA-N-5 cells treated with control solvent or 5 µM RA for 2 hours, 6 hours, 12 hours, and 24 hours, 48 hours, and 4 days. Downregulation was detected from 6 hours after RA treatment. Data were normalized with respect to the expression level of GAPDH mRNA. n=3

3.1.6 Analysis of the binding of activator E2Fs to MYCN promoter during RA treatment by Chromatin immunoprecipitation (ChIP)

To examine the binding of E2F transcription factors, E2F1, E2F2, and E2F3 (activator E2Fs), to the MYCN promoter, ChIP assays were performed. Chromatin lysate was prepared from neuroblastoma LA-N-5 cells treated with control solvent or 5 μM RA for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. As shown in figure 11, the binding of these proteins to the MYCN promoter did not change during RA treatment.



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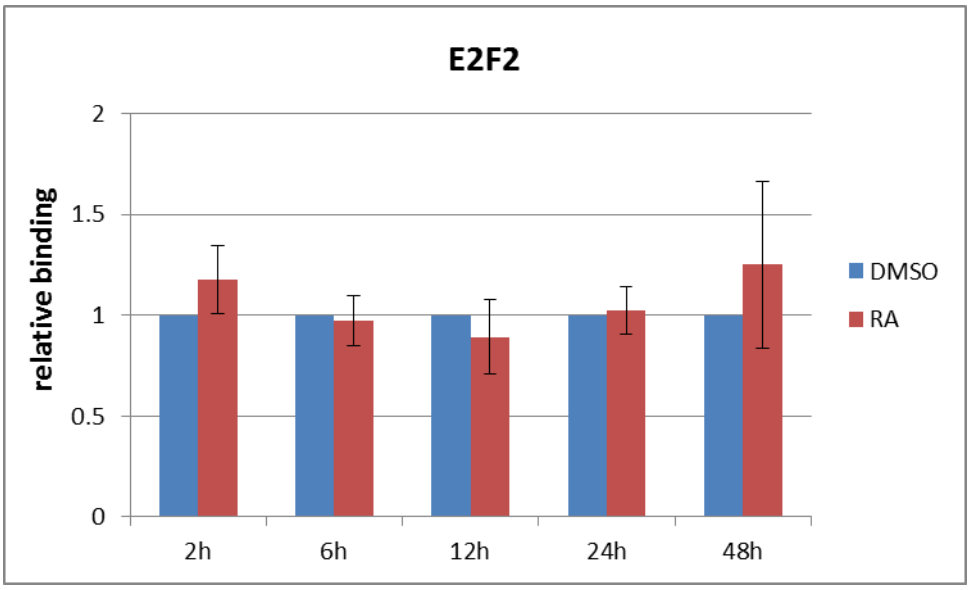

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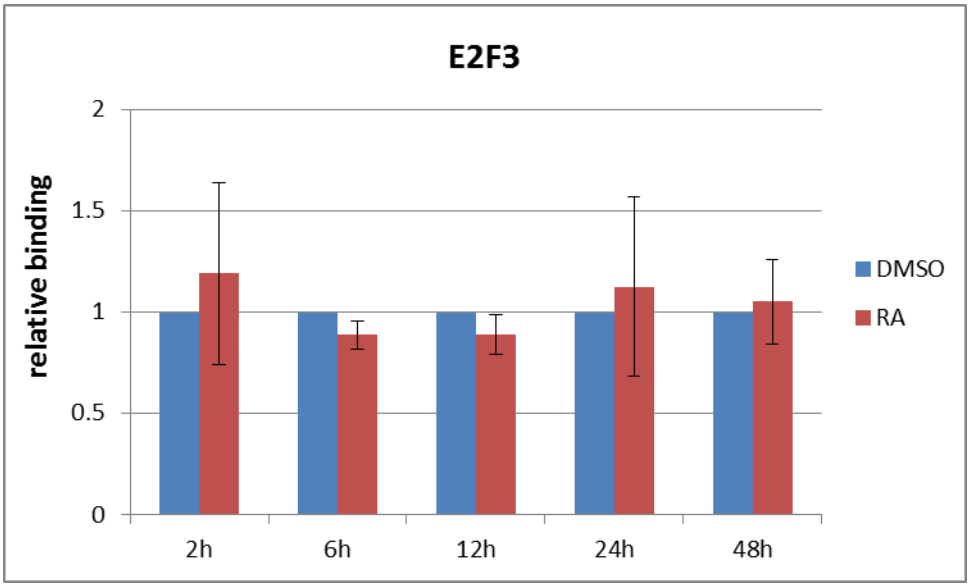
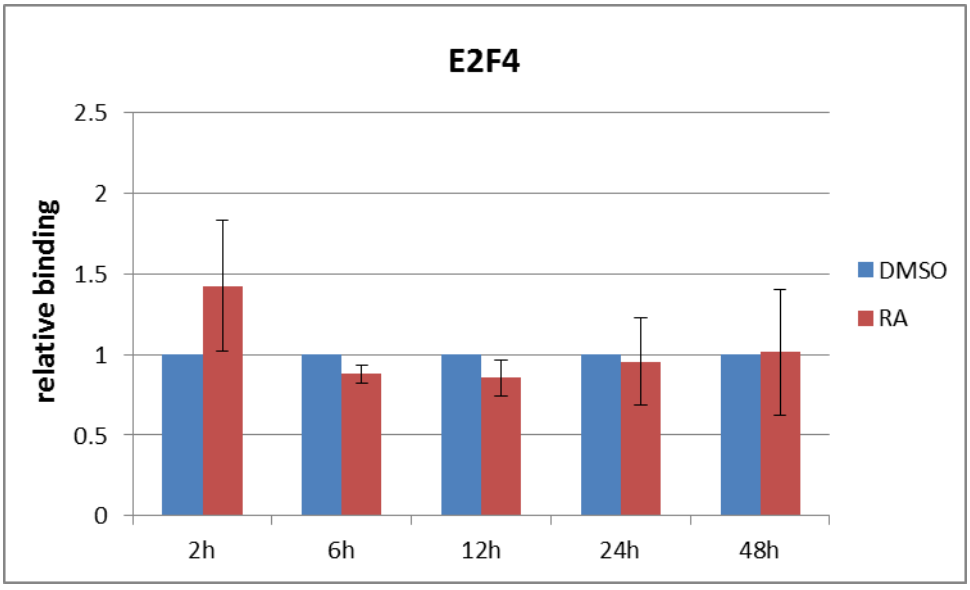


Figure 11. Relative binding of activator E2Fs to the MYCN promoter. Chromatin lysate was prepared from neuroblastoma LA-N-5 cells. Cells were treated with control solvent or 5 µM RA for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. The bindings of E2F1 (a), E2F2 (b), and E2F3 (c) to the MYCN promoter did not change during RA treatment. The DNA output from assays were analyzed by real-time PCR. The binding of E2Fs to the MYCN promoter was calculated by via the delta delta Ct comparative method and expressed relative to control. n=3

3.1.7 Analysis of the binding of repressor E2Fs to the MYCN promoter during RA treatment by Chromatin immunoprecipitation

To examine the binding of the repressor E2Fs, E2F4 and E2F5, to the MYCN promoter, ChIP assays were performed. Chromatin lysate was prepared from neuroblastoma LA-N-5 cells treated with control solvent or 5 μM RA for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. As shown in figure 12, the binding of these proteins to the MYCN promoter did not change during RA treatment.

a



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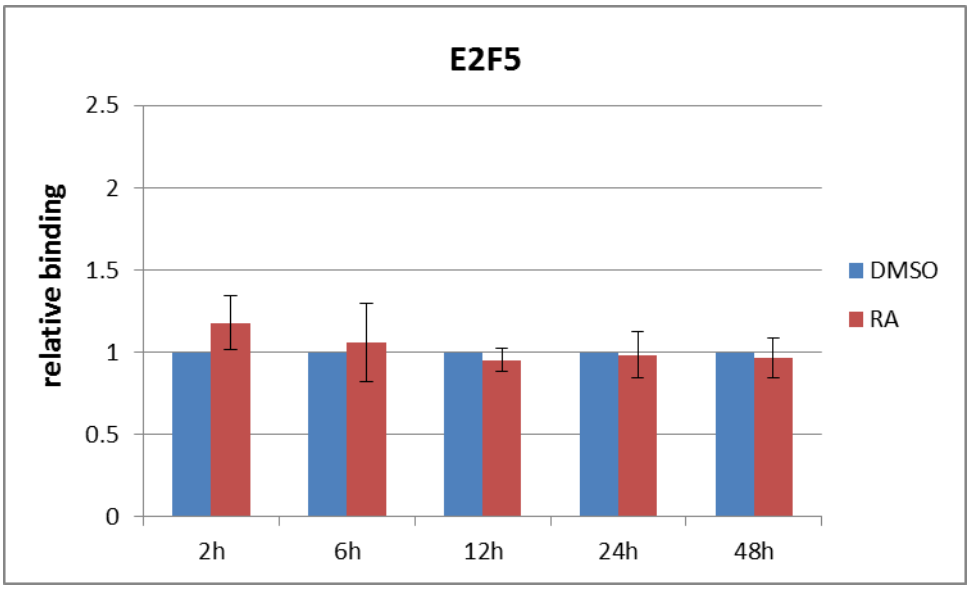


Figure 12. Relative binding of repressor E2Fs to the MYCN promoter. Chromatin lysate was prepared from neuroblastoma LA-N-5 cells. Cells were treated with control solvent or 5 µM RA for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. The binding of E2F4 (a) and E2F5 (b) to the MYCN promoter did not change during RA treatment. The DNA output from assays were analyzed by real-time PCR. The binding of E2Fs to the MYCN promoter was calculated by via the delta delta Ct comparative method and expressed relative to control. n=3

3.1.8 Conclusion and discussion

According to the results of morphological observation, Western blot, and RT-qPCR, it was considered that the key molecular events for the repression of MYCN expression should occur within 6 hours after RA treatment. But the binding of activator E2Fs and repressor E2Fs onto the MYCN promoter did not change during RA treatment. These results indicate that the classic model of E2F-Rb interaction does not apply to MYCN downregulation by RA in neuroblastoma and E2Fs are not key factors. Therefore, the research which focuses on Rb protein should be done.

3.2 Specific Aim 2. Investigate the interaction between Rb tumor suppressor proteins and the complex of E2F proteins and DNA sequence within the MYCN promoter in neuroblastoma during RA treatment.

3.2.1 Hypothesis

The binding of Rb tumor suppressor proteins to the complex of E2F proteins and DNA sequence within the MYCN promoter increases in neuroblastoma during RA treatment.

3.2.2 Rationale

E2Fs play a critical role in cell cycle progression by regulating transcription in response to mitogenic stimulation [209, 237]. On the other hand, the retinoblastoma tumor suppressor protein, Rb and other Rb family proteins, p107 and p130 negatively regulates cell cycle progression by interacting with E2F proteins and repressing their activities [202, 208, 219, 223, 224, 238, 239]. Also, it is well-known that Rb activity is regulated by cyclin/CDK complexes through phosphorylation [208, 213-216, 219-222]. Recent studies suggest that phosphorylation at specific site (Ser608, Ser612, Ser780, Ser795, Ser807/811, Thr821/826) are involved in cell cycle progression and RA affects phosphorylation at several sites [234, 240-245].

As previously reported as the result of specific aim1, the binding of E2Fs to the MYCN promoter did not change while RA downregulates MYCN expression.

Therefore, the binding of Rb family proteins onto the MYCN promoter and its phosphorylation state during RA treatment must be investigated.

3.2.3 Analysis of Rb binding to the MYCN promoter during RA treatment by Chromatin immunoprecipitation

To examine the binding of Rb to the MYCN promoter, ChIP assay was performed. Chromatin lysate was prepared from neuroblastoma LA-N-5 cells treated with control solvent or 5 μM RA for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. Not as we expected, the binding of Rb to the MYCN promoter did not increase during RA treatment (Figure 13).

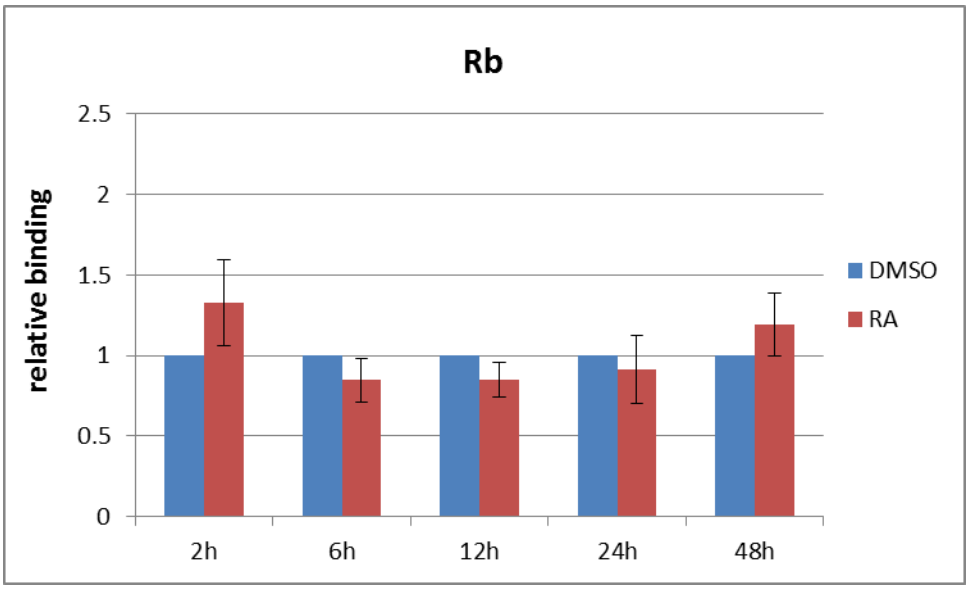
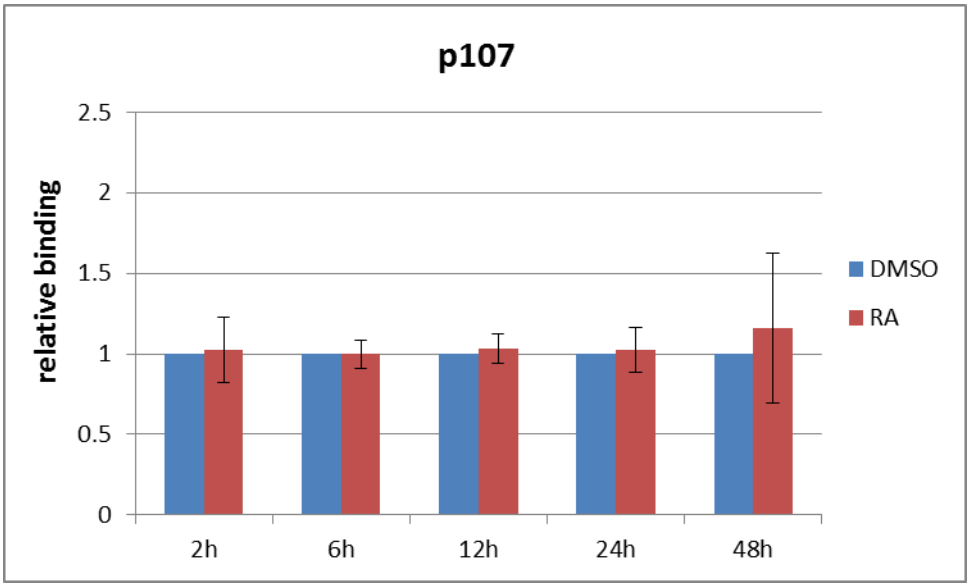


Figure 13. Relative binding of Rb to the MYCN promoter.
Chromatin lysate was prepared from neuroblastoma LA-N-5 cells. Cells were treated with control solvent or 5 µM RA for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. The binding of Rb to the MYCN promoter did not change during RA treatment. The DNA output from assays were analyzed by RT-qPCR. The binding of Rb to the MYCN promoter was calculated by via the delta delta Ct comparative method and expressed relative to control. n=3

3.2.4 Analysis of binding of other Rb family proteins, p107 and p130 to the MYCN promoter during RA treatment by Chromatin immunoprecipitation

To examine the binding of other Rb family proteins, p107 and p130 to the MYCN promoter, ChIP assays were performed. Chromatin lysate was prepared from neuroblastoma LA-N-5 cells treated with control solvent or 5 μM RA for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. The binding of these proteins to the MYCN promoter did not change during RA treatment (Figure 14).

a



b

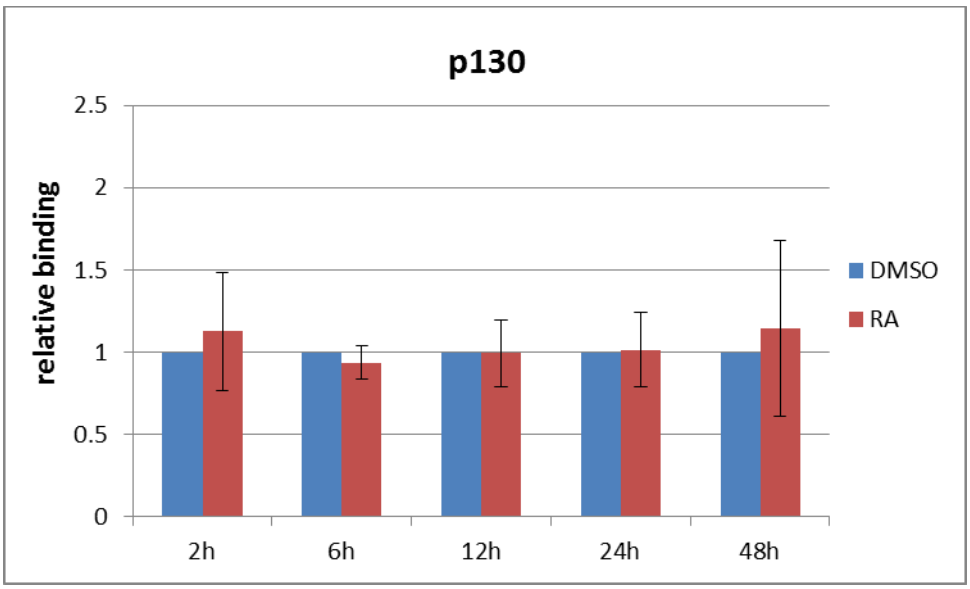
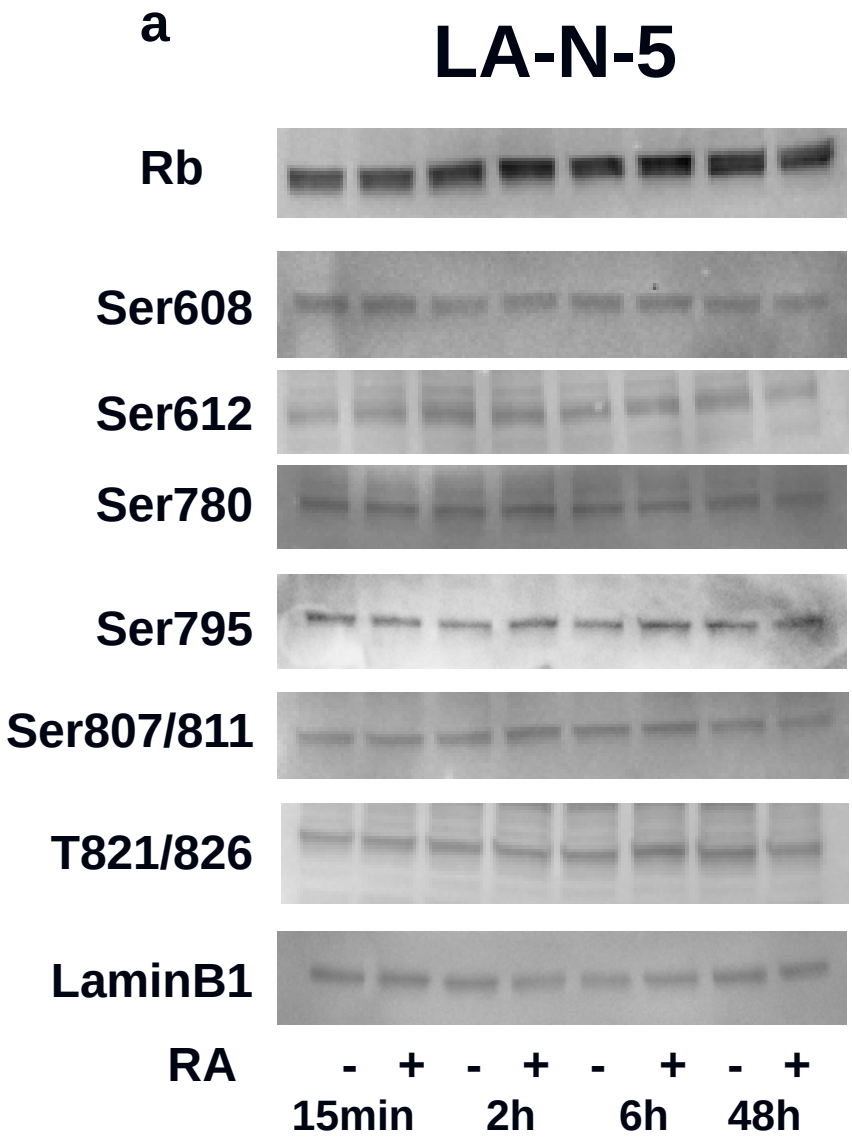


Figure 14. Relative binding of Rb family proteins to the MYCN promoter. Chromatin lysate was prepared from neuroblastoma LA-N-5 cells. Cells were treated with control solvent or 5 µM RA for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. The binding of p107 (a) and p130 (b) to the MYCN promoter did not change during RA treatment. The DNA output from assays were analyzed by RT-qPCR. The binding of these proteins to the MYCN promoter was calculated by via the delta delta Ct comparative method and expressed relative to control. n=3

3.2.5 Analysis of Rb phosphorylation by Western blot

To examine the Rb phosphorylation state, Western blots with antibodies which are specific for each phosphorylated Rb (Ser608, Ser612, Ser780, Ser795, Ser807/811, Thr821/826) were performed. MYCN-amplified neuroblastoma cell line, LA-N-5 and non-MYCN-amplified neuroblastoma cell line, SH-SY5Y were cultured with control solvent or 5 μM RA for indicated periods and protein was extracted. As shown in figure 15, not as we expected, the amount of total Rb and phosphorylation at each specific phosphorylation site did not change in both cell lines.


a
LA-N-5
Rb
Ser608
Ser612
Ser780
Ser795
Ser807/811
T821/826
LaminB1
RA
- + - + - + - +
15min 2h 6h 48h

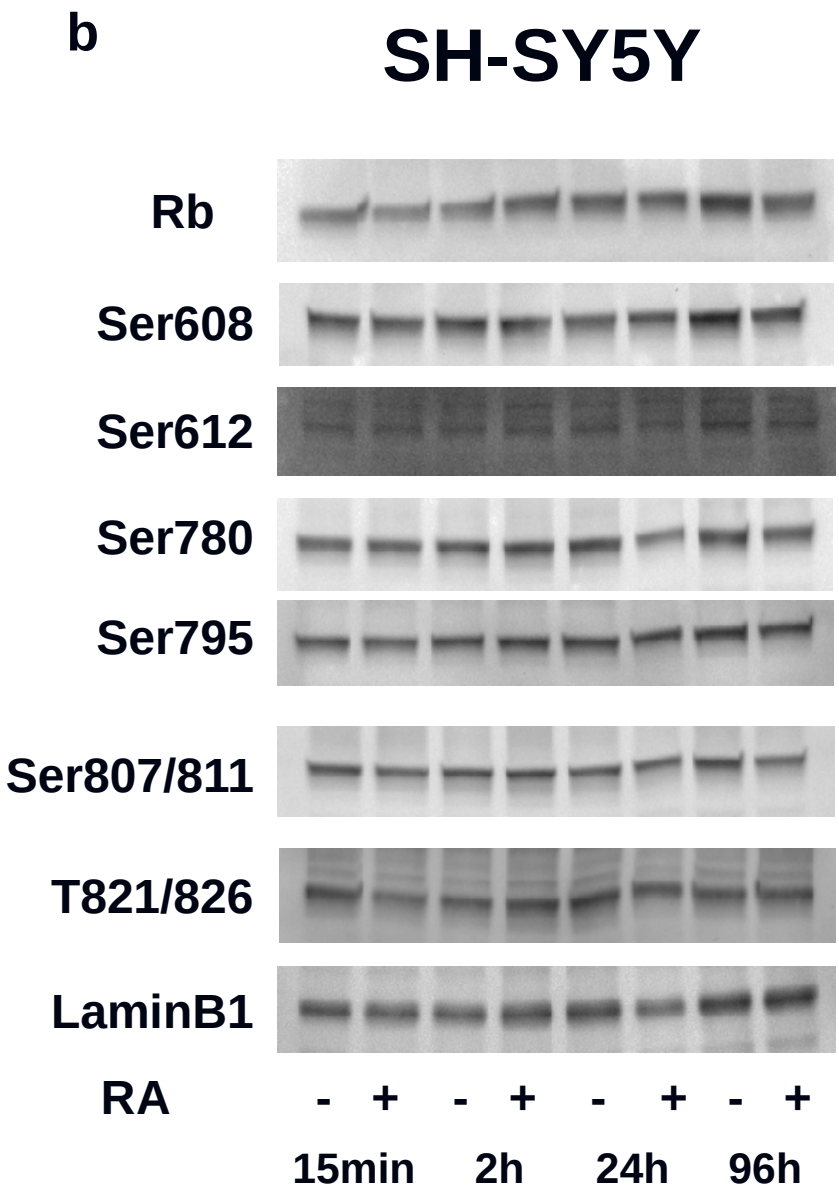


Figure 15. RA does not affect Rb phosphorylation state.
MYCN-amplified neuroblastoma cell line, LA-N-5 (a) and non-MYCN-amplified neuroblastoma cell line, SH-SY5Y (b) were treated with control solvent or 5 µM RA for the indicated periods. Cells were harvested for Western blot with indicated antibodies for Rb or phosphorylated Rb at each specific phosphorylation site.

3.2.6 Conclusion and discussion

In this specific aim, the movement of Rb family proteins, Rb, p107, and p130 was examined. As we hypothesized, we expected that the binding of these tumor suppressor proteins onto the MYCN promoter increase and the phosphorylation of Rb decreases during RA treatment. However, ChIP assays revealed that the binding of these proteins to the MYCN promoter did not change. Also, no changes of Rb phosphorylation were detected by Western blot with antibodies for phosphorylated Rb at each specific phosphorylation site. These findings imply that Rb and other Rb family proteins are not key molecules for MYCN downregulation in neuroblastoma.

3.3 Specific Aim 3. Determine whether Rb is essential key factor for the downregulation of MYCN oncogene and the induction of cell differentiation by RA in neuroblastoma.

3.3.1 Hypothesis

Tumor suppressor gene, Rb is the essential key molecule which is involved in the transcriptional repression of MYCN oncogene and cell differentiation in neuroblastoma during RA treatment.

3.3.2 Rationale

Rb is the suppressor protein which interacts with E2Fs and represses their transcriptional activity. As described as the result of specific aim 2, the Rb binding onto the MYCN promoter did not change while RA downregulated MYCN expression. Next, in order to determine whether Rb is essential key factor for MYCN downregulation and the induction of cell differentiation by RA, whether RA still represses the MYCN transcription and induce cell differentiation without the presence of Rb protein should be examined.

3.3.3 Determine whether RA represses MYCN promoter activity in the presence of Rb siRNA

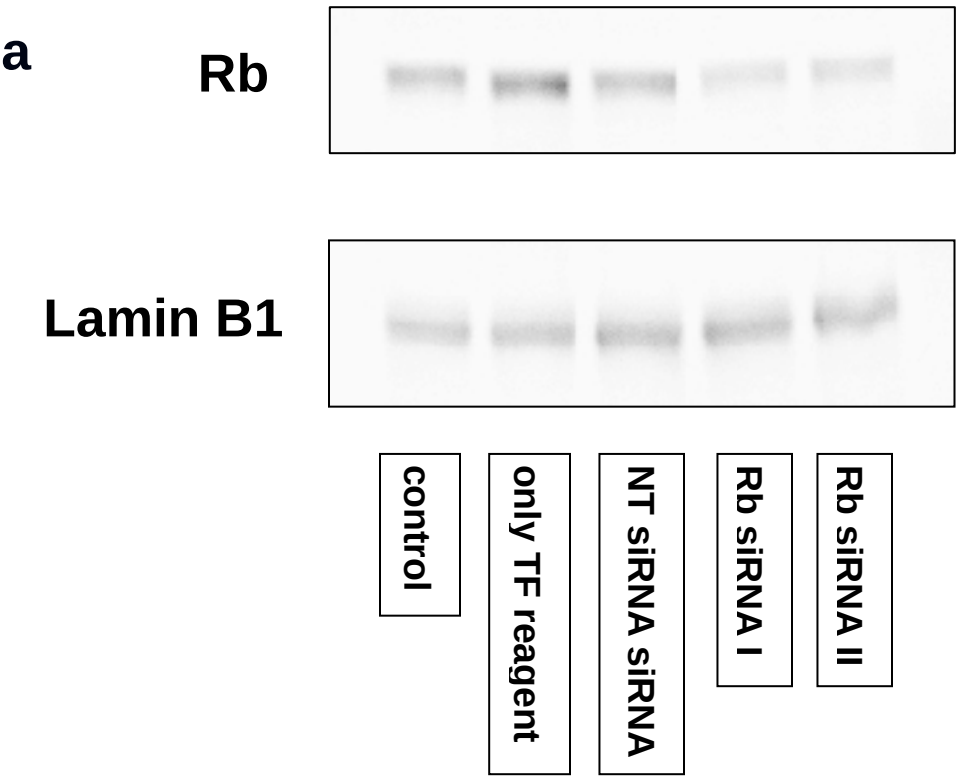
To determine whether Rb is essential key factor for the downregulation of MYCN oncogene by RA, luciferase reporter assays were performed following the transfection of Rb siRNA or control siRNA (non-targeting siRNA, NT siRNA).

First of all, the effect of siRNA transfection on Rb expression in neuroblastoma was examined. Neuroblastoma cell line, SH-SY5Y cells were plated in 6-well plate and after 2 days, control siRNA, Rb siRNA I, and Rb siRNA II were transfected into these cells. As controls, cells were treated without any reagent and siRNA (control), or with only transfection reagent, but not siRNA (only TF reagent). As shown in figure 16A and B, transfection of Rb siRNA I resulted in significant reduction of Rb protein (60% reduction), but not control siRNA (NT siRNA).

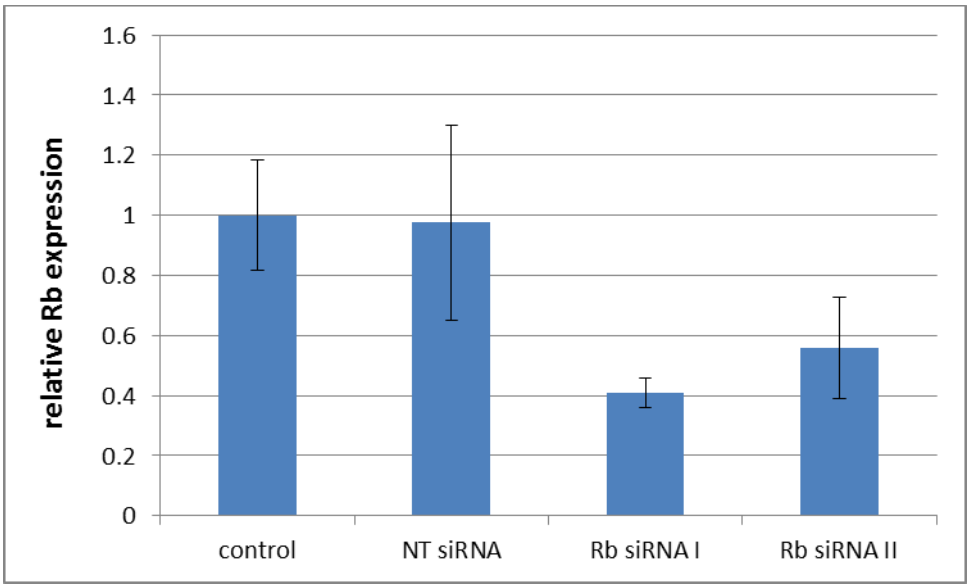
Next, the effect of Rb siRNA I and control siRNA on MYCN promoter activity was examined. SH-SY5Y cells were plated in 6-well plate and after 2 days, control siRNA or Rb siRNA I were transfected into these cells. 2 days after siRNA transfection, two luciferase plasmids, Renilla luciferase vector driven by MYCN promoter (4.5 MYCN-pGL4.70 Rhluc) and 50 ng of the pGL4.10 firefly luciferase vector as control for transfection efficiency were transfected. After 3 days, cells were harvested and luciferase assay was performed to examine MYCN promoter activity. As shown in figure 16C, knockdown of Rb did not affect MYCN promoter activity.

Lastly, the effect of Rb knockdown on MYCN repression by RA was examined. 1 day after transfection of two plasmids following siRNA transfection described previously, media with control solvent or 5 μM RA were added and SH-SY5Y cells were cultured for 2days. After harvest, luciferase assay was performed to examine MYCN promoter activity. As shown in figure 16D, as compared with controls, RA did not repress MYCN promoter activity in cells which were transfected with Rb siRNA I. This result indicates that Rb knockdown impaired the repression of MYCN promoter activity by RA.

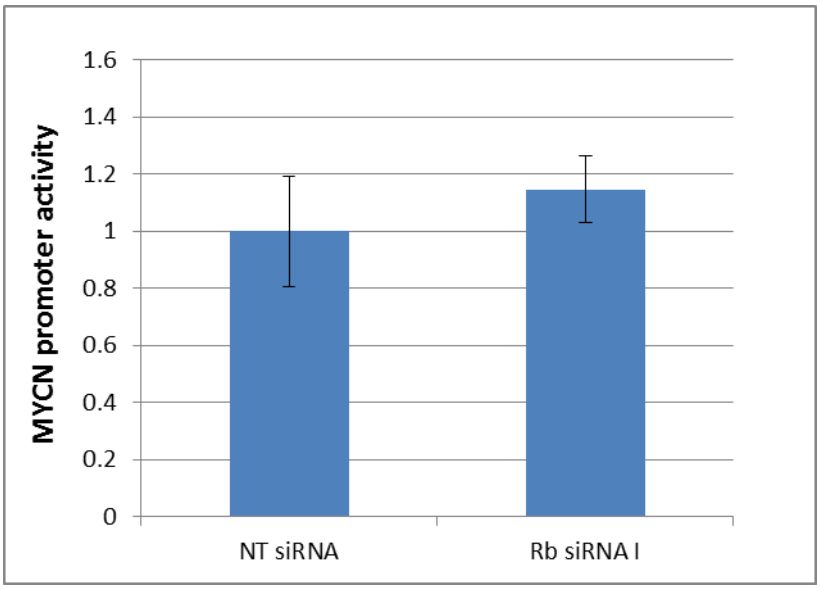
a


Rb
Lamin B1
control
only TF reagent
NT siRNA siRNA
Rb siRNA I
Rb siRNA II

b


relative Rb expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
NT siRNA
Rb siRNA I
Rb siRNA II

c



d

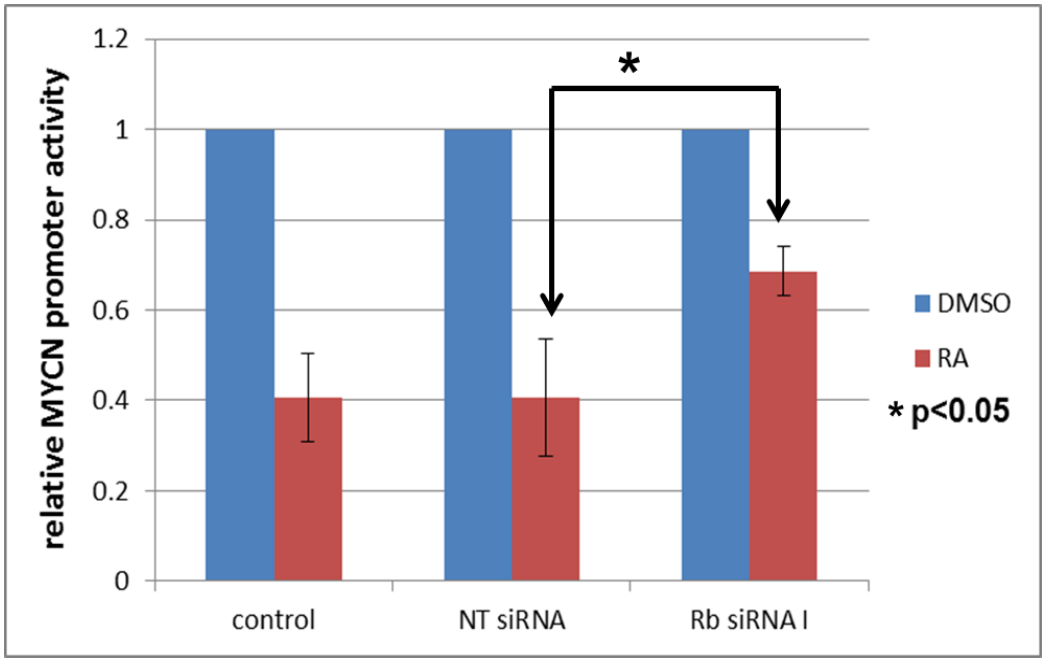


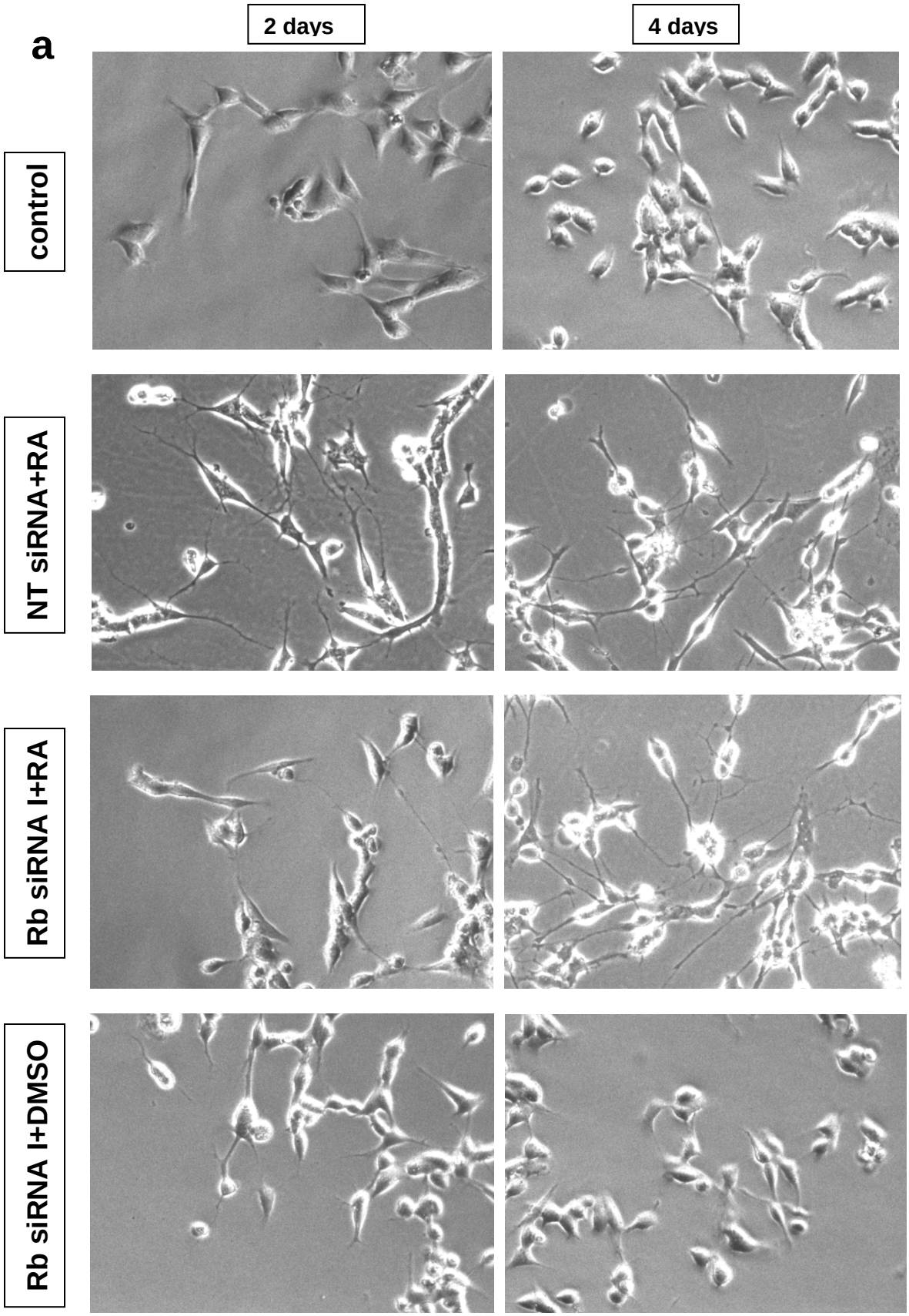
Figure 16. Effect of Rb siRNA transfection in SH-SY5Y.
(a) Western blot analysis of Rb protein. (b) Densitometric analysis of Rb expression, normalized by control (untreated). (c) Effect of control siRNA and Rb siRNA I on MYCN promoter activity. (d) Effect of Rb knockdown on the repression of MYCN by RA. Results are expressed as the mean±SD of at least three independent experiments.

3.3.4 Determine the Rb is essential for the induction of cell differentiation of neuroblastoma by RA

To determine whether Rb is essential, not only for the downregulation of MYCN oncogene, but also cell differentiation by RA in neuroblastoma, the importance of Rb in cell differentiation by RA was examined following the transfection of Rb siRNA or control siRNA (non-targeting siRNA).

1.6×10^5 SH-SY5Y cells were plated in 6-well plate and after 2 days, Rb siRNA or control siRNA were transfected into these cells. 3 days after siRNA transfection, cells were cultured in medium with 5 μM RA or control solvent for 4 days. In 4 independent fields, cells were observed by microscope and cell with neurite longer than 100 μm was counted as differentiated [246, 247].

As shown in figure 17, 2 days after RA treatment, about 70% of control siRNA-transfected cells treated with RA were differentiated. However, only 40% of Rb siRNA-transfected cells treated with RA were differentiated. On the other hand, Rb siRNA-transfected cells cultured without RA were not differentiated. This result indicates that the transient knockdown of Rb impaired the induction of cell differentiation significantly, and therefore, Rb is involved in cell differentiation by RA in neuroblastoma.


a
2 days
4 days
control
NT siRNA+RA
Rb siRNA I+RA
Rb siRNA I+DMSO

b

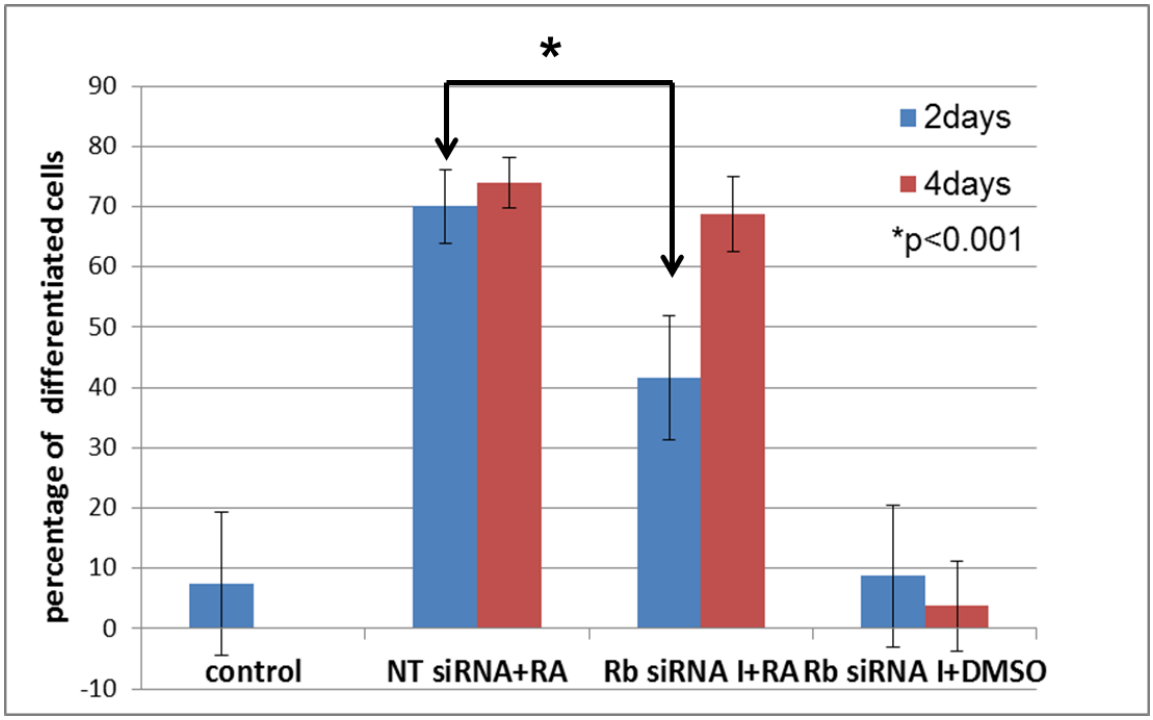


Figure 17. Rb knockdown impaired the induction of cell differentiation in SH-SY5Y by RA.
Neuroblastoma cell line, SH-SY5Y cells were cultured in different conditions, without RA and siRNA (control), with 5 µM RA and NT siRNA, with 5 µM RA and Rb siRNA I, and with control solvent (DMSO) and Rb siRNA I for 4 days. (a) Photographed cell morphology. (b) Percentage of differentiated cells. Values are the means±SD of at least four independent fields.

3.3.5 Conclusion and discussion

In this specific aim, reporter assays with RNA interference revealed that Rb knockdown impaired the repression of MYCN promoter activity by RA. This result indicates that Rb is involved in the RA induced-downregulation of MYCN oncogene in neuroblastoma, although the results in specific aim 2 imply that Rb is not key molecule.

Analysis of cell differentiation with siRNA also revealed that Rb knockdown impaired the induction of cell differentiation in neuroblastoma by RA. One recent study which revealed that Rb, especially phosphorylation of Rb at Ser612 is essential for the induction of cell differentiation in neuroblastoma by RA supports our result [234].

As shown in figure 16C, as compared with control siRNA, Rb siRNA transfection did not affect MYCN promoter activity without the presence of RA. RA is able to repress the MYCN in the presence of Rb (figure 16D). ChIP assays in specific aim 2 revealed that the Rb binding to the MYCN promoter does not change during RA treatment. Taken together, these findings indicate that Rb itself does not repress the MYCN expression, but essential for RA-induced downregulation of MYCN by acting as the connector for the real repressor which represses the MYCN expression responding to RA treatment in neuroblastoma.

CHAPTER 4. CONCLUSION AND DISCUSSION

Results in this study displayed that Rb is essential, but indicated that it is not a main repressor protein for the downregulation of MYCN oncogene.

Initially we hypothesized that Rb is the key molecule mediating the downregulation of MYCN in neuroblastoma by RA. RA was shown to induce the growth arrest of breast cancer cells through decreasing PKC alpha expression and ERK MAPK phosphorylation which decreases the phosphorylation of Rb [240, 248]. This report supports our initial hypothesis and suggests same or similar molecular events occur in neuroblastoma. Similarly, many new studies suggest the utility of cyclin/CDK inhibitor for neuroblastoma treatment, because inhibition of cyclin/CDK leads to the growth arrest of neuroblastoma cells through the activation of Rb [244, 249]. However, the data presented here do not correlate with either of these reports.

Experiments in specific aim 1 revealed that the key molecular events for MYCN repression by RA should occur before or around 6 hours after RA treatment. However, the binding of activator E2Fs (E2F1, E2F2, and E2F3) and repressor E2Fs (E2F4 and E2F5) at the MYCN promoter did not change during RA treatment. These results indicated that the interaction of E2F proteins with the MYCN promoter is not the key event for the downregulation of MYCN by RA in neuroblastoma.

In specific aim 2, ChIP assays revealed something unexpected. The binding of Rb and other Rb family proteins, p107 and p130 at the MYCN promoter did not change during RA treatment. Also, no changes of Rb phosphorylation state were

detected. These results implied that Rb is not a key molecule for MYCN downregulation by RA in neuroblastoma.

Lastly, however, experiments with RNA interference in specific aim 3 revealed that Rb knockdown impaired both of the repression of MYCN promoter activity and the induction of cell differentiation in neuroblastoma by RA. Those data indicate that Rb is involved in MYCN repression by RA in neuroblastoma.

Rb is the first tumor suppressor gene found that regulates cell cycle progression [250]. However, numerous new studies are still ongoing in order to uncover its unknown functions and biological active forms for cell cycle control, differentiation, and DNA damage repair. Many of these studies that examine posttranslational modifications, such as phosphorylation, acetylation, methylation, SUMOylation, and ubiquitinylation, are still not well-understood. For example, in 2010, Pickard et al. discovered that acetylation of Rb is required for the differentiation of keratinocytes [233]. Also, in 2014, Hattori et al. reported the findings that differ from previous concept of Rb phosphorylation. They found that the phosphorylation of Rb at Ser612 is essential for RA-induced cell differentiation in neuroblastoma and this phosphorylation does not impair the Rb binding to E2F [234]. Therefore, other posttranslational modifications of Rb are possibly involved in the RA-induced MYCN downregulation, despite the fact that no changes in phosphorylation were detected in our study. Accordingly, further studies which focus on the posttranslational modifications of Rb in neuroblastoma with RA treatment may be done.

Again, our results indicate that Rb is an essential factor, but not the main repressor for the downregulation of MYCN by RA in neuroblastoma. Even without RA treatment, there is a high probability that Rb is essentially located at the MYCN promoter. In other words, Rb is not changed during RA treatment for the MYCN repression. However, it is still essential for MYCN repression by RA. This indicates that Rb is not the real repressor, but involved in MYCN repression, possibly by acting as the connector for the primary repressor and RA treatment exerts this real repressor which represses MYCN expression. With the gathered information, two models are proposed (figure 18 and 19). The first model is that RA treatment places the primary repressor on Rb-E2F complex at the MYCN promoter (figure 18). The second model is that RA treatment activates the primary repressor by inactivating the molecule which inhibits the primary repressor (figure 19). Nuclear receptors such as RARs and vitamin D receptors (VDRs) were shown to inhibit the activity of some proteins through the direct interaction in a ligand-dependent manner [251-253]. Although correlation between the alteration in RAR/RXR and RA-resistance in neuroblastoma was not seen, the expression level of RARβ has correlation with good outcome in neuroblastoma [83, 138]. Also, exogenous RARβ expression sensitizes neuroblastoma to RA treatment [254]. Thus, the complex such as RA and RAR is possibly involved in RA-induced MYCN repression by acting as inhibitor for the inhibitor (shown as xxx in figure 19) of the primary repressor (shown as X in figure 18 and 19). Again, in both models, Rb is an essential part of the repressor complex as a connecter. Next, the question is, ”What is Rb serving to connect?”

To date, over 100 Rb binding proteins were reported [255] and Rb interacts with chromatin-remodeling enzymes for transcriptional repression, such as histone deacetylases (HDACs) [223, 224], histone methylases (SUV39H1, SUV420H1, SUV420H2) [207, 231, 235], the methyl-lysine binding protein HP1 [230], and DNA methyltransferase, (DNMT1) [256]. Rb recruits these enzymes to modify chromatin structure and control the expression of target genes. Interestingly, in the first two articles which reported the recruitment of the histone deacethylase by Rb in 1998, the histone deacethylase inhibitor, tricostatin A (TSA) inhibited the transcriptional repression by Rb-histone deacetylase complex. Their results indicate that the existence of Rb at the promoter is not sufficient for transcriptional repression, and that the repressive effect of Rb may be mediated through HDAC and not directly caused by Rb. These data supports our conclusion.

In order to identify the real repressor in neuroblastoma, several experiments may be done. As previously described, many proteins may form the repressor complex with Rb, therefore, ChIP assays with antibodies for those proteins found in previous reports may reveal the partner of Rb during RA treatment in neuroblastoma. Also, Western blot with antibodies for candidate proteins following the immunoprecipitation (IP) with Rb antibody with samples which are treated with and without RA may provide new information. IP with Rb antibody followed by SDS-PAGE and protein characterizations, such as mass spectrometry, X-ray crystallography, cryo-electron microscopy, and NMR may be

done. Gene deletion or knockdown with RA treatment should also be done for the verification.

The goal of this study is to uncover the molecular mechanism through which RA downregulates MYCN oncogene in neuroblastoma and apply this knowledge towards better clinical application for neuroblastoma patients. With the results in this study and new information which may be provided by the proposed experiments, several suggestions may be proposed. For example, gene diagnosis for the genes which are involved in RA-induced MYCN downregulation may be done. It is because the gene diagnostic test would help neuroblastoma patients avoid ineffective treatments which could reduce the well-being of patients. Neuroblastoma tumors, and even neuroblastoma cell lines often show RA-resistance [130, 169, 257]. This resistance is possibly attributed to the abnormality of genes which are involved in MYCN repression by RA, including chromatin remodeling enzymes and even Rb, because the abnormalities of the Rb gene do exist in neuroblastoma, though they are rare [258].

Another suggestion may be considered for the pharmacological therapy. HDAC inhibitors show potential as new drugs for neuroblastoma, because they increase the expression of tumor suppressor genes such as $p21^{WAF1/CIP1}$ and $p27^{Kip1}$, and induce the growth arrest, differentiation and apoptosis in neuroblastoma [189, 191, 192, 259]. However, in the case that the real repressor which we model is HDAC, we should avoid the combination of RA and HDAC inhibitors for the treatment, because HDAC inhibitors could cancel the effect of RA.

In conclusion, this study revealed a small part of the mechanism through which RA downregulates the expression of MYCN oncogene. Our findings may be applied towards better clinical applications for neuroblastoma patients, including better usage of RA and gene therapy. Therefore, further studies, especially identification of the elusive primary repressor which of MYCN expression following RA treatment in neuroblastoma still require further work in the future.

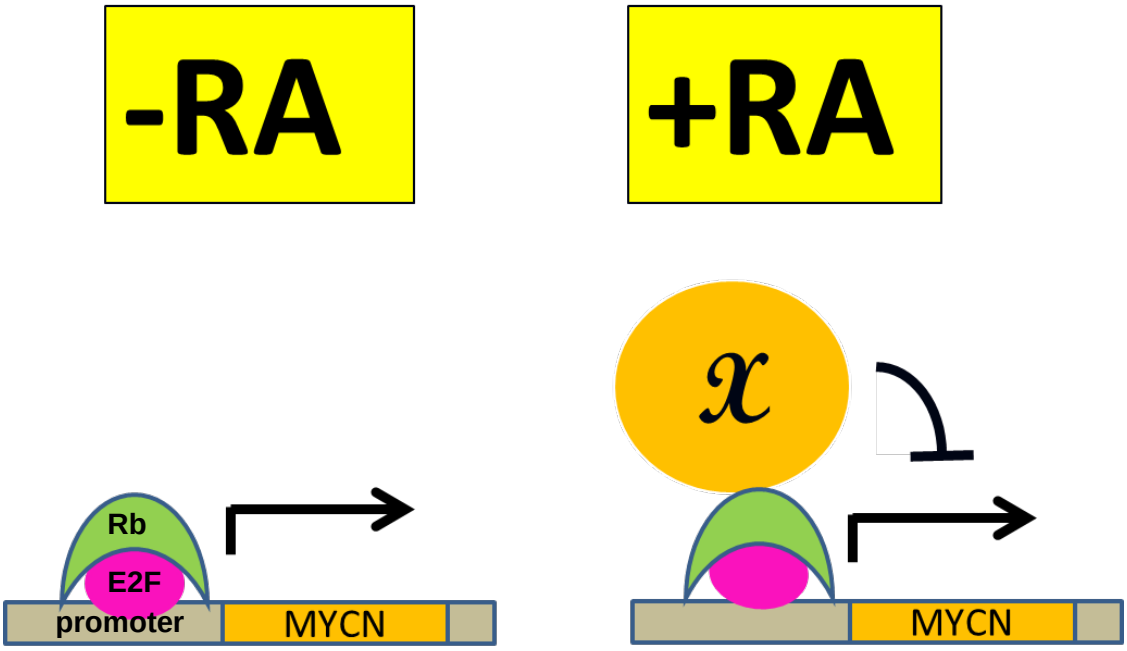


Figure 18. First proposed model of the downregulation of MYCN by RA.
With RA treatment, the real repressor (X) interacts with Rb-E2F complex and represses the MYCN expression.

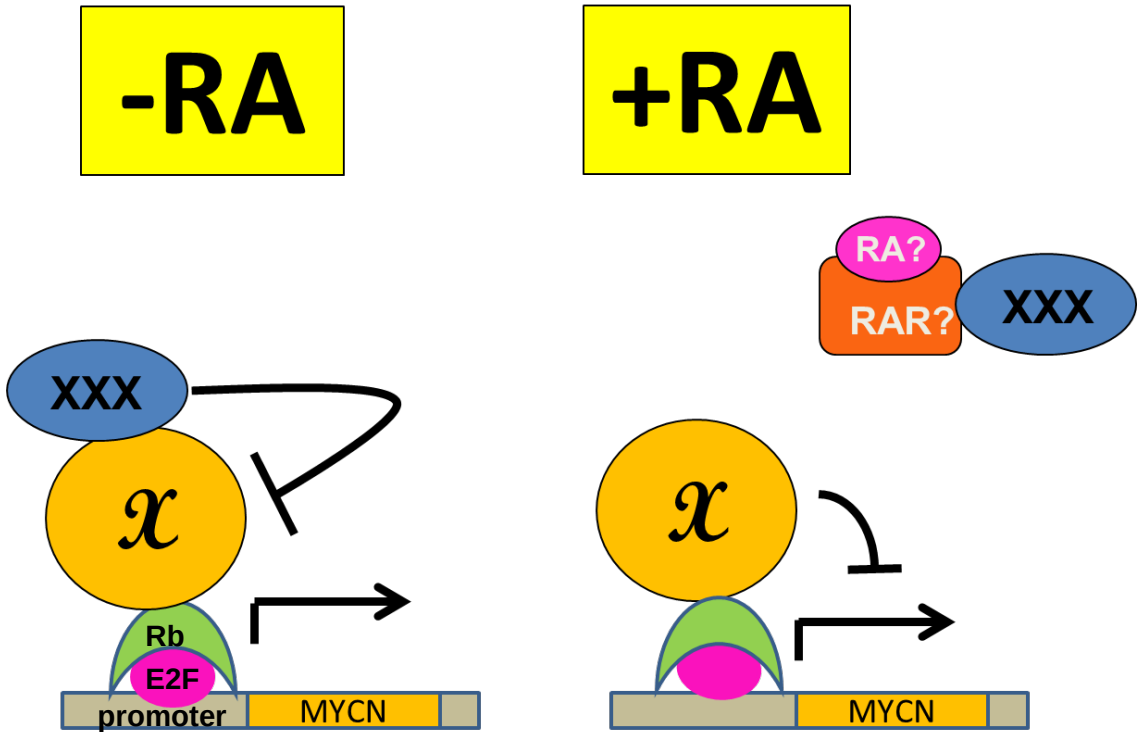


Figure 19. Second proposed model of the downregulation of MYCN by RA.
RA treatment activates the primary repressor (X) by sequestering the molecule (xxx) which inhibits the primary repressor at the MYCN promoter.

CHAPTER 5. MATERIALS AND METHODS

Tissue culture

Tissue culture reagents were purchased from Mediatech (Herdon, VA). The MYCN-amplified neuroblastoma cell line, LA-N-5 was cultured in Iscove's modification of DMEM (Mediatech, Herdon, VA) containing 6 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS) with or without antibiotics at 37℃ and 5% CO_2. The non-MYCN-amplified neuroblastoma cell line, SH-SY5Y was cultured in Dulbecco's Modification of Eagle's Medium (Mediatech, Herdon, VA) containing 6 mM glutamine and 10% FCS with or without antibiotics at 37℃ and 5% CO_2.

Retinoic acid

All-trans RA was purchased from Sigma (St Louis, MO) and was dissolved in dimethyl sulfoxide to a concentration of 10 mM. The stock solution was kept at -80℃.

Protein extraction

After rinse with PBS, cells were collected and pelleted for 5 minutes at 1000 × g at 4℃. Cell pellet was resuspended in protein extracting buffer containing protease inhibitors and phosphatase inhibitors (Roche, Indianapolis, IN).

Supernatant was collected and protein concentration was measured by BCA assay (Pierce, Rockford, IL) according to the manufacturer's instruction.

Western blot

MYCN monoclonal antibody NCM II 100 was kindly provided by Naohiko Ikegaki. The Lamin B1 antibody was purchased from Imgenex (Sorrento Valley, CA). The antibodies for Rb, phospho-Rb Ser608, Ser780, Ser795, and Ser807/811 were purchased from Cell Signaling technology (Danvers, MA). The antibody for phospho-Rb Ser612 was purchased from MBL Bion (Des Plaines, IL). The antibody for phospho-Rb Thr821/826 was purchased from Santa Cruz biotechnology (Dallas, TX). The horseradish peroxidase-conjugated donkey anti-rabbit antibody and the horseradish peroxidase-conjugated goat anti-mouse antibody were purchased from Amersham Biosciences (Piscataway,NJ). 35 µg of extracted protein lysate was run in SDS-PAGE gel and transferred to Immobilon-P (Millipore, Billerica, MA) membrane. Then, immunoblotting with antibodies were performed. The bands were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and quantified by ImageJ (NIH).

RNA extraction

After RA treatment or control treatment, cells were lysed with Trizol (Life technologies, Carlsbad, CA). Next, chloroform was added and aqueous phase was isolated. Finally RNA was precipitated with isopropyl alcohol. Total RNA concentration was measured by NanoDrop 2000 (Thermo Scientific, Rockford, IL).

RT-qPCR

1 µg of total RNA was converted to 20 µl of cDNA with random hexamers and Invitrogen High Capacity cDNA RT Kit with RNase Inhibitor. Realtime PCR reactions were performed in triplicate using reverse transcription reaction and iQ SYBR Green Supermix (BioRad, Hercues, CA) on CFX96 Touch^{TM} Real-Time PCR Detection System (Biorad). Total PCR reaction volume was 20 µL with 300 nM of each primer (forward primer: 5'-CCGGGCATGATCTGCAA-3', reverse primer: 5'-CCGCCGAAGTAGAAGTCATCTT-3'). The PCR profile was 95℃ for 5 minutes followed by 30 cycles of 95℃ for 15 seconds and 60℃ for 1 minute. Melt curve analysis was done in order to ensure only one PCR product was being produced. The relative amount of MYCN was normalized with housekeeping gene, GAPDH, using the cycle threshold (Ct). And the fold change (delta delta Ct) was calculated by comparing the delta Ct value of each experimental condition to the control treatment. All data points represent the average of three experiments.

Chromatin precipitation (ChIP)

Cells were plated and treated with 5 µM RA or solvent in 175 cm^2 flasks for each time point, 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours. Upon harvest, cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature. Then, glycine for the final concentration of 125 mM was added to stop cross-link. After 5 minutes incubation, cells were scraped and pelleted in 50 ml tube. After harvest, ChIP assays were performed with SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) (Cell Signaling Technology, Danvers, MA) following manufacturer's protocol. Antibody for E2F1 was purchased from Cell Signaling Technology. Antibodies for E2F2, E2F3, E2F4, E2F5, Rb, p107, and p130 and normal rabbit serum were purchased from Santa Cruz Biotechnology (Dallas, Texas).

Real-time PCR reactions were performed in triplicate using iQ SYBR Green Supermix (BioRad, Hercules, CA) on CFX96 Touch™ Real-Time PCR Detection System (Biorad). 2 µL of aliquots of the co-precipitated DNA elutes or negative control were added to 20 µL PCR reactions containing 300 nM of each primer. Primers, E2FChips (5'-AATGACAAGCAATTGCCAGGC-3') and E2FChipas were used to amplify a region of the MYCN promoter. This experiment was repeated 3 times and all data points represent the average of three experiments.

RNA interference (RNAi)

Control (non-targeting) siRNA was obtained from Santa Cruz biotechnology (Dallas, TX). Rb siRNA I and Rb siRNA II were obtained from Cell signaling technology (Danvers, MI). Cells were plated in 6-well plate and 100 nM of each siRNA were transfected using 7.5 µL of Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY) according to the manufacturers' protocol.

Luciferase reporter assay

1 µg of the pGL4.70 Renilla luciferase vector driven by MYCN promoter (4.5 MYCN pGL4.70 Rhluc) and 50 ng of the pGL4.10 firefly luciferase vector as control for transfection efficiency were transfected into SH-SY5Y cells with 3 µL of GeneJammer transfection reagent (Agilent Technologies, Santa Clara, CA) according to manufacturer's instruction. Dual Luciferase® Reporter Assay (Promega, Madison, WI) was performed following manufacturers' protocol. Relative light intensities were quantified with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). All data points represent the average of at least three independent assays.

Analysis of cell differentiation

Cell with the neurite longer than 100 µm were counted as differentiated cell and expressed as percentage of total number of cells counted [246, 247]. Cells were counted in at least four independent fields.

Statistical analysis

Statistical analysis was performed in Microsoft Excel by two-tailed student's t-test assuming equal variance.

CHAPTER 6. REFERENCES

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