Doctoral Thesis

Non-genomic Actions of Diterpenoid Acids: with Special Reference to Differentiation Induction of Human Neuroblastoma and Hepatoma Cells

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CONTENTS

ABBREVIATIONS

2-PCPA	*trans*-2-phenylcyclopropylamine
4EGI	inhibitor of eIF4E/eIF4G interaction
9CRA	9-*cis* retinoic acid
ACR	acyclic retinoid
AOF	amine oxidase (flavin-containig)
APP	amyloid-β precursor protein
ATCC	American Type Culture Collection
ATRA	all-*trans* retinoic acid
BDNF	brain-derived neurotrophic factor
BINAP	2,2'-bis(diphenylphosphino)-1,1'-binaphthyl
BLAST	Basic Local Alignment Search Tool
BLBP	brain lipid binding protein
bp	base pairs
CCD	charge coupled device
CCND1	cyclin D1
CDK	cyclin-dependent kinase
cDNA	complementary DNA
ChAT	choline acetyltransferase
ChIP	chromatin immunoprecipitation
ChIP-seq	chromatin immunoprecipitation sequencing
CHX	cycloheximide
CoA	coenzyme A
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
CRABP	cellular retinoic acid binding protein
DMAPP	dimethylallyl diphosphate
DME	Dulbecco's modified Eagle's
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DP	DNA-binding partner of E2Fs
DR	direct repeat
eIF	eukaryotic translation initiation factor
ES	embryonic stem
FA	farnesoic acid
FABP	fatty acid binding protein
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum

FBXO	F-box protein for other domain
FPP	farnesyl diphosphate
FPPS	farnesyl diphosphate synthase
G1	gap phase 1 in cell cycle
G2	gap phase 2 in cell cycle
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial cell derived neurotrophic factor
GGA	geranylgeranoic acid
GGal	geranylgeranial or geranylgeranyl aldehyde
GGOH	geranylgeraniol
GGPP	geranylgeranyl diphosphate
GGPPS	geranylgeranyl diphosphate synthase
GluR	glutamate receptor
GPP	geranyl diphosphate
GSK	glycogen synthase kinase
H3K4	histone H3 lysine-4
HDAC	histone deacetylase
HK	hexokinase
HMG-CoA	3-hydroxy-3-methyglutaryl-CoA
HMT	histone methyltransferase
HOX	homeobox
IC_{50}	inhibitory concentration at a half maximum
IPP	isopentenyl diphosphate
JARID	Jumonji, AT rich interactive domain
JMJD	Jumonji domain
kDa	kilodalton
KDM	lysine (K)-specific demethylase
K_i	inhibitory constant for enzyme reaction
M	mitosis phase in cell cycle
MAO	monoamine oxidase
MeCP	methyl CpG binding protein
MEP	methylerythritol phosphate
miRNA	microRNA
mRNA	messenger RNA
MVA	mevalonic acid
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor of kappa-light polypeptide gene enhancer in B-cells
NGF	nerve growth factor

NR	nuclear receptor
NT	neurotrophin
NTF	neurotrophic factor
NTR	neurotrophin receptor
NTRK	neurotrophic tyrosine kinase, receptor
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with Tween 20
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phospholipase C
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PTB	polypyrimidine tract binding protein
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
Pu	purine
PVDF	polyvinylidene difluoride
RAR	retinoic acid receptor
RARE	retinoic acid response element
RB	retinoblastoma protein, ret proto-oncogene
RET	ret (rearranged during transfection) proto-oncogene
RIPA	radio immunoprecipitation assay
RNA	ribonucleic acid
RNAi	RNA interference
ROR	retinoic acid receptor-related orphan receptor
RORE	ROR-response element
RPI-1	ret protein inhibitor-1
rRNA	ribosomal RNA
RT-qPCR	reverse transcription and quantitative polymerase chain reaction
RTT	Rett syndrome
RXR	retinoid X receptor
RXRE	retinoid X response element
S	DNA synthesis phase
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Shc	Src homology 2 domain containing
siRNA	small interfering RNA
SMCY	selected mouse cDNA on Y

SUMO	small ubiquitin-related modifier
SWI/SNF	switch/sucrose nonfermentable
$T_{1/2}$	half-life
TFDP	transcription factor DP-1
TLX	trophoblast-lymphocyte cross-reaction
TNF	tumor necrosis factor
Tirs	tris(hydroxymethyl)aminomethane
TR	testicular receptor
TYH	tyrosine hydroxylase
UTR	untranslated region
UV	ultraviolet
ZF-Jmj	zinc finger Jumonji

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Chapter I

GENERAL INTRODUCTION

Despite a plethora of studies establish “genomic actions” of diterpenoid acids, the actions partially explain the mechanisms of broader biological effects of diterpenoid acids. Hence, “non-genomic actions” become prospective research topics during the last decade. In this thesis, we describe differentiation-inducing effects of a diterpenoid geranylgeranoic acid, and discuss novel “non-genomic actions” of the diterpenoid acid in human neuroblastoma and hepatoma cells in comparison with another diterpenoid acid, retinoic acid.

I. 1. Terpenoids biosynthesis

Terpenoids, also known as isoprenoids, a class of natural organic compounds consisting of the five-carbon unit called “isoprene”, are synthesized ubiquitously and play essential roles among eubacteria, archaebacteria and eukaryotes including human. They are derived through condensations of isoprene compound isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Two distinct and independent pathways exist to biosynthesize IPP: the classical mevalonate (MVA) pathway (**Fig. I-1**) [Chaykin et al, 1958; Spurgeon & Porter, 1981] and a mevalonate-independent methylerythritol phosphate (MEP) pathway [Surmacz & Swiezewska, 2011]. Although MEP pathway is limited to photosynthetic plants and bacteria, the MVA pathway is an important cellular metabolic pathway present ubiquitously in all organisms. Acetyl-CoA is generated in a cell, which is added another acetyl-CoA and becomes acetoacetyl-CoA in a reaction catalyzed by thiolase. Produced and pooled IPP are converted to DMAPP by an IPP isomerase. The enzymes of the MVA pathway have been studied from a number of organisms, including humans. 3-Hydroxy-3-methyglutaryl-CoA (HMG-CoA) reductase, the best-characterized and rate-limiting enzyme in

the cholesterol synthetic pathway, is the target of the statin class of cholesterol-lowering drugs [Alberts et al, 1980], the treatment of cardiovascular disease, and inflammatory processes [Liao & Laufs, 2005]. It is important for the production of DMAPP and IPP, which serve as the basis for the biosynthesis of molecules used in processes as diverse as isoprenoid synthesis, protein prenylation, cell membrane maintenance and N-glycosylation. It is also a part of steroid biosynthesis.

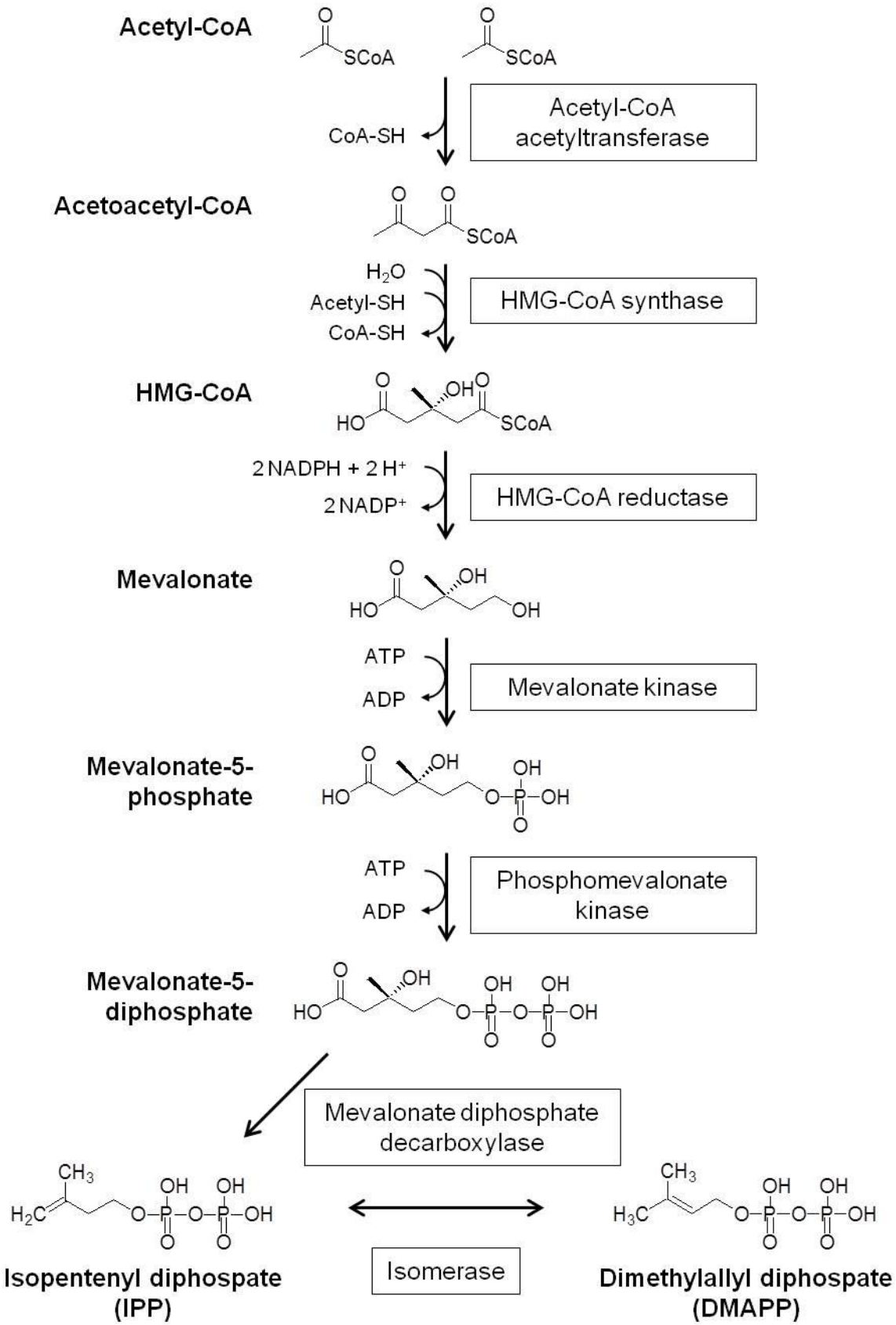

Fig. I-1. The mevalonate (MVA) pathway.

Beginning with the C_5 molecule DMAPP, a series of C_{10} GPP (geranyl diphosphate), C_{15} FPP (farnesyl diphosphate), C_{20} GGPP (geranylgeranyl diphosphate), and higher-molecular-weight isoprenoid diphosphates are formed by addition of C_5 IPP to the growing chain (**Fig. I-2**). GPP and FPP are produced through a "tail-to-head" addition of IPP by farnesyl diphosphate synthase (FPPS). The metabolic pathway from FPP branches into two, which is decided by FPPS and its interacting membrane enzymes. The major pathway is steroidogenesis. FPPS interacts with enzyme squalene synthase that produces squalene by a "tail-to-tail" condensation of 2 molecules of FPP. Squalene is a hydrocarbon and a triterpene, and is a natural and vital part of the synthesis of cholesterol, steroid hormones, and vitamin D in the human body. Another pathway is polyprenoid synthesis or non-steroidogenesis, where FPPS interacts with geranylgeranyl diphosphate synthase (GGPPS) that produces GGPP by prenyltransfer reaction from IPP to FPP. When described in more detail, there are two alternative pathways to produce GGPP [Bansal & Vaidya, 1994]. One pathway produces all-*trans* GGPP by *trans*-prenyltransferase interacting with FPPS. Such a course that produces all-*trans* GGPP is called "non-steroid" pathway. In plants, all-*trans* GGPP is also the precursor of carotenoids, gibberellins, tocopherols, and chlorophylls. Bimolecular condensation of GGPP in a tail-to-tail manner composes phytoene, a tetraterpene starting material for hundreds of carotenoids in plant cells but no phytoene synthase has been found in animal cells. In other words, the end products of all-*trans* GGPP include ubiquinone, chlorophyll or carotenoids. Contrastively in animal cells, all-*trans* GGPP is discovered with almost all organ tissues but no phytoene synthase has been found. Another one is *cis*-polyprenol pathway, where FPPS interacts with *cis*-prenyltransferase that produces 2-*cis* GGPP. The end products of

2-*cis* GGPP include polyprenols and their 2,3-dihydroderivatives, dolichols (C_{80-100}). Although significant amounts of polyprenols and dolichols are produced and detected in mammalian tissues, biological implication of these metabolites is scarcely understood, except for glycosyl-carrier function in glycoprotein and glycolipid synthesis [Surmacz & Swiezewska, 2011].

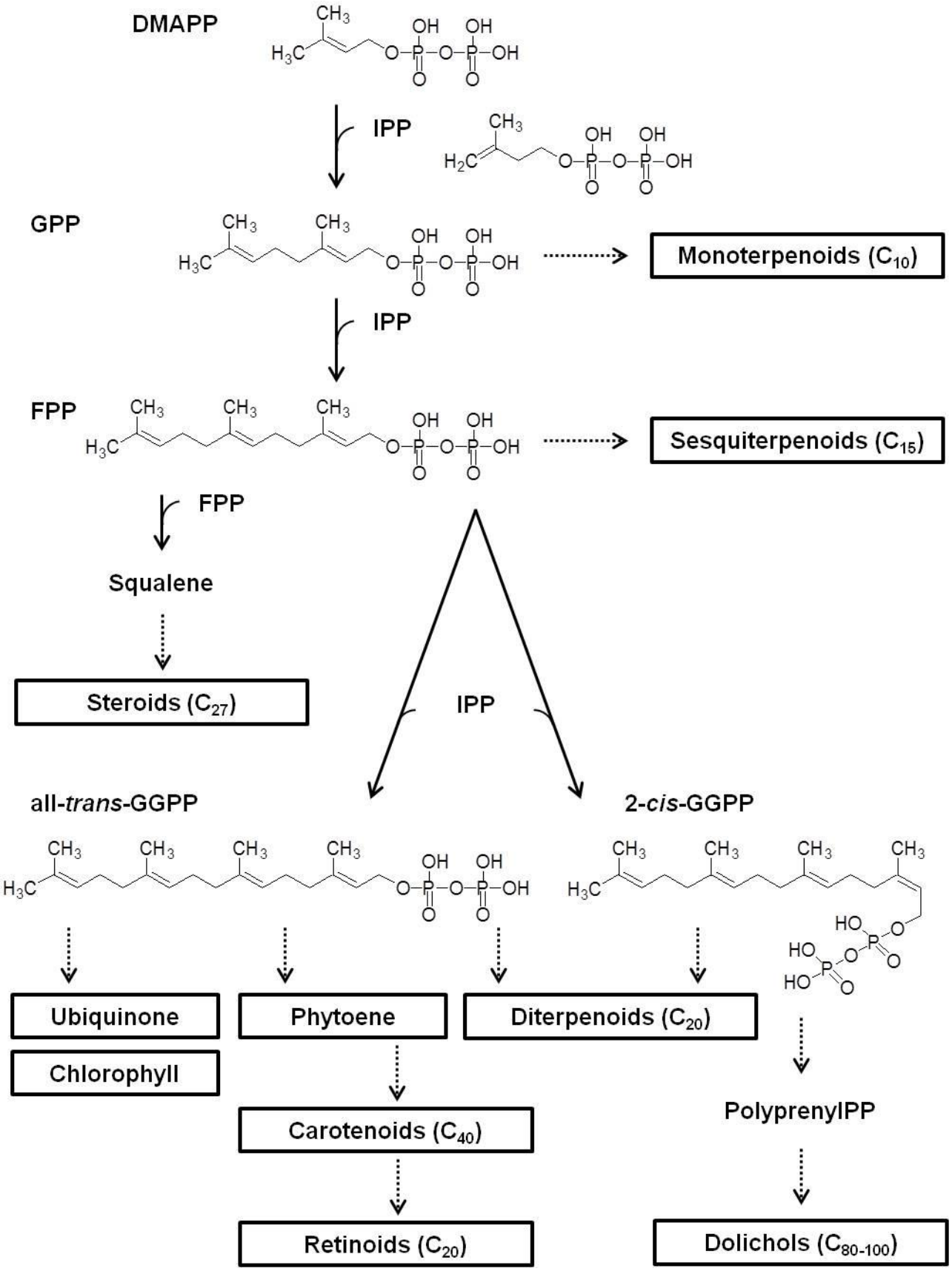

Fig. I-2. Schematic diagram of the isoprenoid production.

Endogenous isoprenoid metabolites, including monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes conteinig retinoids (C_{20}), sterols (C_{30}), carotenoids (C_{40}), ubiquinones (C_{50}), and dolichols ($C_{80\text{-}100}$) have physiological roles in plants and animals. Specifically, isoprenoids are involved in various steps of biological processes such as compartmentation, enzyme reaction, signal transduction, and even social activity. Furthermore exogenous terpenoids play as nutrient and/or medicinal compounds. Vitamin A is chemically a part of retinoids (C_{20}) and its metabolite all-*trans* retinoic acid has high activity of biological effects in particular (see later). In addition to providing one or two molecules of retinoids by β-carotene monooxygenase, some carotenoids (C_{40}) indicate associations between human health and alleviating metabolic diseases. Various other terpenoids are on the list of agents have the potential to treat disease, or some of them have entered clinical trial or therapy.

GGPP is one of the key isoprenoid intermediates to be allocated to the synthesis of various end products necessary for plant growth and defense. Recent observations have led to the identification of new biologically active compounds including farnesol and geranylgeraniol (GGOH), which can be derived by dephosphorylation of FPP and GGPP, respectively. Mitake and Shidoji suggested that a diterpenoid acid, GGA (geranylgeranoic acid) could be enzymatically derived from GGOH in mammal cells (**Fig. I-3**) [Mitake & Shidoji, 2012]. Inasmuch as the chemically-synthesized GGA shows antitumor and cell-differentiation inducing effects, a future detailed study may warrant novel biological roles of the intermediates of MVA pathway. In other words, GGPP can be exo- and/or endogenous source of physiologic active diterpenoid acid.

In conclusion, metabolites of isoprenoid pathway are crucially important for health promotion. However, the detailed metabolic pathway map and the molecular based evidence of these effects still remain unclear. Or clarification of the molecular mechanism is requisite.

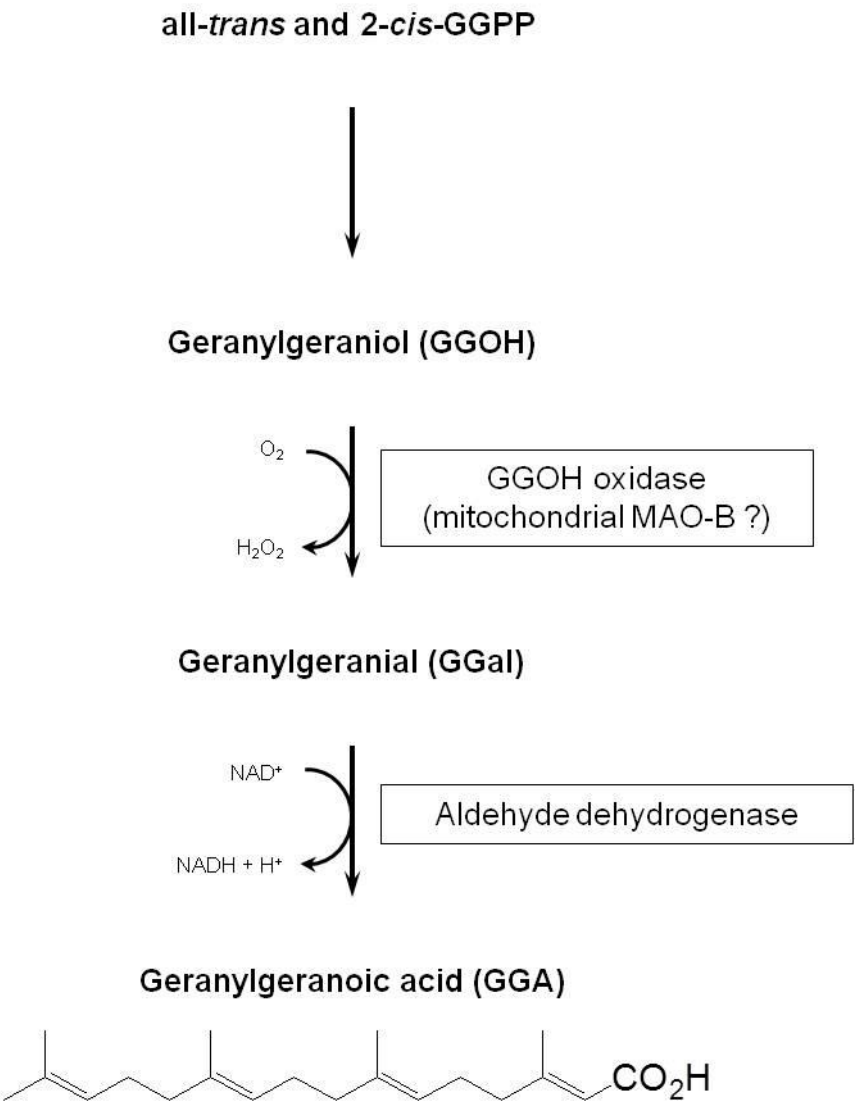

Fig. I-3. Enzymatic biosynthesis of GGA from GGPP.

I. 2. Retinoid effects; genomic and non-genomic regulations of gene expression

Most widely and deeply recognized diterpenoid are retinoids including 11-*cis* retinal and all-*trans* retinoic acid (ATRA), which has multiple biological activities in development, maintenance of epithelial tissues, vision, immune response and carcinogenesis (**Fig. I-4**). According to analysis of previous studies, retinoic acid binds to nuclear receptor proteins in the steroid and thyroid hormone receptor superfamily, so-called retinoic acid receptors (RARs). Three types of RARs gene (*RARA*, *RARB* and *RARG*) have been cloned [Ruberte, 1994], moreover, several isoforms generated by differential promoter usage and alternative splicing have been identified. RARs form heterodimers with retinoid X receptors (RXRs), which also belong to the superfamily and consist of three subtypes RXRα (coded by *RXRA*), RXRβ (coded by *RXRB*) and RXRγ (coded by *RXRG*), and these dimers act as transcriptional trans-regulatory factors. Recent studies have shown that retinoic acid binds to not only RARs but also peroxisome proliferator-activated receptors (PPAR) β/δ (but not the other PPARα and γ) [Al Tanoury et al, 2013], which a member of the nuclear receptor superfamily. Moreover, some in vitro studies suggest that retinoic acid signaling could be mediated by other receptors such as RORα, COUP-TFII or TR2/4 [Al Tanoury et al, 2013].

Nuclear retinoid receptors bind genome wide retinoid response elements through contact with coregulator, which are common for RARs and PPARs. The classical retinoid response elements are composed of a direct repeat of the motif 5'-PuG (G/T) TCA spaced by 0 (DR0), 1 (DR1), 2 (DR2), 5 (DR5), or 8 (DR8) base pairs [Al Tanoury et al, 2013]. Therefore, retinoic acid is considered to regulate gene expression via ligand-dependent nuclear receptor activation, which we call genomic action of retinoic acid in this thesis.

However, during the last decade, this scenario became more complicated with the discovery that retinoic acid also has extranuclear and nontranscriptional effects which influences expression of target genes, so-called nongenomic effects [Al Tanoury et al, 2013]. Indeed, several laboratories reported that retinoic acid activates rapidly the p38 mitogen-activated protein kinase, the p42/p44 extracellular signal-regulated kinase and the Janus kinase / signal transducer and activator of transcription 5 signaling cascade [Al Tanoury et al, 2013]. Moreover, it was demonstrated that, in response to retinoic acid, unconventional cytosolic located RARα triggers rapid local translation of the postsynaptic glutamate receptor GluR1 and subsequently an increase in synaptic strength [Al Tanoury et al, 2013]. Today, it is admitted that, in addition to the above classical genomic effects, retinoic acid also has number of nongenomic effects [Al Tanoury et al, 2013].

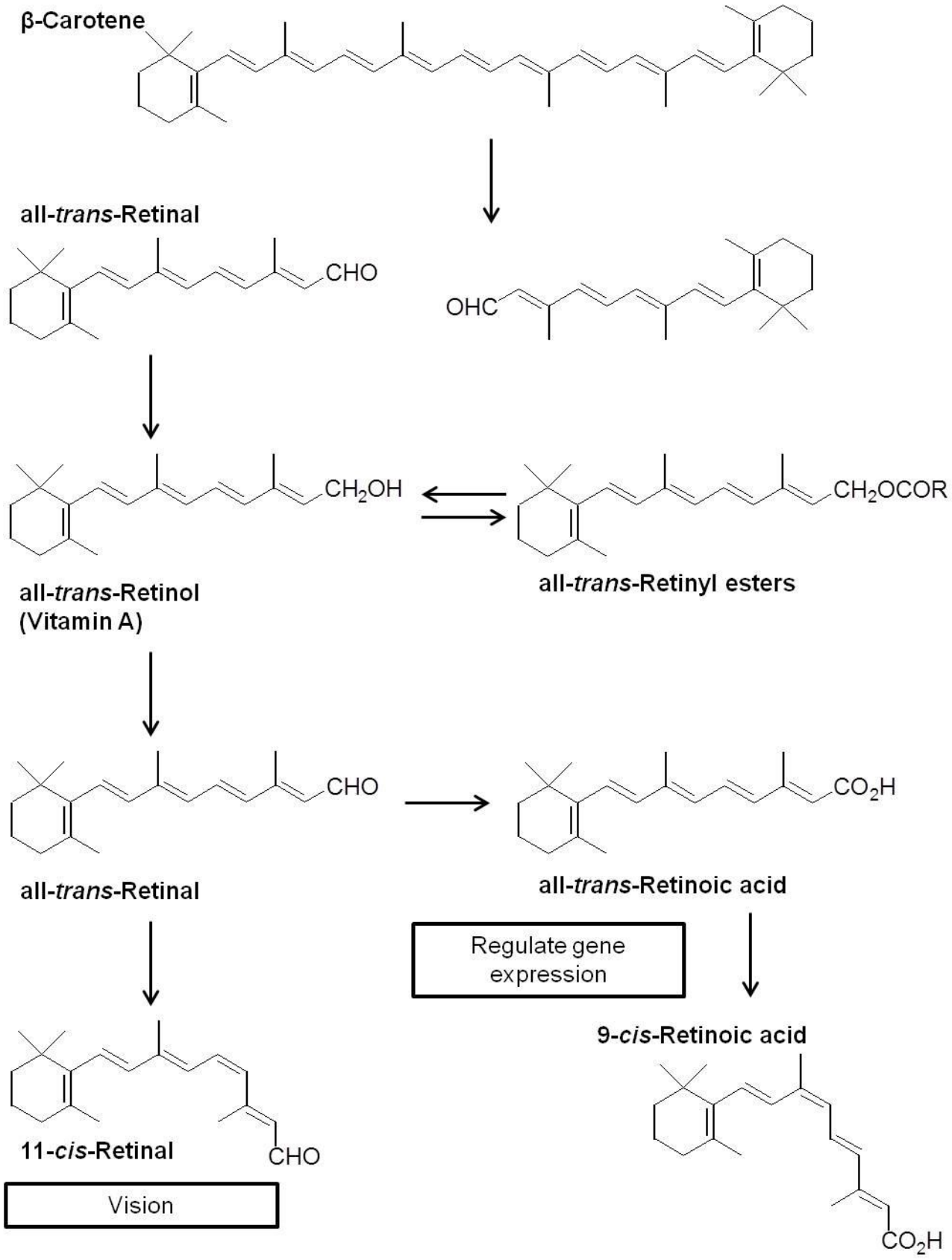

Fig. I-4. Vitamin A production and its metabolites.

I. 3. Acyclic retinoids

Today, several compounds that do not fit with the chemical atructure of retinoic acid but are much more active in several assays have been synthesized, and now, retinol, retinoic acid, other active metabolites and active synthetic compounds are grouped as “retinoids” [Al Tanoury et al, 2013]. Retinoids including ATRA, 9-*cis* retinoic acid (9CRA) and other retinoic acid derivatives are clinically utilized as chemotherapeutic agents for acute promyelocytic leukemia, but their side effects are sometimes so serious that it becomes difficult to continue administration of the retinoids [Yob & Pochi, 1987]. Therefore, synthetic retinoids without serious side effects are now certainly desirable especially in cancer prevention field. In this point of view, a promising synthetic retinoid with few side effects has been developed in Japan.

It has been shown that hepatocytes and neurons are both targets for retinoic acid in terms of the gene expression of intracellular retinoic acid binding protein (CRABP), RARs, and RXRs. For as much as GGA is a potent ligand for CRABP, RAR, and RXR and it acts as a potential agonist of natural retinoids [Muto et al, 1981; Araki et al, 1995; Yamada et al, 1994], GGA and its biological active derivatives have been called as “acyclic retinoids”. However, the acid shows some different properties from those of retinoic acid. For example, GGA induced cell death in human hepatoma-derived cells, whereas ATRA and 9CRA did not [Nakamura et al, 1995]. In addition, although like natural retinoids, acyclic retinoids show a strong antitumor activity, their toxicity is much less than that of retinoic acid [Muto & Moriwaki, 1984; Moriwaki et al, 1988]. Furthermore, recent studies on GGA have reported several cytological actions such as an incomplete autophagic response [Okamoto et al, 2011] and phospholipid hydroperoxide glutathione peroxidase

preventable cell death [Shidoji et al, 2006], both of which are impossible to be explained by nuclear retinoid receptor-mediated pathways or genomic actions of retinoids.

I. 4. Brief outline of the thesis

In this thesis, we describe some biological effects of diterpenoid acids, particularly GGA and ATRA (**Chapters I and VI**). Their biological actions described here are categorized into either genomic (**Chapter III**) or non-genomic actions. The genomic actions described in this thesis consist of 2 categories, which are conveyed through nuclear retinoid receptors or other orphan receptors. The non-genomic actions are further subdivided to structural changes of chromatin through histone modification, or so-called "epigenetic effects" (**Chapter IV**) and transcriptional regulation through signal transduction including phosphorylation cascade (**Chapter II, V**). A schematic diagram for brief outline for the thesis is illustrated in **Fig. I-5**.

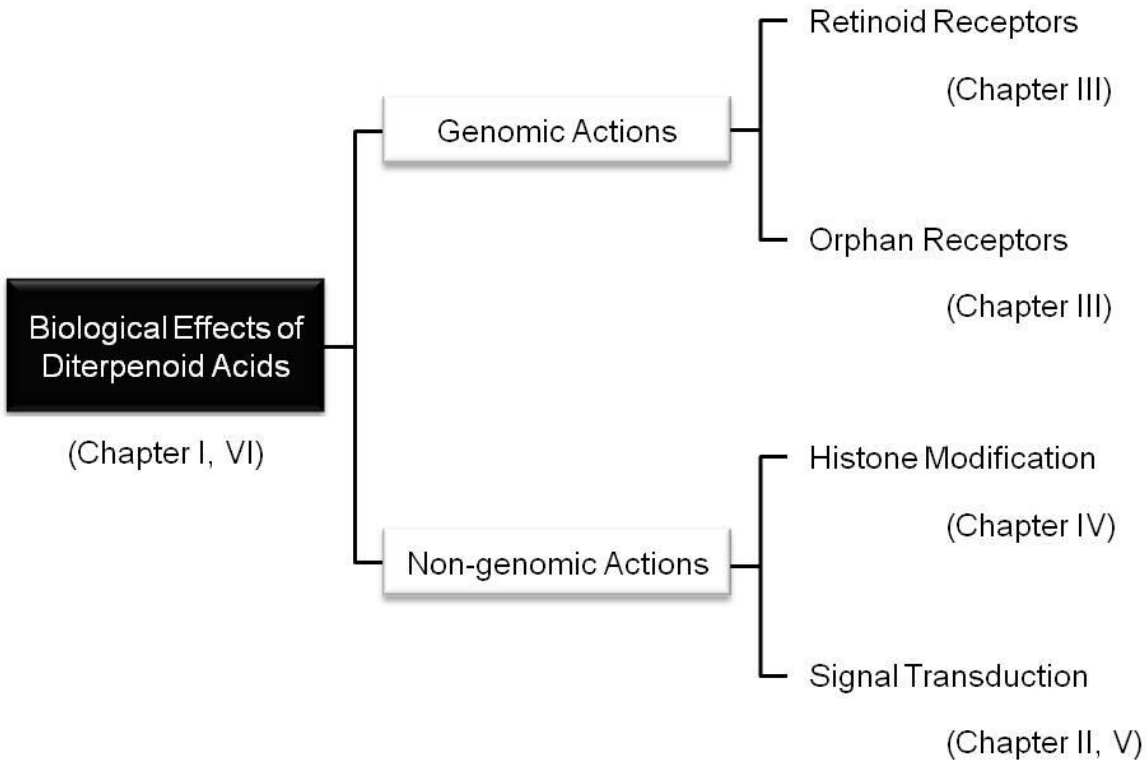

Fig. I-5. Schematic diagram for brief outline of the thesis.

Chapter II

RAPID DOWNREGULATION OF CYCLIN D1

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Rapid downregulation of cyclin D1 induced by geranylgeranoic acid in human hepatoma cells.

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II. 1. Abstract

Geranylgeranoic acid (GGA) and its derivatives are currently under development as chemopreventive agents against second primary hepatoma in Japan. We aimed to evaluate chemoprevention targets of GGA and a surrogate marker of chemopreventive response to clarify the molecular mechanism of hepatoma chemoprevention with GGA. Human hepatoma-derived cell lines such as HuH-7, PLC/PRF/5, and HepG-2, were treated with GGA and its derivatives. Cellular dynamics of several cell-cycle-related proteins were assessed by either immunoblotting or immunofluorescence method. The cellular expression of cyclin D1 protein was suppressed immediately after GGA treatment. This reduction was partially blocked by pretreatment with 26S proteasome inhibitor MG-132, indicating that proteasomal degradation was involved in GGA-induced disappearance of cyclin D1. A phosphorylation of retinoblastoma protein (RB) at serine 780, a target site of cyclin D1-dependent kinase 4, was rapidly decreased in GGA-treated HuH-7 cells. Furthermore, subcellular fractionation, immunoblotting, and immunofluorescence revealed GGA-induced nuclear accumulation of RB. These results strongly suggest that cyclin D1 may be a target of chemopreventive GGA in human hepatoma cells. GGA-induced rapid repression of cyclin D1, and a consequent dephosphorylation and nuclear translocation of RB, may influence cell cycle progression and may be relevant to GGA-induced cell death mechanisms.

II. 2. Introduction

II. 2. 1. Cell cycle and its related proteins

Normal cells as they are proliferating and renewing are going through different phase, in a process referred as the cell cycle (**Fig. II-1**). From the G1 phase where the cell has 2 sets of its genome, it goes through the DNA-synthesis, S-phase, over to the G2 phase where its DNA content becomes doubled. Finally the cell enters the mitosis, M-phase to give two daughter cells with identical genome. During its life a cell can be exposed to various DNA damaging agents such as UV irradiation. Interestingly it has been observed that following DNA damage the cell is able to arrest either at the G1/S transition or at the G2/M in order to initiate DNA repair before the cell goes on [Hartwell & Weinert, 1989]. The role of these 2 checkpoints is to avoid the propagation of mutagenic lesions to the daughter cells.

E2F

Regulation of cell cycle results in complex interaction with cell-cycle related proteins. Cell-cycle related proteins that regulate G1/S transition are shown in **Fig. II-2**. The E2F transcription factor family members are the key regulators of cell proliferation. For example, E2Fs control the cell cycle by regulating the expression of number of genes, whose products are required for the S-phase entry and cell cycle progression [Helin et al, 1998]. E2F1 promotes cell cycle by regulating critical regulator genes involved in the DNA replication and G1/S transition [Bracken et al, 2004].

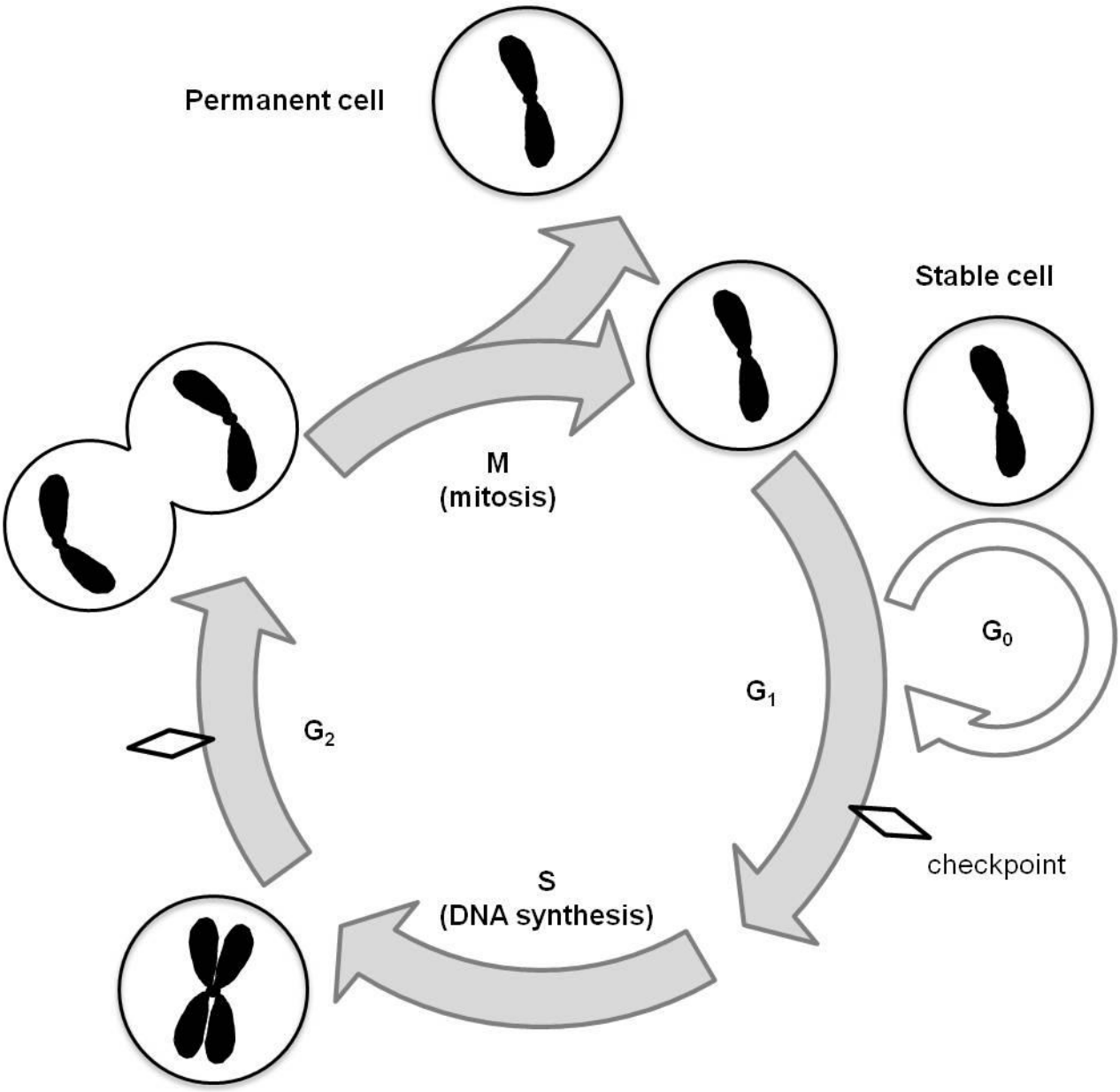

Fig. II-1. Cell cycle.

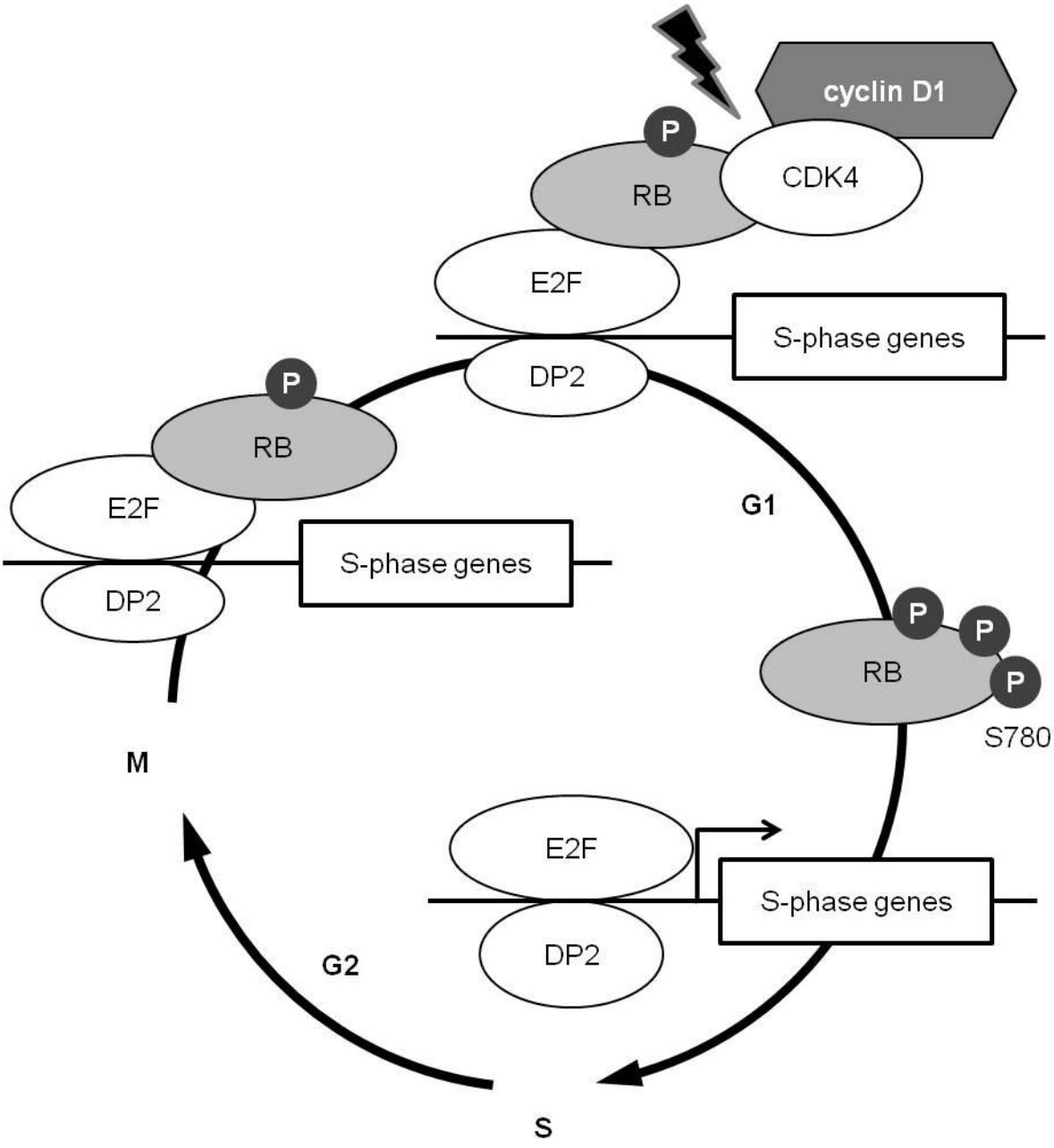

Fig. II-2. Genes related G1/S transition.

The transcriptionally active forms of E2F are a collection of heterodimeric protein complexes [Girling et al, 1993], each composed of one E2F protein family subunit and one DP (a DNA-binding partner of E2Fs) protein family subunit. The DP family contains two well-characterized members, TFDP1 and -2. These two proteins share high homology in the DNA binding/hetero-dimerization domain but diverge from each other in the C-terminus [Girling et al, 1993]. Due to the lack of a transactivation domain, DP proteins themselves have no transcriptional activity. Instead, they exert a regulatory function by dimerizing with E2F proteins. In fact, the hetero-dimerization of E2F-DP is essential for both high affinities of DNA binding and efficient transcriptional regulation by E2Fs [Helin et al, 1993]. As heterodimers, the E2F-DP complexes bind to the consensus E2F DNA recognition site TTT(C/G)GCGC(C/G) identified in a large number of cellular promoters. This could lead to either activation or repression of the target genes, depending on the specific E2F members involved. E2F1 to -3, for example, usually lead to the activation of genes critical for DNA synthesis and cell cycle progression. E2F4 and -5, on the other hand, recruit RB and related proteins to E2F-regulated promoters and actively repress gene expression [Dimova & Dyson, 2005].

RB

The initial functional characterization of the retinoblastoma protein (RB) following the seminal discovery of the *RB* gene as first tumor suppressor focused on its role as a central regulator of cell cycle progression. RB tumor suppressor function was originally thought to be largely due to its capacity to arrest cells in G1 by inhibiting the activity of E2F transcriptional factors [Riley et al, 1994]. It is now believed that RB has many

cellular roles in addition to serving as a G1 checkpoint, including control of cellular differentiation during embryogenesis and in adult tissue, regulation of apoptotic cell death, maintenance of permanent cell-cycle arrest and preservation of chromosomal stability [Zheng & Lee, 2001].

Cyclins

Cyclins are key molecules in cell-cycle control because of their specific and periodic expression during cell cycle progression. The D-type cyclins (cyclin D1, D2, and D3) complex with CDK (cyclin-dependent kinase) 4 and CDK6 and thereby regulate transition from the G1 phase into the S phase by phosphorylation and inactivation of RB [Holnthoner et al, 2002]. The activity of the cyclin D/CDK complexes is negatively regulated by the CDK inhibitors [Holnthoner et al, 2002]. Cyclin E binds to CDK2, which is required for the transition from G1 to S phase of the cell cycle that determines cell division. As the cell passes from G1 into S phase, cyclin A in turn associates with CDK2, replacing cyclin E. Cyclin B is necessary for the progression of the cells into and out of M phase of the cell cycle by a formation of the cyclin B/CDK1 complex.

II. 2. 2. Cyclin D1 as a potent chemoprevention target

Recently it has been demonstrated that nuclear cyclin D1 could be a potential oncogene product because it may promote tumorigenic growth [Kim & Diehl, 2009]. The early onset of deregulation of the *cyclin D1* (*CCND1*) gene, or aberrant cyclin D1 expression in premalignant tissues relative to normal tissues, implies that it is an attractive target for cancer chemoprevention [Piechocki et al, 2007]. In fact, it was found some

years ago that high intra-lesional cyclin D1 protein expression was linked to shorter cancer-free survival [Papadimitrakopoulou, Izzo et al, 2009]. Furthermore, a specific polymorphism of *CCND1* (G/A870) located at the splice junction of exon 4/5, which is proposed to influence the relative amounts of this spliced shorter isoform, is reported to be associated with the aggressive phenotype to esophageal adenocarcinoma [Izzo et al, 2007]. *CCND1* genotype and cyclin D1 protein expression are now expected to be important risk markers for laryngeal cancer [Papadimitrakopoulou, Izzo et al, 2009].

II. 2. 3. Non-genomic actions of retinoids, posttranslational downregulation of cyclin D1

Successful clinical cancer chemoprevention studies have so far been conducted with retinoid [Lippman et al, 1990], although it has been found in randomized phase III intergroup chemoprevention trials that it did not reduce second primary tumors [Papadimitrakopoulou, Lee et al, 2009]. Pioneering work by Dmitrovsky's group proposed that posttranslational downregulation of cyclin D1 by ATRA, a natural retinoid, may be a chemoprevention mechanism [Langenfeld et al, 1997]. In this decade, an important concept has been established, namely that ATRA is a promising compound playing a key role in the rapid downregulation of cyclin D1 by inducing enhanced proteasomal proteolysis [Dragnev et al, 2007]. Another successful example of clinical cancer chemoprevention has been provided by unique acyclic retinoid (ACR or Peretinoin) [Muto et al, 1996; 1999]. Serious side effects are often an unavoidable problem with any cancer therapeutic agent, including retinoids. During retinoid therapy for promyelocytic leukemia, some of the patients will suffer from ATRA syndrome, such as weight gain, dyspnea, fever, respiratory distress, pulmonary infiltrates,

episodic hypotension, and acute renal failure, which sometimes may cause lethal events [Larson et al, 2003]. Therefore, prior to becoming retinoid resistant, many patients have to withdraw from the drug or stop taking it [Ortega et al, 2005]. In comparison with a natural retinoid such as ATRA, it has been established that ACR or Peretinoin is a far safer retinoid. Muto's group has developed acyclic retinoid as safe chemicals that have been proven efficient for cancer chemoprevention [Muto et al, 1996; 1999]. In fact, the efficacy of acyclic retinoid in the prevention of second primary hepatoma has been demonstrated in a placebo-controlled double-blinded and randomized phase II clinical trial involving postoperative hepatoma patients with few side effects [Muto et al, 1996, 1999]. Subsequently, it was revealed that oral administration of Peretinoin (600 mg/d) for 12 mo significantly increased the 5-yr survival rate in those patients, without any side effects after radical therapy for primary hepatoma [Muto et al, 1996, 1999]. Downregulation of cyclin D1 has also been repeatedly reported with the use of this novel synthetic retinoid in several cellular systems [Shimizu et al, 2004a; 2004b; Suzui et al, 2006]. However, the molecular mechanism underlying the downregulation of cyclin D1 is thought to be quite different from ATRA-induced posttranslational suppression of cyclin D1. It has been demonstrated that proteasomal inhibitor did not rescue an ACR-induced decrease in the cellular level of cyclin D1 protein, in contrast to its preventive effect on ATRA-induced downregulation [Suzui et al, 2006]. Furthermore, ACR took more than 2 d to induce downregulation of cyclin D1 gene expression at its transcriptional level. A natural mother compound of Peretinoin is GGA, which consists of 4-isoprene units in a straight chain and has a carboxylic group at its tail terminus [Shidoji & Ogawa, 2004]. In the past 2 decades, Peretinoin or 4,5-didehydroGGA has been proven to suppress carcinogenesis in experimental

animals [Moriwaki et al, 1988] and has been demonstrated to be an efficient chemical in the prevention of second primary hepatoma in a phase II clinical trial [Muto et al, 1998]. GGA and 4,5-didehydroGGA were initially screened as acyclic retinoid to bind to the cellular retinoic acid-binding protein [Muto et al, 1981]. Furthermore, both compounds possess ligand activities for the retinoid receptor (retinoic acid receptor and retinoid X receptor) in the reporter assay [Yamada et al, 1994] and also show efficient activity in the induction of cell death in human hepatoma-derived cell lines [Shidoji et al, 2006].

II. 2. 4. Aim of the study

In the present study, we aimed to determine whether GGA is able to downregulate the cellular level of cyclin D1 in comparison with ATRA-induced proteasomal degradation of cyclin D1 protein.

II. 3. Results

II. 3. 1. Rapid decrease in cyclin D1 content after GGA treatment in HuH-7 cells

To observe the changes of cyclin D1 expression by GGA treatments, immunoblotting analysis was performed. In HuH-7 cells, cyclin D1 was clearly detected before GGA treatment. Cyclin D1 rapidly disappeared over a period of 2 h after the addition of GGA, and the GGA -treated cells did not accumulate any detectable cyclin D1 protein during the experiment (**Fig. II-3A**). A rapid reduction in cyclin D1 content induced by GGA also occurred in the other hepatoma-derived cell lines, PLC/PRF/5 and HepG2 (**Fig. II-3A**). **Fig. II-3B** shows that the downregulation of cyclin D1 is specific for GGA, in other words neither geranylgeraniol (a terminal carboxyl group of GGA reduced to an alcohol group) nor farnesoic acid (the number of isoprenoids units in GGA is reduced from 4 to 3) decreased cyclin D1 level in these 3 cell lines. ATRA was active in reducing the cyclin D1 contents but was less active than GGA.

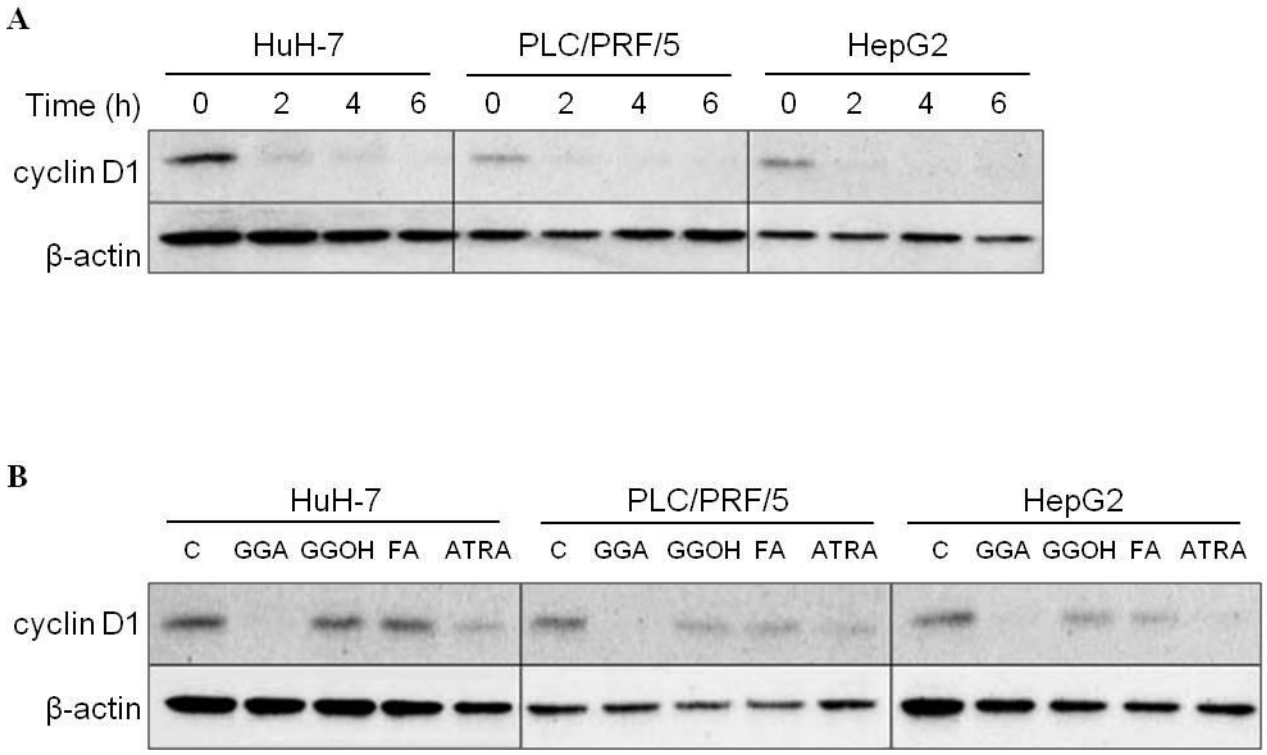

Fig. II-3. Downregulation of the cellular cyclin D1 level in hepatoma-derived cell lines by treatment with GGA.

A: Total cell lysates from HuH-7, PLC/PRF/5, or HepG2 cells treated with 10 μM GGA for the indicated times were analyzed by immunoblot with anti-cyclin D1, and the same membrane was reprobed with anti-β-actin as loading control. **B**: Cyclin D1 was measured by immunoblot with the lysates from HuH-7, PLC/PRF/5, or HepG2 cells treated for 2 h with ethanol (C), 10 μM GGA, 10 μM geranylgeraniol (GGOH), 10 μM farnesoic acid (FA), or 10 μM all-*trans* retinoic acid (ATRA) in medium. Actin-β was reprobed as a loading control.

In HuH-7 cells, the reduction of the cyclin D1 contents had already started at 30 min after the addition of GGA and exhibited a half-life $T_{1/2}$ of 22 min (**Fig. II-4**). GGA downregulated the cellular level of cyclin D1 in a dose-dependent manner with an IC_{50} of 6.8 μM **(Fig. II-5A**). The inhibitory effect of GGA was specific for cyclin D1 protein, due to the fact that the cellular levels of other cyclins such as cyclin B1 and cyclin E were not significantly changed after GGA treatment, at any concentrations tested herein (**Fig. II-5B**).

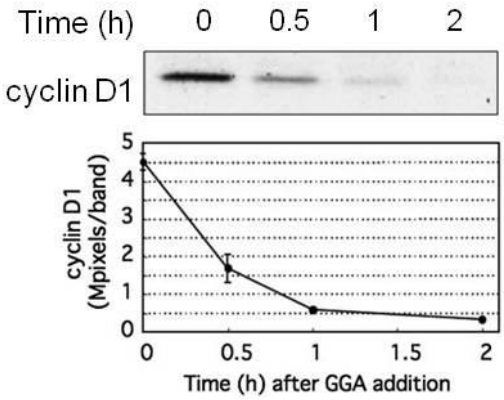

Fig. II-4. Rapid decrease in cellular cyclin D1 levels after GGA treatment in HuH-7 cells.

Total cell lysates from cells treated with 10 μM GGA for the indicated times were analyzed on immunoblot with anti-cyclin D1 antibody. The calculated density (mean ± SD, *n* = 3) of each band was plotted against sampling time.

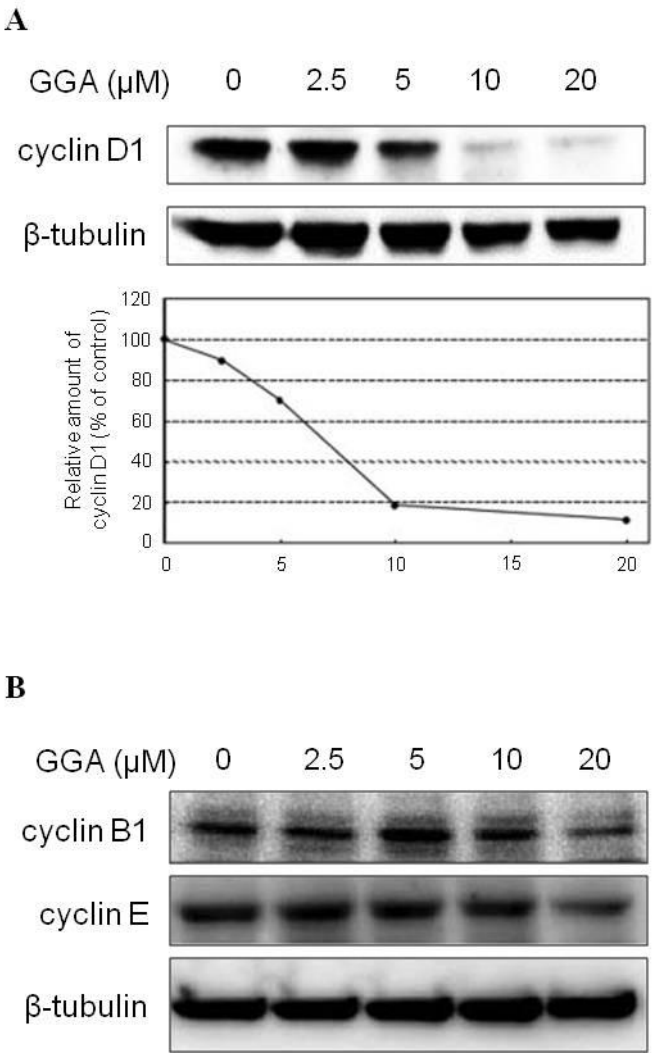

Fig. II-5. Dose effects of GGA on cyclins in HuH-7 cells.

A: Cyclin D1 was measured using immunoblot with the lysates from the cells treated for 1 h with the indicated concentrations of GGA in medium. The same membrane was reprobed with anti-β3-tubulin as loading control. The calculated density of each band was plotted against final concentrations of GGA. **B**: Cyclin B1 and E were measured with loading control of β3-tubulin.

II. 3. 2. GGA-induced downregulation of RB protein in HuH-7 cells

To examine whether or not GGA had an effect on RB expression, immunoblotting was performed. As shown in **Fig. II-6 (upper panel)**, the doublet bands for RB were detected at the positions around 105 kDa in control HuH-7 cells. The upper band, possibly phosphorylated forms of RB, was continuously decreased until 6 h and had disappeared at 16 h after GGA treatment. In contrast, the lower band, unphosphorylated RB, was gradually decreased after GGA treatment with a transient increase at 2h. In the literature, it is well established that RB is phosphorylated at serine-780 (Ser708) by protein kinase CDK4 whose activity is dependent on cyclin D1. Therefore, to examine whether or not the cellular level of RB phosphorylated at Ser780 was changed after GGA treatment, phosphorylated-Ser780-RB specific antibody was employed. As shown in **Fig. II-6 (lower panel)**, the cellular level of the phospho-RB was immediately downregulated by GGA treatment.

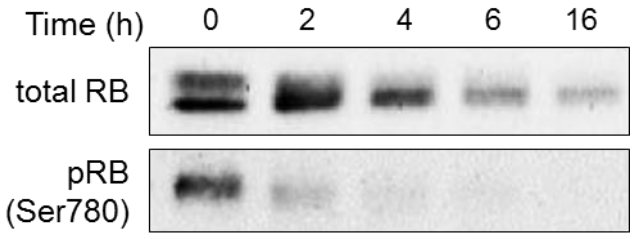

Fig. II-6. Effects of GGA on the phosphorylation of retinoblastoma protein (RB).

RB in the total cell lysates from HuH-7 cells treated with 10 μM GGA for the indicated times was measured using immunoblot with anti-RB or anti-phosphoRB (serine-780). pRB; phosphorylated RB.

In some cancer cell lines, RB proteins have been reported to aberrantly accumulate in their cytoplasmic space. In HuH-7 cells, RB was detected both in the nuclear and cytoplasmic fractions prior to the addition of GGA. After GGA treatment, the density of the nuclear RB band was increased at 6 h and the cytoplasmic RB was gradually and continuously decreased (**Fig. II-7A**). Nucleocytoplasmic localization was demonstrated using an immunofluorescence technique, which revealed both cytoplasmic and nuclear localization of RB in untreated cells (**Fig. II-7B**). The fluorescence intensity of the nuclear RB was increased, and the cytoplasmic RB fluorescence was gradually decreased after GGA treatment.

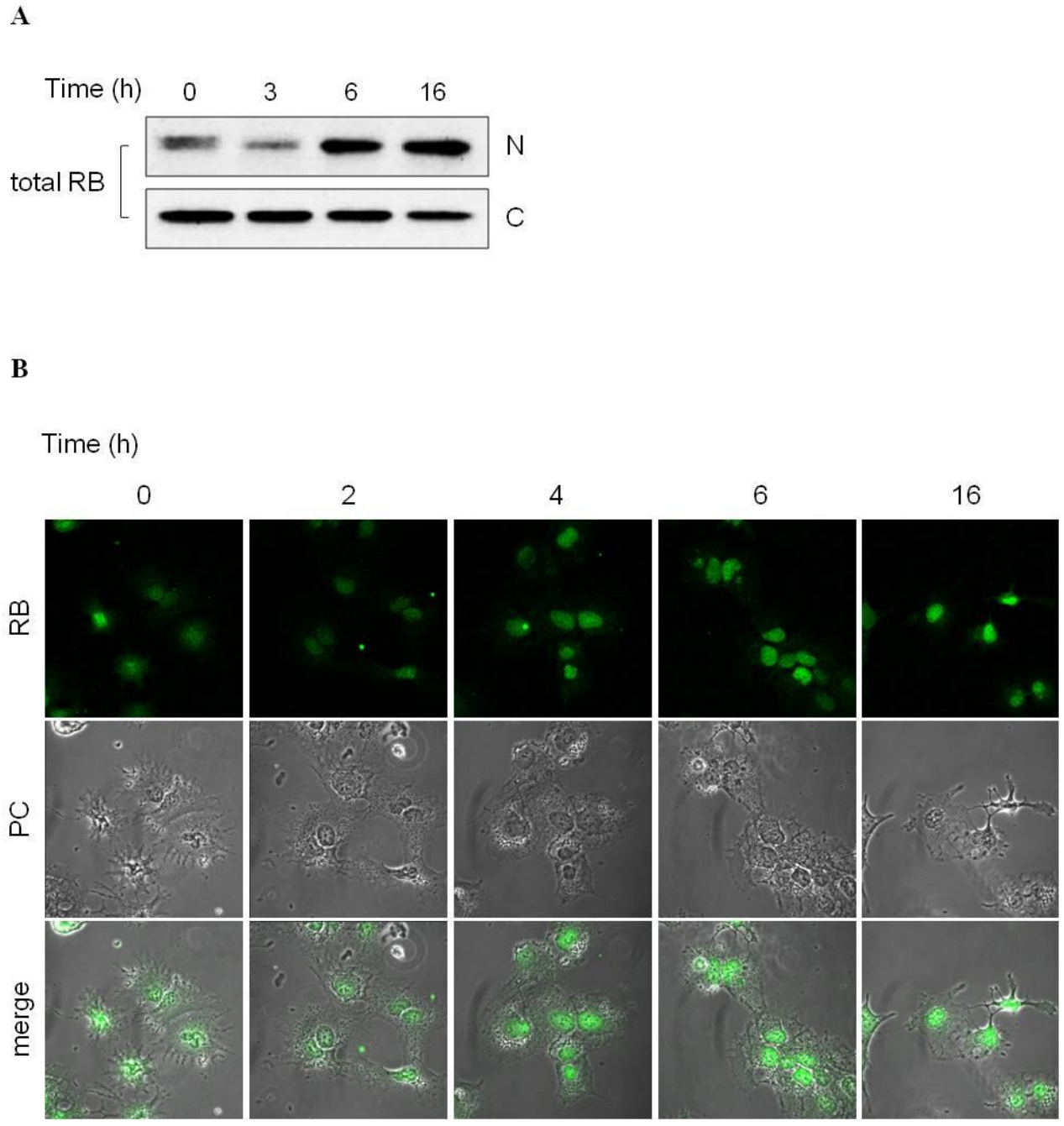

Fig. II-7. Effects of GGA on subcellular localization of RB.

A: RB was measured using immunoblot with the cytoplasmic extracts or nuclear extracts from HuH-7 cells treated with 10 μM GGA for the indicated times. **B**: Immunofluorescence images of RB with HuH-7 cells treated with 10 μM GGA for the indicated times. PC; phase-contrast image, Merge; immunofluorescence image + phase-contrast image.

II. 3. 3. GGA-induced downregulation of E2F1 expression in HuH-7 cells

Two molecular species of E2F1 were found on the immunoblot. Both bands were continuously decreased during GGA addition in a time-dependent manner (**Fig. II-8A**). However, a small change in *E2F1* mRNA expression level was observed (**Fig. II-8B**).

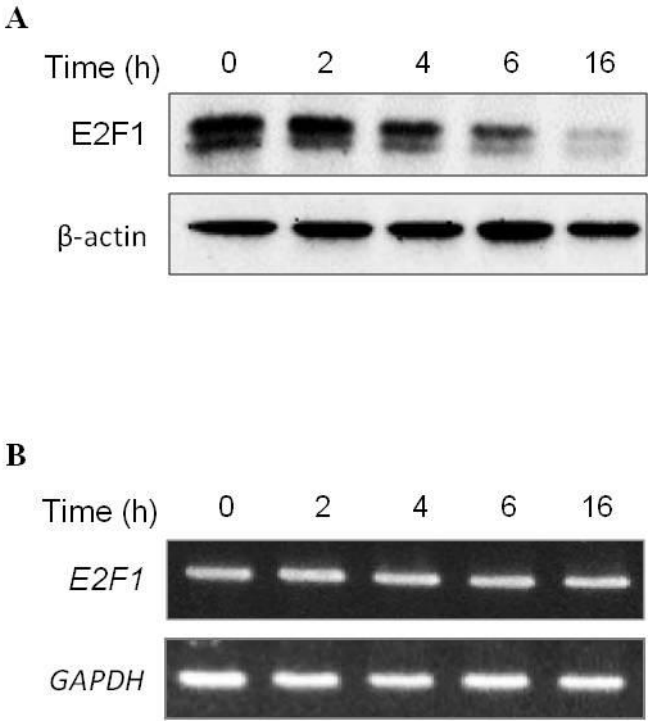

Fig. II-8. Effects of GGA on cellular expression of E2F1.

A: E2F1 in the tonal cell lysates from HuH-7 cells treated with 10μM GGA for the indicated times was measured using immunoblot with anti-E2F1, and the same membrane was reprobed with anti-β-actin as loading control. **B**: Semi-quantitative RT-PCR was performed to measure *E2F1* mRNA levels of HuH-7 cells treated with 10 μM GGA for the indicated times. *GAPDH* mRNA was used as a loading control.

II. 3. 4. Mode of action for GGA to downregulate cyclin D1 levels in HuH-7 cells

Weinstein's group have reported that ACR (Peretinoin or 4,5-didehydroGGA) downregulates *cyclin D1 (CCND1*) gene expression at the transcriptional level [Shimizu et al, 2004a; 2004b; Suzuki et al, 2006]. Since GGA is one of the acyclic retinoids, we first examined the cellular level of *CCND1* mRNA. **Fig. II-9A** clearly shows that no downregulation of the *CCND1* mRNA level was induced by GGA treatment. In the literature, Dmitrovsky's group have established that ATRA is active in inducing proteasomal degradation of cyclin D1, resulting in its downregulation [Dragnev et al, 2007; Feng et al, 2007; Freemantle et al, 2007; 2009; Langenfeid et al, 1997]. Since ATRA was also active in decreasing the cyclin D1 level to some extent in our system (**Fig. II-3B**), the preventive effect of MG132, a proteasome inhibitor, on GGA-induced dowregulation of cyclin D1 was examined. As a result, 30-min preincubation with MG132 definitely protected the GGA-induced downregulation of cyclin D1, but its effect was apparently partial (**Fig. II-9B**). In the presence of MG132 alone at 1 h, as expected HuH-7 cells time-dependently accumulated cyclin D1, at the cellular level (**Fig. II-9C, upper panel**). At the same time, ATRA did not provide any additional effect on MG132-induced accumulation of cyclin D1 (**Fig. II-9C, lower panel**), whereas GGA evidently inhibited MG132-induced accumulation of cyclin D1 (**Fig. II-9C, middle panel**). Next, the inhibitory effect of GGA on translation of the *CCND1* gene was tested. When translation of the *CCND1* gene was stopped with cycloheximide, the intracellular half-life of cyclin D1 protein was calculated to be 20 min (**Fig. II-9D, upper panel**). No additional effect of GGA was detected on the cycloheximide-induced downregulation of cellular cyclin D1 (**Fig. 2-9D middle panel**), whereas ATRA further enhanced the downregulation of cellular cyclin

D1 protein level in the presence of cycloheximide (**Fig. II-9D, lower panel**).

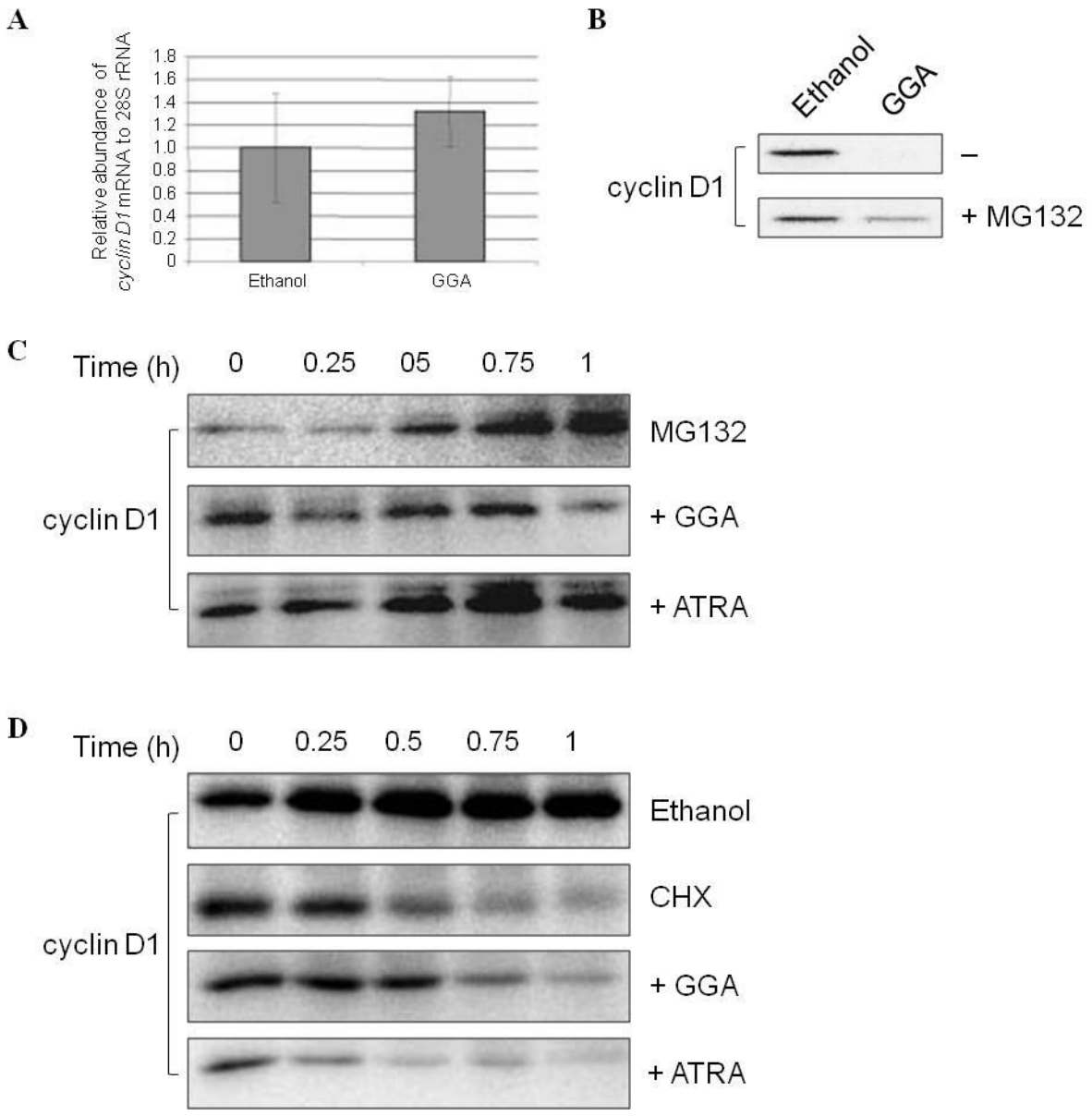

Fig. II-9. Suppression of cyclin D1 synthesis by GGA treatment.

A: Reverse transcription and quantitative real-time polymerase chain reactions was performed for *cyclin D1* mRNA in total RNA from HuH-7 cells treated for 1 h with ethanol or 10 μM GGA, and the relative abundance was calculated against the amounts of *28S* rRNA. **B**: Cyclin D1 levels were measured using immunoblot, with total cell lysates from 30-min MG132-pretreated or nontreated HuH-7 cells, treated for 2 h with ethanol or 10 μM GGA. **C**: Cyclin D1 levels were analyzed using immunoblots with total cell lysates from HuH-7 cells treated with 20 μM MG132, in the presence or absence of 10 μM GGA or 10 μM all-*trans* retinoic acid (ATRA) for the indicated times. **D**: Cyclin D1 levels were analyzed using immunoblots, with total cell lysates from HuH-7 cells treated with 50 μg/ml cycloheximide (CHX), in the presence or absence of 10 μM GGA (+ GGA) or 10 μM ATRA (+ ATRA) for the indicated times.

II. 3. 5. Reversibility of GGA-induced downregulation of cyclin D1 in HuH-7 cells

To examine whether or not GGA-induced downregulation could be reversed after removing GGA from culture medium, immunoblotting was performed with anti-cyclin D1 antibody. At 1h after removal of GGA in medium, cyclin D1 band, which had disappeared after 1-h pretreatment with GGA, was back, and the density of the bands even exceeded over the original level at 6 h after removal of GGA (**Fig. II-10**).

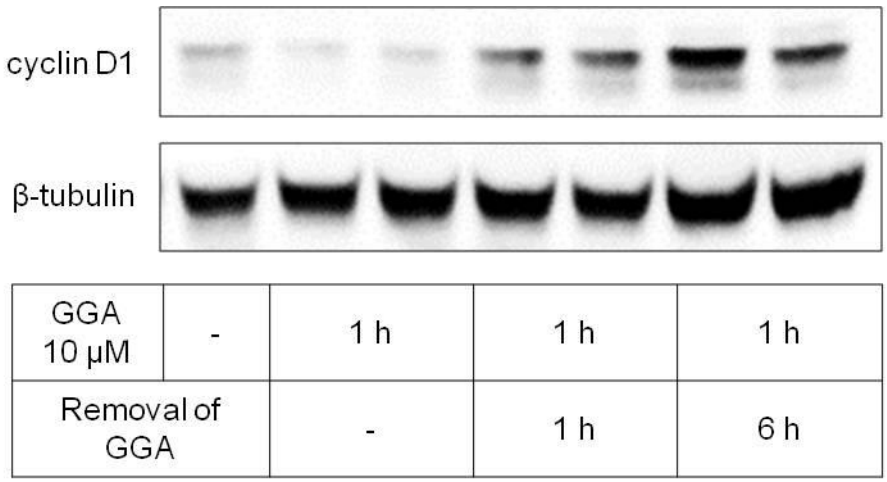

Fig. II-10. Effect of GGA removal on expression of cyclin D1 in HuH-7 cells.

Cells were treated with 10 μM GGA for 1 h and consecutively incubated in culture medium alone for 1 or 6 h. Cyclin D1 levels were analyzed using immunoblots with total cell lysates. The same membrane was reprobed with anti-β3-tubulin as loading control.

II. 4. Discussion

In the present study, we found for the first time that a diterpenoid acid, GGA induced a rapid downregulation of cyclin D1 in human hepatoma-derived cell lines. And it was also found that the phosphorylated RB (at Ser780) immediately disappeared and a gradual decrease in E2F1 level occurred in HuH-7 cells after GGA treatment. In addition, it was unexpectedly shown that GGA induced a subsequent nuclear translocation of the cytoplasmic RB protein. These findings are all consistent with previous findings that GGA induced growth suppression in hepatoma cells.

Among the GGA-induced changes found in the present study, we focused on the rapid downregulation of cyclin D1, because cyclin D1 is upstream signal of the RB/E2F1 signal transduction pathway for cell growth. At the cellular level, cyclin D1 may be directly modulated by GGA. In other words, cyclin D1 may be a non-genomic target of GGA when it exerts a cancer chemopreventive action. It is well established that cyclin D1 is one of the important regulatory proteins that promotes G1-to-S phase progression in many different cell types [Alao et al, 2007]. In fact, most tumor cells show a higher expression level of the *CCND1* gene, and overexpression of cyclin D1 is known to correlate with the early onset of cancer and the risk of tumor progression and metastasis of clinical cancer cells, including hepatoma [Zhang et al, 2002].

In considering the molecular mechanism of GGA-induced downregulation of cyclin D1 (**Fig. II-11**), it is noteworthy that cyclin D1 is, in general, a highly labile or rapid-turnover protein with a half-life of 20-30 min. In addition, proteolysis of cyclin D1 requires polyubiqutination by F-box protein FBXO31 [Santra et al, 2009], which targets cyclin D1 to the 26S proteasome [Takahashi-Yanaga et al, 2008]. A number of

therapeutic agents have been observed to induce cyclin D1 degradation in vitro through proteasome degradation. Among them, ATRA, a diterpenoid acid similar to GGA, is well known to induce the enhanced proteasomal degradation of cyclin D1 [Dragnev et al, 2007; Feng et al, 2007; Freemantle et al, 2007; 2009; Langenfeld et al, 1997] as well as in the literature the first described posttranscriptional downregulation of fibronection gene expression by ATRA treatment [Scita et al, 1996]. Therefore, we were very much interested in whether GGA was able to enhance the proteasomal degradation of cyclin D1. Although, as expected, ATRA in the presence of a proteasome inhibitor, MG132, was totally inactive in decreasing the cyclin D1 level, the inhibitor was completely unable to facilitate the accumulation of cellular cyclin D1 in the presence of GGA. This result indicated that GGA may induce a putative protease degradation of cyclin D1 through an ubiquitin-independent or antizyme-dependent process [Newman et al, 2004], although the present study failed to show any enhancement of cyclin D1 proteolysis after GGA treatment (**Fig. II-9C**). In other words, the MG132 experiment demonstrated that GGA maintained or slightly decrease the initial cellular level of cyclin D1, even while proteasomal degradation was blocked by MG132.

GGA is one of the acyclic retinoids that possess no cyclic structure in their chemical formula and show ligand activity for nuclear retinoid receptors [Araki et al, 1995]. According to Weinstein's group, the most scrutinized acyclic retinoid so far is 4,5-didehydroGGA (ACR or Peretinoin), which downregulates *CCND1* gene expression at the transcriptional level with cells in culture, including hepatoma-derived cell lines [Shimizu et al, 2004a, 2004b, Suzuki et al, 2006]. Recently, the suppressive effect of 4,5-didehydroGGA on *CCND1* gene expression was shown in vivo in diethylnitrosoamine-induced liver tumor of obese and

diabetic mice [Shimizu et al, 2011]. As compared with GGA tested in the present study, 4,5-didehydroGGA downregulates the *CCND1* transcript and protein levels more slowly over a period of 24 to 48 h even in the similar in vitro systems [Shimizu et al, 2004a, 2004b, Suzuki et al, 2006]. After taking into consideration that 4,5-didehydroGGA or Peretinoin required such a long time to reduce the *CCND1* transcript level, GGA-induced rapid downregulation of cyclin D1 protein may not be transcriptional but posttranscriptional. Indeed, the cellular level of *CCND1* mRNA still remained at the initial level at 1 h after GGA treatment (**Fig. II-9A**). Furthermore, as mentioned earlier, the proteasomal degradation of cyclin D1 was not enhanced by GGA in the presence of cycloheximide (**Fig. II-10D**).

In this context, we finally speculated that the apparent synthetic rate of cyclin D1 protein should be reduced after GGA treatment. When the cells were incubated with MG132, with the assumption that cyclin D1 is degraded solely through proteasomal proteolysis, the accumulation rate of cellular cyclin D1 should reflect the synthetic rate. Interestingly, after blocking of the proteolysis of cyclin D1 with MG132 treatment, a time-dependent accumulation of cyclin D1 was completely prevented by co-treatment with GGA (**Fig. II-10D**). This result strongly suggests that the impaired translational synthesis of cyclin D1 protein occurred immediately after the addition of GGA. In sharp contrast, ATRA treatment had no significant effect on MG132-induced accumulation of cyclin D1 protein, indicating that ATRA may not be involved in translational control of the *CCND1* gene (**Fig. II-11**).

It could easily be considered that a rapid decrease in cyclin D1 content after GGA addition may be conveyed by a cellular RNA interference (RNAi) mechanism. The microRNAs that are able to repress the

translation of the *CCND1* gene have so far been reported to be miR-34a, miR-16, miR-193, miR-200b, miR-17, and miR-20 [Yu et al, 2010; Xia et al, 2010; Qin et al, 2010; Chen et al, 2010; Jiang et al, 2009; Sun et al, 2008]. These microRNAs potentially bind to the 3'-untranslated region (UTR) of *CCND1* mRNA and repress translation of the *CCND1* gene. Among these miRNAs, we were interested in looking at the cellular levels of miR-17, consisting of a miR 17-92 polycistronic cluster of 7 miRNAs, some of which are known to knock down *E2F1* gene expression. In the present experiments, the cellular levels of E2F1 protein were gradually reduced after GGA treatment (**Fig. II-8**). However, both matured form and pri-miR 17 were unexpectedly downregulated by GGA (unpublished result). To our knowledge, transcription and maturation of cellular miRNA will take around 1 h [Davies et al, 2008], so that it may be difficult for any miRNAs to downregulate cyclin D1 over a period of 15 min.

Another possible mechanism for rapid downregulation of cyclin D1 is the potential blocking of a specific nuclear export of *CCND1* mRNA by GGA due to inhibition of eukaryotic translation initiation factor eIF4E bound to the 3' UTR of *CCND1* mRNA. A recent report on the small molecule 4EGI-1 supports the idea of reducing the level of cyclin D by inhibiting eIF4e/eIF4G interaction in human cancer cells [Fan et al, 2010]. Taken together, GGA might regulate the subcellular distribution of *CCND1* mRNA (**Fig. II-11**).

We also have to mention some cell biological consequences from GGA-induced downregulation of cyclin D1. It is well established that cyclin D1 forms holoenzymes as an oncogenic component with CDK4 and CDK6, which phosphorylates nuclear localized tumor suppressor RB protein to release the E2F1 transcription factor. This factor can then activate some of the genes essential for the G1-S transition [Jiao et

al, 2008]. In the present study, as expected the phosphorylated RB (at serine 780) had disappeared by 2 h after GGA treatment, probably because of the downregulation of cyclin D1.

In the present study, the cytoplasmic RB was detected in HuH-7 and GGA reduced the cytoplasmic RB level but increased the nuclear content of RB. In other words, GGA produced nuclear translocation of the cytoplasmic RB protein. In recent studies, it has been demonstrated that deregulated CDK activity, often associated with hyperphosphorylation of RB, might alter RB subcellular localization and thereby compromise its tumor suppressor function [Jiao et al, 2008]. These studies presented evidence that the pharmacological inhibition of CDK activity allowed the cytoplasmically mislocalized RB to accumulate in the nucleus, providing a reasonable explanation for GGA-induced relocalization of RB to the nucleus in HuH-7 cells. Another possible mechanism for GGA-induced nuclear translocation of the cytoplasmic RB is CDK independent. Most recently, Iwao and Shidoji reported that GGA induced translocation of tumor-suppressive protein p53 accumulated in cytoplasm to nuclei through de-sequestration of the cytoplasmic p53 [Iwao & Shidoji, 2014]. It suggests that GGA induced nuclear transport of RB protein by cyclin D1 dependent/independent manner. However, the influence on tumor repressive effects of GGA-induced reduction of the cytoplasmic RB level and accumulation of the nuclear RB were unknown.

Finally, we would like to discuss the biological significance of GGA-induced rapid downregulation of cyclin D1 in cancer chemoprevention, especially in the prevention of hepatoma. Amplification, polymorphism, and/or overexpression of the *CCND1* gene have been found in human hepatomas [Nishida et al, 1994, Zhang et al, 2002], and in addition downregulation of FBXO31 E3 ubiquitin ligase for cyclin D1

[Huang et al, 2010]. Besides hepatoma, cyclin D1 is also frequently overexpressed in a variety of other human carcinomas [Kim et al, 2009]. These findings suggest that aberrant expression of cyclin D1 protein may play an important role in the development of human hepatoma and other carcinomas. Indeed, overexpression of cyclin D1 is sufficient to initiate hepatocarcinogenesis in transgenic mice [Deane et al, 2001]. Thus, cyclin D1 can function as an oncogene product in the liver, and is therefore a potential target for hepatoma prevention and therapy.

The concept of oncogene addiction, a term coined by Bernard Weinstein, refers to the dependence of a cancer cell on one overactive gene or pathway for cell survival and growth. The source of Weinstein's theory was his observation that only partial blocking of cyclin D1, a key component of the Rb/E2F1 pathway, was sufficient to arrest the growth of cancer cells that were overexpressing the protein [Arber et al, 1997, Weinstein et al, 1997]. Over the last decade, researchers have accumulated further evidence for "oncogene addiction". They are now studying how such addiction takes place and identifying the candidate genes in the different tumors.

II. 5. Conclusion

We were able to demonstrate that GGA induced rapid downregulation of cyclin D1 in several human hepatoma cell lines. This may be conveyed by non-genomic actions of GGA, and may be a plausible mechanism involved in hepatoma chemoprevention.

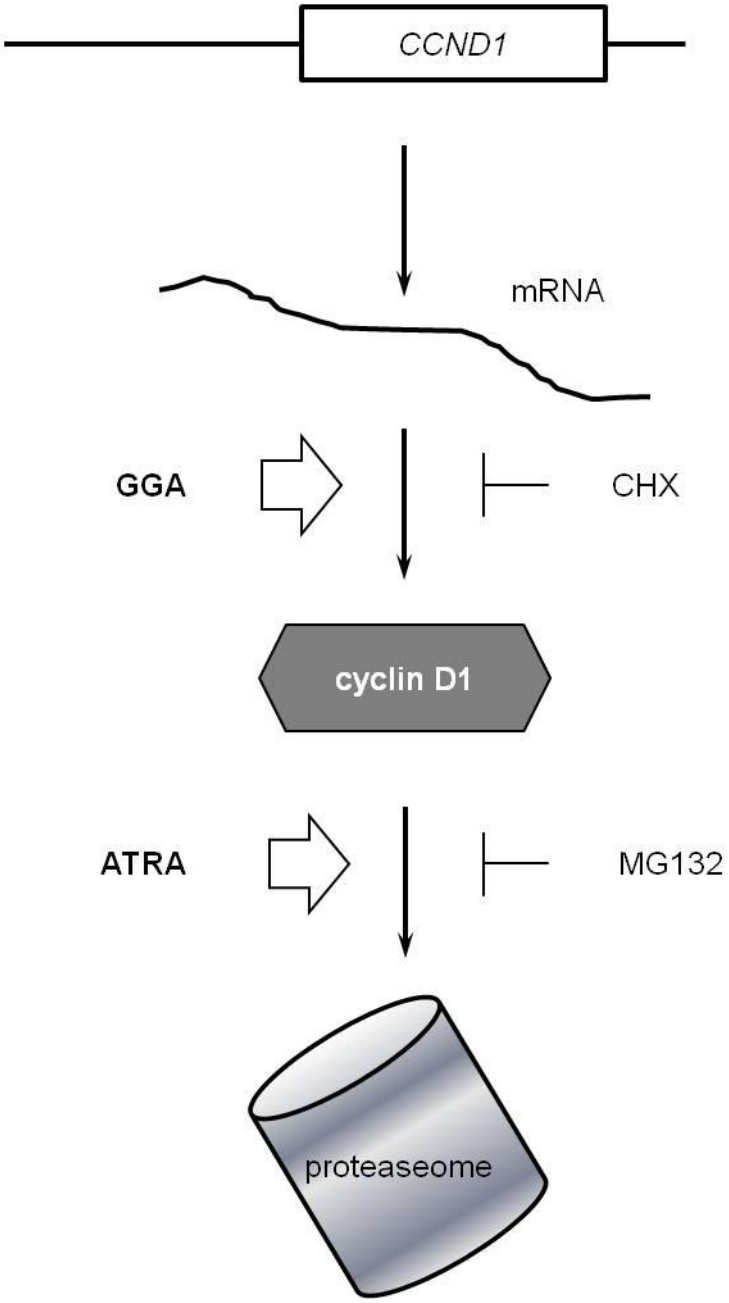

Fig. II-11. Hypothesis for molecular mechanism of GGA effect on cyclin D1.

Chapter III

UPREGULATION OF NEUROTROPHIC TYROSINE KINASE, RECEPTOR, TYPE 2

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Yoshihiro Shidoji

Reversible upregulation of tropomyosin-related kinase receptor B by geranylgeranoic acid in human neuroblastoma SH-SY5Y cells.

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III. 1. Abstract

All-*trans* retinoic acid (ATRA) plays crucial roles in cell survival and differentiation of neuroblastoma cells. In the present study, we investigated the effects of geranylgeranoic acid (GGA), an acyclic retinoid, on differentiation and neurotrophic tyrosine kinase, receptor, type 2 (*NTRK2*) gene expression in SH-SY5Y human neuroblastoma cells in comparison with ATRA. GGA induced growth suppression and neural differentiation to the same extent as ATRA. Two variants (145 and 95 kDa) of the NTRK2 protein were dramatically increased by GGA treatment, comparable to the effect of ATRA. Following 6- to 8-d GGA treatment, the effect of GGA on NTRK2 was reversed after 2–4 d of its removal, whereas the effect of ATRA was irreversible under the same conditions. Both GGA and ATRA upregulated the cellular levels of three major *NTRK2* messenger RNA splice variants in a time-dependent manner. Time-dependent induction of cell cycle-related genes, such as cyclin D1 and retinoblastoma protein, and amplification of the neural progenitor cell marker, brain lipid binding protein, were suppressed by GGA treatment and were completely abolished by ATRA. ATRA and GGA induced retinoic acid receptor β (RARβ) expression, whereas the time-dependent expression of both RARα and RARγ was abolished by ATRA, but not by GGA. Our results suggest that GGA may be able to restore neuronal properties of SH-SY5Y human neuroblastoma cells in a similar but not identical way to ATRA.

III. 2. Introduction

III. 2. 1. Neurotrophins and their receptors

Neurotrophins (NTs) play crucial roles in the development of the central nervous system, influencing proliferation, differentiation, survival and death of neuronal and non-neuronal cells. NTs include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NTF3) and neurotrophin 4 (NTF4). NTs mediate their cellular effects through the actions of two different receptors, the neurotrophic tyrosine kinase, receptor (NTRK, previously known as Trk; tropomyosin-related kinase receptor) and p75 neurotrophin receptor (p75NTR), a member of the TNF (tumor necrosis factor) receptor superfamily (**Fig. III-1**). NGF binds most specifically to NTRK1, BDNF and NTF4 to NTRK2, and NTF3 to NTRK3. All NTs can bind to p75NTR with low affinity, enhancing the activation of NTRK receptors [Bothwell et al, 1995] (**Fig. III-2**).

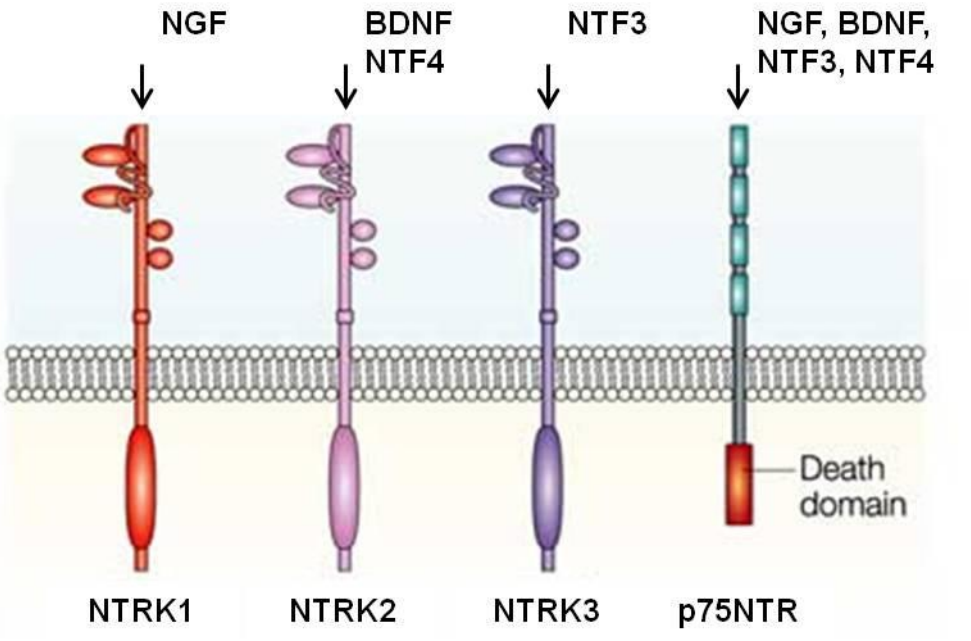

Fig. III-1. Schematic diagram of neurotrophins and their cell-surface membrane receptors.

[modified from Chao, 2003]

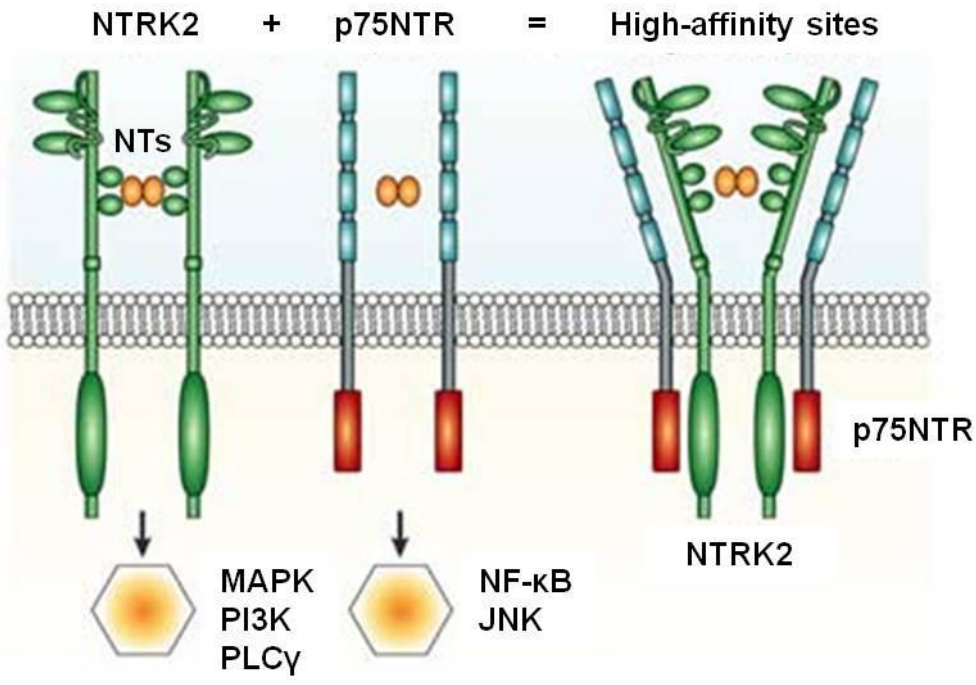

Fig. III-2. Crosstalk between NTRK2 and p75NTR with NTs.

[modified from Chao, 2003]

JNK; mitogen-activated protein kinase 8, MAPK; mitogen-activated protein kinase 14, NF-κB; nuclear factor of kappa-light polypeptide gene enhancer in B-cells, PI3K; phosphatidylinositol-4,5-bisphosphate 3- kinase, PLCγ; phospholipase C gamma

III. 2. 2. BDNF

In the pathophysiology of depression, BDNF and neurogenesis have an important function [Nibuya et al, 1995; Santarelli et al, 2003; Monteggia et al, 2007; Adachi et al, 2008]. In addition, electroconvulsive seizures [Altar et al, 2004] and administration of antidepressant drugs can increase hippocampal BDNF levels [Nibuya et al, 1995]. Moreover, Taliaz et al. reported that reduced BDNF protein levels, in hippocampal sub-regions, could reduce neurogenesis in vivo and affected behaviors associated with depression [Taliaz et al, 2010]. Furthermore, several studies show that BDNF regulates synaptic transmission, synaptic plasticity and synaptic growth [Lu et al, 2013]. Since the synaptic dysfunction is a key pathophysiological hallmark in neurodegenerative disorders, BDNF may provide the synaptic repair therapies for neurodegenerative disorders, including Alzheimer's disease [Lu et al, 2013].

NTRK2 is expressed in three major splice variants [Middlemas et al, 1991]. Full-length receptors (NTRK2-FL) possess an intracellular tyrosine kinase domain as well as an extracellular ligand-binding domain. The two truncated receptors, NTRK2-T1 and NTRK2-Shc, lack tyrosine kinase activity [Klein et al, 1990] (**Fig. III-2**). NTRK2-T1 has a direct functional role in mediating calcium release from intracellular stores [Rose et al, 2003]. NTRK2-Shc contains an SHC (Src homology 2 domain containing) transforming protein binding domain and is predominantly expressed in the brain, probably as a negative regulator of NTRK2 signaling [Zuccato et al, 2008]. Consequently, impairment of the BDNF/NTRK2 signaling system (**Fig. III-3**) is considered to associate with neurodevelopmental and neurodegenerative diseases [Yoshii & Constantine-Paton, 2010]. In other words, proper maintenance of the BDNF/NTRK2 signaling system may

be important in prevention of neurodevelopmental/ neurodegenerative disorders, or neuroblastoma.

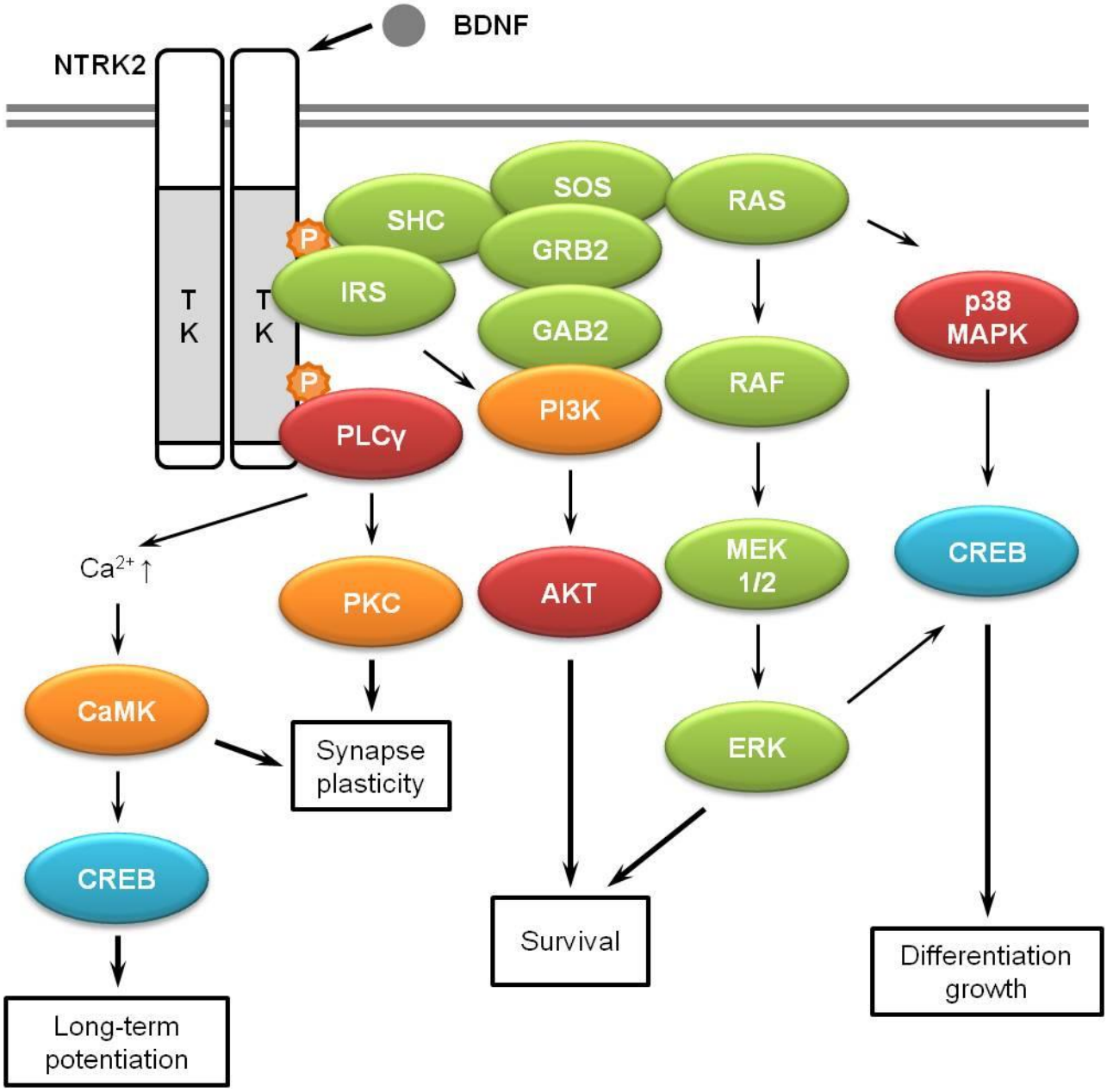

Fig. III-3. BDNF/NTRK2 signaling pathways.

AKT; RAC-alpha serin-threonine-protein kinase, CaMK; calcium/calmodulin-dependent protein kinase, CREB; cyclic AMP-responsive element-binding protein, ERK; mitogen-activated protein kinase 1, GAB2; GRB2-associated binding protein 2, GRB2; growth factor receptor-bound protein 2, IRS; insulin receptor substrate, MEK; mitogen-activated protein kinase kinase, p38MAPK; mitogen-activated protein kinase 14, PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase, RAF; RAF proto-oncogene serin/threonin-protein kinase, RAS; rat sarcoma viral oncogen homolog, SHC; SHC-transforming protein 1, SOS; son of sevenless homolog, TK; tyrosine kinase

III. 2. 3. Differentiation-inducing effect of ATRA on neuroblastoma SH-SY5Y cells

It is well known that all-*trans* retinoic acid (ATRA) predisposes undifferentiated SH-SY5Y neuroblastoma cells to BDNF treatment; for example, ATRA-treated SH-SY5Y cells produce and secrete β-amyloid precursor protein (APP) upon BDNF treatment; however, naive SH-SY5Y cells do not produce APP after exposure to BDNF alone [Ruiz-Leon & Pascual, 2003]. These results indicate that ATRA shifts BDNF-insensitive SH-SY5Y cells to BDNF-responsive SH-SY5Y cells. In fact, many researchers have reported that *NTRK2* gene expression is upregulated by ATRA treatment in SH-SY5Y cells [Kaplan et al, 1993; Hu & Koo, 1998; Edsjo et al, 2003; Ruiz-Leon & Pascual, 2003; Hecht et al, 2005; Holback et al, 2005; Kou et al, 2008; Nishida et al, 2008], however, a couple of exceptional papers demonstrated that ATRA decreased *NTRK2* mRNA levels in SH-SY5Y cells [Enrhard et al, 1993; Chen et al, 2010].

III. 2. 4. Metabolites of the mevalonate pathway in neurons

Neuron is one of the second most active cells that are producing cholesterol from the mevalonate pathway after hepatocyte. In neurons, a fraction of cholesterol is metabolized and eliminated by the enzyme cholesterol 24-hydroxylase, as shown in **Fig. III-4**. The mevalonate pathway in neurons produces neuronal steroids as well as nonsterol isoprenoids including geranylgeraniol (GGOH). Recently, Kotti's group have shown that constant production of GGOH in neuronal cells is required for long-term potentiation but not via protein geranylgeranylation [Kotti et al, 2006; 2008; Russell et al, 2009]. GGOH may be metabolically oxidized to GGA, which can be produced either by a two-step oxidation of GGOH [Mitake & Shidoji, 2011]

or by a one-step oxidation of geranylgeranial (GGal) produced by prenylcysteine lyase from geranylgeranylated proteins [Lu et al, 2006], suggesting that GGA may be a physiological metabolite in neurons. Furthermore, Shidoji & Ogawa identified natural GGA in some medical herbs [Shidoji & Ogawa, 2004], hence, one can speculate that GGA, either exogenously ingested from foods or de novo synthesized from GGOH/GGal, may participate in neuronal functions through upregulation of the BDNF/NTRK2 signal transduction pathway.

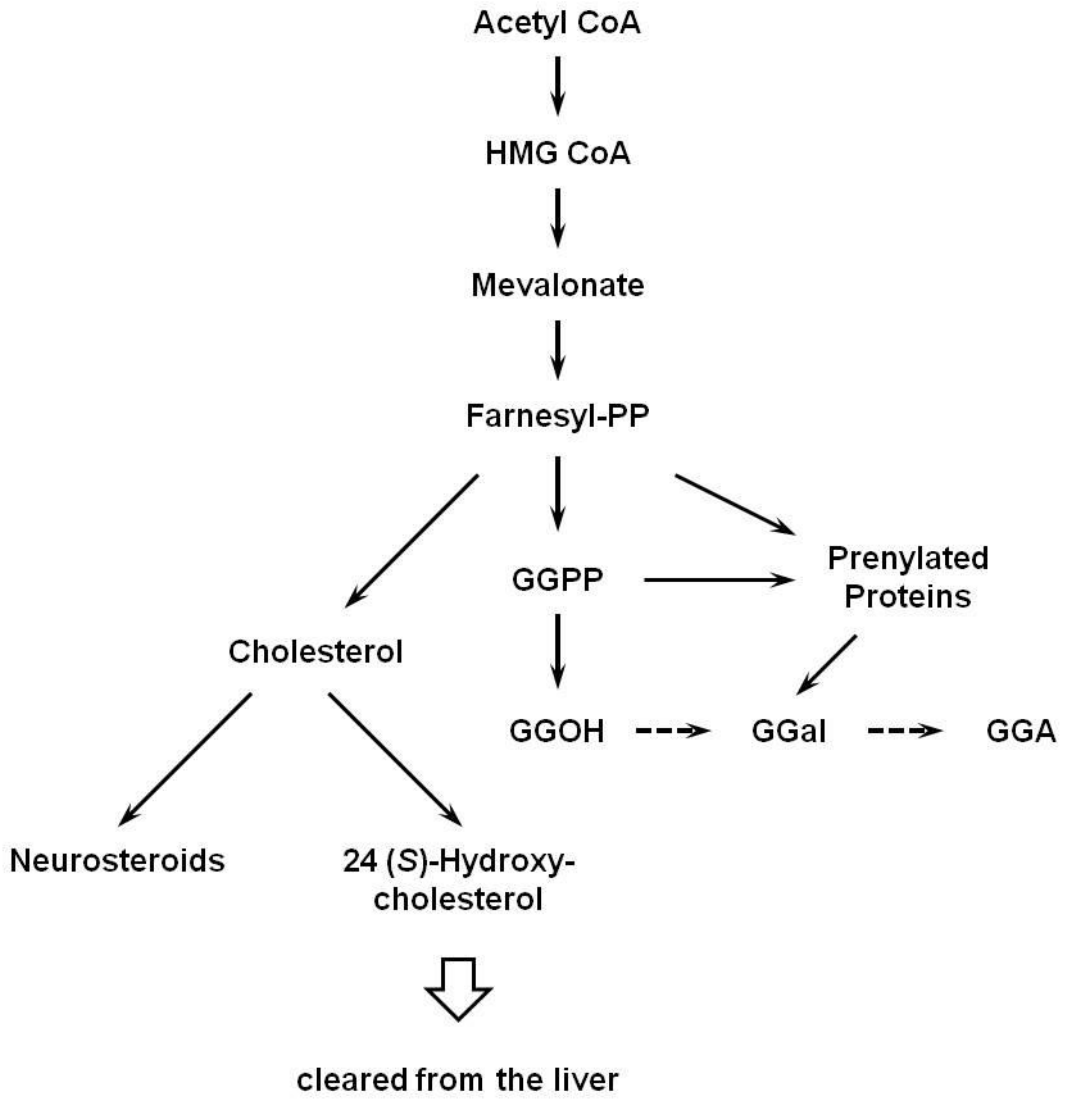

Fig. III-4. Isoprenoid metabolism in mammalian brain cells including steroid and nonsterol isoprenoids.
[Sakane & Shidoji, 2011]

III. 2. 5. Aims of the study

In this study, we investigated the GGA-mediated protective effects on regulation of BDNF and/or NTRK2 in SH-SY5Y neuroblastoma cells, to pave the way toward efficient prevention or suppression of neuroblastoma, using natural safe compounds.

III. 3. Results

III. 3. 1. Proliferation profile of SH-SY5Y cells with GGA

Compared with the vehicle (ethanol) control, ATRA treatment at 10 μM virtually inhibited proliferation of SH-SY5Y cells during the entire experiment for 10 d (**Fig. III-5**). The same concentration of GGA also significantly suppressed cell proliferation, although slightly less so than ATRA (**Fig. III-5**).

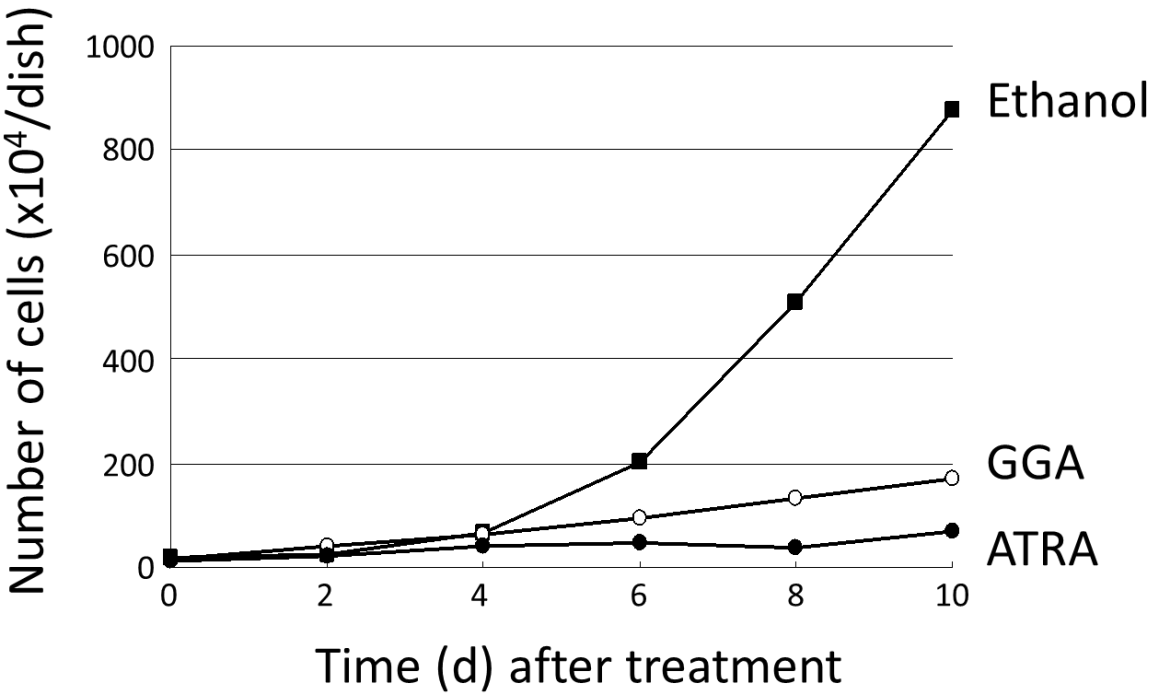

Fig. III-5. Effect of GGA on proliferation of SH-SY5Y cells.

Cells were treated with ethanol (closed squares), 10 μM GGA (open circles) or 10 μM ATRA (closed circles) for the indicated days. Viable cell numbers per dish were calculated by detaching cells at the indicated time points and counting them on a hemocytometer. The experiment was performed in singlicate.

III. 3. 2. Suppression of growth-related gene expression by GGA in SH-SY5Y cells

The cellular expression levels of cyclin D1 increased in a time-dependent manner in control ethanol treated cells (**Fig. III-6A**). The time-dependent upregulation of cyclin D1 was completely suppressed by ATRA treatment, and protein levels remained at initial levels after 10-d treatment. Similarly, GGA suppressed their upregulation (**Fig. III-6A**).

The same response was observed with total and phosphorylated RB protein levels (**Fig. III-6B**). These results are consistent with the proliferation data shown in **Fig. III-5**.

In contrast, the time-dependent expression of cyclin E, another cell cycle-related protein, was not influenced by either ATRA or GGA treatment (**Fig. III-6C**).

Expression of BLBP/FABP7, a marker for neural stem cells and proliferating glial progenitor cells [Shi et al, 2008], was examined in SH-SY5Y cells. BLBP/FABP7 protein expression was induced in a time-dependent manner in control ethanol-treated cells during 10-d culture, and 10 μM GGA suppressed this time-dependent induction (**Fig. III-6D**). Treatment with 10 μM ATRA completely abolished the induction of the protein.

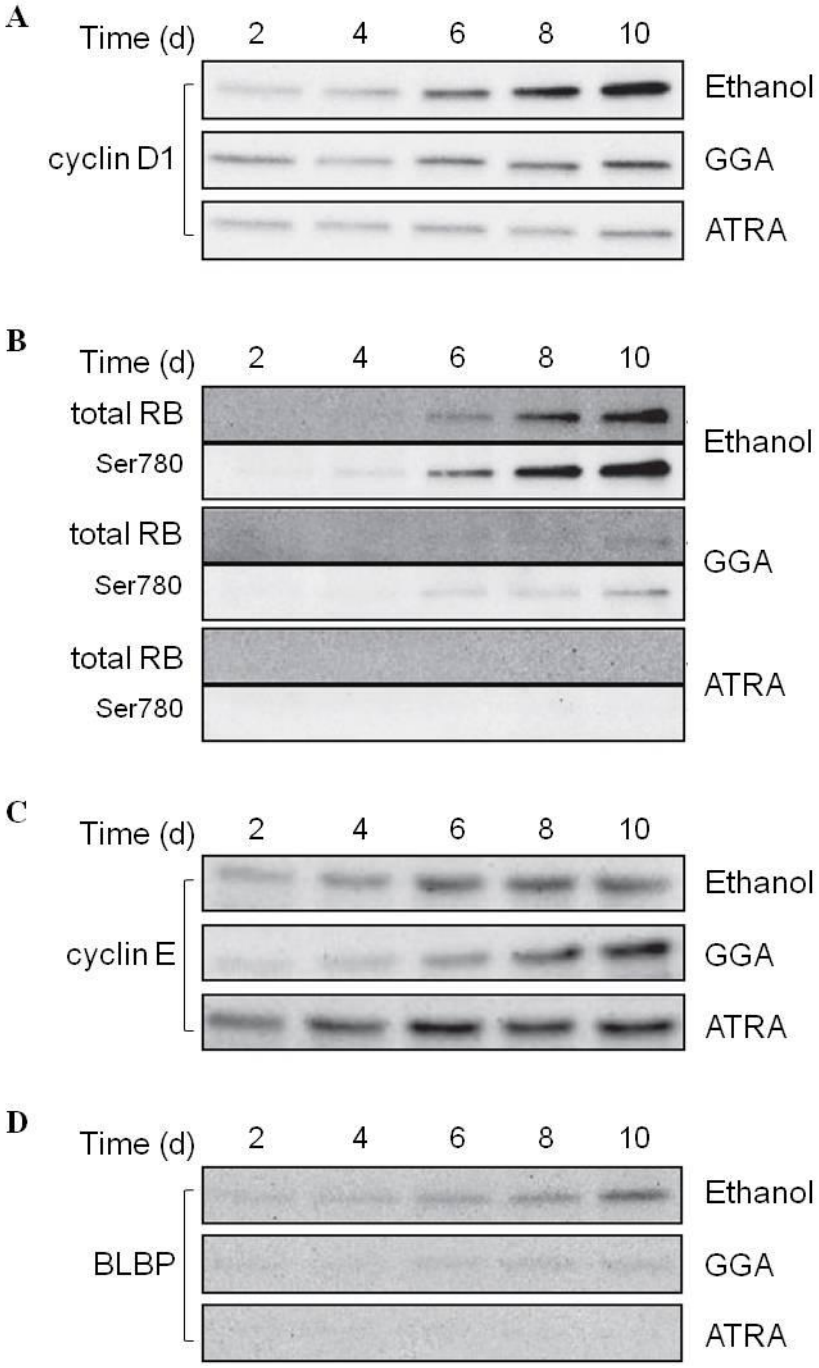

Fig. III-6. Downregulation of cell cycle-related gene expression by GGA.

SH-SY5Y cells were treated with ethanol, 10 μM GGA, or 10 μM ATRA for the indicated number of days Ten micrograms of total protein from the cell lysates was separated by SDS-PAGE and analyzed by immunoblotting with either anti-cyclin D1 (**A**), anti-RB and anti-phosphorylated RB, specific for phosphorylated Ser-780 of RB (**B**), anti-cyclin E (**C**) or anti-BLBP (**D**) antibodies.

III. 3. 3. Morphologic alterations of SH-SY5Y cells by GGA

It has been well established that ATRA inhibits cellular proliferation and induces neurite outgrowth in SH-SY5Y cells. In the present study, control ethanol-treated cells grew and clumped together, forming a ‘‘neurosphere-like’’ structure and did not extend neurite outgrowth (**Fig. III-7**). However, when treated with ATRA (10 μM) for 4 d, cells attached to the dish and dispersed without any cellular aggregation, and neurite-like elongation and its branches were prominently observed (**Fig. III-7** and **Fig. III-8**). It was also clearly observed that 10 μM GGA prevented the formation of cellular aggregates and produced elongation of neurite-like structures after 4-d treatment, almost to the same extent as ATRA. These effects were observed until 10 d after addition of either ATRA or GGA.

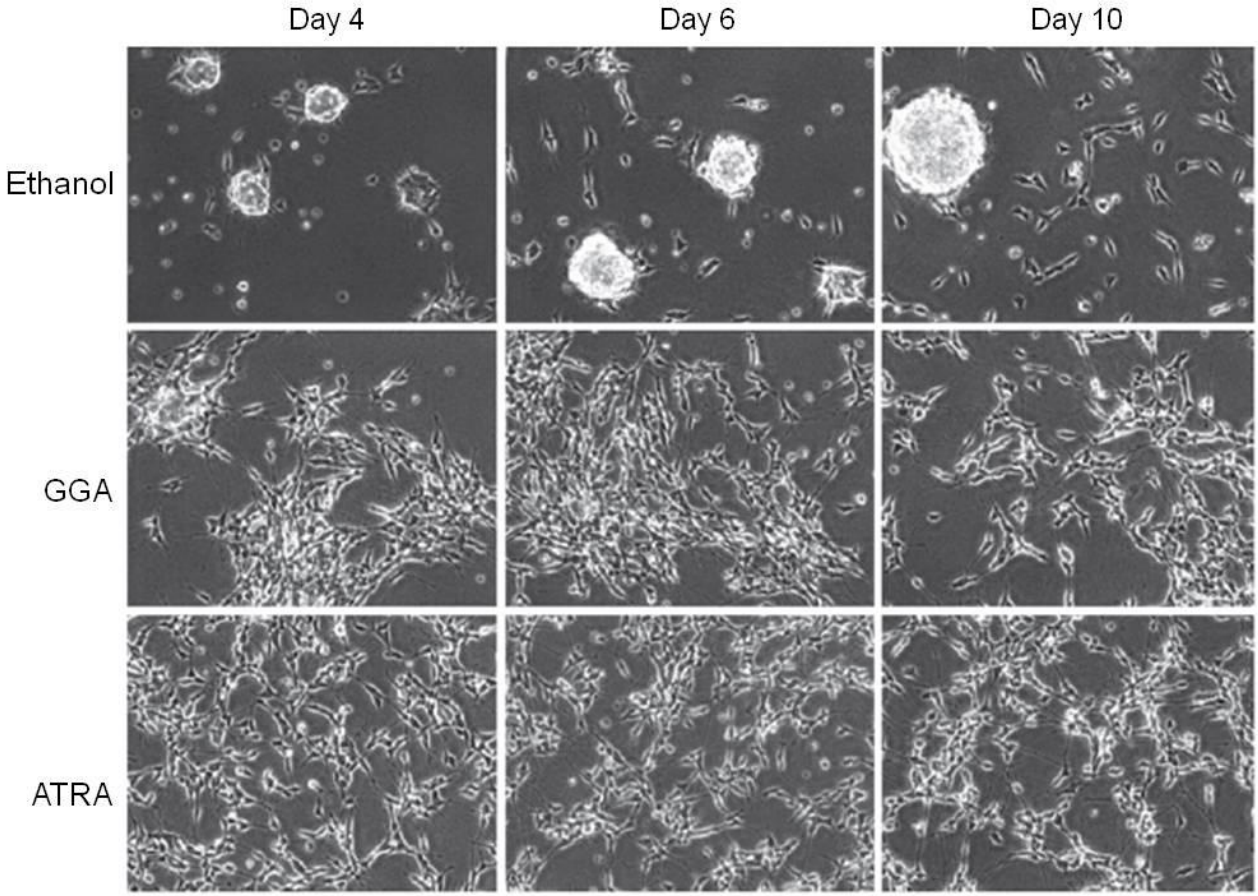

Fig. III-7. Effect of GGA on morphology of SH-SY5Y cells.

Cells were treated with ethanol, 10 μM GGA or 10 μM ATRA for indicated number of days. Phase-contrast images were collected on CCD camera connected to an inverted microscope.

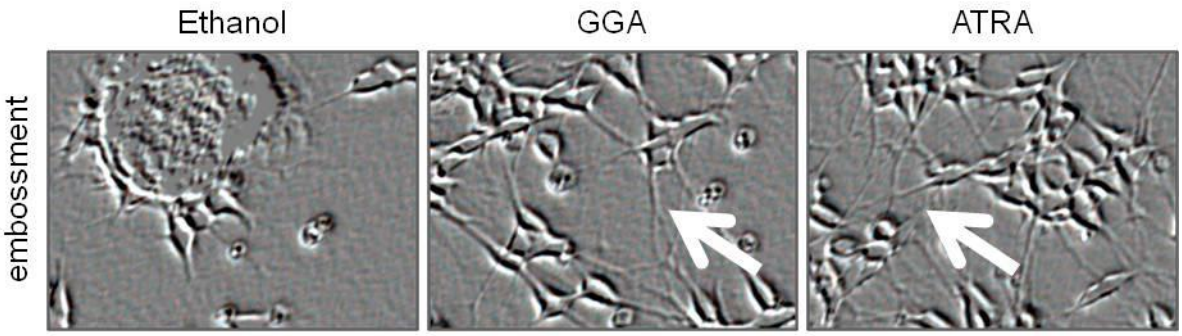

Fig. III-8. Embossment images.

Phase-contrast images on day 6 were cropped and emboss-processed. Arrows indicate neurite-like structures.

III. 3. 4. Downregulation of hexokinase-2 (HK2) with GGA in SH-SY5Y cells

Hexokinase-1 (HK1), brain form hexokinase and HK2, muscle form hexokinase were both detected in control ethanol-treated cells, and increased in a time-dependent manner in control ethanol treated cells (**Fig. III-9**). The time-dependent upregulation of HK2 was completely suppressed by ATRA treatment, and protein levels remained at initial levels after 10-d treatment. Similarly, GGA suppressed their upregulation. In contrast, HK1 expression showed no change during ATRA or GGA treatment.

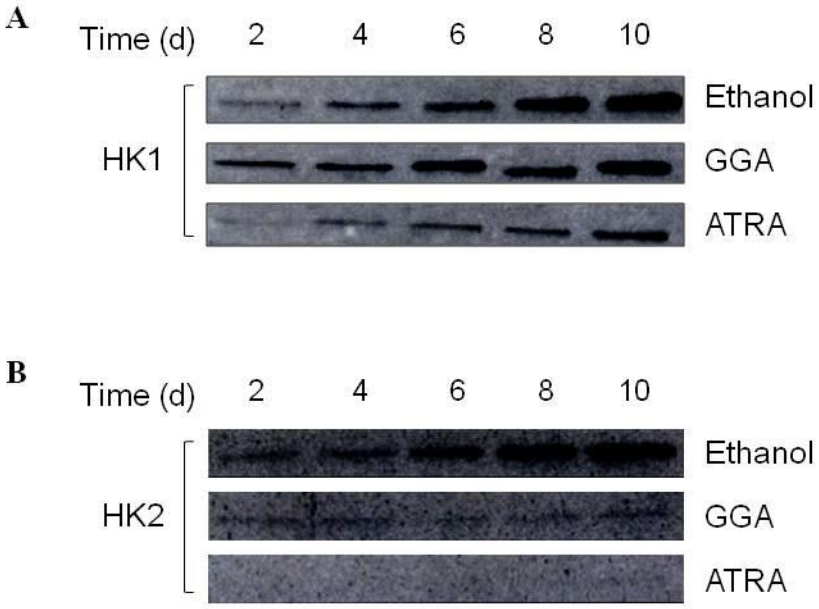

Fig. III-9. Effects of GGA on hexokinases (HKs) expression.

SH-SY5Y cells were treated with ethanol, 10 μM GGA, or 10 μM ATRA for the indicated number of days Ten micrograms of total protein from the cell lysates was separated by SDS-PAGE and analyzed by immunoblotting with either anti-HK1(**A**) or anti-HK2 (**B**) antibodies.

III. 3. 5. Expression of neurotransmitter synthesizing enzymes tyrosine hydroxylase (*TYH*) and choline acetyltransferase (*ChAT*) in SH-SY5Y cells

The expression of the *TYH* and *ChAT* genes was examined during the 10 μM GGA or ATRA treatment. As a result, *TYH* expression was apparently upregulated 2-fold by GGA treatment and (**Fig. III-10A**), whereas *ChAT* expression did not change at all after GGA treatment (**Fig. III-10B**).

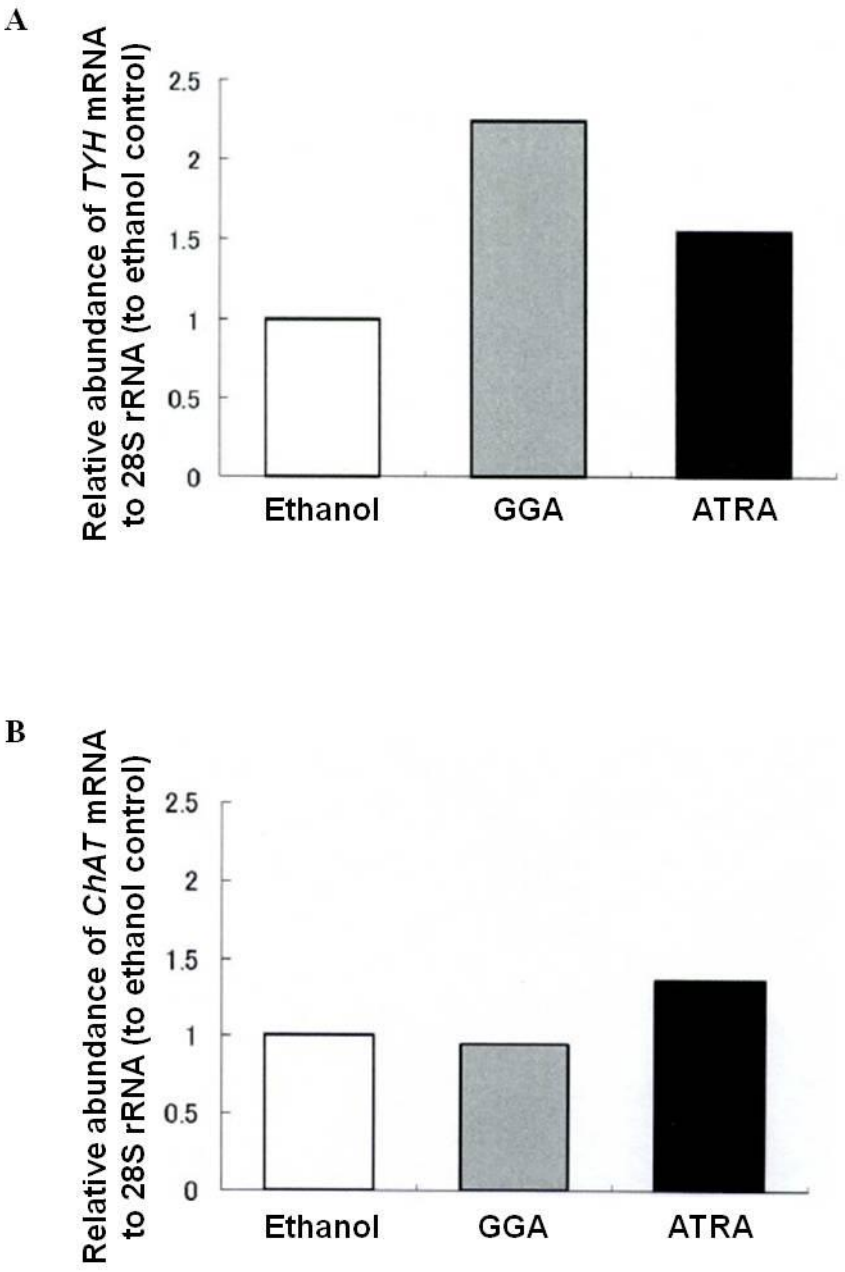

Fig. III-10. Effects of GGA on *TYH* and *ChAT* expression.

Cells were treated with 10 μM GGA, 10 μM ATRA or vehicle alone for 4 d. *TYH* (**A**) and *ChAT* (**B**) mRNA levels were analyzed by quantitative RT-PCR with 28S rRNA levels as an internal control. The experiment was performed in singlicate.

III. 3. 6. Expression of NTRK2 in SH-SY5Y cells

Next, we examined the effect of GGA on the expression of NTRK2 by using the immunoblotting. **Figure III-11** clearly shows that both GGA and ATRA at 10 μM dramatically increased the cellular expression of NTRK2 protein as doublet bands in a time-dependent manner. The upper band of the full-length NTRK2 protein (145 kDa) and the lower band of the truncated NTRK2 (95 kDa) were both induced by both drugs.

We then measured the splice variant levels of *NTRK2* mRNAs. All three variants measured herein were upregulated most efficiently by ATRA treatment, and their upregulation was unexpectedly fast (**Fig. III-12**). By day 2 of treatment, ATRA had already increased the cellular levels of each variant by several hundred folds. The extent of GGA-induced upregulation of the *NTRK2-FL* and *NTRK2-T-Shc* splice variants was almost one-third of the ATRA-induced upregulation of these variants, respectively, while the mRNA levels of the *NTRK2-T1* variant in GGA-treated cells were comparable to those of ATRA-treated cells.

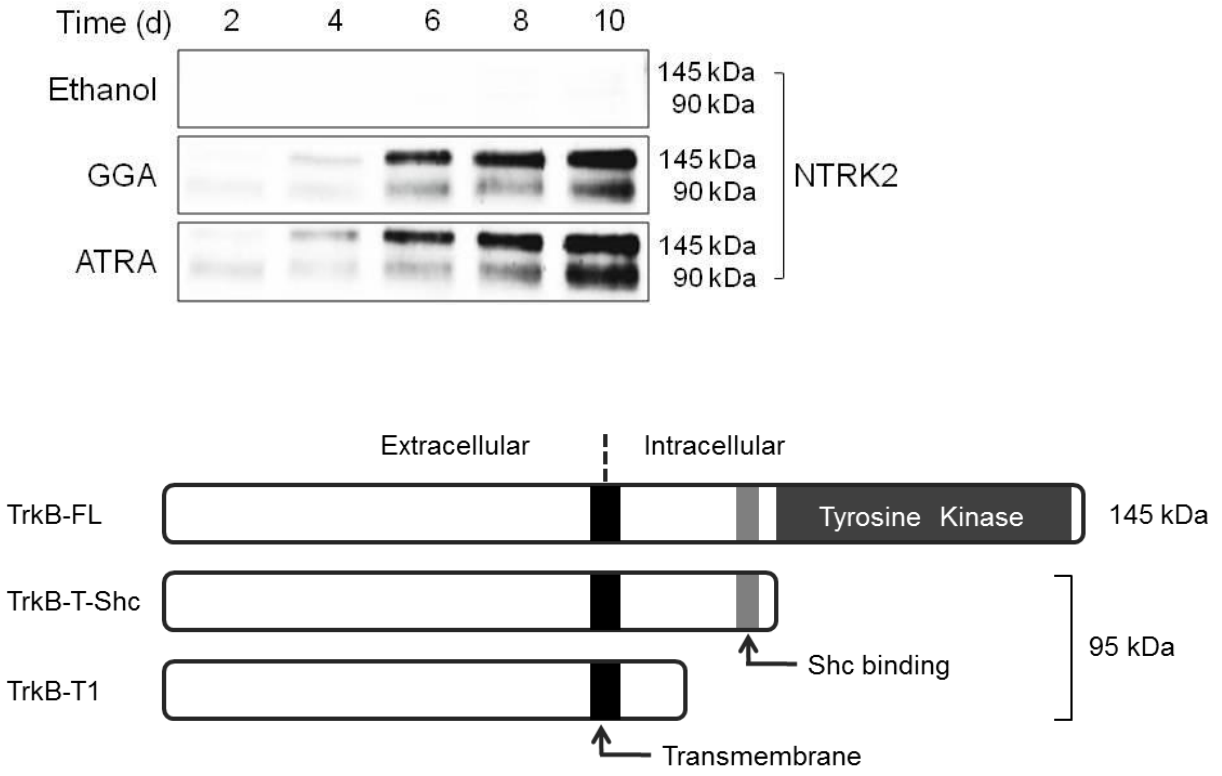

Fig. III-11. Effects of GGA on NTRK2 expression in SH-SY5Y cells.

Cells were treated with ethanol, 10 μM GGA or 10 μM ATRA for indicated number of days. NTRK2 protein levels were analyzed by immunoblotting with anti-NTRK2/TrkB antibody. Lower panel indicates the schematic structure of NTRK2 subtypes.

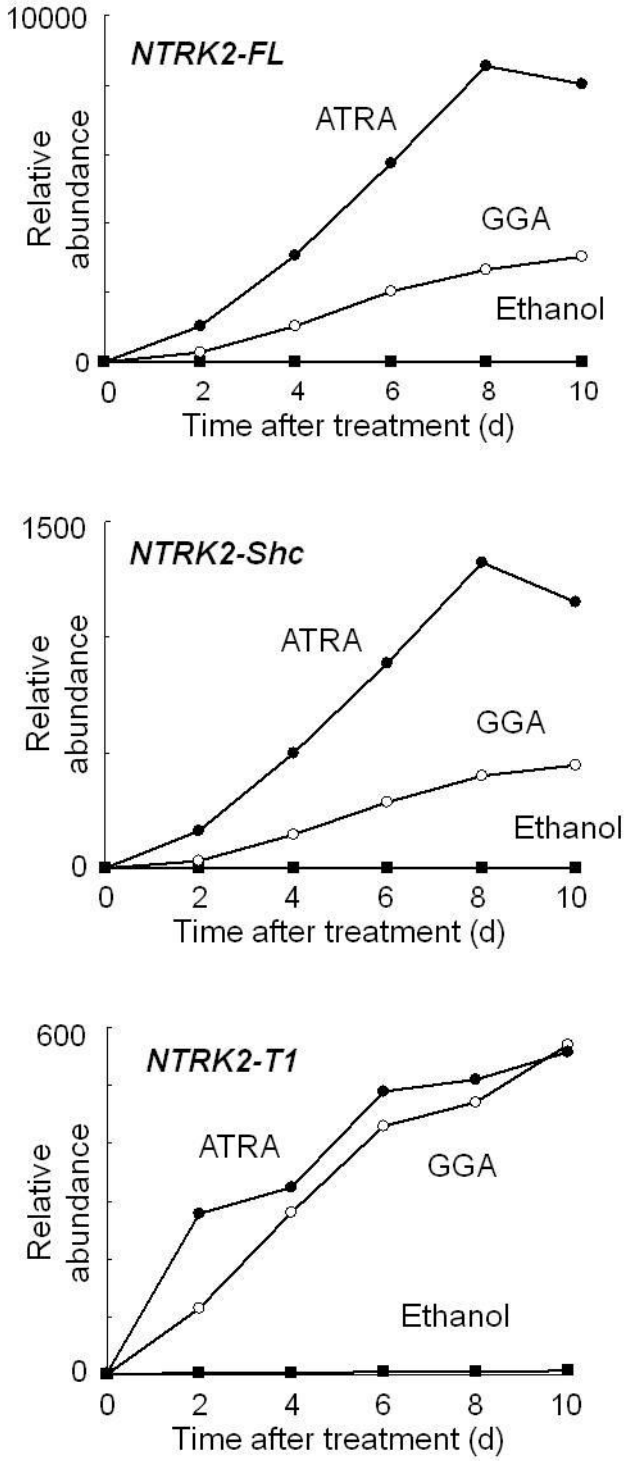

Fig. III-12. Effects of GGA on *NTRK2* splice variant expression in SH-SY5Y cells.

NTRK2-FL, *NTRK2-Shc* and *NTRK2-T1* mRNA levels were individually determined by quantitative RT-PCR with the corresponding specific primer sets. Cells were treated with ethanol (closed squares), 10 μM GGA (open circles) or 10 μM ATRA (closed circles) for the indicated number of days. The experiment was performed in singlicate.

Figure III-13 shows the reversibility of GGA-induced upregulation of *NTRK2* gene expression. GGA (10 μM) and ATRA (10 μM) reproducibly increased the cellular levels of NTRK2 in a time-dependent manner, in comparison with ethanol control. Surprisingly, 4-d removal of GGA after 8-d pre-treatment completely erased the cellular induced NTRK2 protein, whereas 8-d removal of ATRA after 4-d pre-treatment maintained the induced level of NTRK2.

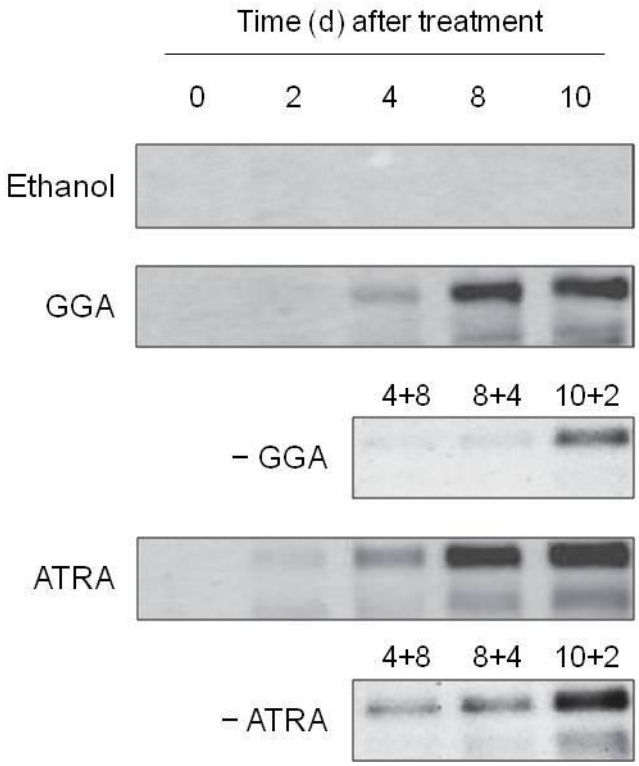

Fig. III-13. Effects of GGA removal on NTRK2 expression in SH-SY5Y cells.

Cells were treated with ethanol, 10 μM GGA or 10 μM ATRA for 4, 8 or 10 d and consecutively incubated in either the same drug-containing media or culture medium alone for 8, 4 or 2 d, respectively. Ten micrograms of total protein from whole cell lysates was separated by SDS-PAGE and immunoblotted with anti-NTRK2 antibody.

III. 3. 7. Expression of nuclear retinoid receptors in SH-SY5Y cells

RARα and RARγ expression were detectable in control ethanol-treated cells and upregulated in a time-dependent manner, whereas ATRA-treated cells showed suppressed expression of these proteins during the experiment (**Fig. III-14**). In contrast, GGA treatment kept the time-dependent expression levels of RARα and RARγ. GGA and ATRA remarkably induced cellular expression of RARβ. A time-dependent increase in RXRα and RXRγ expression was observed in ethanol-treated cells (**Fig. III-14**). ATRA (10 μM) completely blocked the time-dependent increase of RXRα and RXRγ expression, but 10 μM GGA only partially inhibited it.

To examine whether or not ATRA and GGA regulate RARβ expression at the transcript level, *RARB* mRNA was measured. **Figure III-15** shows the *RARB* gene expression was induced at transcript level by ATRA treatment in a time-dependent manner. Treatment with 10 μM GGA also upregulated the expression, although slightly less so than ATRA.

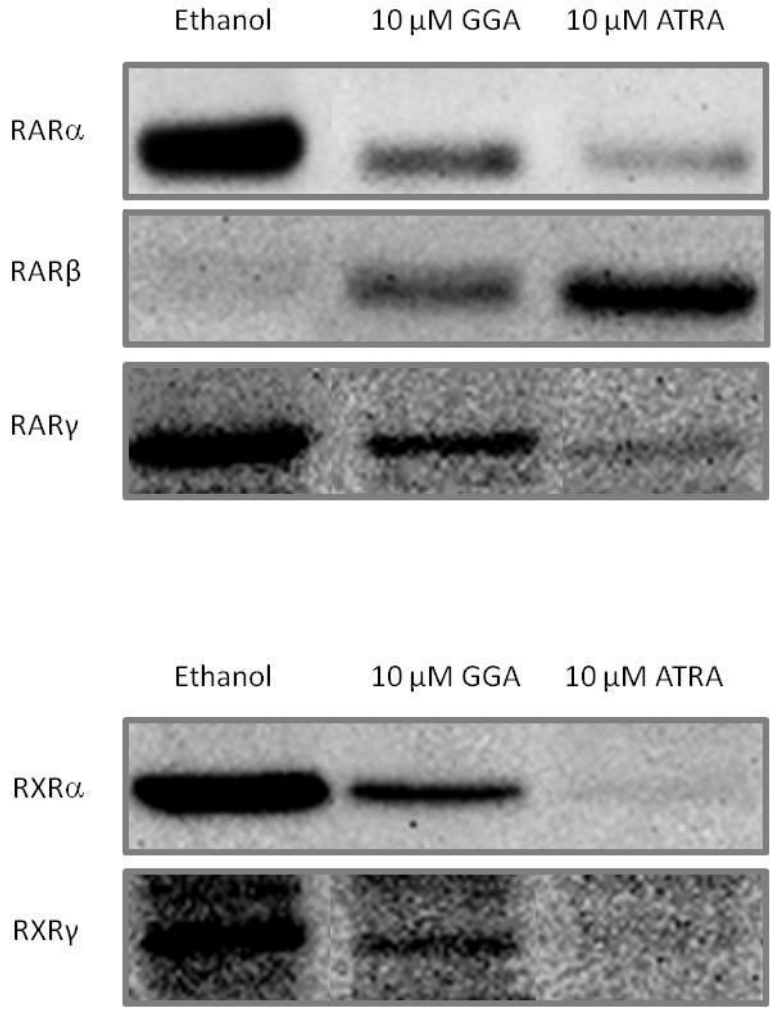

Fig. III-14. Effects of GGA on retinoid receptors expression in SH-SY5Y cells.

Cells were treated with ethanol 10 μM GGA or 10 μM ATRA for 10 d. RAR/RXR protein levels in 10 μg total protein of whole cell lysates were analyzed by immunoblotting with specific antibodies.

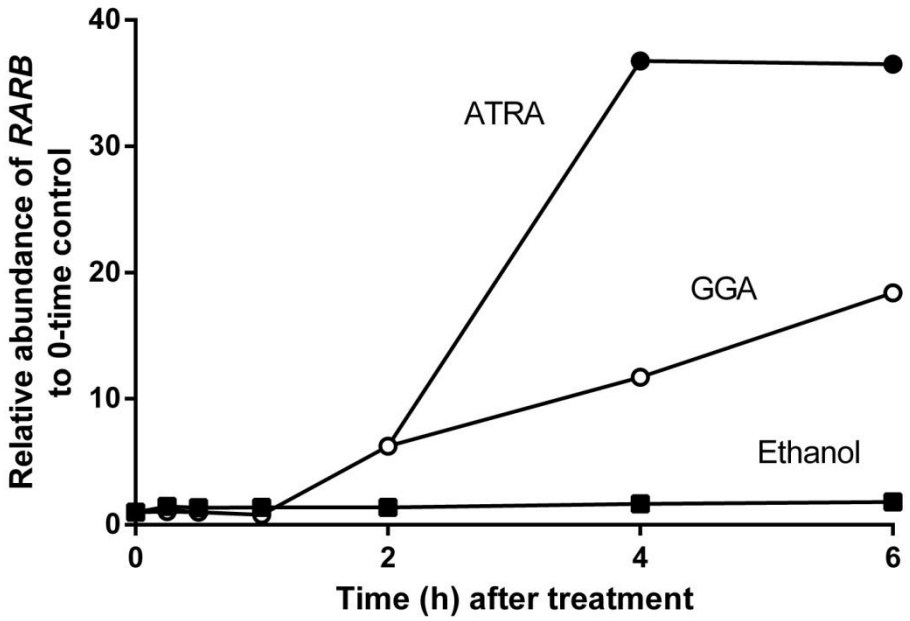

Fig. III-15. Effects of GGA on *RARB* expression in SH-SY5Y cells.

RARB mRNA levels was individually determined by quantitative RT-PCR with the corresponding specific primer set. Cells were treated with ethanol (closed squares), 10 μM GGA (open circles) or 10 μM ATRA (closed circles) for the indicated time. The experiment was performed in singlicate.

III. 3. 8. Effect of overdosing or suppression of *RARB* on *NTRK2* expression induced by GGA

In order to clarify whether nuclear receptor-mediated genomic actions of GGA are involved in the upregulation of the *NTRK2* gene, we performed a knockdown experiment by using siRARB. As shown in **Fig. III-16**, *RARB* gene expression was effectively knocked down by siRARB transfection. However, GGA-induced upregulation of *NTRK2* gene expression was not influenced by siRARB treatment, suggesting that RARβ may not play a role in the transcriptional regulation of the *NTRK2* gene.

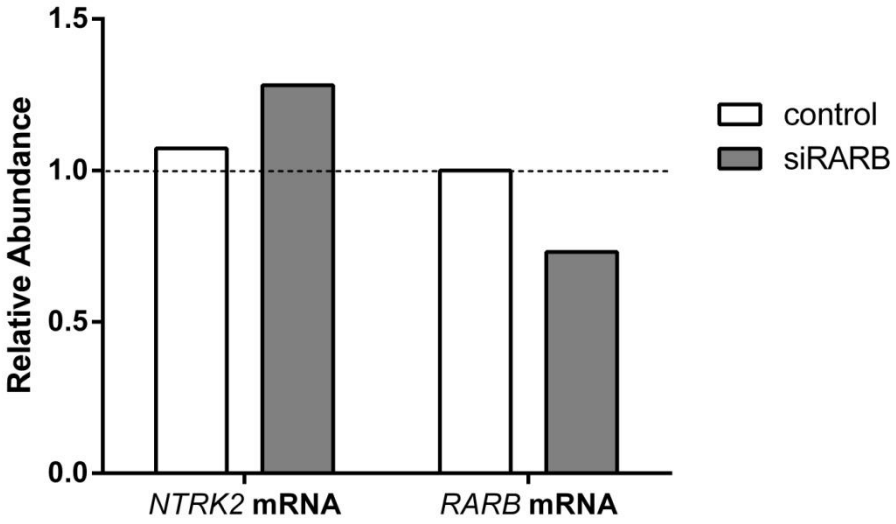

Fig. III-16. Effects of *RARB* knockdown on *NTRK2* gene expression in SH-SY5Y cells.

Small interfering RNAs (400 pmoles per 20 cm^2) were transfected 24 h before treatment. Transfected cells were treated with 10 μM GGA for 24 h. *RARB* and *NTRK2* mRNA levels were individually determined by quantitative RT-PCR with the corresponding specific primer sets. The experiment was performed in duplicate.

On the other hand, gene-overdosing experiment was further performed to confirm that ectopic expression of the *RARB* gene is unable to induce the expression of the *NTRK2* gene. As a result, despite pRShRARB transfection gave an extreme overexpression (2 x10^5 fold) of the *RARB* gene, GGA-induced upregulation of the *NTRK2* gene was not influenced by 2 x10^5-fold overexpression of the *RARB* gene (**Fig. III-17**).

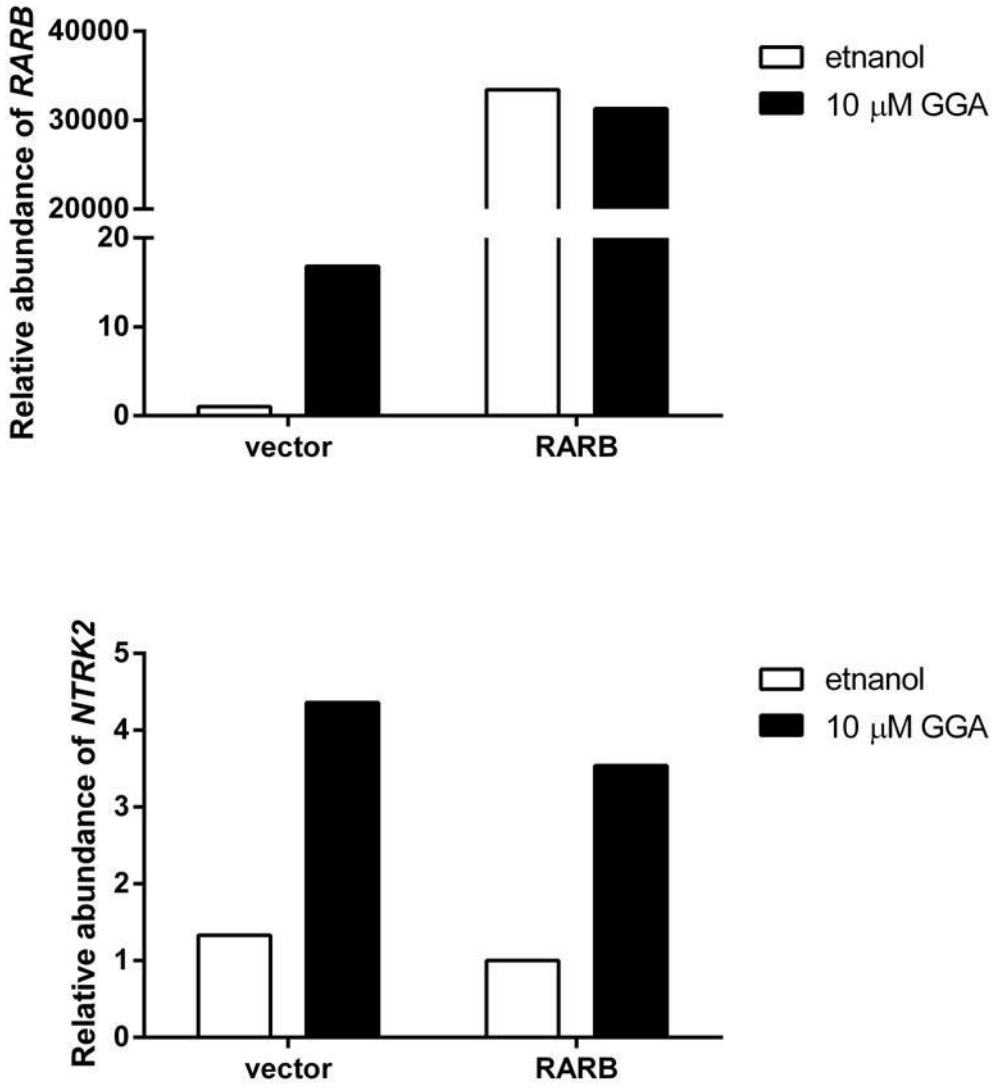

Fig. III-17. Effects of *RARB* gene dosage on *NTRK2* gene expression in SH-SY5Y cells.

pRShRARB or pRS vector (0.2 μg per 0.3 cm^2) were transfected 24 h before treatment. Transfected cells were treated with ethanol or 10 μM GGA for 6 h. *RARB* and *NTRK2* mRNA levels were individually determined by quantitative RT-PCR with the corresponding specific primer sets. The experiment was performed in duplicate.

III. 4. Discussion

We have shown herein that GGA, a safe chemopreventive natural isoprenoid for human hepatoma, is prominently active in inducing growth retardation and *NTRK2* gene expression at both mRNA and protein levels in human neuroblastoma-derived SH-SY5Y cells, comparable to the effects of ATRA. Taking into account that ATRA shows serious and even lethal side effects in clinical therapy of promyelocytic leukemia patients [Larson RS, 2003], we hope that GGA, a natural dietary ingredient, could become a safe alternative to ATRA to protect neurons from carcinogenesis.

In the literature, ATRA has been known to inhibit cellular proliferation and to induce neurite outgrowth in SH-SY5Y cells; hence, ATRA-treated SH-SY5Y cells are extensively used as a differentiate dopaminergic or cholinergic neuronal model [Xie et al, 2010]. Therefore, it is remarkable that GGA had differentiation-inducing effects (inhibition of cellular proliferation; **Fig. III-5**, and induction of neurite outgrowth; **Fig. III-7, 8**) on these neuroblastoma cells, comparable to ATRA.

The cellular levels of cyclin D1, its target RB, and the phosphorylated RB were all significantly reduced by GGA (**Fig. III-6**). These data indicate that GGA-induced G1-arrest may be involved in growth retardation of SH-SY5Y cells. However, it is interesting that cyclin E was not downregulated by either GGA or ATRA treatment, suggesting that GGA and ATRA might induce an impairment in G1-progression, but not in G1/S-transition.

The expression of BLBP/FABP7 is known to be associated with malignancy of glioma cells [Mita et al, 2007] and proliferation and invasiveness of melanoma [Goto et al, 2010]. Therefore, the GGA- or

ATRA-induced reduction of BLBP/FABP7 expression may indicate antitumor effects of GGA and ATRA against neuroblastoma, although dietary supplementation of docosahexaenoic acid-rich oil upregulated BLBP/FABP7 gene expression in experimental brain tumors [Nasrollahzadeh et al, 2008].

A classic study on ATRA-induced differentiation in SH-SY5Y cells demonstrated that treatment with ATRA resulted in cells that were more substrate adherent and exhibited neurite outgrowth [Kaplan et al, 1993]. Similarly, in the present study, we found that GGA-treated cells, as well as ATRA-treated cells, as observed by the lack of cell aggregates (**Fig. III-7**). Furthermore, neurite outgrowth in GGA-treated cells demonstrates morphological characteristics of neural differentiation in these cells (**Fig. III-8**).

ATRA is a well-known inducer of *NTRK2* gene expression at transcription level [Kaplan et al, 1993]. The present study confirmed this and reemphasized that ATRA is a strong inducer of the full-length as well as the truncated forms of NTRK2 protein species and significantly upregulated cellular mRNA levels corresponding to *NTRK2-FL*, *NTRK2-T1*, and *NTRK2-Shc* variants. Surprisingly, GGA showed almost the same effect on NTRK2 gene expression (**Fig. III-12** and **Fig. III-13**). Namely, GGA was shown as a potent inducer of *NTRK2* gene expression at both transcription and protein levels, comparable to ATRA. However, the induction mode was different between ATRA and GGA. Although 8-d removal of ATRA did not affect the previous 4-d induced level of NTRK2 protein, 4-d removal of GGA eliminated the preceding 8-d induced level of the protein, suggesting that ATRA may irreversibly induce *NTRK2* gene expression, whereas GGA may have a reversible effect (**Fig. III-14**). Besides transcriptional regulation, ATRA is known to be involved also in translational regulation, in which ATRA induces the internal ribosomal entry site transactivating

factor or polypyrimidine tract binding protein (PTB1) to recruit the translational machinery for *NTRK2* mRNA [Timmerman et al, 2007]. This combinatorial mechanism of transcriptional and translational upregulation may construct an irreversible effect of ATRA on *NTRK2* gene expression. However, currently we are unaware whether GGA is able to induce PTB1. As for clinical aspects, the reversibility of the effect of GGA on NTRK2 expression may constrain a patient to take the drug for a longer time, although the reversibility of the effect of GGA on NTRK2 expression in vivo remains unknown.

GGA is an acyclic retinoid, which possesses ligand activity for nuclear retinoid receptors [Araki et al, 1995]. ATRA has been believed to act through its nuclear receptors in biological processes, thus we assessed the cellular expression of retinoid receptors such as RARα, RARβ, and RARγ (**Fig. III-10**). RARα and RARγ expression were increased in a time-dependent manner in ethanol control cells, while no RARβ was detected in these cells during the entire experimental period. This expression profile of RARs in SH-SY5Y cells is consistent with previous reports [Carpentier et al, 1997; Joshi et al, 2006]. However, unlike previous reports on ATRA induced upregulation of retinoid receptors [Carpentier et al, 1997; Joshi et al, 2006], ATRA clearly suppressed the time-dependent expression increase of both RARα and RARγ. The suppressive effect of GGA on these two proteins was minor. On the other hand, GGA and ATRA both strikingly induced the cellular expression of RARβ. *RARB2* gene promoters are well known to possess a retinoic acid response element (RARE) motif, and indeed ATRA increases *RARB* mRNA in a variety of cell lines including neuroblastoma [Cheung et al, 2003]. Yamada et al also showed that both 4,5-didehydroGGA and ATRA induced upregulation of *RARB* gene expression in hepatoma cell lines [Yamada Y, 1994].

We almost exclude the possibility that RARβ may be involved in the upregulation of the *NTRK2* gene upon GGA or ATRA treatment, so far there has been no report of a RARE motif at the 5'-upstream region of the *NTRK2* gene. Therefore, in the next chapter, we tried to analyze the non-genomic actions of GGA on the cellular expression of the *NTRK2* gene.

III. 5. Conclusion

We found for the first time that GGA dramatically induced a time-dependent expression of the *NTRK2* gene at protein as well as mRNA levels. The effect was comparable to that of ATRA; however, in contrast to ATRA, it was reversible. These GGA-mediated effects on *NTRK2* gene expression in SH-SY5Y cells are likely to shed light on diet-controlled intervention against neuroblastoma.

Chapter IV

INHIBITION OF LYSINE-SPECIFIC DEMETHYLASE 1A

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Akimori Wada[2]
Hiroshi Sagami[3]
Yoshihiro Shidoji[1]

Inhibition of lysine-specific demethylase 1 by the acyclic diterpenoid geranylgeranoic acid and its derivatives.

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IV. 1. Abstract

Lysine-specific demethylase 1A (KDM1A) is upregulated in many cancers, especially neuroblastoma. We set out to explore whether geranylgeranoic acid (GGA) inhibits KDM1A activity by using recombinant human KDM1A. GGA inhibited KDM1A activity with IC_{50} similar to that of the clinically used drug tranylcypromine. In human neuroblastoma SH-SY5Y cells, GGA induced NTRK2 gene expression alongside upregulation of histone H3 with dimethylated lysine-4 in the regulatory regions of the *NTRK2* gene. Dihydrogenation of GGA reinforced the KDM1A-inhibitory effect in a position-dependent manner. The inhibitory effects of dihydro-derivatives of GGA on recombinant KDM1A strongly correlated with the induction of *NTRK2* gene expression in SH-SY5Y cells. These data demonstrate for the first time the efficient KDM1A-inhibitor activity of GGA and its derivatives, providing a novel prospect of preventing cancer onset by using GGA to regulate epigenetic modification.

IV. 2. Introduction

IV. 2. 1. Epigenetic regulatory mechanisms

In the last several years, many researchers have reported that non-genomic regulation of gene expression during development, particularly neurogenesis. First of all, here we describe overview of "epigenetic regulation" such as DNA methylation, histone modification and non-coding RNAs.

DNA methylation

DNA methylation is a key epigenetic modification in the mammalian genomes known to be involved in many biological processes to regulate nucleotide structure. In mammals, methylation of cytosine base on DNA strands is a heritable epigenetic modification that occurs mostly at the CpG dinucleotides other than the CpGs in CpG islands [Weber et al, 2005]. Because CpG islands are found in approximately 40% of promoters of mammalian genomes, methylation of the CpGs in CpG islands should be reversible by cellular regulatory mechanism. In the last decade, it has become extremely attractive given its involvement in a diverse range of cellular functions including tissue-specific gene expression, cell differentiation [Jackson-Grusby et al, 2001], development [Okada et al, 1999] and reprogramming [Dobbs et al, 2013], genomic imprinting, X chromosome inactivation, and regulation of chromatin structure, disease states and circadian rhythm [Brid et al, 2002; Peaston et al, 2006, Bollati et al, 2010; Faulk et al, 2011; Suzuki et al, 2008]. Notably, the epigenome contains hypervariable regions that could be a source of cellular diversity [Suzuki et al, 2008] or could underlie disease states [Boumber & Issa, 2011]. Such hypervariability might be

influenced by metabolite fluctuations, temperature variation, and other environmental agents that exert their action on chromatin-modifying enzymes and gene regulation [Waterland et al, 2004; Cropley et al, 2006; Gilbert et al, 2007; Chinnusamy et al, 2009]. A clear example of how environment plays an important role in shaping the epigenome is represented by monozygotic twins, who are epigenetically indistinguishable during the early year of life but with age exhibit remarkable differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation, affecting their gene-expression portrait [Fraga et al, 2005].

Histone modification

Nucleosomes, the basic repeating unit of chromatin, consist of ~147 bp DNA wound around a histone core containing two copies of the histone proteins H2A, H2B, H3, and H4 [Luger et al, 1997]. Histones undergo dynamic post-translational modifications (PTMs) on specific residues, most of which are contained on the flexible N-terminal tail that protrudes from the nucleosomal surface [Cosgrove, 2007]. It has been hypothesized that PTMs may form a “histone code” in which particular marks or combinations of marks elicit a specific physiological response by regulating chromatin structure [Jenuwein & Allis, 2001]. PTMs may perform these tasks by directly altering the chemical environment of the surrounding chromatin or through the action of other proteins that bind to these marks, termed readers. Readers may contain or recruit effector proteins, forming a signaling scaffold to alter chromatin function and consequently mediate processes such as gene expression, apoptosis, and DNA damage repair [Jenuwein & Allis, 2001].

In human cells, many activities essential for cell survival, such as DNA transcription, synthesis and repair, are mediated by dynamic changes in nucleosome structure that facilitate access of DNA-binding proteins to double-stranded DNA [Clapier et al, 2009]. Proteins that regulate the change in nucleosome structure are called chromatin-regulating proteins. These proteins can be classified into two groups that take part in distinct mechanisms: histone modification and chromatin remodeling. Some histone modifiers attach substrates, such as phosphate, poly-ADP-ribosyl, acetyl, methyl, SUMOyl and ubiquityl groups to histone tails by covalent interaction, whereas other histone modifiers detach these groups from previously modified histones. Chromatin remodelers usually function as complexes that change nucleosome assembly (e.g. by forming a DNA-loop or sliding a nucleosome) in an ATP-dependent manner.

Histone lysine methylation has been widely accepted as a key epigenetic modification. Whereas acetylation of lysine residues causes neutralization of the basic amine group, the methylation does not change the charge of lysine residues and thus has a minimal direct charge effect on DNA-histone association. Rather, the different methylation status of specific histone lysine can serve as a unique platform for recruiting methylation “reader” proteins that activate or repress genes’ transcriptional activity through hydrophobic interaction. In general, histone H3 lysine-4 (H3K4), H3K36, and H3K79 methylation are gene activation marks, whereas H3K9 and H4K27 methylation are gene-repressive modifications [Wozniak & Strahl, 2014].

H3K4me3 occupies as many as 75% of all human gene promoters in several cell types (e.g., ES cells), indicating that it plays a critical role in mammalian gene expression [Pan et al, 2007; Zhao et al, 2007]. In fact, H3K4me3 is required to induce critical developmental genes in animals, including several mammals,

and is important for animal embryonic development [Shilafard et al, 2012]. H3K4me3 levels are positively correlated with gene expression levels [Barski et al, 2007; Pokholok et al, 2005].

Lysine-specific demethylase 1A (KDM1A)

Although H3K4me3 is clearly associated with actively transcribed genes, studies have demonstrated that H3K4me3 is localized around the transcription initiation sites of numerous unexpressed genes in human ES cells, primary hepatocytes, and several other cell types [Pan et al, 2007; Zhao et al, 2007; Guenther et al, 2007]. In particular, it frequently co-resides with the repressive mark H3K27me3 in the promoters of critical differentiation-specific genes [e.g., *Homeobox* (*HOX*) gene clusters] that are transcriptionally inactive in ES cells [Pan et al, 2007; Zhao et al, 2007; Bernstein et al, 2007; Mikkelsen et al 2006]. It has been proposed that the "bivalent" domains, composed of H3K4me3 and H3K27me3, may maintain differentiation-specific gene promoters in a repressive status in self-renewing stem cells but be poised for prompt gene activation upon differentiation stimuli [Bernstein et al, 2007]. Consistent with this, many bivalent genes have increased H3K4me3 levels and decreased H3K27me3 levels while being transcriptionally activated during differentiation. Interestingly, recent studies demonstrated that most bivalent domains are occupied by lysine-specific demethylase 1A (KDM1A) [Adamo et al, 2011; Whyte et al, 2012], indicating that it plays a role in maintaining low levels of dimethylated H3K4 (H3K4me2) that often co-exist with H3K4me3. For these reasons, H3K4me3 is classified as a chromatin landmark for transcriptionally active genes in ES cells [Guenther et al, 2007].

Most H3K4me3-containing promoters are also occupied by H3K9/H3K14 acetylation [Guenther et al, 2007]. In transcriptionally active genes, H3K36me3 and H3K79me2 are significantly enriched downstream of H3K4me3-containing promoters: H3K36me3 peaks toward the 3′ end of genes in gene bodies, whereas H3K79me2 is located toward the 5′ end [Guenther et al, 2007]. Therefore, H3K4me3 likely cooperates with other histone marks for gene activation. The combinatorial arrangement of H3K4me3 and other histone marks may support, at least in part, the "histone code" hypothesis [Jenuwein et al, 2001].

H3K4me2 decorates genomic regions independently of H3K4me3, although most of it overlaps with H3K4me3 near the transcription start sites [Bernstein et al, 2005]. H3K4me2 may have an antagonistic effect on DNA methylation [Weber et al, 2007]. Monomethylated H3K4 (H3K4me1) also co-occupies regions near the start sites with H3K4me3. Apart from the transcription start sites, H3K4me1, together with H3K27 acetylation, specifies enhancer regions [Visel et al, 2009; Heintzman et al, 2009]. In summary, H3K4me1, H3K4me2 and H3K4me3 have commonality in gene activation, although their subsets play distinct roles in modulating chromatin function.

The reversibility of histone methylation was not clear until the discovery of the first histone demethylase KDM1A (also known as LSD1) in 2004 [Shi et al, 2004]. Subsequently, more than dozen of human histone lysine-specific demethylase genes have been cloned (**Table IV-1**). A new class of the F-box protein family was reported as KDM2A that specifically demethylates both mono- and di-methylated lysine-36 of histone H3 (H3K36me1/me2). Another new class of zinc finger proteins containing JmjC-domain was identified that KDM3A/JMJD1A can demethylate methylated lysine residues in histone (H3K9me3) [Goda et al, 2013],

KDM4A/JMJD2A for methylated lysine residues H3K9/36 [Black et al, 2013], KDM5C/JARID1C for H3K4me3 [Grafodatskaya et al, 2013], and KDM6A/JMJD3 for H3K27 [Shahhoseini et al, 2013]. These members of KDM super-family play important roles in gene transcription in various cells during development and homeostasis.

Among them, as mentioned above numerous studies in ES cells and neural stem cells strongly suggest that KDM1A is a key histone methylation modifier in transcriptional regulation for cell fate determination. *KDM1A*-null mice are embryonic lethal around E6.5, and *KDM1A*-deficient mouse ES cells demonstrate increased cell death and impaired differentiation, such as embryoid body formation defects [Wang et al, 2007]. It has been reported that KDM1A is also required for neural stem cell proliferation; it is recruited to chromatin by the nuclear orphan receptor NR2E1/TLX to repress negative cell cycle regulators, including *p21*, *p53*, *POU5F1* or *OCT3/4* and *PTEN*, resulting in continuous proliferation of neural stem cells [Sun et al, 2010; Wang et al, 2012].

Table IV-1. Tentative classification of histone lysine demethylases (KDMs) in NCBI gene database

Official Symbol	Other Aliases	Gene Location	Histone Substrates	Family	References
KDM1A	LSD1, AOF2	1p36.12	H3K4me1/me2, H3K9me1/me2	AOF	Shi et al, 2004,
KDM1B	LSD2, AOF1	6p22.3	H3K4me1/me2	AOF	Fang et al, 2010
KDM2A	FBXL11, JHDM1A	11q13.1	H3K36me1/me2	F-box	Tanaka et al, 2010
KDM2B	FBXL10, JHMD2A	12q24.31	H3K4me3, H3K36me1/me2	F-box	Tsukada et al, 2006; Frescac et al, 2007
KDM3A	JMJD1A, TSGA	2p11.2	H3K9me1/me2	ZF-Jmj	Yamane et al, 2006
KDM3B	JMJD1B, C5orf7	5q31	H3K9me1/me2	ZF-Jmj	Kim et al, 2012
JMJD1C	TRIP8	10q21.3	H3K9me1/me2	ZF-Jmj	Wolf et al, 2007
KDM4A	JMJD2A, JMJD2	1p34.1	H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3	JMJD2	Klose et al, 2006; Whetstine et al, 2006
KDM4B	JMJD2B	19p13.3	H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3	JMJD2	Shi et al, 2011
KDM4C	JMJD2C, GASC1	9p24-p23	H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3	JMJD2	Cloos et al, 2006
KDM4D	JMJD2D	11q21	H3K9me2/me3, H1.4K26me2/me3	JMJD2	Shin & Janknecht, 2007
KDM4E	JMJD2E	11q21	H3K9me2/me3, H3K56me3	JMJD2	Thalhammer et al, 2011
KDM5A	JARID1A, RBBP2	12p13.33	H3K4me2/me3	JARID1	Hayakawa et al, 2007
KDM5B	JARID1B, PLU-1	1q32.1	H3K4me2/me3	JARID1	Yang et al, 2007
KDM5C	SMCX, JARID1C	Xp11.22-p11.21	H3K4me2/me3	SMCY	Iwase et al, 2007; Tahiliani et al, 2007
KDM5D	HY; HYA; SMCY	Yq11	H3K4me2/me3	SMCY	Akimoto et al, 2008

IV. 2. 2. Epigenetic regulation in cancer

Epigenetic alterations are key causes of aberrant gene function leading to cancer [Sharma et al, 2010; Varier et al, 2011]. Global hypomethylation of genomic DNA is commonly observed in cancerous cells, leading to genomic instability and activation of growth-promoting genes. Conversely, site-specific hypermethylation of promoter region CpG islands is also typical of cancers, causing silencing of tumor suppressor genes such as *p53* and *RB*. The CpG methylation is required for mammalian development and is also often perturbed in human cancers [Weber et al, 2005].

Additionally, histone modification is typical alterations of chromosome structure in cancerous cells. For a famous example, increased histone deacetylase activity in tumor cells results in a global loss of acetylated histones and subsequent nucleosome remodeling. But in this thesis, we have paid attention to another histone modification, methylation.

As repeatedly described, KDM1A is capable of removing dimethyl and monomethyl groups on H3K4me2 and H3K4me1, as well as methyl groups on non-histone proteins such as a tumor suppressor p53 and DNA methyltransferase 1 [Forneris et al, 2009]. Overexpression of KDM1A is frequently observed in prostate, breast, and bladder cancers, and especially neuroblastoma, where it correlates directly with adverse clinical outcome and inversely with differentiation [Schulte et al, 2009]. Thus, KDM1A enzyme inhibitors are of clinical interest for their anticancer role as well as their potential application in other human diseases that exhibit deregulated gene expression.

IV. 2. 3. KDM1A as a candidate target of isoprenoids

KDM1A is a flavin adenine dinucleotide (FAD)-containing enzyme belonging to the amine oxidase superfamily [Forneris et al, 2008]. The structural homology between KDM1A and monoamine oxidase-B (MAOB), a clinically validated pharmacological target, suggests that KDM1A is a druggable target. Indeed, screening of known MAO inhibitors has uncovered sub-millimolar KDM1A inhibitors among which the best known is the antidepressant drug tranylcypromine (*trans*-2-phenylcyclopropylamine or 2-PCPA). The drug acts as an irreversible inhibitor forming a covalent adduct with the FAD cofactor of KDM1A with a *K*i value of 242 μM [Schmidt & McCafferty, 2007]. Besides the antidepressant, the terpene *trans, trans*-farnesol, a component of tobacco smoke, is well known as a potent, reversible, specific inhibitor of mammalian MAOB [Khalil et al, 2006]. *K*i values of farnesol for the inhibition of human, baboon, monkey, dog, rat, and mouse liver MAOB are within the range of 0.5–5 μM. Although three-dimensional structures of MAOB-farnesol [Hubalek et al, 2005], MAOB-tranylcypromine [Bonivento et al, 2010], and KDM1A-tranylcypromine [Binda et al, 2010] complexes have been determined to sub-molecular resolution, no published structure of the KDM1A-farnesol complex is available. Furthermore, while several candidate KDM1A inhibitor compounds have been synthesized based on the structures of KDM1A and MAOB in complexes with the antidepressant tranylcypromine, limited studies on the structure-activity relationship of farnesol analogs with human KDM1A have been reported to date.

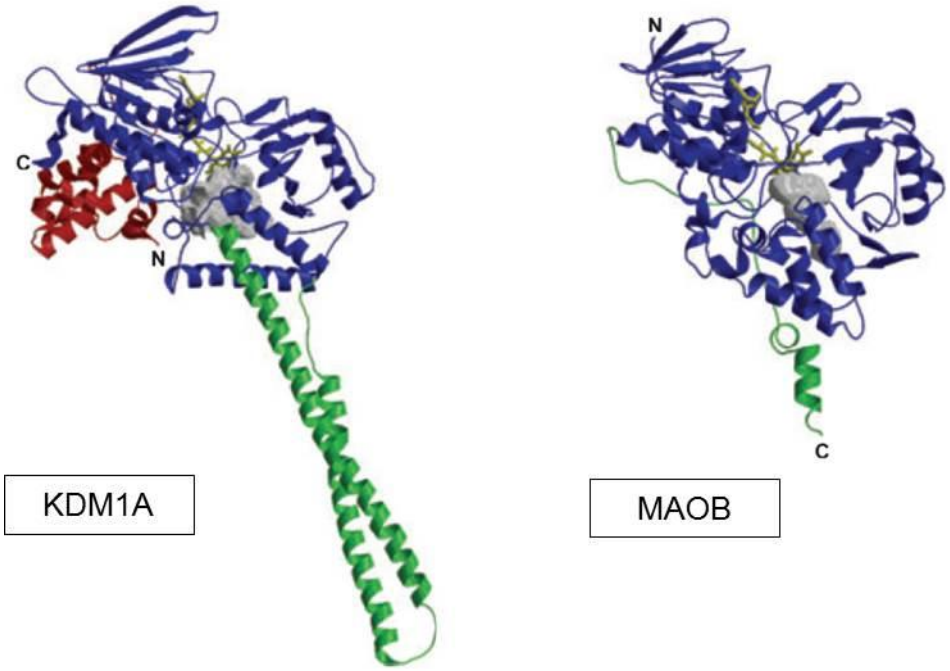

Fig. IV-1. 3D-structure of KDM1A and MAOB.

IV. 2. 4. Aim of the study

Here, we analyze whether GGA and its dihydro-derivatives are potent inhibitors of recombinant human KDM1A. Furthermore, we are going to present that these compounds induce *NTRK2* gene expression via upregulation of H3K4me2 in the putative promoter regions of the *NTRK2* gene in human neuroblastoma SH-SY5Y cells.

IV. 3. Results

IV. 3. 1. Inhibition of KDM1A activity by farnesol

First, farnesol was confirmed as a micromolar inhibitor of human MAOB. Farnesol inhibited recombinant MAOB enzyme with an IC_{50} value of 2 μM (**Fig. IV-2, upper panel**). This IC_{50} is close to the reported *K*i value for farnesol with human MAOB [Khalil et al, 2006]. Farnesol did not inhibit MAOA, rather a slight activation was observed with increasing concentrations of farnesol. **Fig. IV-2, lower panel**, shows that farnesol also inhibited recombinant human KDM1A activity in a dose-dependent manner with IC_{50} of approximately 120 μM when the first 21 amino acid-containing peptide of the N-terminal tail of histone H3 was used as substrate. This indicates that farnesol is also a weak inhibitor for human KDM1A.

Conversion of the terminal functional group of farnesol from alcohol either to a carboxyl, amide, or amine group did not enhance inhibitor activity at 100 μM, whereas the standard inhibitor tranylcypromine efficiently inhibited KDM1A activity at the same concentration (**Fig. VI-3**).

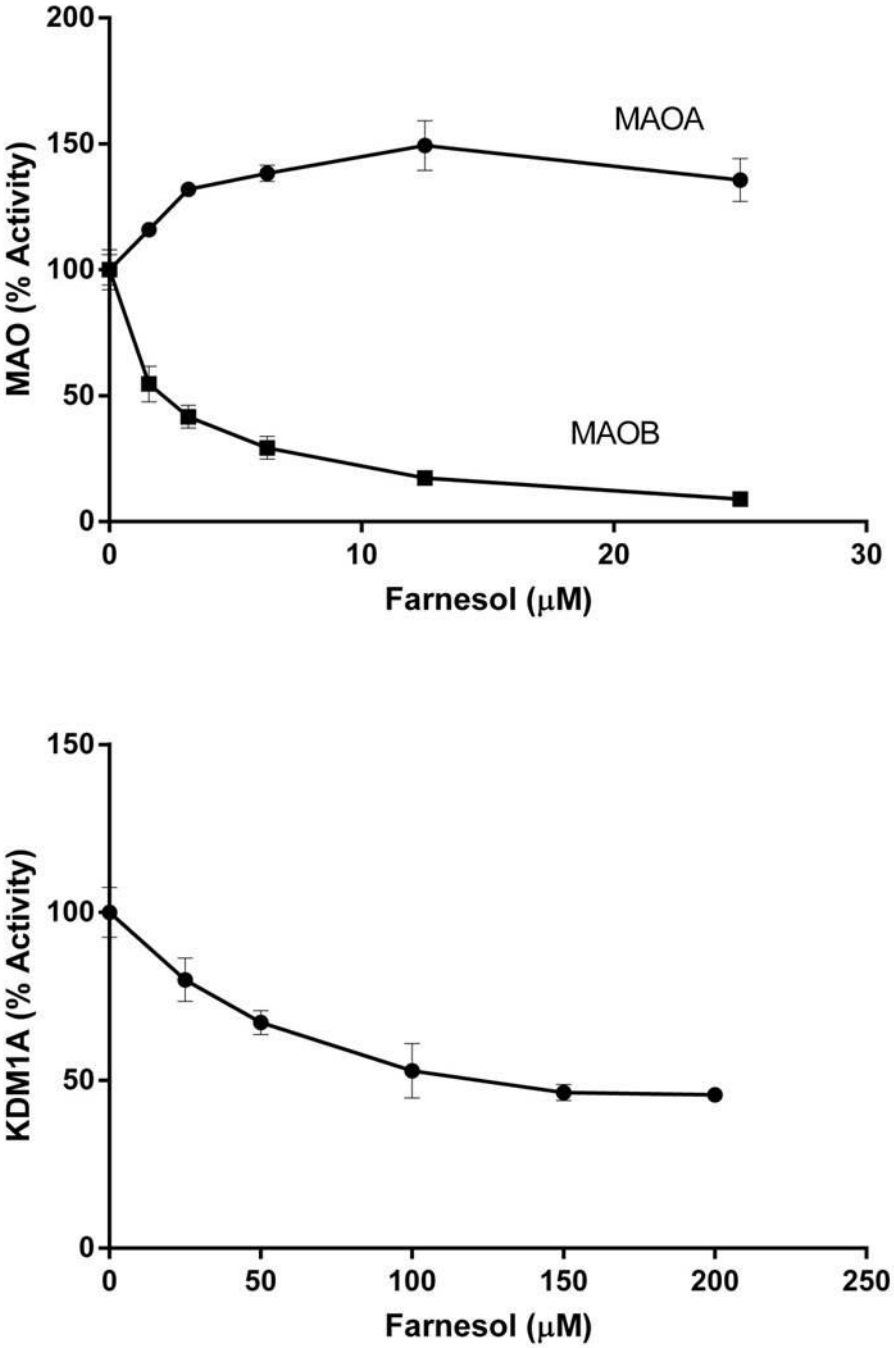

Fig. IV-2. Inhibition of recombinant human MAOB and KDM1A activities with farnesol.

Farnesol was pre-incubated with enzymes for 1 h on ice. The enzyme reaction was performed for 1 h (MAO) or 30 min (KDM1A) at 37°C. Results were presented as the mean ± SD (*n* = 3).

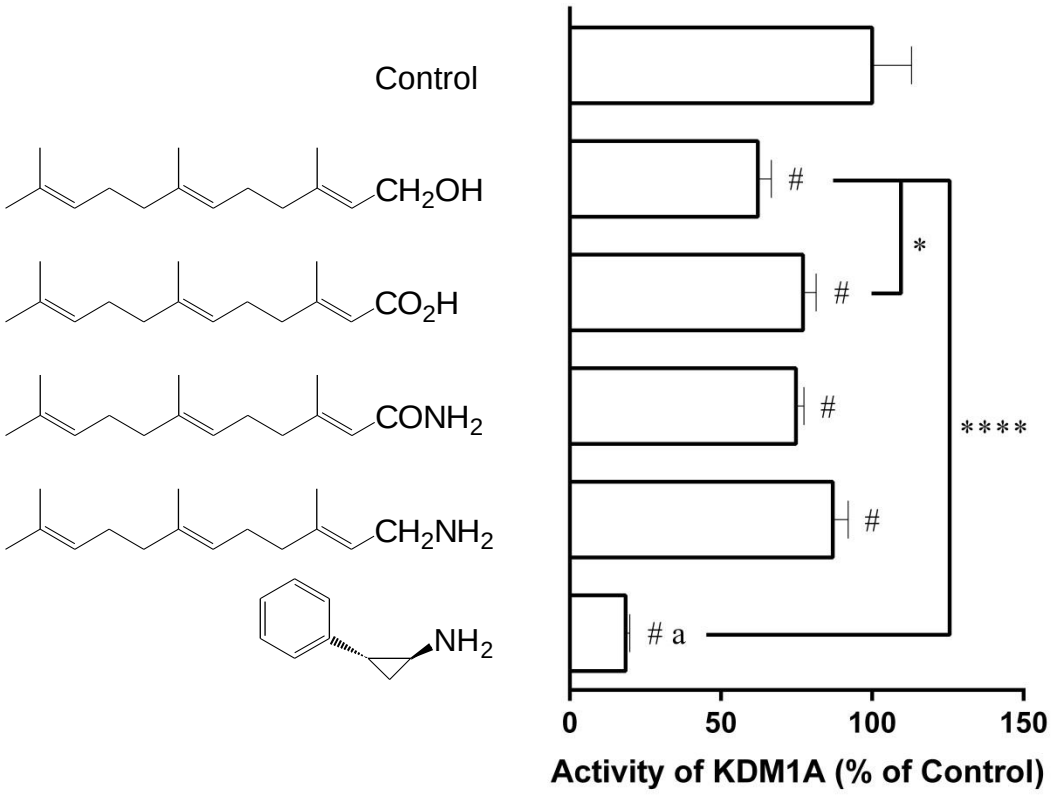

Fig. IV-3. KDM1A inhibitory effects of farnesol derivatives.

Farnesol derivatives were assessed at 100 μM. *trans*-2-phenylcyclopropylamine (tranylcypromine; 2-PCPA) was used as a positive control. Results were presented as the mean ± SD (n = 3) and analyzed by Tukey's multiple comparisons test, #; significant vs. control, a; significant vs. farnesol derivatives, *p < 0.05, ****p < 0.0001.

IV. 3. 2. Effects of isoprenoid chain length on KDM1A inhibitory activity

When the terminal functional group of polyisoprenoids was alcohol, the number of isoprene units in each molecule, from two (C_{10}) to four (C_{20}), did not affect KDM1A inhibitory activity (**Fig. IV-4A**). Conversely, when a carboxyl terminal group was present, increasing the number of isoprene unit from two (C_{10}) to four (C_{20}) significantly increased KDM1A inhibitory activity. Consequently, C_{20}-GGA was by far the most potent inhibitor of KDM1A tested. The IC_{50} of C_{20}-GGA was 47 μM (**Fig. IV-4B**), and the mode of the inhibition was non-competitive (**Fig. IV-5**).

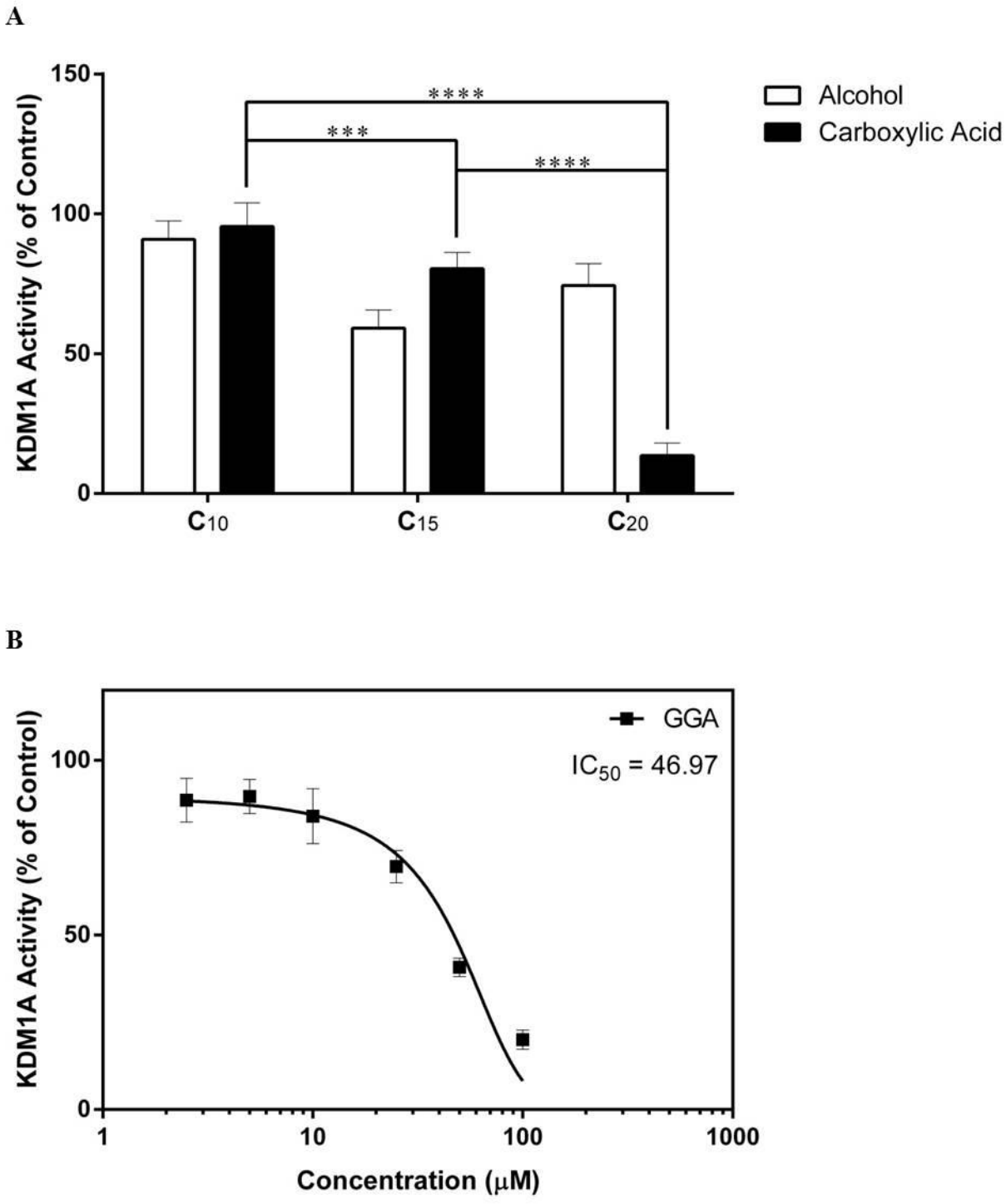

Fig. IV-4. Isoprenoid chain length-dependent inhibition of KDM1A activity by polyprenoic acids. (**A**) Each inhibitor was used at a concentration of 100 μM. Data are expressed as the mean ± SD (n = 5) and analyzed by Tukey's multiple comparisons test, ***p < 0.001, ****p < 0.0001. (**B**) Dose-dependent inhibition of KDM1 by geranylgeranois acid. Data are expressed as mean ± SD (n = 4).

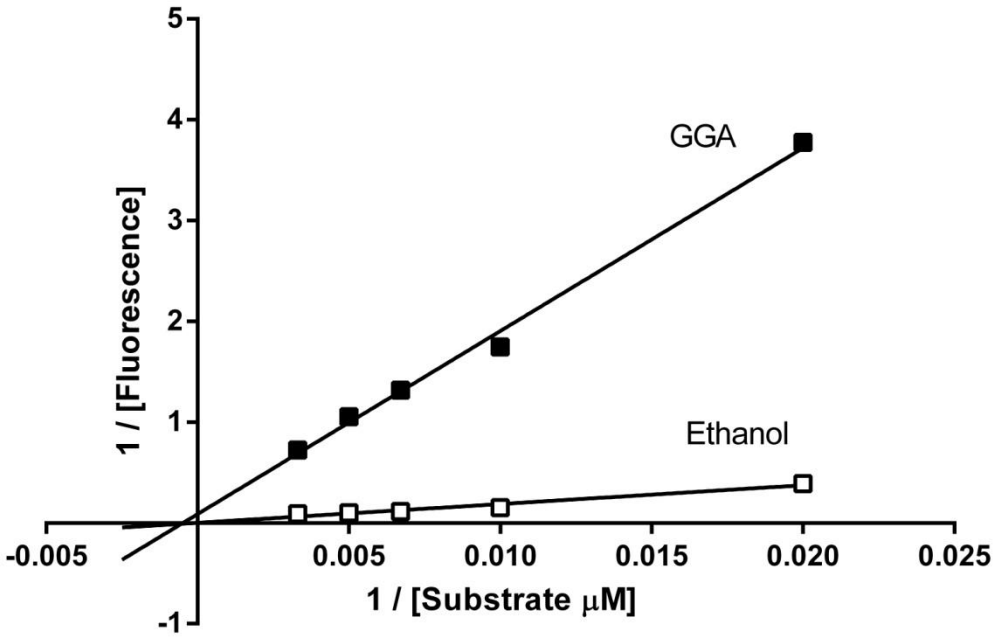

Fig. IV-5. GGA inhibits KDM1A in a non-competitive fashion.

Ethanol or GGA were pre-incubated with KDM1A for 1 h on ice. The aliquot enzyme reaction with indicated concentration of substrate was performed for 30 min at 37°C. The experiment was performed in duplicate

IV. 3. 3. Effect of dihydrogenation on KDM1A-inhibitory activity of GGA

Dihydrogenation of isoprene units on GGA reinforced its inhibitory activity, dependent on the site of hydrogenation (**Fig. IV-6**). From most to least potent, the KDM1A-inhibitory activity of GGA dihydro-derivatives was as follows: 14,15- > 10,11- > 6,7- > (*S*)2,3- > (*R*)2,3-dihydroGGA. This suggests that a steric structure of a straight tetra-isoprenoid chain is important for inhibition of hKDM1A activity. The most potent inhibitor, 14,15-dihydroGGA, had an IC_{50} of 22 μM, comparable to that of 2-PCPA (23 μM; **Fig. IV-7**).

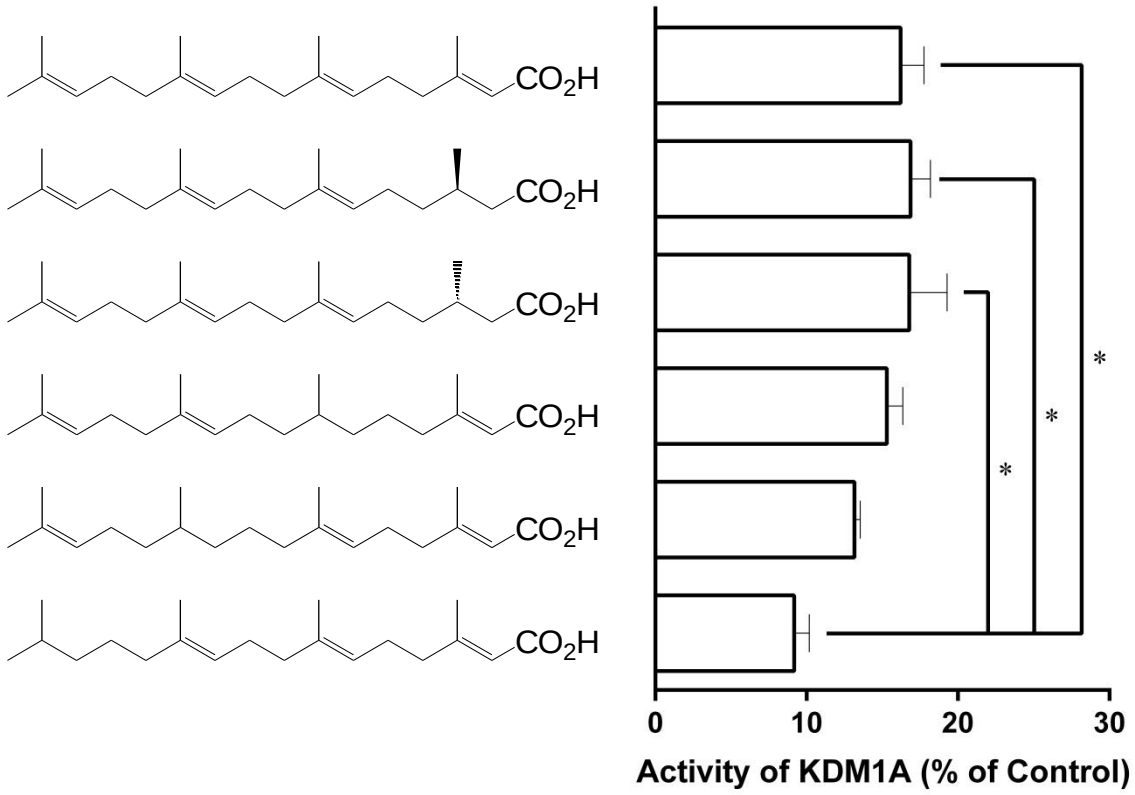

Fig. IV-6. Effect of dehydrogenation on the KDM1A-inhibitory activity of GGA.

Dihydro-GGAs (100 μM each) were pre-incubated with hKDM1A and KDM1A enzyme activity was measured. Data are presented as mean ± SD (n = 3) and analyzed by Tukey's multiple comparisons test, *$p < 0.05$.

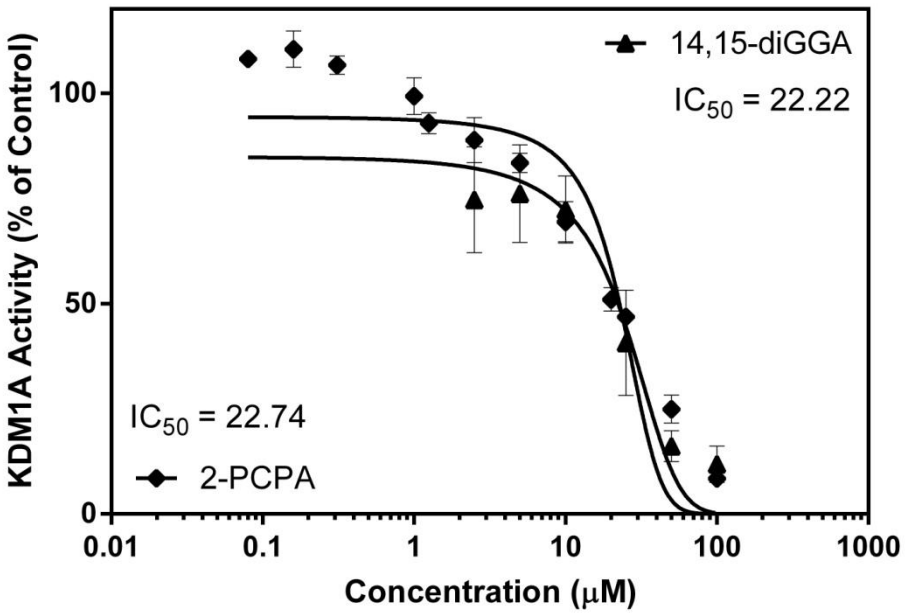

Fig. IV-7. Concentration dependence of the KDM1A-inhibitory activity of 14,15-dihydroGGA.

KDM1-inhibitory effects of 14,15-dihydroGGA and 2-PCPA at the indicated concentrations. Results are presented as the mean ± SD (n = 5).

IV. 3. 4. Upregulation of H3K4me2 bound to the promoter region of the *NTRK2* gene by GGA

In **Chapter III**, we describe that GGA upregulates cellular levels of three major *NTRK2* messenger RNA splice variants, as well as two variant forms (145 and 95 kDa) of the NTRK2 protein in human neuroblastoma SH-SY5Y cells. Here, we show that GGA upregulated NTRK2 expression at 4 h after the treatment (**Fig. IV-8**). ChIP-seq technology revealed neuron-specific upregulation of H3K4me3 bound to putative P1 (from -885 to -689) and P2 (around +1515) promoter regions of the *NTRK2* gene as depicted in **Fig. IV-9, upper panel**. Therefore, the next experiment was conducted with H3K4me2 ChIP assay with PCR primers targeted to the P1 and P2 promoter regions. GGA treatment significantly increased the cellular levels of H3K4me2 bound to both promoter regions of the *NTRK2* gene (**Fig. IV-9, lower panel**).

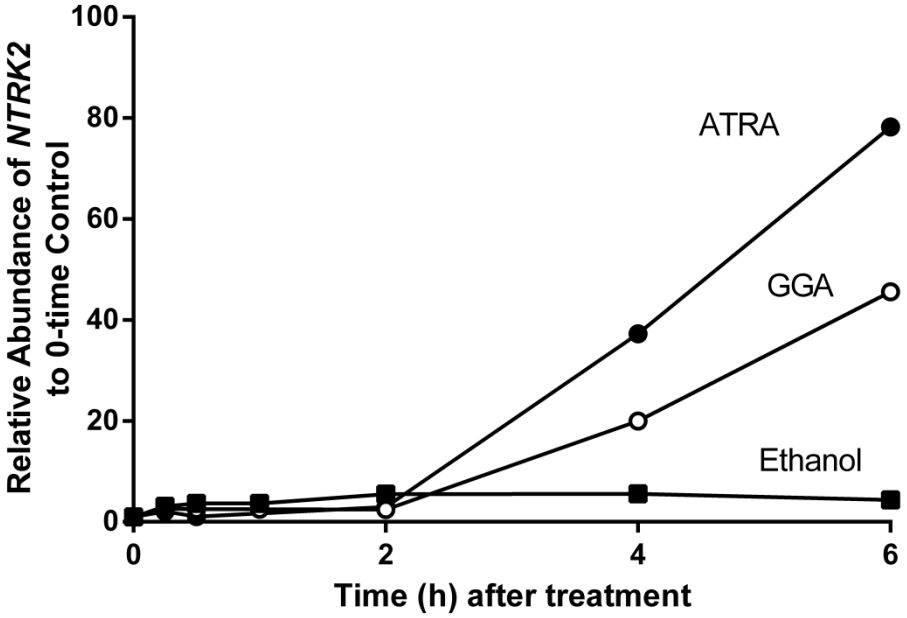

Fig. IV-8. Time-dependent upregulation of the *NTRK2* gene by GGA treatment.

SH-SY5Y cells were treated with ethanol (closed square), 10 μM GGA (open circle) or 10 μM ATRA (closed circle) for the indicated times. *NTRK2* mRNA levels was analyzed by quantitative RT-PCR with 28S rRNA levels as an internal control. The experiment was performed in singlicate.

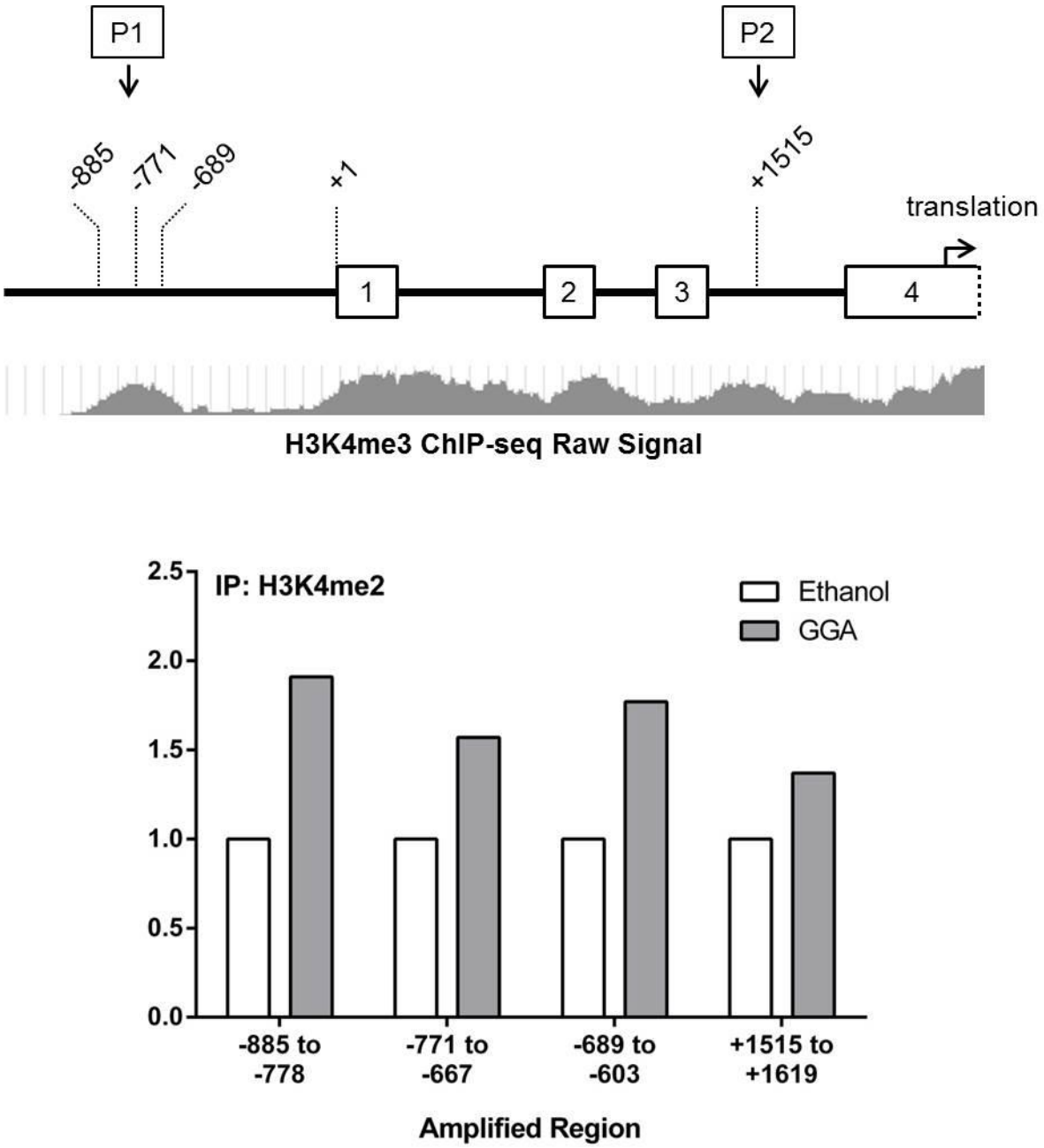

Fig. IV-9. GGA induced dimethylated H3K4 bound to promoter regions of the *NTRK2* gene.

(**Upper panel**) Schematic representation of the upstream portion of the *NTRK2* gene with H3K4me3 status. +1 indicates the transcriptional start site. H3K4me3 ChIP-seq Raw Signal image was adopted from the UCSC Genome Browser. (**Lower panel**) GGA treatment induced de-methylated H3K4 bound to promoter region of the *NTRK2* gene. Cells were treated with 10 μM GGA for 2 h, and ChIP assay was performed. The data were normalized to input DNA. The experiment was performed in singlicate.

The recombinant-hKDM1A-inhibitory effects of several dihydro-derivatives of GGA correlated with their ability to induce *NTRK2* gene expression in SH-SY5Y cells (**Fig. IV-10**). This suggests that dihydroGGA-induced inhibition of KDM1A activity may be responsible for the drug-induced upregulation of *NTRK2* gene expression.

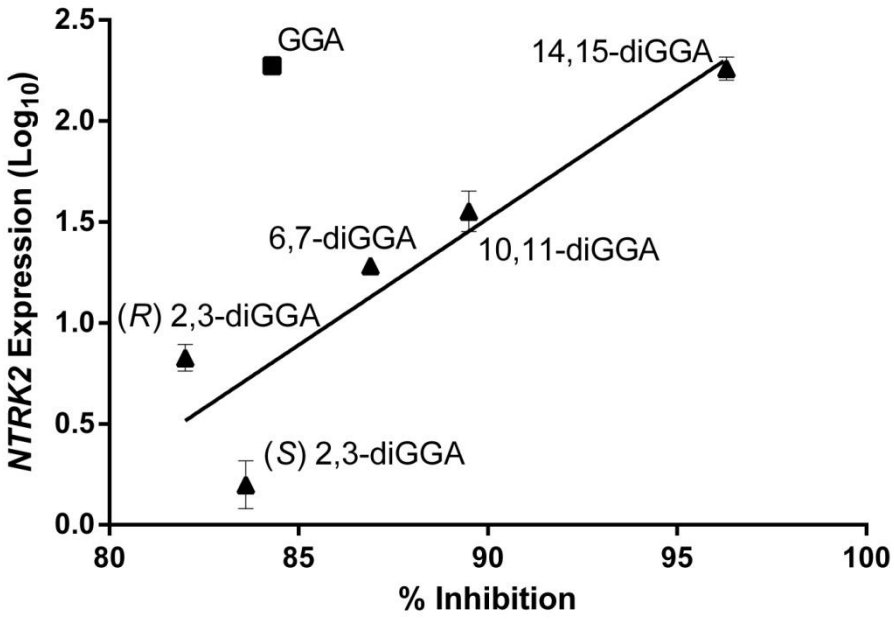

Fig. IV-10. Correlation between KDM1A inhibition and *NTRK2* expression levels.

NTRK2 mRNA levels were analyzed by RT-qPCR after 48 h treatment with 10 μM dihydroGGAs. Results are presented as the mean ± SD (n = 4). diGGA is an abbreviation of dihydroGGA.

IV. 4. Discussion

The present study examines the inhibitory effect of the acyclic diterpenoid GGA on the epigenetic regulator enzyme KDM1A. The rationale for conducting this work stems from the observations that the antidepressant tranylcypromine, an efficient MAO inhibitor, can also inhibit KDM1A, a member of the FAD-containing amine oxidase superfamily and that farnesol, an acyclic sesquiterpenoid found in tobacco smoke, is a competitive inhibitor of human MAOB. In this context, farnesol was expected to inhibit KDM1A activity, which it did in a dose-dependent manner. Furthermore, data presented in this study clearly demonstrates that GGA is a more potent inhibitor of human KDM1A than farnesol and that this inhibition occurs in a non-competitive fashion.

Interestingly, dihydro-derivatives of GGA were more potent inhibitors of KDM1A than GGA. Of the dihydro-derivatives tested, 14,15-dihydroGGA was the most potent inhibitor with an IC_{50} of 22 μM, comparable with that (23 μM) of the clinically-used inhibitor tranylcypromine. Although tranylcypromine is used for treatment of depression and is proven to be safe, it is a purely synthetic chemical and may be impractical to take for prolonged periods of time to prevent carcinogenesis. In contrast, the natural diterpenoid GGA and 14,15-dihydroGGA are branched chain fatty acids, with which human cells are familiar as components of the mevalonate-pathway.

As described in **Chapter III**, we recently reported that GGA suppressed cellular proliferation and induced neuronal differentiation and upregulation of neuron-specific *NTRK2* gene expression in human neuroblastoma SH-SY5Y cells. This cell line is known to strongly express KDM1A [Schulte et al, 2009].

Schulte and colleagues found that tranylcypromine-mediated inhibition of KDM1A reprograms the transcriptome of SH-SY5Y cells and administration of the drug inhibits neuroblastoma xenograft growth in mice [Schulte et al, 2009]. Assuming that GGA is able to inhibit KDM1A activity resulting in global upregulation of H3K4me2 levels within cells, GGA treatment expectedly increased cellular H3K4me2 levels present around the P1 and P2 promoter regions of the *NTRK2* gene in human neuroblastoma SH-SY5Y cells. Such histone modifications, in general, cause transcriptional activation of the gene. In the present study, a time-dependent activation of *NTRK2* gene expression was indeed demonstrated after GGA treatment. As neuronal differentiation involves increased cellular levels of H3K4me3 around the P1 and P2 promoter regions of the *NTRK2* gene, GGA treatment altered neuroblastoma cells from an undifferentiated to a differentiated state via upregulation of H3K4me2 levels around the P1 and P2 regions of the *NTRK2* gene. The relationship is further supported by a significant correlation between the KDM1A-inhibitory effects and the *NTRK2* gene-upregulating effects of the dihydro-GGAs.

The initiation and progression of cancer, traditionally seen as a genetic disease, is now known to involve epigenetic abnormalities along with genetic alterations. A new terminology ‘‘histone onco-modifications’’ has been proposed to describe the posttranslational modifications of histones that have been linked to carcinogenesis. KDM1A, which belongs to a flavin-dependent amine oxidase (AOF) superfamily (**Table IV-1**), consists of a growing number of transcriptional complexes that are implicated in carcinogenesis [Hou et al, 2010]. Hence, there has been an increased effort to identify or design KDM1A inhibitors that could function as antitumor epigenetic therapeutic agents [Forneris et al, 2008]. Here, KDM1A inhibition with

small molecule inhibitors resulted in growth inhibition of tumor cells in vitro and an increase in global H3K4me2 methylation.

IV. 5. Conclusion

The present study clearly demonstrates that GGA and its dihydro-derivatives represent most promising cancer-preventive epigenetic therapeutic agents targeting KDM1A, and further work is warranted to this end.

Chapter V

RET KINASE SIGNAL TRANSDUCTION

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Yoshihiro Shidoji

Geranylgeranoic acid induces neurotrophin tyrosine kinase receptor, type 2 expression through activation of RET in neuroblastoma SH-SY5Y cells (in preparation)

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V. 1. Abstract

Besides NTRK2, another cell-surface receptor for neurotrophic factor GDNF, *RET* kinase gene expression is known to be upregulated by ATRA treatment and a cross-talk between RET and NTRK2 is reported during neuronal differentiation of neuroblastoma cells. On the other hand, methyl CpG binding protein 2 (MeCP2) is an epigenetic regulator of gene expression that is essential for normal brain development. MeCP2 causes changes in chromatin structure through interactions with corepressors to act as a transcriptional modulator. Here we show that natural diterpenoid geranylgeranoic acid (GGA)-induced phosphorylation of MeCP2 and consequent upregulation of *RET* gene expression, which is thought to be prevented by binding of MeCP2 to promoter region of the gene. Furthermore, GGA-induced upregulation of *RET* gene expression was cancelled by co-treatment with RET receptor tyrosine kinase inhibitor, RPI-1, suggesting that RET receptor tyrosine kinase-ignited phosphorylation cascade may contribute in phosphorylation of MeCP2. The present results demonstrate the effect of GGA on signal transduction may be related with differentiation of neuroblastoma SH-SY5Y cells.

V. 2. Introduction

V. 2. 1. RET is a key factor in neuronal differentiation

The ret proto-oncogene (*RET*) encodes a transmembrane receptor tyrosine kinase. RET is required for the development of the kidney, and the enteric and central nervous system [Schuchardt et al, 1994]. The *RET* gene is often rearranged and constitutively activated in a large proportion of papillary thyroid carcinomas [Grieco et al, 1990], and germ-line point mutations in *RET* is responsible for the dominantly inherited cancer syndromes multiple endocrine neoplasia types 2A and B [Mulligan et al, 1993; Hofstra et al, 1994], associated with medullary thyroid carcinomas, pheochromocytomas and ganglioneuromas. Moreover, deletions or loss-of-function mutations in *RET* have been associated with Hirschsprung's disease [Edery et al, 1994], which results in a lack of neurons in distal segments of the enteric nervous systems and colon aganglionosis.

RET kinase is activated by binding to a ligand complex formed by the glial cell line-derived neurotrophic factor (GDNF) family members of neurotrophic factors such as GDNF, neurturin, persephin, and artemin, bound to its cognate four different GDNF-family receptor-α (GFRα1-4) glycosylphosphatidylinositol-linked co-receptors. RET can mediate various signaling pathways to promote cell survival, migration and differentiation, and neurite outgrowth.

In retinoic acid-induced neuroblastoma differentiations, RET is critical component of transcriptional alteration [Oppenheimer et al, 2007], suggesting a key role of RET in this process. Angrisano et al reported that ATRA induced *RET* gene transcriptional activation through chromatin and DNA methylation dynamics

in neuroblastoma cells [Angrisano et al, 2011]. For more details; ATRA induces H3K4 trimethylations in *RET* promoter region and displacement of MeCP2/HDAC1/Sin3A complex from *RET* enhancer facilitating transcription. Furthermore, expression of active RET results in cell growth suppression and expression of a neuronal phenotype in neuroblastoma cells without ATRA treatment [D'Alessio et al, 1995]. These studies suggest that RET activation can mediate ATRA actions in neuroblastoma differentiation.

V. 2. 2. MeCP2 is required for regulation of *RET* transcription

Methyl CpG binding protein 2 (MeCP2) is one of epigenetic regulators of gene expression that is essential for normal brain development. MeCP2 binds to methylated cytosines of CpGs in the mammalian genome and modulates transcription, thereby translating these epigenetic marks (methylation of CpGs) into changes in gene expression. In general, epigenetic regulation plays an instrumental role in the development and maturation of the mammalian brain. Consequently, some mutations in the X-linked gene *MECP2* have severe consequences for neuronal development and can cause the neurodevelopmental disorder Rett syndrome (RTT) [Amir et al, 1999]. By using MeCP2-null mice and their primary neurons, MeCP2 is shown to be required also for global heterochromatic and nucleolar changes during activity-dependent neuronal maturation [Singleton et al, 2011]. Indeed, morphologically immature neurons are a hallmark of MeCP2 deficiency in the brain [Armstrong et al, 1995]. The loss of MeCP2 function in the brain is predicted to not only interfere with the proper regulation of these gene expression changes, but also alter the morphological changes in neuronal nuclei, which leads to the neurodevelopmental phenotypes associated with RTT.

MeCP2 is known to mediate gene silencing by causing changes in chromatin structure through interactions with histone deacetylase [Jones et al, 1998]. MeCP2 binds to methylated CpG sites in gene promoters, where it complexes with other repressors, including histone deacetylases (for example, HDAC1) and histone methyltransferases (HMT), and the co-repressor Sin3a (**Fig. V-1**) [Fen and Nestler, 2010]. MeCP2 may also directly or indirectly interact with chromatin-modifying enzymes, such as histone H3K9 methyltransferase [Fuks et al, 2003], SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 [Harikrishnan et al, 2005], and SWI2/SNF2 DNA helicase/ATPase [Nan et al, 2007].

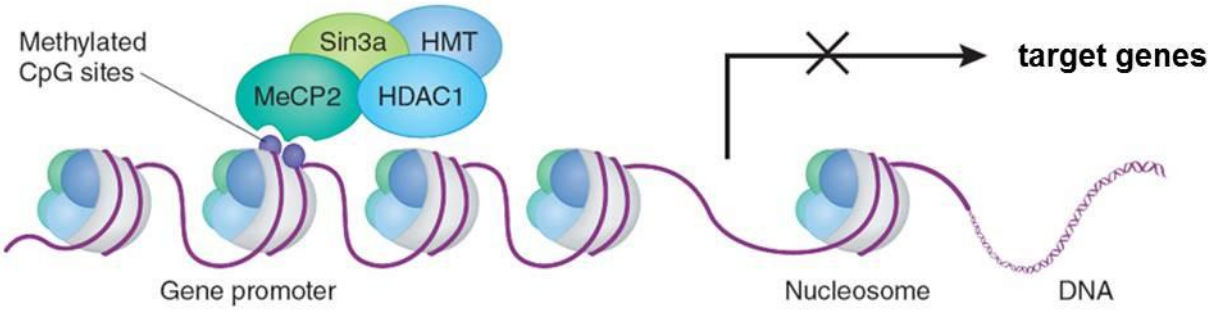

Fig. V-1. MeCP2-mediated regulation of gene expression.

[slightly modified from Feng and Nestler, 2010]

HDAC1; histone deacetylase 1, HMT; histone methyltransferase, MeCP2; methyl CpG binding protein 2

Previous studies on the regulation of MeCP2 focused on its phosphorylation at two sites, serine 80 (S80) and serine 421 (S421). According to the literatures, MeCP2 (S80) in neurons is lost and in turn MeCP2 (S421) appears after stimulation of neuronal activity. MeCP2 (S80) has been reported to be required for dynamic function of adult neurons [Tao et al, 2009]. However, there are conflicting reports on the role of MeCP2 (S421) in activity-dependent gene expression [Zhou et al, 2006, Cohen et al, 2011], making the functional consequence of phosphorylation at this site unclear at this moment. Zhou et al claimed that by triggering MeCP2 phosphorylation (S421), neuronal activity regulates a program of gene expression that mediates nervous system maturation, whereas Cohen et al proposed that the phosphorylation of MeCP2 (S421) appears not to regulate the expression of specific genes; rather, MeCP2 functions as a histone-like factor whose phosphorylation may facilitate a genome-wide response of chromatin to neuronal activity during nervous system development.

Posttranslational modifications, for example, phosphorylation, are a potential mechanism to provide localized functional specificity to the genome-widely distributed MeCP2. This may allow an individual MeCP2 molecule to act as either a transcriptional activator or a repressor, depending on the specific posttranslational modifications it contains. However, similar to total MeCP2, MeCP2 (S421) is enriched throughout the genome, and MeCP2 functions as a histone-like factor whose phosphorylation may facilitate a genome-wide response of chromatin to neuronal activity during nervous system development as mentioned above [Cohen et al, 2011].

Recently, numerous potential posttranslational modification sites on MeCP2 molecule including

phosphorylation, acetylation, and ubiquitylation were demonstrated in SH-SY5Y cells (**Fig. V-2**) [Gonzales et al, 2012]. They also reported that phosphorylation of S80 and/or S229 of MeCP2 is important for regulating interactions between MeCP2 with distinct combinations of cofactors. Particularly, MeCP2 (S229) was specifically enriched at the *RET* gene promoter and was expected to be required for regulation of *RET* transcription in response to ATRA [Gonzales et al, 2012].

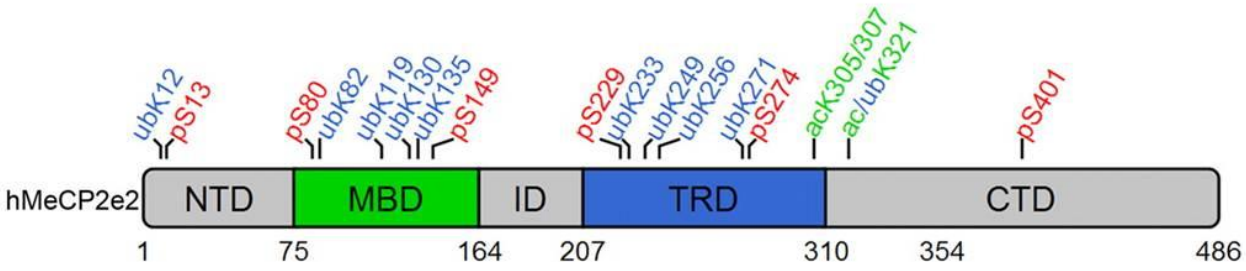

Fig. V-2. Potential posttranslational modifications in MeCP2.

[Gonzales et al, 2012]

NTD; N-terminal domain, MBD; methyl-DNA binding domain, ID; interdomain, TRD; transcriptional repression domain, CTD; C-terminal domain

V. 2. 3. Aims of the study

In this study, we investigates the effects of GGA on MeCP2 phosphorylation and consequent upregulation of *RET*, a silencing target of MeCP2.

V. 3. Results

V. 3. 1. Phosphorylation of MeCP2 by GGA treatment

First of all, we performed immunoblotting and Phos-tag® immunoblotting with nuclear extracts from SH-SY5Y cells to detect MeCP2 phosphorylations. As shown in **Figure V-3, left panel,** MeCP2 was detected in non-treated control cell. GGA or ATRA increased nuclear MeCP2 protein level at 2 h after treatment. GGA or ATRA increased nuclear MeCP2 protein level at 2 h after treatment.

Due to its unique phosphate-binding characteristics, Phos-tag® gel causes band sift of proteins phosphorylated at any sites. In other words, phosphorylated proteins can be detected as slower migrating species on immunoblot using PAGE gel containing appropriate amount of Phos-tag® acrylamide. Phosphorylated MeCP2 was detected on a Phos-tag® immunoblot (**Fig. V-3, right panel**). Upon GGA or ATRA treatment, hyper-phosphorylation of MeCP2 occurred immediately.

Although molecular species were not identified yet, it is worthwhile to mention that a smaller molecular sized band of MeCP2 was also increased, but phosphorylation looked not enhanced by GGA or ATRA treatment. It may be raised by a splice variant of MeCP2-e2 that encodes a slightly shorter protein translated from an ATG in exon-2 [Kerr et al, 2012].

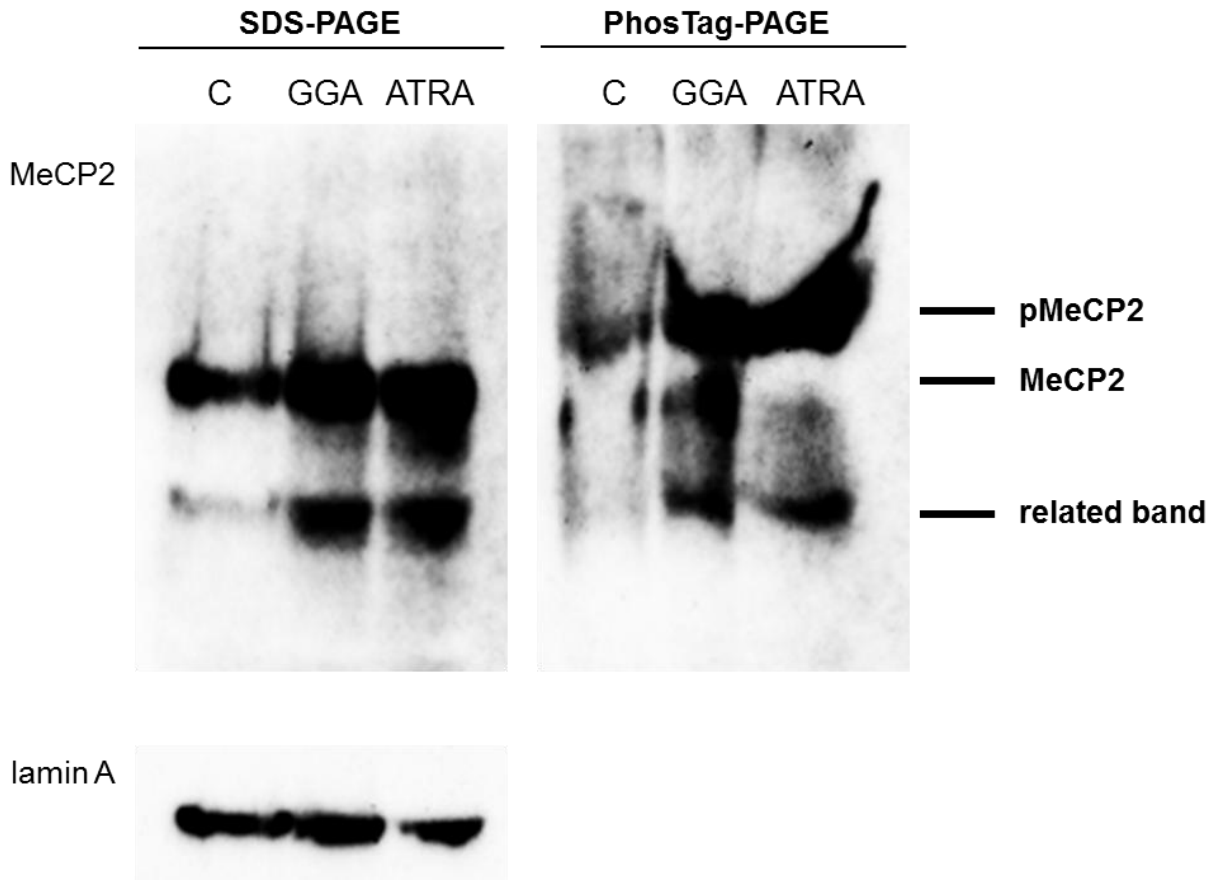

Fig. V-3. Phosphorylation of MeCP2 by GGA treatment.

SH-SY5Y cells were treated with ethanol, 10 μM GGA or 10 μM ATRA for 2h, and nuclei were extracted. Twelve micrograms of nuclear proteins were separated with SDS-PAGE or PhosTag-PAGE and MeCP2 were detected with specific antibody. Lamin A, a nuclear membrane bound protein was detected as a loading control.

V. 3. 2. Upregulation of RET expression by GGA treatment

We next examined whether or not GGA is able to upregulate *RET* gene expression. As a result, **Figure V-4** shows that GGA clearly induced upregulation of the *RET* gene both at mRNA (**Fig. V-4A)** and protein levels (**Fig. V-4B**) in a time-dependent manner.

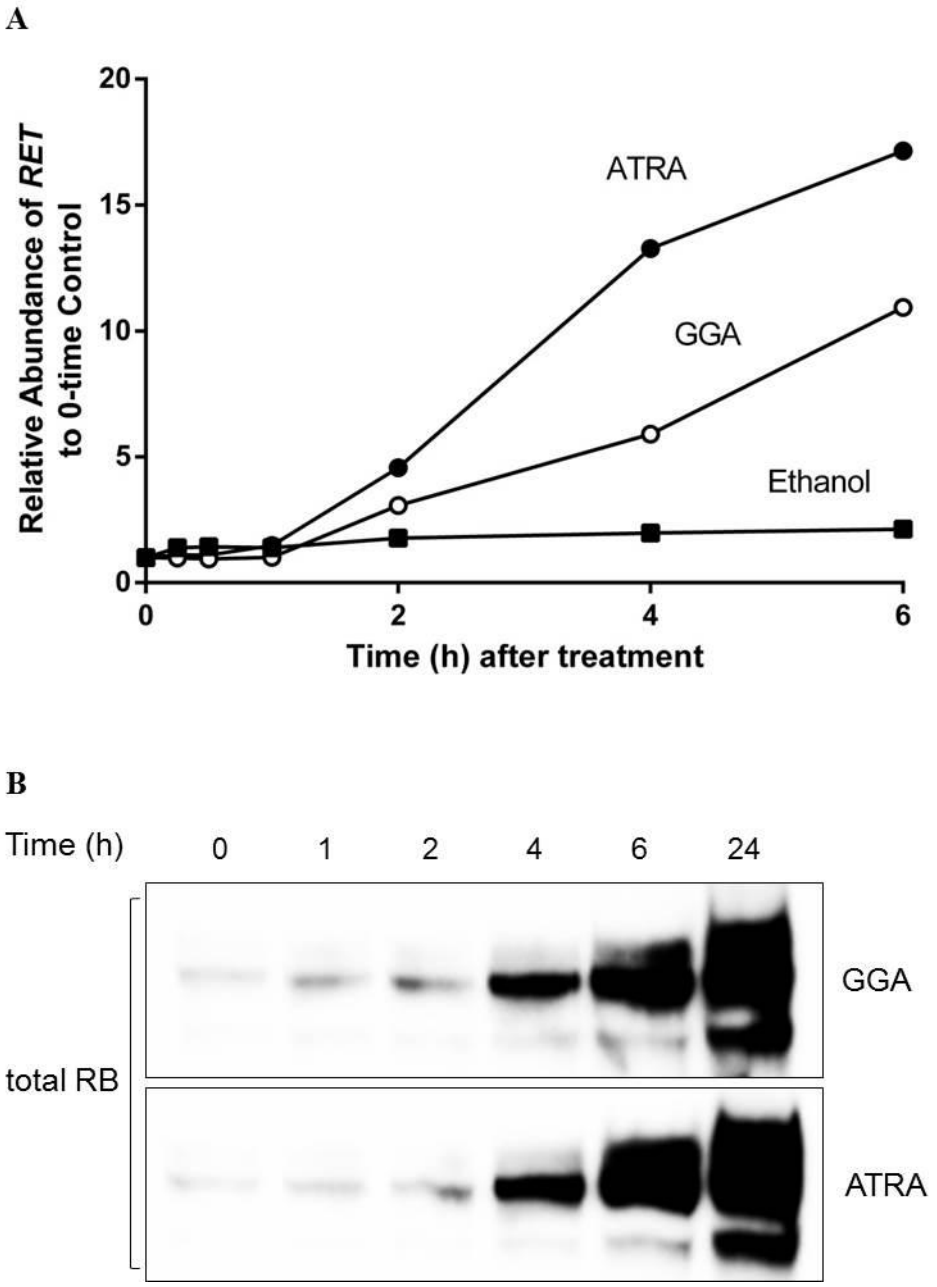

Fig. V-4. Time-dependent upregulation of *RET* expression with GGA.

(**A**) SH-SY5Y cells were treated with ethanol (closed squire), 10 μM GGA (open circle) or 10 μM ATRA (closed circle) for the indicated times. *RET* mRNA levels was analyzed by quantitative RT-PCR with 28S rRNA levels as an internal control. The experiment was performed in singlicate. (**B**) Ten micrograms of total protein from the cell lysates was separated by SDS-PAGE and analyzed by immunoblotting.

V. 3. 3. Induction of lysine-4 methylation of histone H3 in upstream region of the *RET* gene

In the literatures, ATRA has been reported to indeed increase H3K4me3 level at the promoter region as the main mark of RET activation [Angrisano et al, 2010]. In this context, we were very much interested in changes of H3K4 methylation status at the promoter region of the *RET* gene after GGA treatment. As a result, di-methylated H3K4 was broadly increased around upstream regions including enhancer, intervening sequence, HOXB5 binding site and promoter of the *RET* gene after 2-h GGA treatment and it was maintained by 14 h (**Fig. V-5**). In a sharp contrast, an increment of H3K4me3 was detected only in the promoter region at 14 h after GGA treatment (**Fig. V-5**).

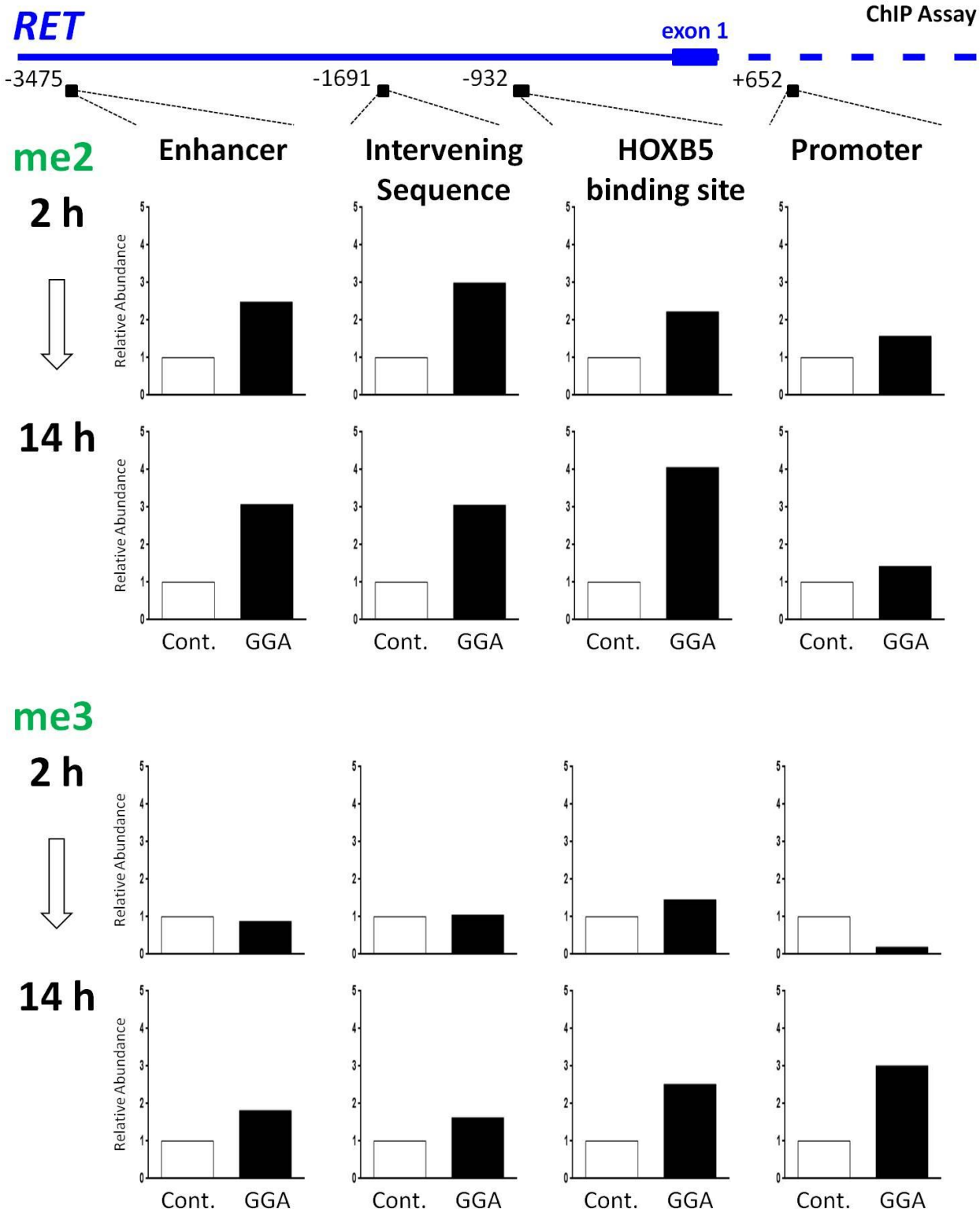

Fig. V-5. Induction of lysine-4-methylations of histone H3 at upstream of the *RET* gene by GGA. SH-SY5Y cells were treated with 10 μM GGA for 2 or 14 h, and ChIP assay was performed. Results were normalized by input DNA. The experiment was performed in singlicate.

V. 3. 4. Knockdown of *RET* attenuated GGA-induced upregulation of *NTRK2* expression

Consequently, suppression of *RET* gene expression with siRNA specific for *RET* (siRET) evidently attenuated cellular protein levels of RET in the presence of GGA in medium (**Fig. V-6, upper panel**). Furthermore, a transient transfection of SH-SY5Y cells with siRET clearly prevented not only GGA-induced increment of *RET* mRNA, but also GGA-induced increment of *NTRK2* mRNA levels (**Fig. V-6, lower panel**).

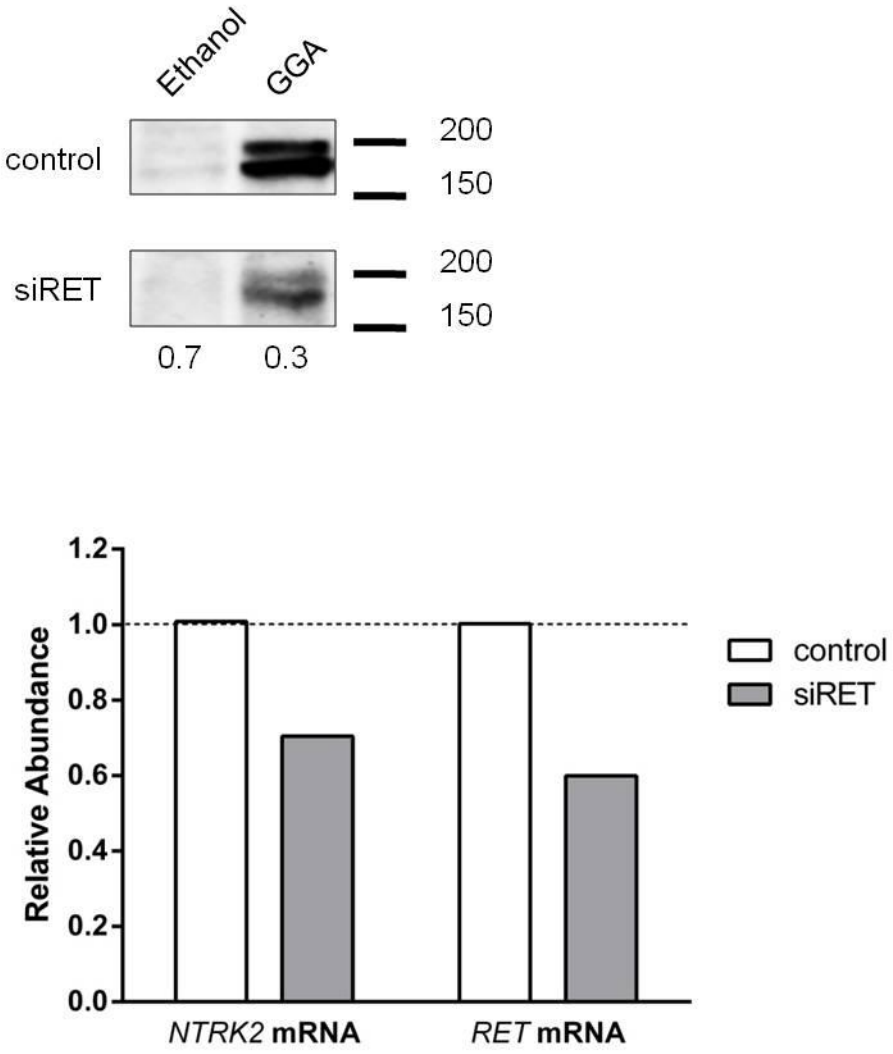

Fig. V-6. Knockdown of the *RET* gene attenuated *NTRK2* mRNA level in the presence of GGA. Small interfering RNAs (400 pmol per 20 cm^2) were transfected 24 h before treatment. Transfected cells were treated with 10 μM GGA for 24 h. RET were detected by immunoblotting (**upper panel**). The ratios to control were indicated. *NTRK2* and *RET* mRNA levels were individually determined by quantitative RT-PCR with the corresponding specific primer sets (**lower panel**). The experiment was performed in duplicate.

V. 3. 5. Effect of RET kinase inhibitor on GGA-induced *NTRK2* upregulation

Finally, we analyzed a role of RET-ignited signaling transduction of phosphorylation cascade by using RET tyrosine kinase inhibitor, RPI-1. Co-treatment with RPI-1 of SH-SY5Ycells totally cancelled GGA (or ATRA)-induced upregulation of *NTRK2* gene expression in dose- and time-dependent manners (**Fig. V-7**), which strongly indicates RET kinase activity can mediate GGA-induced upregulation of *NTRK2* gene expression.

Unexpectedly and interestingly, RPI-1 also inhibited *RET* gene upregulation caused by GGA treatment in time- and dose-dependent manners (**Fig. V-8**). IC_{50} (inhibitory concentration at a half maximum) of RPI-1 for *RET* gene expression was approximately 50 μM, which is fairly higher than its IC_{50} (~7.5 μM) for *NTRK2* gene expression. As mentioned above, higher concentrations of RPI-1 may non-specifically inhibit other tyrosine kinase so that RPI-1-mediated inhibition of *RET* gene upregulation may not be due to a putative hypercycle of *RET* gene expression. GDNF is a paracrine rather than autocrine peptide. Thus, it is unlikely that GDNF/RET signaling system forms a hypercycle in SH-SY5Y cells in culture.

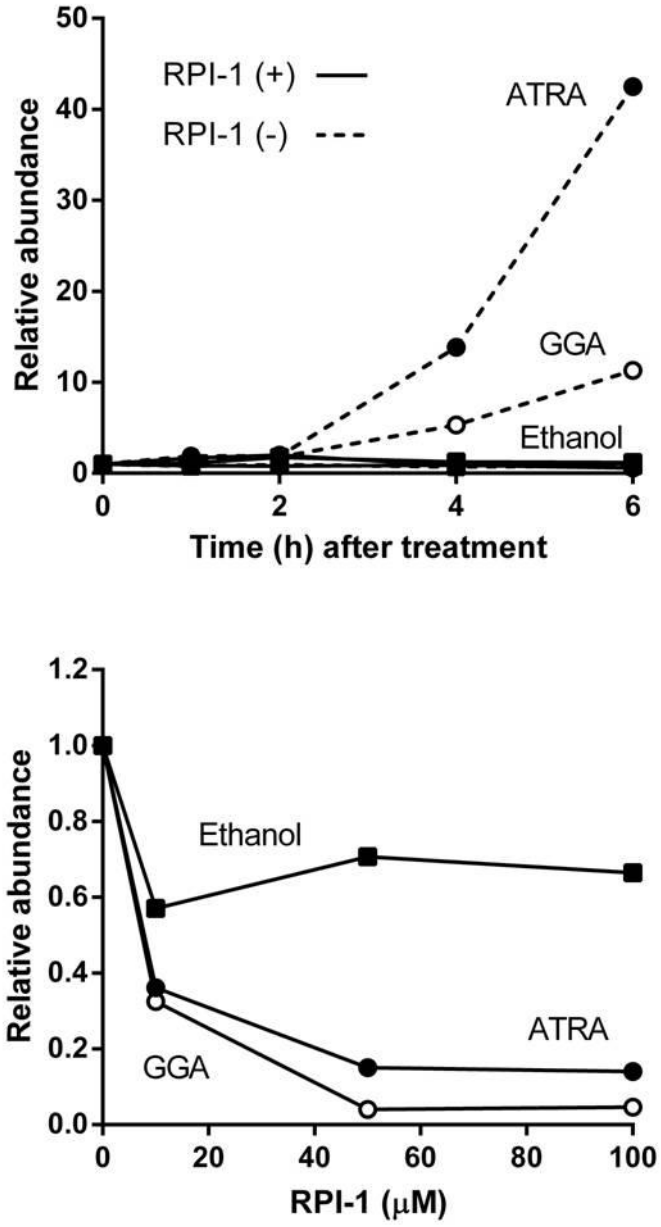

Fig. V-7. Effect of RET kinase inhibitor RPI-1 on GGA-induced *NTRK2* upregulation.

NTRK2 mRNA levels were analyzed by quantitative RT-PCR with 28S rRNA levels as an internal control. SH-SY5Y cells were treated with ethanol (closed squire), 10 μM GGA (open circle) or 10 μM ATRA (closed circle) in present/absent RPI-1 for 4 h (**upper panel**) or indicated times (**lower panel**). The experiment was performed in singlicate.

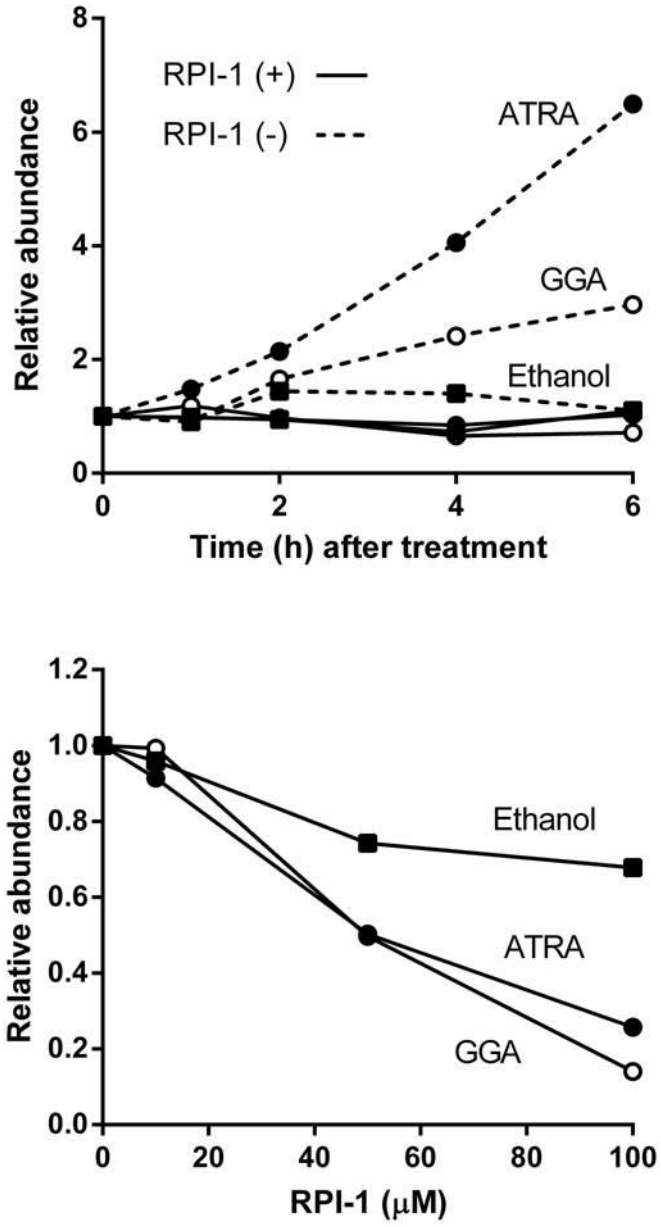

Fig. V-8. Effect of RET kinase inhibitor RPI-1 on GGA-induced *RET* upregulation.

RET mRNA levels were analyzed by quantitative RT-PCR with 28S rRNA levels as an internal control. SH-SY5Y cells were treated with ethanol (closed squire), 10 μM GGA (open circle) or 10 μM ATRA (closed circle) in present/absent RPI-1 for 4 h (**upper panel**) or indicated times (**lower panel**). The experiment was performed in singlicate.

V. 4. Discussion

In **Chapter III**, we described a dramatic upregulation of *NTRK2* gene expression occurred after GGA or ATRA treatment, but it has been proven that the *NTRK2* gene is not a target gene of retinoid receptors by dry and wet experiments; i.e. a computer-aided search of possible retinoids-response elements, and knockdown and/or transient transfection of the *RARB* gene.

In **Chapter IV**, however, we were able to demonstrate GGA (or ATRA)-induced alterations of histone H3K4 methylation status in the chromatin around the promoter regions of the *NTRK2* gene, which may partly participate GGA (ATRA)-induced transcriptional activation of the *NTRK2* gene. This means that GGA or ATRA may cause "epigenetic" effects of the *NTRK2* gene through histone modifications. Then, we had a next question how GGA or ATRA modifies histone H3K4 methylations. And the answer is that GGA or ATRA directly inhibits histone lysine-specific demethylase 1A (KDM1A) that specifically demethylates H3K4me2 and H3K4me1. This is it! We thought at that moment this was a final answer to the question how GGA or ATRA can induce a transcriptional activation of the *NTRK2* gene.

However, some strange feelings remained a little bit in our minds. That is, ATRA is a stronger inducer of *NTRK2* gene expression than GGA, but the inhibitory activity of ATRA against recombinant hKDM1A is weaker than GGA, implying that GGA (or ATRA)-mediated direct inhibition of KDM1A could not be sole explanatory variable of the upregulation of *NTRK2* gene expression. We had to explore some other mechanisms underlying GGA (or ATRA)-induced upregulation of *NTRK2* gene expression to complete this thesis.

Besides NTRK2, another cell-surface receptor for neurotrophic factor GDNF (glial cell-derived neurotrophic factor), RET tyrosine kinase gene expression has been repeatedly reported to be also upregulated by ATRA treatment [Angrisano et al, 2011] and a cross-talk between RET and NTRK2 is reported during neuronal differentiation of neuroblastoma cells [Esposito et al. 2008]. Therefore, *RET* gene expression may be connected to *NTRK2* gene expression at molecular level.

MeCP2 is found in the developing and adult brain in humans, with the highest levels occurring in mature neuronal nuclei, where MeCP2 levels increase with postnatal age [Balmer et al, 2003; Shahbazian et al, 2002], suggesting that MeCP2 might play some important roles also in the function of mature neurons. We were very much interested whether MeCP2 may abandon a transcriptional repressor role during GGA or ATRA treatment, because RET is a silencing target of MeCP2. For example, Angrisano et al reported that ATRA induces the release of MeCP2 from methylated *RET* enhancer region, which results in upregulation of *RET* gene expression [Angrisano et al, 2011]. In the present study, we demonstrated that GGA and ATRA increased MeCP2 and after 2 h treatment. Furthermore, RET expression was upregulated by GGA or ATRA treatment in the time dependent manner. These results suggest that GGA infects RET regulation through attenuate silencing effect of MeCP2.

In **Chapter IV**, we found GGA (or ATRA)-induced enhancement of histone H3K4 methylation in the promoter regions of the *NTRK2* gene, which is expected to cause transcriptional activation of the *NTRK2* gene. In this chapter, we showed that di-methylated H3K4 was broadly increased around upstream regions including enhancer, intervening sequence, HOXB5 binding site and promoter of the *RET* gene after 2-h GGA

treatment, and di-methylated H3K4 was detected only in the promoter region at 14 h after treatment (**Fig. V-5**). In this experiment, we did not do with ATRA but our findings are consistent with the previous report that ATRA increased H3K4me3 level at the promoter region of the *RET* gene [Angrisano et al, 2010]. Considering the temporal sequence effects of GGA, however, trimethylation of histone H3K4 at the *RET* gene promoter could not be an initial cause of *RET* gene expression. That is to say, the cellular mRNA levels for the *RET* gene were already upregulated at 2 h after GGA treatment (**Fig. V-4**), when the H3K4me3 levels were conversely basal at the *RET* gene promoter (**Fig. V-5**). On the other hand, GGA-induced upregulation of *NTRK2* mRNA level preceded GGA-induced increment in H3K4me3 level at *RET* gene promoter. This would suggest that *NTRK2* is a downstream component of GGA signaling than *RET*.

In the literature, a crosstalk between RET and NTRK2 means that NTRK2 promotes RET phosphorylation by a mechanism that does not require GDNF, a definitive ligand for RET complex, indicating that NTRK2 is an upstream component rather than RET in neuronal differentiation [Esposito et al, 2008]. However, the present study implied that RET may be rather upstream than NTRK2. Accordingly, we set out knockdown experiments of either the *RET* or *NTRK2* gene in order to examine whether or not there is a cross-talk between RET and NTRK2 during GGA-induced neuronal differentiation in SH-SY5Y cells and determine which gene product is upstream in GGA signaling. As a result, a transient transfection of SH-SY5Y cells with siRET clearly prevented not only GGA-induced increment of *RET* mRNA, but also GGA-induced increment of *NTRK2* mRNA levels (**Fig. V-6, lower panel**).

Because the nucleotide sequence of commercially-available siRET used herein is not available for us, we

are unaware of its off-target effects, but assuming that the commercial siRET used here is specific for *RET* mRNA, siRET-induced attenuation of *NTRK2* mRNA levels strongly suggests that there must be a certain crosstalk between RET and NTRK2 during GGA treatment. If such is the case, RET will be upstream and NTRK2 will be downstream or RET signal will go to NTRK2. In agreement with this speculation, a commercial siNTRK2 used in the present study specifically downregulated the cellular levels of *NTRK2* mRNA, but it was unable to decrease the cellular levels of *RET* mRNA (*data not shown*).

However, Esposito et al showed a completely opposite direction of signal flow that a knockdown of *NTRK2* gene with siNTRK2, a custom-made nucleotide, prevented activation of RET tyrosine kinase activity and a custom-made siRET did not downregulate the cellular levels of NTRK2 in ATRA-treated SH-SY5Y cells [Esposito et al, 2008]. We do not so far have a reasonable explanation for this discrepancy, but we are tentatively speculating that time-dependent changes might cause fluctuations in direction of differentiation signaling.

Finally, at least by our hands, RET was shown an upstream signal that induces *NTRK2* gene expression in GGA signaling. Furthermore, inhibition of RET activation reduced GGA-induced *NTRK2* upregulation by co-treatment with RET inhibitor RPI-1. However, since specificity of RPI-1 inhibitor for RET kinase has not been extensively explored yet and RPI-1 also reduced the phosphorylation of MET (hepatocyte growth factor receptor kinase), discoidin domain receptor tyrosine kinase 1 (or NTRK4), and PLCG1 (phospholipase C, gamma 1) [Caccia et al, 2010], we cannot exclude a possibility that RPI-1 may inhibit NTRK2 kinase activity and therefore downregulate *NTRK2* gene expression by breaking autocatalytic hypercycle of *NTRK2*

gene expression. Furthermore, taking into account that the cellular mRNA of BDNF, a neurotrophic ligand for NTRK2, was also upregulated in GGA-treated SH-SY5Y cells (data not shown), the formation of functional autocrine loop of BDNF/NTRK2 signaling is rather feasible.

In the presence of GGA or ATRA, lower concentrations (20 μM) of RPI-1 blocked the drug-induced upregulation of NTRK2 gene expression, which probably means that RPI-1 inhibited active RET tyrosine kinase. If so, we must assume that a ligand-free activation of RET tyrosine kinase by either GGA or ATRA treatment.

V. 5. Conclusions

In this chapter, we showed the non-genomic actions of GGA on signal transduction, phosphorylation of MeCP2 and activation of a tyrosine kinase RET in neuroblastoma SH-SY5Y cells. We also described that either knockdown of the *RET* gene or RET tyrosine kinase inhibitor blocked GGA-induced upregulation of *NTRK2* gene expression.

Chapter VI

GENERAL DISCUSSION

In this thesis, we scrutinized biological effects of some diterpenoid acids by using human hepatoma and neuroblastoma cells in culture, in terms of growth suppression, induction of differentiation, and particularly gene expression. Besides ATRA, two other diterpenoid acids such as GGA and Peretinoin (4,5-didehydroGGA) have been shown to provide several cell-biological effects on gene expression, which can be conceptually divided into two main classes of genomic and non-genomic actions (**Fig. I-5**). In this thesis, we herein define genomic actions of diterpenoid acids as "any actions of each diterpenoid acids through their genomic response elements with nuclear receptors" and their non-genomic actions are defined as "any actions of each diterpenoid acids through other factors than their genomic response elements".

VI. 1. Genomic actions

VI. 1. 1. Retinoid receptors

RARB

ATRA is the most characterized diterpenoid acid as a potent ligand for nuclear retinoid receptors. And it is fully established that ATRA works to transactivate its target genes including RARB by binding to the ligand-binding domain of RARs. In this regards, it is important to notice that GGA and Peretinoin as well as ATRA were active to induce the reporter gene expression through either retinoic acid response element of the *RARB* gene (RAREβ) or a synthetic retinoid X response element (RXRE) [Araki et al, 1996]. Therefore, we tested whether GGA and its derivatives could induce the upregulation of *RARB* gene expression in human neuroblastoma cells to confirm the genomic action of GGA and its derivatives. As expected, GGA induced

upregulation of *RARB* gene expression in SH-SY5Y cells to a lesser extent than ATRA, which strongly indicates that GGA worked as a potent ligand for RAR in human neuroblastoma SH-SY5Y cells, probably through RAREβ. This is a sort of evidence that GGA has a genomic action through RARβ.

However, RARβ seems not to be directly involved in upregulation of *NTRK2* gene expression, because neither knockdown of the *RARB* gene suppressed the *NTRK2* gene expression (**Fig. III-17**), nor extensive dosage (2×10^5 fold) of the *RARB* gene into SH-SY5Y cells upregulated the *NTRK2* gene expression (**Fig. III-18**). In other words, up-and-down changes of *RARB* gene expression did not affect GGA-induced upregulation of *NTRK2* gene expression, such that GGA as well as ATRA significantly and constantly increased *NTRK2* gene expression to the same extent. As a consequence, we have reached a conclusion that the diterpenoid acid-induced upregulation of *RARB* gene expression is independent of *NTRK2* gene expression.

Other RARs and RXRs

Other than the *RARB* gene, ATRA and GGA both unexpectedly induced the downregulation of RARα, RARγ, RXRα, and RXRγ at their cellular protein levels. ATRA (or GGA)-induced expression profile of RARs and RXRs in SH-SY5Y cells is mostly inconsistent with previous reports [Carpentier et al, 1997; Joshi et al, 2006]. In their papers, treatment with ATRA upregulated RARα, RARγ, and RXRβ and definitely induced a drastic increase of RARβ (both at the RNA and protein level) in SH-SY5Y cells [Carpentier et al, 1997; Joshi et al, 2006]. In the case of RARβ, our present data is consistent with their findings in the

literatures. But, we never found any increment in the cellular RARα, RARγ, and RXRβ levels. A major difference between their and our experiments is exposure time of retinoids to the cells, 24 – 48 h vs. 2 – 10 d, which may be responsible to produce such differences in retinoid receptors gene expression in the treated cells. Indeed, Figure 1 of Carpentier group's paper clearly tells that ATRA-induced upregulation of RARα, β, γ, and RXRβ in 24 h was significantly reversed in the next 24 h [Carpentier et al, 1997], which means that 2-d treatment with ATRA gave little changes in retinoid receptor expression in their experimental condition. The same holds true for the present study that no salient downregulation of RARα expression was observed on day 2 with either GGA or ATRA treatment, in comparison to dramatic suppression of RARα expression on day 10 [Sakane & Shidoji, 2011]. In this context, we might miss a transient surge of RARα expression after GGA or ATRA treatment.

On the other hand, in accordance with our findings, Chen et al reported that downregulation of RXRα, a nuclear receptor that can suppress NF-κB activity, mediates the elevation of cyclooxygenase-2 expression and prostaglandin E2 production in senescent macrophages after ATRA treatment [Chen et al, 2013]. In the present study, although ATRA almost diminished the expression of RXRα, but the suppressive effect of GGA was intermediate (**Fig. III-15**). Proteasomal degradation of retinoid receptors may be enhanced after ATRA or GGA treatment. At this moment, we are unable to explain how ATRA or GGA induces the degradation of retinoid receptors, it might contribute to side-effects of ATRA found in clinical studies, because GGA shows less side-effects.

VI. 1. 2. Orphan receptors

RAR-related Orphan Receptor-α (RORα)

RARs and RXRs belong to nuclear receptor (NR) superfamily that consists of 48 known members. In NR superfamily, RARs are referred as NR subfamily 1 (NR1) and RXRs as NR subfamily 2 (NR2). Among NR1 subfamily, two members of RORα and PPARα were interesting for us to test the activity that mediates ATRA and GGA effects on *NTRK2* gene expression, because both receptors are expressed in SH-SY5Y cells and are recognized as an orphan receptor that its endogenous ligand is still unidentified.

TFSEARCH, a freeware that searches transcription factor binding sites, detected two ROR-response element (RORE) candidate sequences within 2,500-bp upstream of the *NTRK2* gene. Furthermore, as mentioned above, we demonstrated *RORA* expression in SH-SY5Y cells, although the cellular levels of *RORA* mRNA were not changed after ATRA or GGA treatment (data not shown). Dual luciferase assay with RORE construct and *RORA* expression vector surprisingly demonstrated dose-dependent antagonistic effect of GGA in human hepatoma HuH-7 cell system. Although it is potentially possible to have a speculation that this antagonistic effect of GGA may participate in GGA-induced upregulation of *NTRK2* gene expression, we must demonstrate clear evidence that RORα is acting to suppress *NTRK2* gene expression.

Since nuclear receptor activity depends on cofactors consisting of activator/repressor complex, we have to test ligand activity of GGA on RORα and target gene expression in SH-SY5Y cells to determine genomic action of GGA on *NTRK2* gene expression in these cells. Therefore, we still have an open question whether genomic effect of ATRA or GGA is involved in the upregulation of *NTRK2* gene expression.

Peroxisome Proliferator-activated Receptor-α (PPARα)

Peroxisome proliferator-activated receptors (PPARs) of NR1 subfamily comprise three members of isotypes, PPARα (NR1C1), PPARδ (NR1C2) and PPARγ (NR1C3), which bind to specific DNA response elements (PPRE), as heterodimers with RXR. In the brain PPARα looks especially intriguing, since it is selectively expressed in certain brain areas and neuronal/glial populations, and modulates antioxidant responses, neurotransmission, neuroinflammation, neurogenesis, and glial cell proliferation/differentiation [Fidaleo et al, 2014]. A very recent study has shown that PPARα and its candidate ligands, including oleoylethanoloamide and palmitoylethanolamide, are involved in physiological and pathological responses, such as satiety, memory consolidation, and modulation of pain perception [Fidaleo et al, 2014]. The protective role of PPARα agonists in neurodegenerative diseases and in neuropsychiatric disorders makes manipulation of this pathway highly attractive as therapeutic strategy for neuropathological conditions [Fidaleo et al, 2014].

By structural analogy, GGA is a prospective ligand on PPARs. PPARα can mediate the genomic action of GGA on *NTRK2* gene expression, because we have preliminary data, which suggest the ligand activity of GGA on PPARα by dual luciferase assay with DR-1 type PPRE in human hepatoma HuH-7 cell system. But, unfortunately, TFSEARCH failed to detect any PPRE sequence in 5000-bp upstream region of the *NTRK2* gene. Therefore, one can easily conclude that it is not likely that PPARs play a transcriptional role in *NTRK2* gene expression.

In total, we may have to demonstrate ligand activities of GGA for several other nuclear orphan receptors

in target condition and cells, in order to illustrate whole genomic actions of GGA or ATRA in hepatoma and neuroblastoma cells. Lastly and most importantly, it is necessary to precisely determine that the genomic actions are central or not on hepatic and neuronal differentiation. In the next section, we discuss the non-genomic actions of GGA and ATRA.

VI. 2. Non-genomic actions

While the role of genetic events of DNA mutations is well established, DNA methylation and histone modifications are key causes of aberrant gene function leading to cancer and de-differentiation. In general, abnormality of DNA modifications occurs in malignant cells; global hypomethylation of genomic DNA is commonly observed in cancerous cells, leading to genomic instability, activation of growth-promoting genes [Sharma et al, 2010] and concomitant hypermethylation of the CpG islands in functional genes leads to suppression of tumor-suppressor genes in tumor cells [Varier et al, 2011]. But these DNA modifications are rather slower process than histone modifications. Therefore, histone modifications are applicable to regulatory process with signaling molecules. It has been well known that both DNA and histone modifications cause reversible alterations of chromatin structure between open and closed status.

VI. 2. 1. Histone modification

H3K4 methylation

LSD1 or KDM1A is capable of removing dimethyl and monomethyl groups on lysine 4 of histone H3

(H3K4me2 and H3K4me1, respectively), as well as methyl groups on non-histone proteins such as a tumor suppressor p53 and DNA methyltransferase-1. Nuclear receptor recruitment depends on chromatin structure around enhancer/suppressor region of its target genes. Downregulation of KDM1A results in supply of substrate H3K4me2 to histone methyltransferase, a regulator of transcriptionally active chromatin or open-structured chromatin. H3K4me2 in the upstream region of the *NTRK2* and *RET* genes was increased by GGA treatment (**Fig. IV-10, Fig. V-5**), suggesting that GGA upregulated these two genes expression through induction of open-structured chromatin.

The next question arises how GGA or ATRA increases H3K4me2 around the upstream regions of the *NTRK2* and *RET* genes. In this regards, it is most interesting that significant upregulation of *KDM1A* gene expression has been reported in clinical samples of hepatoma [Magerl et al, 2010; Zhao et al, 2012; 2013] and neuroblastoma [Schulte et al, 2009; Xu et al, 2013]. We speculated that GGA might have an ability to inhibit KDM1A activity, because farnesol can inhibit monoamine oxidase-B that is highly homologous flavin-enzyme to KDM1A in its 3D structure. Indeed, GGA showed inhibitory activity against recombinant KDM1A and further position-differently dihydrogenated GGAs inhibited the KDM1A activity to the different degree. The intensity of the inhibitory activity of these dihydroGGA derivatives clearly corresponded to upregulation of *NTRK2* gene expression in SH-SY5Y cells, strongly suggesting that GGA induces H3K4 trimethylation through direct inhibition of KDM1A activity and consequently upregulates transcription of the target *NTRK2* and *RET* genes by making these chromatin structures open.

VI. 2. 2. Signal transduction effects

Finally, we addressed a question why GGA-induced upregulation of gene expression is specific for both the *NTRK2* and *RET* genes. In similar to histone modifications, the canonical signaling pathways are regulated by posttranslational modifications, including phosphorylation cascades, of accessory components in gene-specific transcriptional regulators to make GGA effects specific for the target genes.

In **Chapter V**, by using knockdown technique, we demonstrated that RET was an upstream signal of NTRK2 (**Fig. V-6**). In other words, GGA could not upregulate *NTRK2* gene expression unless RET was activated by GGA treatment. Both *RET* and *NTRK2* genes are known suppressed by MeCP2 that binds to methylated CpG islands on the upstream regions of the genes in neuroblastoma cells [Gozales et al, 2012; Abuhatzira et al, 2007]. When SH-SY5Y cells are treated with GGA or ATRA, MeCP2 might be highly phosphorylated and then one can easily predict that suppressive complex will be released from closed chromatin (**Fig. VI-1**). After considering the present finding that RET kinase inhibitor decreased GGA-induced upregulation of both *RET* and *NTRK2* genes, we can reasonably assume that GGA stimulates RET kinase activity and its downstream signal transduction mediates phosphorylation of MeCP2 protein (**Fig. V-6**).

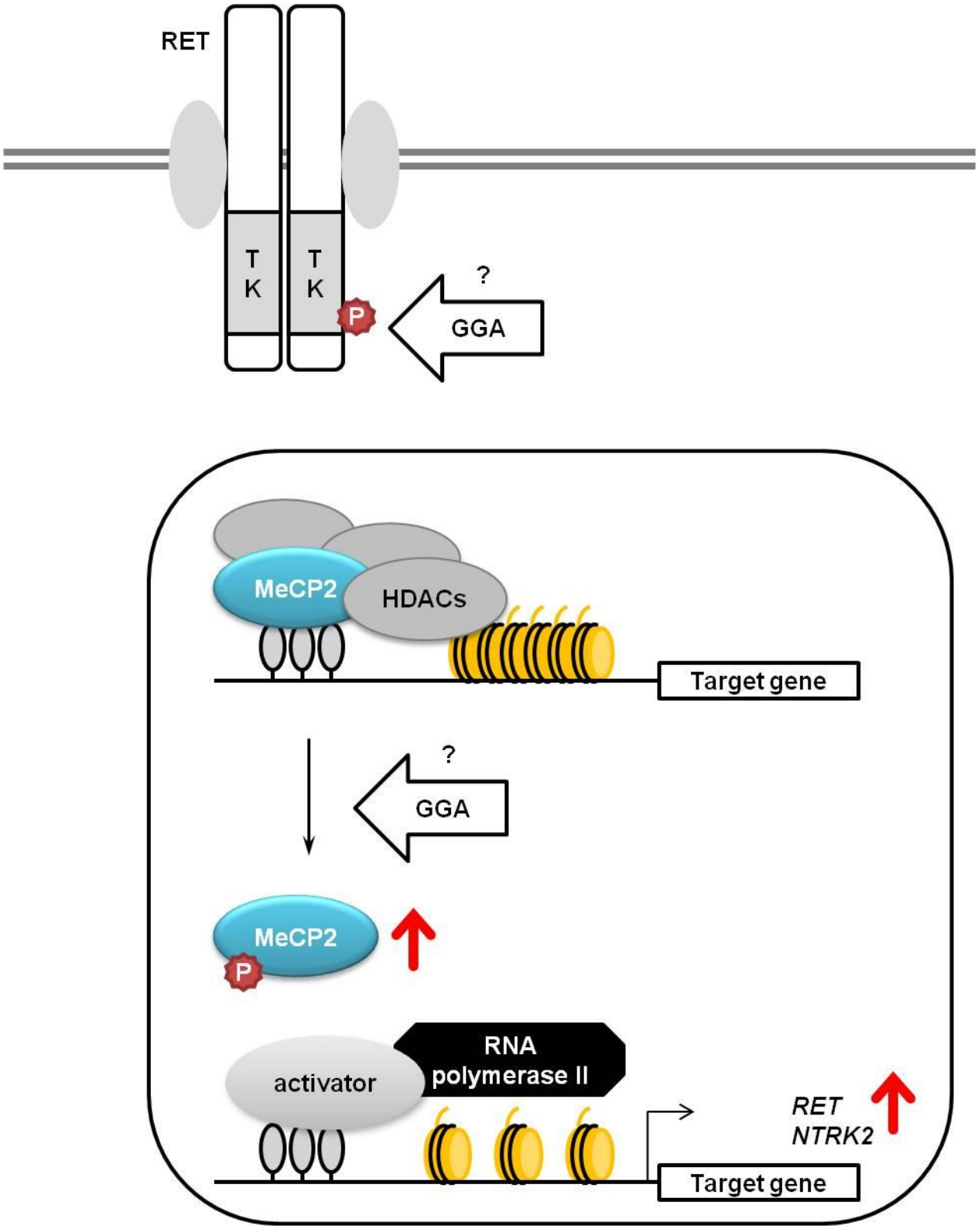

Fig. VI-1. Working hypothesis for molecular mechanism how GGA act in *NTRK2* gene expression.

HDAC; histone deacetylase, MeCP2; methyl CpG binding protein 2, TK; tyrosine kinase

VI. 3. Implications

Accumulating evidence suggests that KDM1A is a cancer therapeutic target, and its inhibitors are currently identified as chemotherapeutic agents. Concrete evidence for natural compound, GGA, as a KDM1A inhibitor will pave a road to chemoprevention of cancer and neurodegenerative diseases. Since Peretinoin (4,5-didehydroGGA), but not ATRA has been proven to show few side effects in clinical trials, GGA and its derivatives may provide groundwork of nutritional prevention and medications as a clinical diterpenoid in future lifestyle and preventive medicine. Inasmuch as many developed countries including Japan are facing to very super-aged society, highly efficient nutritional prevention of cancer and neurodegenerative diseases is required to keep their population in good health.

VI. 4. Conclusions

To be genomic, or to be non-genomic, that is not a question. A question to be addressed is how diterpenoid acids such as GGA, Peretinoin, and ATRA prevent carcinogenesis or induce differentiation. When I finish my thesis work, I must mention that although, in the last two decades, chemopreventive effects of retinoids have been continuously regarded as their *genomic* actions through nuclear retinoid receptors, their *non-genomic* actions should be explored as chemopreventive effects of retinoids, particularly GGA in the future. For future basic science, I would propose that chemopreventive effects of a putative agent should be less genomic and more non-genomic, because genomic actions are generally fundamental in physiology. And I must say that we should search for such natural compounds for effective chemoprevention of cancer and neurodegenerative diseases. At the most end, I hope that diterpenoid acids will be identified and utilized as a nutrient conducive to healthy human life in the near future.

Chapter VII

MATERIALS AND METHODS

VII. 1. Materials

VII. 1. 1. Chemical compounds

Geranylgeranoic acid (GGA), farnesoic acid (FA), farnesyl amine, farnesyl amide and geranic acid were kind gifts from Kuraray (Okayama, Japan).

(*R*)- and (*S*)-2,3-dihydroGGAs were synthesized as follows: x,E,E,E- and x,E,E,Z-geranylgeranoates were obtained by the condensation of farnesyl acetone with triethylphosphonoacetate followed by silica gel column chromatography for purification. Each molecule was then subjected to (*S*)-p-tol-BINAP reduction followed by hydrolysis with KOH [Kodaira et al, 2002].

6,7-DihydroGGA was prepared as follows: Geranylacetone was condensed with triethylphosphonoacetate to yield an unsaturated ester, which was reduced by bis(2-methoxyethoxy)-aluminum hydride in the presence of CuBr to afford the saturated ester. The ester was converted into the corresponding 6,7-dihydroGGA ester according to a previously reported method [Suhara et al, 2010]. Basic hydrolysis of the ester produced the 6,7-dihydroGGA. Similarly, 10,11-dihydroand 14,15-dihydroGGA were prepared from citronellyl bromide and 6-methyl-5-hepten-2-one, respectively.

Geraniol, farnesol, geranylgeraniol, all-*trans* retinoic acid (ATRA), *trans*-2-phenylcyclopropylamine hydrochloride (2-PCPA) and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

A proteasome inhibitor MG132 and RET receptor tyrosine kinase inhibitor RPI-1 were obtained from Calbiochem, Merck Millipore Japan, Tokyo, Japan.

RPI-1 was dissolved in dimethyl sulfoxide (DMSO) and others were in ethanol for treatment.

VII. 1. 2. qPCR primers and siRNAs

Primers are listed in **Table VII-1** and **Table VII-2**.

Table VII-1. Primers for RT-PCR

Gene	Sequence (5'-3')	Temp. (°C)*	Amplicon (bp)	References
Semi-qPCR				
E2F1	Fw: TGTGTGCATGAGTCCATGTGTG Rv: GCAAATCAAAGTGCAGATTGGAG	65	649	Shimonishi et al, 2012
GAPDH	Fw: TGAAGGTCGGAGTCAACGGATTTGGT Rv: CATGTGGGCCATGAGGTCCACCAC	57	983	Shimonishi et al, 2012
qPCR				
BDNF	Fw: TTAGTGACGCGCATGAATGG Rv: TGTGGTTTCGCTGGATAGTAGGT	67	177	-
CHAT	Fw: GGAGATGTTCTGCTGCTATG Rv: GGAGGTGAAACCTAGTGGCA	55	280	Song et al, 2003
CCND1	Fw: CGTGGCCTCTAAGATGAAGG Rv: CTGGCATTTTGGAGAGGAAG	55	185	Shimonishi et al, 2012
NTRK2-FL	Fw: GGTCGTGTTGTGGGAGATTTTC Rv: TTCTTCCTCATGTGGGGCTC	61	177	Sakane & Shidoji, 2011
NTRK2-T1	Fw: TGCGTCTGTGGTGGGATTTTGC Rv: CAACAAGCACCACAGCCCCTTT	62	163	Sakane & Shidoji, 2011
NTRK2-Shc	Fw: GGCATCACCAACAGTCAGCTC Rv: TTATTATCAGGCGGTCTTGGGG	66	69	Sakane & Shidoji, 2011
RARB	Fw: TGAAAATCACAGATCTCCGTAGCA Rv: CCAGGAATTTCCATTTTCAAGGT	59	76	Bohlken et al, 2009
RET	Fw: TGCATCCAGGAGGACACC Rv: TTGAGGTAGACGGTGAGCAG	55	113	-
RNA28S5	Fw: TTAGTGACGCGCATGAATGG Rv: TGTGGTTTCGCTGGATAGTAGGT	55	67	Murphy et al, 2003
TH	Fw: GGAGTTCGGGCTGTGTAAGCA Rv: GACTGGTACGTCTGGTCTTGGTAGG	60	165	-

Table VII-2. Primers for ChIP analysis.

Gene	Sequence (5'-3')	Temp. (°C)*	Amplicon (bp)	References
ChIP				
NTRK2				
-885 to -778	Fw: ATGCTGCAGCCACTAGGGCG Rv: GGGGAAGGAAGGAGTGTGTGCG	61	108	Sakane et al, 2014
-771 to -667	Fw: TCCTCAGGGGCTTCTCCGCT Rv: ACGCGCCAAGGAAGAGACGC	61	105	Sakane et al, 2014
-689 to -603	Fw: TTGGCGTCTCTTCCTTGGCGC Rv: GAGTGGTGAACGGCCAGGCTG	61	87	Sakane et al, 2014
+1515 to +1619	Fw: CCGGCAGTCTCCGCATTCCC Rv: AGCCTGAGGCCAAGGGAGGG	63	105	Sakane et al, 2014
RET				
Enh	Fw: CACCGACCACTTTGCTAACAG Rv: GGTGGTTGGAAGCACAGACT	55	60	Angrisano et al, 2010
IS	Fw: AGGAGCACAGCCCCAGAT Rv: GCCCTTGGCTGACATTGA	55	73	Angrisano et al, 2010
HOXB5 binding site	Fw: ATTCGTGCGGAGAGTTCTGTT Rv: CTGAGCGGGAAAAGGAAAC	61	64	Zhu et al, 2011
Prom	Fw: AACCCCTTCTCAGGTCCAGT Rv: CGAGTAGCGAGGAGGAGGAA	55	114	Angrisano et al, 2010

* Annealing temperature in PCR

Enh; enhancer, IS; intervening sequence, Prom; promoter

Control siRNA-A (sc-37007), *RARB* siRNA (sc-29466) and *RET* siRNA (sc-36404) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

pRShRARB constract was a kind gift from Prof. R.M. Evans (Salk Institute, La Jolla, CA, USA)

VII. 1. 3. Antibodies

Antibodies used in the thesis are listed in **Table VII-3**.

Table VII-3. List of antibodies used in the present study.

Name	Application	Product #	Provider
Actin-beta	IB	#4967	Cell Signaling Technology
BLBP	IB	ab27171	Abcam
Cyclin B1	IB	#4138	Cell Signaling Technology
Cyclin D1	IB	#2922	Cell Signaling Technology
Cyclin E	IB	#4129	Cell Signaling Technology
E2F-1 (clone KH20&KH95)	IB	#05-379	Merck Millipore
Dimethyl-Histone H3 (Lys4) (C64G9)	ChIP	#9725	Cell Signaling Technology
Trimethyl-Histon H3 (Lys4)	ChIP	#07-473	Merck Millipore
Hexokinase I (C35C4)	IB	#2024	Cell Signaling Technology
Hexokinase II	IB	#2106	Cell Signaling Technology
Lamin A (C-terminal)	IB	L1293	Sigma Aldrich
MeCP2 (D4F3)	IB	#3456	Cell Signaling Technology
RARα (C-20)	IB	sc-551	Santa Cruz Biotechnology
RARβ (C-19)	IB	sc-552	Santa Cruz Biotechnology
RARγ (C-19)	IB	sc-550	Santa Cruz Biotechnology
RB (RB1 1F8)	IB, IF	ac24	Abcam
Phospho-RB (Ser 780)	IB	#9307	Santa Cruz Biotechnology
RET (C31B4)	IB	#3223	Cell Signaling Technology
RXRα (D-20)	IB	sc-553	Santa Cruz Biotechnology
RXRβ (Y-20)	IB	sc-555	Santa Cruz Biotechnology
TrkB (H-181)	IB	sc-8316	Santa Cruz Biotechnology
Tubulin-IIIβ	IB	T2200	Sigma Aldrich

IB; immunoblotting, IF; immunofluorescence, ChIP; chromation immunoprecipitation assay

VII. 2. Methods

VII. 2. 1. Cell culture

Hepatoma

The human hepatoma-derived cell lines, HuH-7, PLC/PRF/5, and HepG2, were obtained from RIKEN BioResource Center, Tsukuba, Japan and maintained on 25-cm^2 flasks in Dulbecco's modified Eagle's (DME) medium (Wako Pure Chemical Industries, Osaka, Japan) containing 5% FBS (HyClone, Logan, UT). We inonculated 2×10^4 or 1.2×10^5 HuH-7 cells to a 6-well plate (Thermo Fisher Scientific, Tokyo, Japan) or to a 9.0-cm dish, respectively, and cultured with DME medium containing 5% FBS for 2 d. Thereafter, the cells were washed with Hank's balanced salt solution (Sigma Aldrich, St. Louis, MO) twice, and the medium was replaced by FBS-free DME medium at 2 d prior to the addition of GGA. GGA in ethanol was added at a final concentration of 10 μM. Ethanol (0.1%, v/v) was added as a vehicle control.

Neuroblastoma

Human SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA, USA) were grown in DME medium with 10% heat-inactivated FBS (Hyclone). Cells were seeded in 35-mm dishes at density of 1×10^5 cells/dish. After 2-d incubation, agents were added every 48 h during medium replenishment.

Morphology

Microscopic images were collected by a CoolSNAP charge-coupled device (CCD) camera (Photometrics,

Tucson, AZ, USA) connected to an inverted microscope (IX70; Olympus, Tokyo, Japan).

Proliferation assay

For cell proliferation assays, the trypan blue dye exclusion method was performed. Living cells were detached from the substrate and also dissociated from the neurosphere-like aggregations by trypsinization after a gentle washing of the cells with PBS (-) without eliminating the cellular aggregates to count both attached cells and aggregated cells in total.

Transfection

Plasmid DNA or siRNAs were transient transfected with Lipofectamin2000 (Life Technologies Japan, Tokyo, Japan).

VII. 2. 2. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA isolation was carried out using the High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany), or QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan) with the fully automatic nucleic acid extraction system, Quick Gene 800. Total RNA was quantified by absorbance at 260 nm measured with NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and first-strand complementary DNA (cDNA) was synthesized using Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics GmbH) with random hexamer primer, or Transcriptor Universal cDNA Master

(Roche Diagnostics GmbH). The reverse transcription reactions were performed at 25°C for 10 min followed by 60 min at 50°C, or at 25°C for 5 min followed by 10 min at 60°C.

Semiquantitative PCR was performed in AccuPower PCR Pre-Mix (Bioneer, Daejeon, Korea) on a TaKaRa PCR Thermal Cycler PERSONAL (Tokyo, Japan) under the following conditions. The *E2F1* gene: 30 cycles of denaturation at 95°C for 30 s; annealing and extension at 65°C for 90 s. The *GAPDH* gene: 25 cycles of denaturation at 95°C for 30 s; annealing at 57°C for 30 s; and extension at 72°C for 30 s. Amplicons were separated on 3% agarose gel and stained with SYBR safe (Life Technologies Japan).

Real-time PCR was performed using LightCycler 1.5 (Roche Diagnostics GmbH) and DyNAmo™ Capillary SYBR™ Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences used in this study are presented in Table 1. Gene expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method.

VII. 2. 3. Chromation immunoprecipitation assay (ChIP)

Cross-linked chromatin digestion and immunoprecipitation were carried out with SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instruction. DNA was purified with QIAquick PCR Purification Kit (QIAGEN, Tokyo, Japan) and quantified by real-time PCR with specific primers listed in **Table VII-2**.

VII. 2. 4. SDS-PAGE and immunoblotting

Cells were washed with phosphate-buffered saline, lysed on ice with RIPA buffer [(1% sodium deoxycholate

and 0.1% SDS prepared in 25 mM Tris HCl buffer (pH 7.6)] containing 150 mM NaCl and a protease inhibitor cocktail (Roche Diagnostics GmbH).

Subcellular fractionation was performed using a CelLytic NuCLEAR Extraction Kit (Sigma Aldrich), according to the manufacturer's protocol.

Cell lysates or nuclei extract were denatured with 6× sample buffer (0.4 M Tirs-HCl, pH 6.8, 12% SDS, 45% glycerol, 0.024% bromophenol blue and 10% 2-mercaptoethanol) and boiling at 95°C for 5 min, resolved by SDS-PAGE, and transferred to PVDF membrane. The membrane was incubated with primary and secondly antibodies.

Luminescence was determined by incubation with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) and exposed using LAS-1000 imaging system (GE Healthcare Japan, Tokyo, Japan). Collected images were analyzed by ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA).

VII. 2. 5. Immunofluorescence

Cells were washed with PBS(-) and fixed with 4% paraformaldehyde containing 2% sucrose in PBS(-) for 30 min. After washing with PBS-T, cells were perforated with 5% Triton X100 (Sigma Aldrich), washed 3× with PBS-T, blocked with DME medium containing 5% nonimmune rabbit serum for 1 h, and then incubated with primary antibody in DME medium containing 0.5% nonimmune rabbit serum at 4°C overnight. After washing with PBS-T, cells were incubated with second antibody at room temp for 30 min, washed 3× with

PBS-T, immersed in aqueous mounting medium Permaflour (GE Healthcare Japan) and finally covered with cover glass. Then, cells were observed under a confocal laser-scanning fluorescence microscope system, LSM 510 equipped with Axiovert 200M (Carl Zeiss Japan, Tokyo, Japan).

VII. 2. 6. Monoamine oxidase (MAO) activity and lysine-specific demethylase 1A (KDM1A) inhibitory analysis

MAO

The inhibitory effect of farnesol on MAO activity was measured using MAO-Glo™ Assay (Promega, Madison, WI, USA) and recombinant human MAOA and MAOB (Sigma-Aldrich). Farnesol was dissolved in ethanol to produce a 20-fold diluted solution with MAO Reaction Buffer (Promega) and pre-incubated with enzymes (5 mU) for 1 h on ice. Following oxidization of MAO substrate (40 μM to assay MAOA or 4 μM for MAOB) at 37 °C for 1 h, the methyl ester luciferin, which is produced by the action of MAO on MAO substrate, was reacted with esterase and luciferase for 20 min at room temperature. The produced light was measured with CentroXS3 LB960 (Berthold Japan K.K., Tokyo, Japan).

KDM1A

Determination of inhibitory activity against KDM1A was performed with the LSD1 Inhibitor Screening Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The enzyme reaction was monitored by fluorescence on channel 1 of a LightCycler 1.5 for 30 min.

VII. 2. 7. Statistical analysis

GraphPad Prism version 6.0 for Windows (GraphPad Software, Inc. La Jolla, CA, USA) was used for preparation of data graph and statistic analyses. We consider the result would be statistically significant if a *p* value was found to be less than 0.05.

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Sincerely,

Chiharu S.

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