Functional Study of the Regulation of DNA Topoisomerase II and its Cellular Role in Drug Responsiveness

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THESIS

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LIST OF ABBREVIATIONS

3'/5'-UTR	3'/5'- Untranslated region
Abs.	Absorbance
C_t	Cycle threshold
DMSO	Dimethyl sulfoxide
Doxy	Doxycycline
GFP	Green fluorescent protein
ICEs	Inverted CCAAT Elements
KD	Knock down
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NF-YB	Nuclear factor-YB
PCNA	Proliferating cell nuclear antigen
PI	Propidium iodide
PTEN	Phosphatase and tensin homolog
Sp	Specificity protein

Top2	DNA topoisomerase II
TPA	12-O-tetradecanoylphorbol-13-acetate
RA	Retinoic acid

Chapter 1

1. 1. Summary

Resistance to chemotherapeutic agents often occurs in many human cancers and is a barrier to effective treatment. Such resistance is multifactoral and one is associated with the altered expression of drug's target. DNA topoisomerase II (Top2) is an essential cellular enzyme involved in DNA metabolism and the target of important chemotherapeutic agents, such as doxorubicin, etoposide and teniposide. In mammalian cells, there are two isoforms of DNA topoisomease II, designated Top2α and Top2β. A reduced expression of Top2α is thought to mediate responsiveness to anti-Top2 agents during the development of drug resistance in some cell lines. Our preliminary observation revealed a relation between low Top2α expression and resistance to anti-Top2 agents in our previously selected teniposide-resistant human lymphoblastic leukemia CEM/VM-1-5 cell line. This drug-resistant CEM/VM-1-5 cell line grows much slower than parental drug-sensitive CEM cell line. Furthermore, we observed an inverse correlation between the expression of Top2α and transcription factor NF-YB. Using human cancer cell lines to study drug resistance can help us unravel the molecular mechanisms during the development of drug resistance, further predict treatment outcome and circumvent drug resistance. Therefore, the mechanisms involved in the downregulation of Top2α in drug-resistant cell lines are the focus of this thesis. We **hypothesized** that transcription factor NF-YB mediates Top2α expression in drug-resistant cancer cell lines and three specific aims were proposed to test this hypothesis:

In Aim 1, we successfully knocked down Top2α by RNAi in parental drug-sensitive CEM cell line, which resulted in a decrease in the sensitivity to Top2 poison, etoposide, compared with control CEM cell line. Although evidence from other groups revealed that Top2α is essential for the survival of proliferating cells, our results demonstrated that Top2α-knockdown CEM cells growth was similar to that of control counterpart. This suggests that cells can proliferate with relatively low level of Top2α and there may exist more complicated regulatory mechanisms during the long term selection process of drug-resistant CEM/VM-1-5 cells.

In Aim 2, our question was what causes the reduced expression of Top2α in drug resistant CEM/VM-1-5 cells. Previous work from our laboratory suggested that the transcription factor NF-YB is a negative regulator of Top2α promoter. We have now found that NF-YB protein levels were higher in drug-resistant CEM/VM-1-5 cells, compared with drug-sensitive CEM cells, revealing an inverse correlation between the levels of Top2α and NF-YB expression in CEM and CEM/VM-1-5 cells. We also observed this inverse correlation in drug-sensitive human rhabdomyosarcoma Rh30 cell line and its etoposide-resistant Rh30/v1 subline. These data suggest that the increased NF-YB may be related to or be the cause of reduced Top2α in drug-resistant CEM/VM-1-5 cells and Rh30/v1 cells. To determine the regulatory role of NF-YB in Top2α expression, we further knocked down NF-YB in drug resistant cells. However, Top2α level remained the same, which suggests the indirect regulation of NF-YB on Top2α promoter or NF-YB regulation on the Top2α promoter may involve other cofactors.

Nevertheless, we then asked what causes the upregulation of NF-YB in drug resistant CEM/VM-1-5 and Rh30/v1 cells.

Our data from Aim 1 suggest a complicated regulatory mechanism during the long term selection process of drug-resistant cell lines. Furthermore, recent studies indicate that microRNAs are often aberrantly expressed or mutated in cancer and may mediate drug responsiveness. MicroRNAs function through perfect or near-perfect base pairing with protein coding mRNA 3'-untranslated regions (3'-UTRs) for mRNA degradation or translational repression. Accordingly, in Aim 3 we asked whether microRNAs are involved in Top2α-mediated drug-responsiveness in CEM and CEM/VM-1-5 cells and in Rh30 and Rh30/v1 cells. We have found by microRNA profiling that one particular microRNA, *hsa-miR-485-3p*, is consistently expressed at substantially lower levels in drug resistant CEM/VM-1-5 cells compared with drug sensitive CEM cells. MicroRNA target-predicting algorithms revealed that *hsa-miR-485-3p* has a potential target to 3'-UTR of *NF-YB*. We first validated the binding of *hsa-miR-485-3p* to 3'-UTR of *NF-YB* by luciferase assay. Moreover, ectopic expression of *hsa-miR-485-3p* repressed NF-YB expression and rescued the expression of Top2α. Of more interest, we observed an increase in sensitivity to Top2 poisons in drug-resistant cells ectopically overexpressing *hsa-miR-485-3p.*

To follow up on work in Aim 1 (knock down Top2α by RNAi), in Aim 4 we constructed tetracycline inducible system to turn-on and -off the expression of Top2α shRNA with and without the adding of antibiotic doxycycline (Doxy), respectively. However, we

observed the cytotoxicity effect of doxycycline in CEM cells and all other cancer cell lines tested. The inhibitory effect of doxycycline on cancer cell lines was partly due to apoptosis of the cancer cells. Furthermore, we observed the downregulation of Top2α and upregulation of Top2β in Doxy-treated cells. So far, the regulation and cellular role of Top2β is not clear and our results presented herein suggest that the upregulation of Top2β in Doxy-treated cells is not mediated by differentiation of the cells.

In conclusion, we demonstrated that Top2α is the determinant of sensitivity to anti-Top2 agents in cancer cells and that microRNAs are involved during the development of drug resistance in cancer cells. Ectopic expression of *miR-485-3p* in drug resistant cells led to reduced expression of its target, NF-YB, a corresponding upregulation of Top2α, and increased sensitivity to the Top2 poisons. Furthermore, in the tetracycline inducible system, antibiotic doxycycline inhibited the proliferation of cancer cell lines, along with the downregulation of Top2α and upregulation of Top2β. Doxy-treated cell lines can be used as a model to study the regulation of Top2β which is yet well described.

1.2 Introduction

1.2.1. Overview: Drug resistance in cancer

Cancer is the leading cause of death worldwide, according to World Health Organization, and chemotherapy has been widely used in treatment of cancer. Unfortunately, inherent (*de novo*) and acquired resistance to chemotherapy become an intractable problem in treating most common solid tumors and hematological malignancies [reviewed in 1, 2]. The development of chemoresistance in cancer is considered as a multifactorial phenomenon, reflecting the heterogeneity of cancer cells and can be classified into two major categories (See Figure 1 for an overview of mechanisms of drug resistance). One is the alteration in various pharmacological parameters, including decreased drug absorption, rapid metabolism and renal clearance of drugs after systemic treatment, which partly explains the interpatient variation. The other category of chemoresistance arises as a result of changes in the biology of cancer cells genetically or epigenetically. Therefore, cancer cell lines selected for chemoresistance have become a useful model to study the mechanisms of resistance at the cellular and molecular level. Examples include the enhanced efflux of drugs mediated by ATP-binding cassette (ABC) transporters [3], aberrant methylation of gene promoters results in blocking cancer cell apoptosis, increased repair of DNA damage [reviewed in 4] or alteration of the specific target of drugs. Chemotherapy mainly targets to proliferating cells through inhibition of specific steps of the DNA replication process, therefore, alteration of drug's target, human DNA topoisomerase II (Top2; human gene symbol *Top2*), becomes the major problem encountered during chemoresistance [reviewed in 5].

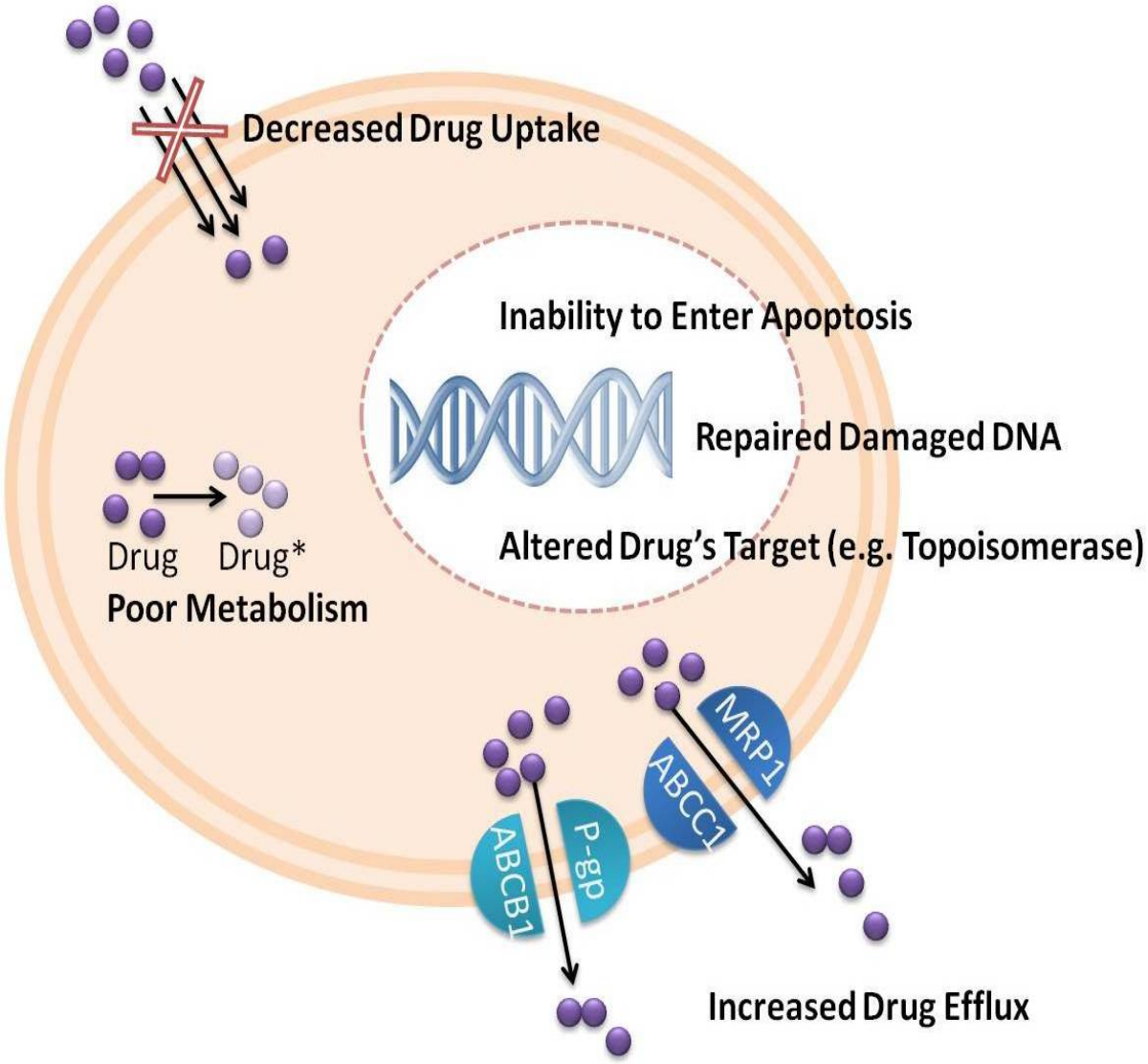


Fig.1. A schematic representation of molecular mechanisms of chemoresistance in a cancer cell
(Adapted from Gottesman Annual Review of Medicine, 2002)

The mechanisms of chemoresistance have been characterized for the past few decades. However, they cannot fully explain the resistant phenomenon in cancer cells. Recent studies have underlined the importance of microRNAs, a group of small noncoding RNAs, in cancer development and their involvement in chemoresistance by regulating gene expression [reviewed in 6]. This thesis focus is on the mechanisms of the regulation of drug's target, DNA topoisomerse II (Top2), during the development of chemoresistance in cancer cells and the involvement of microRNAs in drug responsiveness in cancer cells. A better understanding of chemoresistance in cancer cells can improve prognosis and survival in patients.

1.2.2. Overview: DNA Topoisomerase

DNA topoisomerases are ubiquitous enzymes involved in DNA metabolic processes including replication, transcription, recombination and chromatin remodeling by breaking single or double strands of DNA [reviewed in 7, 8]. While these enzymes are critical to DNA metabolism, they serve as the effective cellular targets for many clinically active anticancer drugs, including camptothecins, etoposide and doxorubicin [reviewed in 5, 9, 10-11]. However, during the treatment course, cancer cells frequently become refractory to the treatments due to the development of drug resistance as described in section 1.2.1. Understanding the regulation of drug's target, DNA topoisomerase, is necessary in order to treat cancers more effectively.

1.2.2.1. DNA Topoisomerase Cellular Function

Chromosome DNA is globally negatively supercoiled in all species [12]. When double-stranded DNA unwinds during transcription and replication, it creates torsional tension by the generating of positively supercoiled DNA, which blocks the progression of DNA metabolism as shown in Figure 2. DNA topoisomerases are enzymes that disentangle the topological problems by introducing transient strand breaks in the positively supercoiled DNA to the energetically more stable state of negatively supercoiled DNA [12-15].

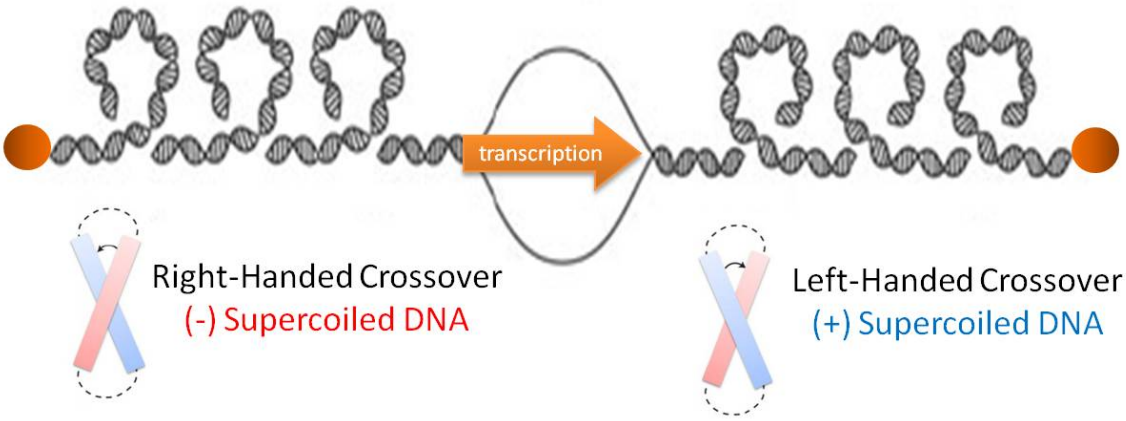


Fig. 2. DNA topology
When double-stranded DNA helix unwinds during transcription, positive supercoiled DNA accumulates ahead of the transcription fork, while the negative supercoiled DNA accumulates behind it. Chromosomal DNA ends are immobilized to the chromosome scaffold (orange spheres).
(Adapted from Schoeffler and Berger, Q Rev Biophys., 2008)

1.2.2.2. DNA Topoisomerase Family

Over the past few decades, many DNA topoisomerases have been discovered [16] and can be classified as type I or type II enzymes, depending on their function: introducing single strand break (type I topoisomerase) or double strand breaks (type II topoisomerase) on double-stranded DNA. Further division into four subfamilies, based on structural and mechanistic similarities, is shown in Table 1.

Table 1. DNA Topoisomerase classifications

Type	Subfamily	Origins	Enzymes	Mechanistic Properties		
				Subunit Structure	DNA cleavage	ATP Dependent
I	IA	E.coli	Bacterial DNA topoisomerases I and III	Monomer	Single Strand	No
		Yeast	Yeast DNA topoisomerase III			
		Mammals	Mammalian DNA topoisomerases IIIα and IIIβ			
	IB	Poxvirus	Poxvirus DNA topoisomerase I			
		Mammals	Mammalian DNA topoisomerases I and mitochondrial DNA topoisomerase I			
II	IIA	E.coli	Bacterial DNA gyrase and DNA topoisomerase IV	Heterotetramer	Double Strand	Yes
		Bacterial phage	Phage T4 DNA topoisomerase	Heterotetramer		
		Yeast	Yeast DNA topoisomerase II	Homodimer		
		Mammals	Mammalian DNA topoisomerases IIα and IIβ	Homodimer		
	IIB	Archaea	Sulfolobus shibatae DNA topoisomerase VI	Heterotetramer		

(Adapted from Champoux JJ., Annu Rev Biochem., 2001., Wang JC., Nat Rev Mol Cell Biol., 2002)

Type IA DNA topoisomerase was the first DNA topoisomerase discovered by James C. Wang in 1971 from E. coli, which could release negatively supercoiled DNA [17]. Subsequent work has led to the identification of type IB DNA topoisomerase [18], type IIA DNA topoisomerases, bacterial DNA gyrase, [19], eukaryotic DNA topoisomerase II [20], and type IIB DNA topoisomerase [21].

A common reaction of both type I and type II DNA topoisomerases is the ability to relax supercoiled DNA. However, DNA metabolic processes also generate knots and catenation of double-stranded DNA as shown in Figure 3. DNA topoisomerase II is the sole enzyme to catalyze the unknotting and decatenation of intact double-stranded DNA. Failure to decatenate during the separation of replicated DNA at mitosis can lead to cell death [22-23]. Furthermore, DNA topoisomerase II is essential for the survival of proliferating cells [reviewed in 24]. Based on the essential role of DNA topoisomerase II in DNA metabolism, in this thesis, we will focus our work on the mechanisms of regulation of DNA topoisomerase II (Top2) in human cancer cell lines and its role in chemoresistance.

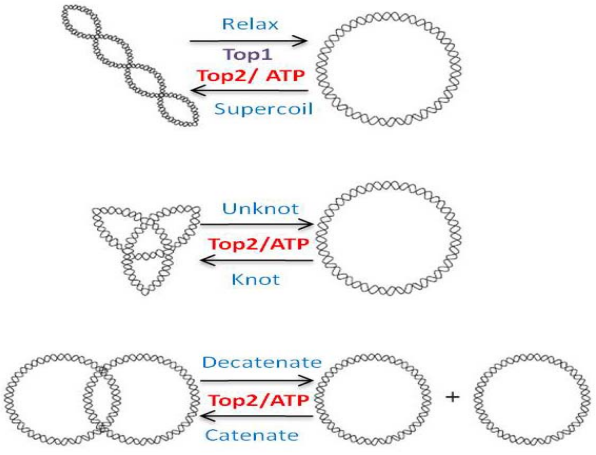


Fig.3. Reactions of DNA Topoisomerases
(Adapted from John L. Nitiss, Current Protocols in Pharmacology, Topoisomerase Assays, 2001)

1.2.2.3. DNA Topoisomerase II (Top2) Biochemistry

Mammalian DNA topoisomerase II (Top2) functions as homodimers in the presence of Mg^{2+} and ATP to generate transient double strand breaks and religate the breaks [5, 7-8, 16]. Briefly, DNA Top2 catalytic mechanism occurs when tyrosyl oxygen of the enzyme attacks DNA phosphodiester bond, forming a covalent enzyme-cleaved DNA complex between each strand of the G (gate) segment. Upon binding of ATP, DNA Top2 undergoes a conformational change that allows the passage of T (transfer) segment of double-stranded DNA. Subsequent religation of the cleaved double-stranded DNA follows the release of T segment [reviewed in 5, 7, 8, 16].

1.2.2.4. DNA Topoisomerase II (Top2) Isoforms

Whereas yeasts have a single gene encoding DNA Top2, higher eukaryotes and mammals have two distinct isoforms of type II topoisomerases, topoisomerase IIα (Top2α: 170 kDa) and topoisomerase IIβ (Top2β: 180 kDa) [25-26]. They are encoded by two different genes located on chromosomes 17q21-22 (*Top2*α) and 3p24 (*Top2*β) [27], which may allow for differential regulation at the transcriptional level. Based on sequence comparisons and crystallographic structure studies, these two isoforms display a high degree of conservation of amino acid sequence (~70%) and each DNA *Top2* isoform has three functional domains [7, 28-30]. The amino-terminal domain has an ATPase function and is highly conserved between species. The central region has breakage and reunion domain, which includes the active site tyrosine residue and is also conserved. The carboxyl-terminal domain is the least conserved between species and *Top2* isoforms and is suggested to be required for nuclear

localization of isoforms [31-32], post-translational modification [33-36] and isoform-specific function [37]. In this thesis, we will emphasize our study on human DNA Top2 in cancers.

1.2.2.5. Expression of DNA Topoisomerase II (Top2)

Despite Top2α and Top2β have similar characteristic to catalyze ATP-dependent DNA strand breaks [38], and both isoforms can complement the loss of the sole Top2 in yeast [39], they have distinct patterns of expression and cellular function.

Mammalian Top2α expression is strictly regulated by cell cycle, with its peak at late S and G2/M phase [40-41]. Subsequent studies of mammalian Top2α also indicate its role in the regulation of cell cycle events such as DNA replication [42], chromosome condensation [43] and segregation [44] in mitotic cells. Top2α knock-out mouse cannot develop at early embryonic stage [45].

In addition to its association with cell cycle, mammalian Top2α expression is correlated to proliferation status. Many studies indicate the upregulation of Top2α during cell proliferation [46-47] and in proliferating tissues [48-50]. Furthermore, higher levels of Top2α are expressed in human tumors compared with normal tissues [51-53].

In contrast, mammalian Top2β expression remains relatively low and constant throughout the cell cycle [40-41], expresses at equal levels in proliferating tissues [48-

50] and is dispensable at cellular level [54-58]. However, increased expression of Top2β has been found in non-proliferating, differentiated cells and tissues [59-65].

Although the cellular role of Top2β is less clear than that of Top2α, recent studies indicate the involvement of Top2β in neuronal development [66-68] and transcriptional regulation [69-71].

1.2.2.6. DNA Topoisomerase II (Top2) and Anti-Cancer Agents

Chemotherapy mainly targets proliferating cells through inhibition of specific steps of the DNA replication process. Since DNA Top2 generates Top2-DNA covalent complexes, a type of DNA damage, during their catalytic cycle, this characteristic has been used to design many anti-Top2 agents, which can be further classified as Top2 poisons and Top2 catalytic inhibitors as listed in Table 2.

Table 2. Anti-Topoisomerase II Agents

Category	Drug Class	Example(s)	Effects
Topoisomerase Poisons	Acridines	m-AMSA (amsacrine)	stabilize cleavable complex
	Actinomycins	Actinomycin D	
	Anthracyclines	Doxorubicin (Adriamycin), Daunomycin	
	Anthracenediones	Mitoxantrone	
	Ellipticines	2-methyl-9-OH- ellipticinium acetate	
	Epipodophyllotoxins	Etoposide (VP-16), Teniposide (VM-26)	
	Isoflavonoids	Genistein	PTK inhibitor and cleavable-complex blocker
Catalytic Inhibitors	Anthracenyl peptides	Merbarone	inhibits cleavable complex formation
	bis-piperazinediones	ICRF-159, 187 (dexrazoxane), and 193	inhibits DNA relaxation and cleavable complex formation

Top2 poisons induce DNA damage by inhibition of DNA religation step where Top2 is covalently bound to DNA with broken strands, forming the ternary Top2-DNA-poison complexes. The drug-induced DNA damages inhibit the essential DNA metabolic processes and can further trigger apoptosis in cells. Top2 poisons represent some of the most important clinically active anticancer agents including doxorubicin and etoposide that are used to treat a variety of cancers [5, 9-11].

The other group of anti-Top2 agents, termed Top2 catalytic inhibitors, kills the cells by inhibiting Top2 catalytic activity. Drugs in this class include merbarone, and bisdioxopiperazines (ICRF-187) [5, 9-11, 72].

1.2.2.7. Resistance to anti-Top2 agents

Anti-Top2 agents are not like most enzyme inhibitors where a drug's action is by suppression of the target enzyme. Anti-Top2 agents exert their effects in a Top2-dependent manner by converting ternary Top2-DNA-drug complexes into a cellular toxin. Therefore, a decrease in the amount or catalytic activity of Top2 leads to decreased sensitivity of cells to anti-Top2 agents [5, 9-11, 72].

Mutations to the coding region of DNA *Top2* in selected drug-resistant cell lines have been documented. Mutations of *Top2*α near or at the tyrosine residule [73-74] or to the N-terminal ATPase domain [74-76] are suggested to inhibit the binding of the drug to the

enzyme, or to interfere with ATP binding/hydrolysis through conformation change. Furthermore, cancer cell lines selected for acquired resistance to anti-Top2 agents *in vitro* often express dysregulation of the amount or catalytic activity of Top2, compared with the drug-sensitive counterpart as listed in Table 3. Low level of DNA Top2 is also associated with drug-resistant phenotype in clinical studies [77-78]; high level of Top2 is associated with chemosensitivity [79-81].

Despite the obvious importance of studying the role of Top2α in drug responsiveness, some evidence suggests a role of Top2β in differentiation. As addressed in section 1.2.2.5., increased expression of Top2β has been found in non-proliferating, differentiated cells and tissues [59-65]. In addition, dysregulation of Top2β by differentiating agent, all-trans retinoic acid (ATAR), in HL-60 cell lines had been shown [64, 82]. Cancer cells stimulated to differentiate grow more slowly than their undifferentiated counterparts [83], which confers a degree of drug resistance relative to proliferating cells.

Moreover, recent studies have indicated the role of microRNAs, a group of small noncoding RNAs, in chemoresistance by regulating gene function. This will be discussed in detail in the section 1.4.

Table 3. Cancer cell lines resistant to Anti-Topoisomerase II agents

Cell Type	Selection Agent	Resistant Cell Line		Reference
human lung cancer cell line SW-1573	doxorubicin		↓ Top2α mRNA and protein	Eijdems EW et al. Br J Cancer. (1995)
human sarcoma cell line MES-SA	doxorubicin		↓ Top2α mRNA and protein	Beketic-Oreskovic L et al. J Natl Cancer Inst. (1995)
human breast cancer cell lines MCF-7	doxorubicin	MCF/7Adp500, MCF7/Ad75	↓ Top2α mRNA	Wosikowski K et al. Clin Cancer Res. (1997)
human stomach-adenocarcinoma cell line MKN-45	doxorubicin	MKN/ADR	↓ Top2α protein	Son YS et al. Cancer Chemother Pharmacol. (1998)
human multiple myeloma RPMI 8226	doxorubicin	Dox1V	↓ Top2α protein	Wang H et al.Biochem Biophys Res Commun. (1997)
human SCLC line, NCI-H209	etoposide (VP-16)	H209/VP	↓ Top2α protein	Jain N et al. Cancer. (1996)
human rhabdomyosarcoma	etoposide	**Rh30/v1**	↓ Top2α protein	Bhat UG et al. Proc Natl Acad Sci U S A. (1999)
human breast cancer cell lines MDA-MB-231	etoposide	MDA-MB-231-VP7	↓ Top2α mRNA	Matsumoto Y et al. Cancer Res. (1997)
human melanoma cell line MeWo	etoposide	MeWo ETO	↓ Top2α, Top2β mRNA and protein	Lage H et al. Br J Cancer. (2000)
human radioresistant glioblastoma cell line T98G	etoposide	T98G-VP(1000)	↓ Top2α mRNA and protein	Matsumoto Y et al. J Neurooncol. (1999)
human erythroleukemic cell line K562	etoposide	K562-VP16	↓ Top2α mRNA	Melixetian MB et al. Leuk Res. (2000)
human monoblastic leukemia cells U-937	etoposide	RERC	↓ Top2α mRNA and protein	Saleem A et al. Cancer Res. (1997)
human leukemia cell line HL-60	mitoxantrone	HL-60/MX2	↓ Top2α mRNA	Harker WG et al. Cancer Res. (1995)
human myelogenous leukaemia line K562	mitoxantrone	K562/Mxn	↓ Top2α protein	Zhou R et al. Med Oncol. (1999)
P388 leukemia cell line	m-AMSA	P388/amsacrine	↓ Top2 protein	Per SR et al. Mol Pharmacol. (1987)
human leukemia cell line HL-60	m-AMSA	HL-60/AMSA	↓ Top2β mRNA	Zwelling LA et al. J Biol Chem. (1989)
human leukaemic CCRF-CEM cells	Genistein	CEM/GN50	↓ Top2β protein	Markovits J et al. Biochem Pharmacol. (1995)
human leukaemic CCRF-CEM cells	teniposide (VM-26)	**CEM/VM-1-5**	↓ Top2α protein	Chen CF et al. Mol Pharmacol. (2011)
Chinese hamster lung cell line DC-3F	9-OH-ellipticine	DC-3F/9-OH-E	↓ Top2α, Top2b mRNA and protein	Khélifa T et al. Cancer Res. (1999)
human leukaemic CCRF-CEM cells	merbarone	CEM/M70-B	↓ Top2α protein and ↑ Top2β protein	Kusumoto H et al. Cancer Res. (1996)

1.2.2.8. Regulation of DNA Topoisomerase II (Top2)

The regulation of gene expression is a multi-step process, including transcriptional, and post-translational modifications, which subsequently reflect the protein expression and phenotype. Transcriptional regulation of mammalian DNA *Top2* is mediated in part by the binding of transcription factors (*trans*-acting factors) to the regulatory regions (*cis*-acting elements) of DNA *Top2* promoter. The structure of the 5'-untranslated region (5'-UTR) of the human *Top2*α promoter has been cloned [84] and characterized in some studies and is represented in Figure 4. The regulatory regions (*cis*-acting elements) of human *Top2*α promoter includes two Sp (specificity protein) binding sites (GC1 and GC2) [85-86], and a region for the binding of Myb [87]. In addition, human *Top2*α promoter has five inverted CCAAT elements (ICEs) which have been shown to have repressive [46, 88] or activating [89] effect on *Top2*α transcription when bound by the transcription factor, nuclear factor-Y (NF-Y) protein complex. ICE1 and ICE3 have also been associated with p53-mediated repression of the human *Top2*α promoter [90-91]

Our previous work suggests that the transcription factor NF-YB (nuclear factor- YB), one subunit of NF-Y protein complex, suppressed the transcription of *Top2*α by binding to ICE3 on human *Top2*α promoter [92]. In this regard, we will further examine the role of NF-YB in the regulation of DNA Top2α. The basis for the disparity of activation or repression of *Top2*α transcription through binding of transcription factor NF-Y to ICEs in *Top2*α promoter is unknown but it suggests that NF-Y protein complex may function differently in cell-type specific manner or by binding to different ICEs. In contrast,

although the human *Top2*β promoter has been cloned [93-94], the transcriptional regulation is less clear.

With regard to post-translational modifications of DNA *Top2*, phosphorylation at C-terminal domain of *Top2* has been postulated to stimulate enzyme catalytic activity [95]. Some phosphorylation sites on human *Top2*α C-terminal domain have been identified as substrates for various kinases, including casein kinase II [96-97], protein kinase C [33, 98], aurora B [99], and Polo-like kinase 1 (Plk1) [100-101] Moreover, recent studies revealed that human *Top2*α and *Top2*β are conjugated by small ubiquitin-like modifier (SUMO) through the C-terminal domains in response to DNA damage [102-103]. SUMO has been suggested to mediate trafficking and function of DNA Topoisomerase II in the chromosome [36].

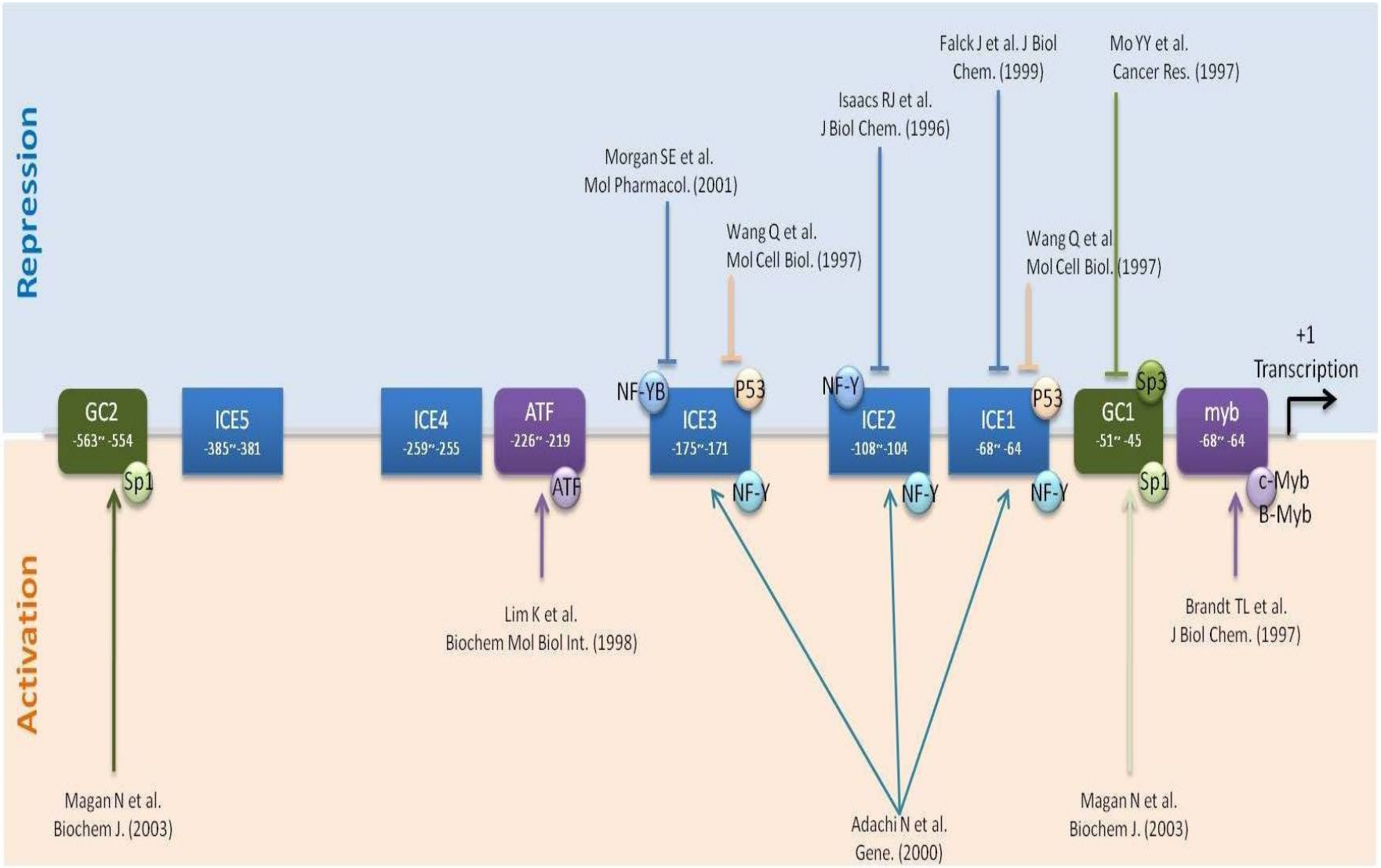


Fig. 4. Relative position of *cis*-acting elements in the human *Top2α* promoter and associated transcription factors

1.3. Transcription Factor, NF-Y (Nuclear Factor-Y)

Transcriptional modification of gene is regulated in part by the binding of transcription factors (*trans*-acting factors) to the regulatory regions (*cis*-acting elements) on the promoters. One such factor, nuclear factor-Y (NF-Y), also known as CCAAT binding factor (CBF) [104-105], is a conserved, sequence-specific transcription factor. It binds to CCAAT elements on the promoters to regulate gene transcription. CCAAT elements are a widespread regulatory elements in most eukaryotic promoters [106].

NF-Y is composed of three subunits, NF-YA, NF-YB, and NF-YC, all necessary for DNA binding. NF-YB and NF-YC dimerize through interaction between complementary histone fold motifs. This dimerization offers a complex surface for NF-YA association and sequence specific DNA binding [104-105]. The regulation of NF-Y protein is not clear but evidence reveals that its mRNA remains constant in growing and differentiated cells [107-108].

NF-Y has been reported to regulate the expression of several cell cycle regulators, including *cyclin B1/B2* [109-110], *cdc25C* [111-112] and *Top2*α [46, 85, 88-89], all containing several CCAAT elements in their promoters. Genome-wide study has also linked NF-Y as a common transcription factor to cell cycle-regulated genes in human cells [113]. NF-Y can function both as a transcription repressor and activator depending

on the cellular context [114-115]. In addition, evidence revealed that the function of NF-Y is negatively regulated by p53 [116-117].

Since our previous work [92] suggested NF-YB as a negative regulator of human *Top2*α at inverted CCAAT element 3 (ICE3) in the *Top2*α promoter, we further studied the mechanism by which NF-YB causes the downregulation of Top2α during the development of drug resistance in this thesis.

1.4. MicroRNA and its Involvement in Chemoresistance

In addition to the chemoresistance mechanisms described in sections 1.2.1 and 1.2.2.6, accumulating evidence revealed that aberrant microRNAs expression correlates with the cancer development and anticancer drug resistance.

MicroRNAs (miRNAs, miRs) comprise a group of endogenous small noncoding RNAs (20–23 nucleotides) that negatively regulate gene expression at the post-transcriptional level through partial complementarity base pairing to the 3′ untranslated region (3'-UTR) of target genes [reviewed in 118, 119-120]. However, some recent cases indicate that miRNAs may also target 5′-UTR of genes [121-122]) or activate gene expression [123-124]. During the past decade, the biogenesis of miRNAs has been well characterized [reviewed in 118, 119-120]. To date, 1733 human miRNAs have been identified according to miRBase (Version 17, Apr. 2011)[125], and they are predicted to regulate the expression of more than 60% of protein-coding genes in the cells [126]. The preliminary identification of miRNA and its target genes is often predicted by computational algorithms [127], then followed by experimental validation. Based on functional studies, evidence has revealed their importance in many biological processes, including development, differentiation, cell proliferation, apoptosis and stress response [128-129]. Since the complementarities between miRNAs and their target genes are imperfect, each single miRNA can impact a large number of targets [130-131]. On the contrary, a single target can also be affected by multiple miRNAs [132].

Since miRNAs are involved in important biological processes, this suggests that aberrant expression of miRNAs is likely to have a role in the development of cancer. The first link between miRNAs and cancer was reported in chronic lymphocytic leukemia (CLL), where the common deletion region on chromosome 13 contains *miR-16* and *miR-15* locations [133]. Subsequently, there is evidence indicating these miRNAs are frequently located close to fragile sites that have been shown to cause DNA instability or in genomic regions associated with cancers [134]. Moreover, several studies have revealed the unique miRNAs expression profiles in tumors and normal tissues [reviewed in 135, 136], which further strengthen the association between miRNAs and cancer.

Dysregulation of miRNAs can contribute to the dysregulation of targeted oncogenes or tumor suppressor genes. In this regard, cancer-associated miRNAs are often classified with tumor suppressor or oncogene properties [137-138]. Oncogenic miRNAs function by targeting tumor suppressor genes, while other miRNAs with tumor suppressor properties can repress oncogene expression and these miRNAs are often downregulated in cancer.

The role of miRNAs in chemosensitivity has recently been studied. MicroRNAs expression profiles revealed altered expression pattern in chemoresistant cancer cell lines as summarized in Table 4. This data suggest that miRNAs have an important role in the development of chemoresistance in cancers of different origins. For example, *miR-214* is highly expressed in the cisplatin-resistant ovarian cell line compared with

parental sensitive ovarian cell line [139]. *miR-214* induced cisplatin resistance in ovarian cell line through negatively regulating PTEN (phosphatase and tensin homolog) expression by binding to its 3'-UTR, leading to down-regulation of PTEN protein and activation of the Akt pathway, which is associated with cell survival and drug resistance.

In this thesis, we compared the miRNA profiling results between drug-resistant CEM/VM-1-5 cells and its parental drug-sensitive CEM cells and examined the role of specific miRNA in drug resistance.

Table 4. miRNAs Involvement in Cancer Chemoresistance

miRNA	Target	Corresponding Drug	Cell Line / Tissue	Human Cancer Type	Reference
miR-21	PDCD4 (Program cell death 4)	Doxorubicin, Paclitaxel	MCF-7	Breast	Bourguignon LY et al., J Biol Chem (2009)
miR-128a	TGFbR1	Letrozole	MCF-7		Masri S et al., Breast Cancer Res Treat (2010)
miR-155	FOXO3a (Forkhead box transcription factor)	Doxorubicin, VP16, Paclitaxel	BT-474		Kong W et al., J Biol Chem (2010)
miR-221, miR-222	ERα	Tamoxifen	MCF-7, T47D and others		Zhao JJ et al., J Biol Chem (2008)
miR-221, miR-222	$p27^{Kip1}$	Tamoxifen	MCF-7		Miller TE et al., J Biol Chem (2008)
miR-328	BCRP/ABCG2	Mitoxantrone	MCF-7		Pan YZ et al., Mol Pharmacol (2009)
miR-345	P-gp/ ABCB1/ MDR1	Cisplatin	MCF-7		Pogribny IP et al., Int J Cancer. (2010)
miR-451	P-gp/ ABCB1/ MDR1	Doxorubicin	MCF-7		Kovalchuk O et al., Mol Cancer Ther (2008)
let-7	IMP1 (IGFII mRNA-binding protein)	Taxol	OVCAR8	Ovarian	Boyerinas B et al., Int J Cancer. (2011)
miR-130a	M-CSF (Colony-stimulating factor)	Cisplatin, Paclitaxel	A2780		Sorrentino A et al., Gynecol Oncol (2008)
miR-214	PTEN	Cisplatin	HIOSE		Yang H et al., Cancer Res (2008)
miR-15b, miR-16	BCL2	Vincristine	SGC7901	Gastric	Xia L et al., Int J Cancer. (2008)
miR-34	BCL2	Doxorubicin, Docetaxel, Gemcitabine	Kato III		Ji Q et al., BMC Cancer (2008)
miR-140	HDAC4 (Histone deacetylase 4)	Methotrexate, 5-fluorouracil (5-FU)	HCT 116	Colorectal	Song B et al., Oncogene (2009)
miR-215	DHFR(Dihydrofolate reductase), TYMS (Thymidylate synthase)	Methotrexate, TYMS inhibitor Tomudex	HCT 116		Song B et al., Mol Cancer (2010)
miR-519c	BCRP/ABCG2	Mitoxantrone	S1		To KK et al., Mol Cell Bio (2008)
miR-34a	Cdk6 (Cell division protein kinase 6), SIRT1 (Silent mating type information regulation homolog)	Cisplatin	T24, TCCSuP and 5637 cells	Bladder	Vinall RL et al., Int J Cancer. (2011)
miR-21	PTEN	Gemcitabine	KMCH-1, Mz-ChA-1, TFK cell lines and human tissue	Cholangiocarcinoma	Meng F et al., Gastroenterology (2006)
miR-141	YAP1 (Yes-associated protein)	Cisplatin	KYSE	Esophageal (ESCC)	Imanaka Y et al., J Hum Genet (2011)
miR-24	DHFR(Dihydrofolate reductase)	Methotrexate	HT1080	Fibrosarcoma	Mishra PJ et al., PNAS (2007)
miR-221, miR-222	PTEN and TIMP3 (Tissue inhibitors of metalloproteinase)	TRAIL (TNF-related apoptosis-inducing ligand)	H460 and human tissue	Lung (NSCLC)	Garofalo M et al., Cancer Cell (2009)
miR-34a	MAGE-A	Cisplatin	R262, UW228 and others	Medulloblastoma	Weeraratne SD et al., Neuro Oncol (2011)
miR-138	H2AX (Histone H2A)	Cisplatin, Camptothecin	U2OS	Osteosarcoma	Wang Y et al., Mol Cancer Res (2011)
miR-148a	MSK1 (Mitogen- and stress-activated kinase 1)	Methotrexate	PC3	Prostate	Fujita Y et al., J Biol Chem (2010)

Chapter 2

2.1. Hypothesis and Specific Aims

Chemoresistance in human cancers remains the main obstacles to effective treatment. Such resistance is multifactoral and one factor is associated with the altered expression of drug's target, DNA topoisomerase II (Top2).

DNA Top2 is an essential cellular enzyme involved in DNA metabolism. Therefore, it becomes an important target for many clinically active chemotherapeutic agents, including doxorubicin and etoposide. These drugs act by forming the ternary Top2-DNA-drug complex, which is a cellular toxin. Human have two isoforms of DNA Top2, Top2α and Top2β. Even though Top2α and Top2β have similar characteristics to catalyze DNA strand breaks, they have distinct patterns of expression and cellular function. Human cancer cell lines selected for acquired resistance to anti-Top2 agents are a useful model to study the mechanisms of chemoresistance. The specific contributions of Top2α and Top2β to anti-Top2 agents are unclear and little is known behind the dysregulation of Top2 during the development of drug resistance in selected cancer cell lines. Altered expression of DNA Top2α has been documented in some cell lines (Table 3), and based on the evidence that 1) DNA Top2α is more sensitive to etoposide, doxorubicin and merbarone than DNA Top2β [38]; 2) DNA Top2α expression is linked to the proliferative status of cells [46-47]; 3) DNA Top2α is required for cell survival [45]; and 4) DNA Top2β is dispensable for cell survival [54-58], I focused my study on **the regulation of DNA Top2α during the development of drug resistance in previously**

established drug resistant CEM/VM-1-5 and Rh30/v1 cells (colored red in Table 3) in the first part of this thesis. The mechanisms that regulate DNA Top2α expression are not fully characterized. Top2α expression is partly mediated by transcription factors binding to its promoter region to activate or repress transcription (Figure 4). Previous work in our lab suggested that the transcription factor NF-YB is a negative regulator of DNA Top2α, working through the *Top2α* promoter [92]. Accordingly, I examined the correlation between transcription factor NF-YB and DNA Top2α in selected drug-resistant cancer cell lines .

Recent studies indicate that miRNAs are often aberrantly expressed in cancer [reviewed in 135, 136] and increasing numbers of studies have revealed the involvement of miRNAs in mediating drug sensitivity and resistance (summarized in Table 4). I further examined the role of miRNAs in mediating chemoresistance in cancer cells based on miRNA profiling data. Therefore, I **hypothesize** that miRNAs are one of the regulating factors in Top2α-meditated drug resistance, possibly through effects on transcription factor NF-YB. To test this hypothesis, I proposed the following specific aims:

1. Determine the role of Top2α in mediating drug sensitivity
2. Examine the role of NF-YB in regulating Top2α
3. Examine the role of microRNAs in Top2α-mediated drug resistance

Based on the Top2α knock-down result from Aim 1, I further studied the cytotoxic effect of antibiotic, doxycycline (Doxy) in human cancer cell lines while establishing tetracycline inducible system to manipulate Top2α expression, followed by the observation of dysregulation of Top2α and Top2β expression in Doxy-treated cancer cells. Despite the obvious importance of studying the role of Top2α in drug responsiveness, some evidence suggests a role of Top2β in differentiation, as addressed in section 1.2.2.5. Cancer cells stimulated to differentiate grow more slowly than their undifferentiated counterparts [83], which confers a degree of drug resistance relative to proliferating cells. In aim 4, I studied **the regulation of Top2β in the context of differentiation**.

4. Examine the cytotoxic effect mediated by antibiotic, doxycycline and its role in DNA Top2α and Top2β expression

2.2. Significance

Results presented in this thesis reveal a novel mechanism of DNA Top2α regulation mediated by transcription facetor NF-YB and *miR-485-3p*. Ectopic expression of *miR-485-3p* in Top2 poison-resistant cells resulted in increased sensitivity of cells to Top2 poisons, suggesting a novel way to improve therapeutic outcomes.

Chapter 3

Material and Methods

3.1. Cell Lines and Culture Conditions

The human cancer cell lines used in this thesis are summarized in Table 5 and were cultured in indicated conditions according to ATCC (American Type Culture Collection) (Rockville, Md.). Teniposide-resistant, acute T-lymphoblastic leukemia cell line CEM/VM-1-5 [140-141] and etoposide-resistant, rhabdomyosarcoma cell line, Rh30/v1 [142] were previously established by continuous incubation of parental cell lines CEM and Rh30 with increasing concentrations of teniposide and etoposide, respectively. Both RPMI 1640 and DMEM medium (BioWhittaker, Inc. Walkersville, MD) were supplemented with 10% FBS (fetal bovine serum)(Gemini, West Sacramento, CA) and 2 mM L-glutamine (BioWhittaker, Inc. Walkersville, MD), while IMDM medium (GIBCO-Invitrogen, Carlsbad, CA) was supplemented with 20% FBS and 4 mM L-glutamine. Suspension cells were maintained in T25 vented flasks, while adherent cells were cultured in 10cm dishes. All cell lines were subcultured twice a week in respective medium and were incubated at 37°C in the humidified chamber containing 5% CO_2/ 95% air. Cell numbers were determined by Coulter Counter Multisizer 3 (Beckman Coulter, Fullerton, CA, USA).

Table 5. Cell Lines and Culture Conditions

Human Cancer Type	Cell Line	Selection Agent	Medium	Growth Properties	Reference
Acute T-lymphoblastic leukemia	CEM		RPMI-1640	Suspension	
	CEM/VM-1-5	Teniposide			Danks MK et al. Cancer Res. (1987), Danks MK et al. Biochemistry. (1988)
Rhabdomyosarcoma	Rh30			Adherent	
	Rh30/v1	Etoposide			Bhat UG et al. Proc Natl Acad Sci U S A. (1999)
Acute promyelocytic leukemia	HL-60		IMDM	Suspension	
Kidney	HEK 293T		DMEM	Adherent	
Breast	MCF-7				
Ovarian	A2780				

3.2. Antibodies

The primary antibodies to Top2α (611326), Top2β (611492) were obtained from BD Biosciences, San Jose, CA, USA. The primary antibodies against Top1 (C-15), NF-YA (G-2), NF-YB (FL-207) and PCNA (PC-5) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. β-actin antibody was from Sigma Chemical Company, St. Louis, MO, USA. The secondary antibodies, anti-mouse, anti-rabbit, and anti-goat IgG peroxidase conjugates were from Jackson ImmunoResearch Laboratories, West Grove, PA, USA.

3.3. Preparation of Protein Extracts

Both total and nuclear protein extracts were collected from ~80% -confluent, logarithmically growing cells. Cells were first washed twice with ice-cold PBS. Total protein extracts were collected and lysed in RIPA buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and proteinase inhibitor (Complete MiniR, Roche, Indianapolis, IN)]. Cell lysates were incubated on ice for 20 minutes, followed by

sonication. After centrifugation (10,000*g,* 20 min at 4ºC), the supernatants were harvested and dispensed into aliquots, stored at -80ºC.

For nuclear protein extracts, cells were washed twice with ice-cold PBS, collected and resuspended in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 6 mM $MgCl_2$), and incubated on ice for 10 minutes and centrifuged (1,200*g*, 5 min at 4°C). The supernatant was removed and the pellet was incubated in buffer A containing 0.5% Nonidet P-40. After 5 min on ice, the nuclei were sedimented (3,300*g*, 15 min at 4°C) and the nuclear pellets were lysed by the addition of ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 2.5 µg/ml pepstatin, 10 µg/ml trypsin inhibitor, and 1 mM benzamidine). The nuclear extracts were incubated on ice for 30 min with occasional vortexing, followed by centrifugation (15,000*g*, 15 min at 4°C), and the cleared supernatants were dispensed into aliquots, and stored at -80ºC.

Protein concentration was measured by Bradford assay [143] (Bio-Rad, Hercules, CA, USA), using BSA (bovine serum albumin, Pierce no. 23210; Rockford, Illinois, USA) as standard.

3.4. Western Blot Analysis

Equal amounts (30~50ug) of protein samples were mixed with an equal volumes of 2x Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and separated on NuPAGE 4–12% bis–tris (Invitrogen, CA, USA) or 4-15% Tris-HCl (Bio-Rad, Hercules, CA, USA) precast acrylamide gradient SDS-PAGE gels. Samples were separated under constant voltage (150V) for 1 hour. Separated protein extracts were electrophoretically transferred onto nitrocellulose membranes (BA85; Schleicher & Schuell, Inc., Keene, NH, USA) at constant voltage (30 V, Invitrogen; 100 V, Bio-Rad) for 1 hour. After blocking with 5% milk in TBST *(*50mM Tris*,* 100mM NaCl, 0.05% Tween 20, pH 7.4) for 1-3 hours, the nitrocellulose membranes were incubated with respective primary antibodies, diluted 1:500 in TBST, except for β-actin (1:5000). Membranes were incubated overnight at 4°C with the primary antibodies. After washing three times for 10 min with TBST, membranes were incubated with 1:5000 dilution of peroxidase conjugated IgG secondary antibodies (anti-mouse for Top2α, Top2β, NF-YA, β-actin and PCNA; anti-rabbit for NF-YB; anti-goat for Top1) for 1 hour. The membranes were then washed with TBST three times for 10 minutes. Bound antibodies were detected using the enhanced chemiluminescence (ECL) detection method (Amersham Corp, Arlington Heights, IL or Thermo SuperSignal, Rockford, IL) according to the manufacturer's instructions. Both X-ray films and the Bio-Rad ChemiDoc™ EQ densitometer were used to detect the chemiluminescent signals. The density of the protein bands was quantified by Bio-Rad Quantity One® software (Bio-Rad Laboratories, Hercules, CA, USA).

3.5. Isolation of Total RNA and Reverse Transcription (RT)-PCR

Total RNAs were extracted from ~80% -confluent, logarithmically growing cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized by using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA). The PCR reactions were carried out for each transcript under the following conditions: 30-40 cycles :94°C 30s, 50-60°C 60s, and 72°C 90s and 1 cycle of 72°C for 10 min. GAPDH mRNA was amplified as an internal control. The following primer sets were used: Top2α (sense: 5'-TCA GAA CAT GGA CCC AGA CA-3'; antisense: 5'-TGC CTC TGC CAG TTT TTC TT-3'); and GAPDH (sense:5'-AAG CTC ACT GGC ATG GCC-3'; and antisense: 5'-CTG TTG CTG TAG CCA AAT TC-3'). The 3′-UTR sequences of NF-YB containing the putative *miR-485-3p* binding site were amplified using the following primers: NF-YB 3′-UTR (sense, 5′-TCT AGA AAG CAA GTG AAA GGT GCC AT-3′; antisense, 5′-TCT AGA ATC ATG AAT TAA CCC AGC CG-3′). To delete the putative *miR-485-3p* binding site, we used the following primers (sense, 5′-TCT AGA AAG CAA GTG AAA GGT GCC AT-3′; antisense, 5′-TCT AGA CCT GAT GCT TGA CTA ATT GAG G-3′), and the sequences were desinated NF-YB 3′-UTR-d.

3.6. Expression Vectors

The sequences for knocking down human Top2α, based on published sequences [144] were shTop2α: sense, 5'-CGC GTC CCC **AAG ACT GTC TGT TGA AAG A**TT CAA GAG A**TC TTT CAA CAG ACA GTC TTG** TTT GGA AAT-3' and antisense, 5'-CGA TTT CCA AAA A**AA GAC TGT CTG TTG AAA GA**T CTC TTG AA**T CTT TCA ACA GAC AGT CTT G**GG GA-3'. The siRNA sequences targeting to human *Top2α* mRNA at nucleotides 204-222 (relative to start codon) are in bold and underlined. The shTop2α was cloned into the pLV-THM vector between ClaI and MluI sites downstream of H1 promoter. The resulting lentiviral vector was called pLV-shTop2α. pLV-THM harbors both the tetracycline operon (tetO) and H1 promoter within 3' LTR/SIN region and the GFP gene as a reporter driven by the EF-1α promoter (Wiznerowicz & Trono, 2003). pLV-shLuc against luciferase gene was used as control.

Control and NF-YB-specific shRNAs in the pLKO.1 puromycin-resistant, lentiviral-based vector were purchased from Sigma (SIGMA Mission shRNA)

The *miR-Crtl* (pCDH-empty vector) and *miR-485-3p* expression vectors (pCDH-*miR-485-3p*) were a gift from Dr. Yin-Yuan Mo (Department of Medical Microbiology, Southern Illinois University School of Medicine, Springfield, Illinois)

The NF-YB 3′-UTR containing the putative *mir-485-3p* binding site and NF-YB 3′-UTR-d with the putative *mir-485-3p* binding site deleted were amplified by PCR and then cloned into the pGL3-thymidine kinase vector at XbaI site. This vector was a generous gifted from Dr. Hyun-Young Jeong (Department of Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, IL). These luciferase reporters containing the NF-YB 3′-UTR with and without the putative *mir-485-3p* binding site are desinated as pGL3-NF-YB-3′-UTR and the pGL3-NF-YB-3′-UTR-d, respectively.

3.7. DNA Sequencing

The PCR products or restriction enzyme-digested expression vectors were separated be electrophoresis gel and purified with QIAquick® Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA). 100 ng DNA were submitted for DNA sequencing and analysis by Big Dye Terminator V3.1 Cycle Sequencing at DNA service facility in the UIC Research Resources Center (www.uic.edu/depts/rrc/dnas/).

3.8. Preparation of Virus Particles

To establish stable cell lines, I generated lentivirus particles for transduction. $6x10^6$ HEK293T cells were seeded in 10cm dish at Day 0, followed by cotransfection with four plasmids: 1) a lentiviral vector with the insert of interest, 2) pMD2.G (expressing envelop protein VSV-G), 3) pMDLg/pRRE (expressing Gag and Pol) and 4) pRSV Rev (expressing Rev) at Day 1 using the calcium phosphate method (The latter three

plasmids were generous gift of Dr. Didier Trono, Department of Genetics and Microbiology, University of Geneva, Geneva, Switzerland). Media were changed at Day 2 and virus particles were harvested at Day 3 by centrifugation (2,500*g*, 10 min) and filtration through 0.45 μm filters to remove cell debris. Filtered virus particles were aliquoted and stored at -80ºC.

3.9. Establishment of Stable Cell Lines

For adherent cell lines, $1x10^5$ cells/well were seeded in 48-well plate one day ahead of transduction and then incubated with 0.5 ml of virus particles. For suspension cells, $1x10^5$ cells were resuspended with 0.5ml of virus particles. Fresh medium was added in accordance with the increasing number of cells in the following days.

CEM cells transduced with pLV-shLuc and pLV-shTop2α virus particles are sorted based on GFP (green fluorescent protein) marker in the expression vector and desinated as CEMshLuc and CEMshTop2α, respectively. Sorting of GFP expressing cells was performed and collected with a flow cytometer in the UIC Research Resources Center (http://www.rrc.uic.edu/fcs).

CEM/VM-1-5 cells transduced with *miR-Crtl* or *miR-485-3p* expression virus particles are defined as CEM/VM-1-5*miR-Crtl* or CEM/VM-1-5*miR-485-3p*, respectively. By the

same token, Rh30/v1 transduced with *miR-Crtl* or *miR-485-3p* expression virus particles are defined as Rh30/v1*miR-Crtl* or Rh30/v1*miR-485-3p*, respectively

3.10. Transient Transfection

Briefly, cells were seeded one day ahead of transfection to reach 80% confluence for optimal transfection. The expression vectors were transfected into cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

3.11. Cell Proliferation Assay and Doubling Time

Cell growth curves were obtained by counting cells grown in 96-well plates using a Coulter Counter Multisizer 3 (Beckman Coulter, Fullerton, CA, USA) with indicated treatments. 5,000 cells/well were seeded in triplicate in a 96-well plate at day 0. Cells were counted each day for 4-6 days. Doubling time was determined graphically and defined as the number of days required for cells in log phase growth to double in number. The experiments were done in triplicate. Cell growth rate or doubling time (DT) was calculated from the exponential growth period as follows: $DT = (T\ 2 - T\ 1)/[\ln_{(\text{cell number at T2/cell number at T1})} / \ln_2]$, where (T 2 − T 1) is the duration of the exponential phase.

3.12. Cytotoxicity Assay

Drug-induced cytotoxicity was measured by the MTT and MTS assays for adherent and suspension cells, respectively. MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and form purple formazan crystals. Medium was then removed and DMSO (dimethyl sulfoxide) was added to dissolve the crystals. The purple-colored solution was measured by spectrophotometer at wavelength 560 nm, as an indicator for viable cells. The formazon formed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was soluble in the medium without extra removal of medium and adding DMSO. MTS assay was used for measuring viability of suspension cells. Its absorbance wavelength is 490 nm.

Exponentially growing cells were seeded in 96-well plates (100 µl/well). Indicated treatments were added to the cells at various concentrations in a final volume of 200 µl/well, and the cells were incubated at 37°C for 72 h. After drug exposure, 20 µl of MTT (5 mg/ml in PBS; Sigma) or 10ul MTS (CellTiter 96 Aqueous One Solution Proliferation Assay System, Promega, Madison, WI) reagents were added to each well and the cells were incubated at 37°C for 3 h. For MTT assay, the plates were centrifuged in a swinging bucket rotor (3,000rpm, 20 min, 4°C). The medium in each well was aspirated and 200 µl of DMSO was added, followed by incubation at 37°C for 10 min. The metabolic activity of the cells for MTT and MTS assay was measured by microplate

reader. Three replicates were measured for each drug concentration and the experiments were done in triplicate. The IC50 value (the half maximal inhibitory concentration) was calculated by GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA).

3.13. Cell Cycle Analysis

Cell cycle analysis was performed by staining the cells with propidium iodide (PI) (Sigma Chemical Company, St. Louis, MO, USA), a membrane-impermeable fluorescent dye which intercalates DNA base pairs. The fluorescent intensity of the dye was related to the DNA content. Cells were fixed with 70% ethanol and permeabilized with 0.1% (v/v) Triton-X to allow entry of PI. Since PI can also bind to double-stranded RNA, cells were treated with RNase for optimal DNA resolution. In the experiments, $1x10^6$ cells were collected, resuspended in ice-cold PBS and then fixed in 70% ethanol/PBS at −20 °C overnight. The fixed cells were washed twice with ice-cold PBS and each sample was resuspended in 500ul staining solution (50 μg/mL of PI (propidium iodide), 2 mg/mL of RNase A, 0.1% (v/v) Triton X-100 in PBS). All reagents were purchased from Sigma. After 30 minutes of incubation at room temperature, cells were analyzed with a flow cytometer in the UIC Research Resources Center (http://www.rrc.uic.edu/fcs).

3.14. Flow Cytometry for Cell Phenotype

$1x10^6$ cells were collected and resuspended in ice-cold PBS. 100ul mixture of fluorochrome-conjugated monoclonal antibodies obtained from BD Biosciences (San Diego, CA, USA) against human CD44 (FITC; cat. #555478) and CD24 (PE; cat. #555428) was added to the each cell suspension at concentrations recommended by the manufacturer and incubated at 4°C in the dark for 30 min. The labeled cells were washed twice with the binding buffer, and then analyzed with a flow cytometer in the UIC Research Resources Center.

3.15. Apoptosis Assay

Apoptosis assay was performed using ApoAlert Annexin V kit (Clontech, Palo Alto, CA). One of the early stages of apoptosis is the translocation of the membrane phospholipid phosphatidylserine from the inner to the outer plasma membrane. The exposed phosphatidylserine can be bound and was detected by annexin-V, which was conjugated with fluorescein. Membrane-impermeable dye, PI, was used as an indicator of dead or dying cells. $1x10^6$ cells were collected, resuspended in 200 µL binding buffer (ApoAlert Annexin V kit) containing 5 µL of annexin V-FITC stock and 10 µL of a 50 µg/mL solution of propidium iodide (PI). After incubation in the dark for 10 minutes at room temperature, samples were analyzed with a flow cytometer in the UIC Research Resources Center (http://www.rrc.uic.edu/fcs).

Apoptosis was also monitored by Hoechst 33342 stained cells by fluorescence microscopy. Hoechst 33342, a type of blue-fluorescence dye, stains the condensed chromatin in apoptotic cells more brightly than normal chromatin.

3.16. Luciferase Assay

Luciferase assay is based on the reaction where the firefly luciferase protein in the presence of ATP and substrate emits light and detected by luminometer [145]. Luciferase assays were carried out in HEK293T cells with Dual-Light system (Applied Biosystems, Foster, CA, USA). $2x10^5$ cells were seeded in 24-well plates in triplicate and transfected with appropriate plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In my experiments, pGL3 luciferase reporters were co-transfected with β-galactosidase reporters, as internal control for transfection efficiency. 48 hours after of transfection, cells were washed twice with ice-cold PBS and lysed in 50 ul of lysis buffer in each well. Cell extracts were collected by centrifugation (14,000rpm, 10 min at 4°C). Dual-light assay was performed using the substrates luciferin and galaton for firefly luciferase and β-galactosidase, respectively. Relative luciferase activities were measured and normalized against β-galactosidase activity. All of the experiments were performed at least three times.

3.17. Statistical Analysis

The difference between two groups was tested using the t-test. Otherwise, one-way analysis of variance (ANOVA) was used for statistical analysis for more than two groups, followed by post-hoc analysis for pairwise, multiple comparisons (Prism software; GraphPad Software, Inc., San Diego, CA). A value of $P < 0.05$ was considered to be statistically significant.

Chapter 4

Specific Aim 1

Determine the Role of Top2α in Mediating Drug Sensitivity

4.1. Background

There are many mechanisms that cause chemoresistance in cancer cells [summerized in Figure 1 and reviewed in 1, 2]. Since interaction with Top2α is the main mechanism of action of anti-Top2 agents, evidence has indicated Top2α as a critical determinant of anti-Top2 agents' sensitivity in human cancer cell (see sections 1.2.2.6 and 1.2.2.7). Dysregulation of Top2α is often observed in drug resistant cancer cells compared with the drug sensitive counterpart (see Table 3). However, little is known behind the regulation mechanisms during the development of chemoresistance. Human cancer cells selected for acquired resistance are therefore used as a model to study this subject. I first examined the expression levels of Top2α in paired Top2 poison sensitive- and resistant- cancer cell lines. Furthermore, I knocked down (KD) Top2α expression in the parental Top2 poison sensitive cancer cell lines by RNA interference (RNAi) technique, and performed functional analysis of drug sensitivity in Top2α-KD cells.

4.2. Results

4.2.1. Expression of Top2α was altered in Anti-Top2 Sensitive- and Resistant-Cancer Cell Lines

I first compared the Top2α expression levels between the human acute T-lymphoblastic leukemia cell line, CEM and its anti-Top2 agents resistant sublines, merbarone resistant CEM-M70-B1 cells, teniposide-resistant (and etoposide cross-resistant) CEM/VM-1-5, and ICRF-187 resistant CEM-ICRF-18 cells, which were previously established in our lab by continuous incubation of CEM cells with increasing concentrations of respective anti-Top2 agents. I found that drug resistant CEM/VM-1-5 expressed reduced Top2α protein compared with parental CEM cell line. However, the Top2α protein levels remain similar in CEM-M70-B1 cells and CEM-ICRF-18 resistant cell lines (Figure 5A). For nuclear protein, PCNA (proliferating cell nuclear antigen) was served as loading control for Western blot. I further confirmed the downregulation of Top2α protein in CEM/VM-1-5 cells in both nuclear and total protein extracts, as measured by Western blot (Figure 5B). Nuclear and total protein extracts were fractionated by different centrifuge speed. The downregulation of *Top2α* at mRNA level was also confirmed by semi-quantified RT-PCR (Figure 5C).

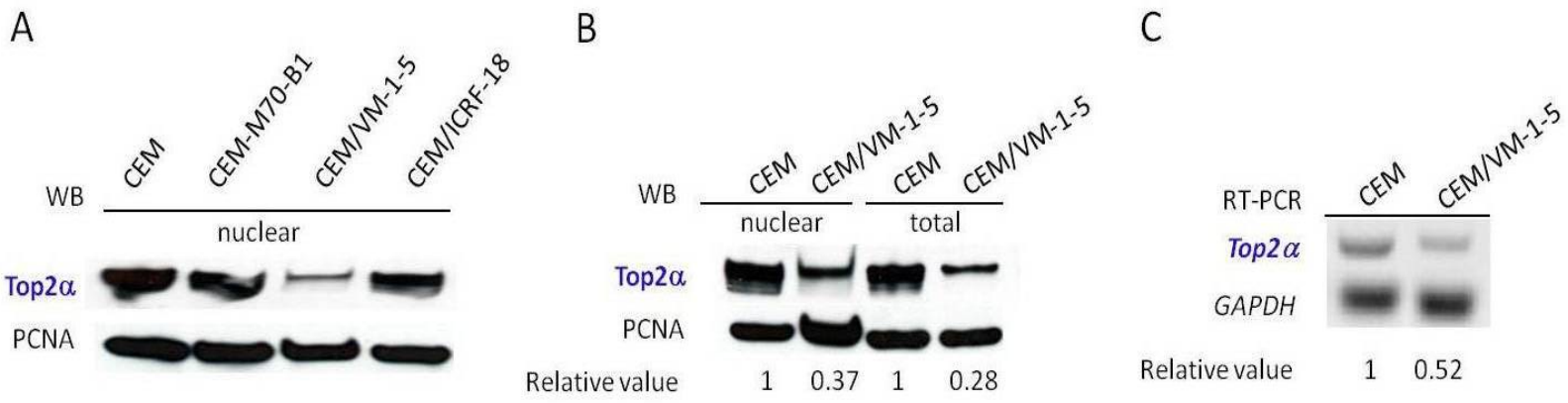


Fig. 5. CEM/VM-15 cells express reduced Top2α protein and mRNA compared to CEM cells

A. Western blot of Top2α protein in CEM and its drug resistant cell sublines, CEM-M70-B1, CEM/VM-1-5 and CEM/ICRF-8
B. Western blot of Top2α protein in CEM and CEM/VM-1-5 cells. Top2α was extracted from nuclear and whole-cell lysates. PCNA served as loading control.
C. RT-PCR of *Top2α* mRNA in CEM and CEM/VM-1-5. *GAPDH* was used as loading contrl.

4.2.2. Suppression of Top2α by RNAi

Preliminary results above suggest that decreased level of Top2α may contribute to teniposide resistance phenotype in CEM/VM-1-5 cells. To better understand the role of Top2α in drug sensitivity, I knocked down its expression in drug sensitive CEM cells by RNAi. Top2α shRNAs targeting two different protein coding regions of *Top2α* mRNA were designed and cloned into pLV-THM lentiviral vector, which has GFP selection marker, and desinated as pLV-THshTop2α-s1 and -s2. These two Top2α shRNA sequences were designed based on previously published sequences [144]. To confirm the knockdown effect, I first transiently transfected these vector-based shRNAs into HEK293T cells, which are ideal for shRNA inhibition [146], and are highly transfectable. I titrated knockdown by using 1, or 3ug of transfected vector with 48 hrs or 72 hrs incubation times. The pLV-THM empty vector was used as a negative control. I found that pLV-THshTop2α-s1 was very effective in suppressing Top2α expression at the protein level with 3ug of transfected vector under 48 hrs of incubation, while pLV-THshTop2α-s2 had less significant effect. Therefore, pLV-THshTop2α-s1 is referred to as pLV-THshTop2α hereafter (Figure 6A).

Since CEM cells were grown in suspension and therefore difficult to transfect, I prepared lentiviral particles carrying pLV-THshTop2α [147] and transduced this virus particles into CEM cells. Resulting cells were referred to as CEMshTop2α. Separately transduced cells expressing shRNA against luciferase (shLuc) were used as a control. To generate stable cell line, transduced CEM cells were sorted by flow cytometry based on the GFP (green fluorescent protein) marker on the expression vector by flow

cytometry. By this method, I found that the transduction efficiency was >99%. I was able to achieve knockdown of Top2α expression to 40% by this vector-based Top2α shRNA in CEM cells compared with control CEM cells (CEMshLuc) as verified by Western blot (Figure 6B and C). However, Top2β and Top1 levels in the cells remained similar in response to Top2α knockdown.

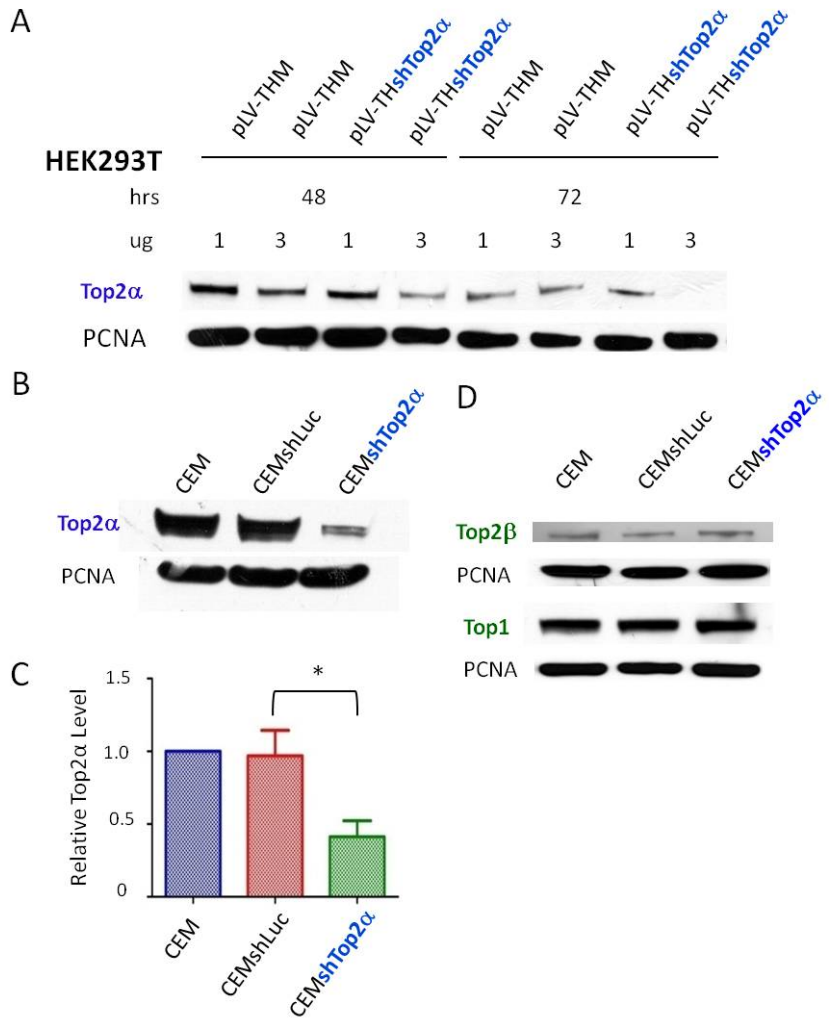


Fig. 6. Knockdown Top2α expression by vector-based Top2α shRNA

A. Western blot of Top2α protein from nuclear extracts in HEK293T cells. Cells were transiently transfected with pLV-THM and pLV-THshTop2α with 1 or 3ug of expression vectors at indicated incubation time, 48 or 72 hrs. PCNA served as loading control.

B. Western blot of Top2α protein from nuclear extracts in CEM and virus transduced CEMshLuc and CEMshTop2α cell lines.

C. Average of Top2α levels from three independent experiments ± S.D. are shown, determined by densitometric scanning on Western blots and normalized to PCNA signal. Expression level in CEM cells was assigned a value of 1 for comparison. *, $p < 0.05$.

D. Western blot of Top1and Top2β protein expression levels from nuclear extracts in CEM and virus transduced CEMshLuc and CEMshTop2a cell lines.

4.2.3. Suppression of Top2α Expression Resulted in Resistance to Top2 poisons

Since anti-Top2 agents are not like most enzyme inhibitors where a drug's action is by suppression of the target enzyme, these agents exert their effects in a Top2-dependent manner by converting Top2-DNA-drug complexes into a cellular toxin (see section 1.2.2.6). Many studies have shown a relationship between Top2α level and sensitivity to anti-Top2 agents (see section 1.2.2.7). Therefore, I asked whether suppression of Top2α alters cell response to Top2 poisons, etoposide and doxorubicin. To answer this question, I performed cytotoxicity assays to examine the effect of Top2α knockdown on drug responsiveness to Top2 poisons. As shown in Figure 7, CEMshTop2α cells were more resistant (~3 fold) to Top2 inhibitors etoposide and doxorubicin, but not vinblastine, a microtubule inhibitor, compared with control CEMshLuc cells, as indicated by increased IC_{50} values, determined by MTT assay. The effects of Top2α knockdown were specific to Top2 inhibitors. However, the Top2 poison, teniposide, selected resistant CEM/VM-1-5 cell line was significant more resistant to Top2 poisons, with IC_{50} 161uM to etoposide, and IC_{50} 290nM to doxorubicin.

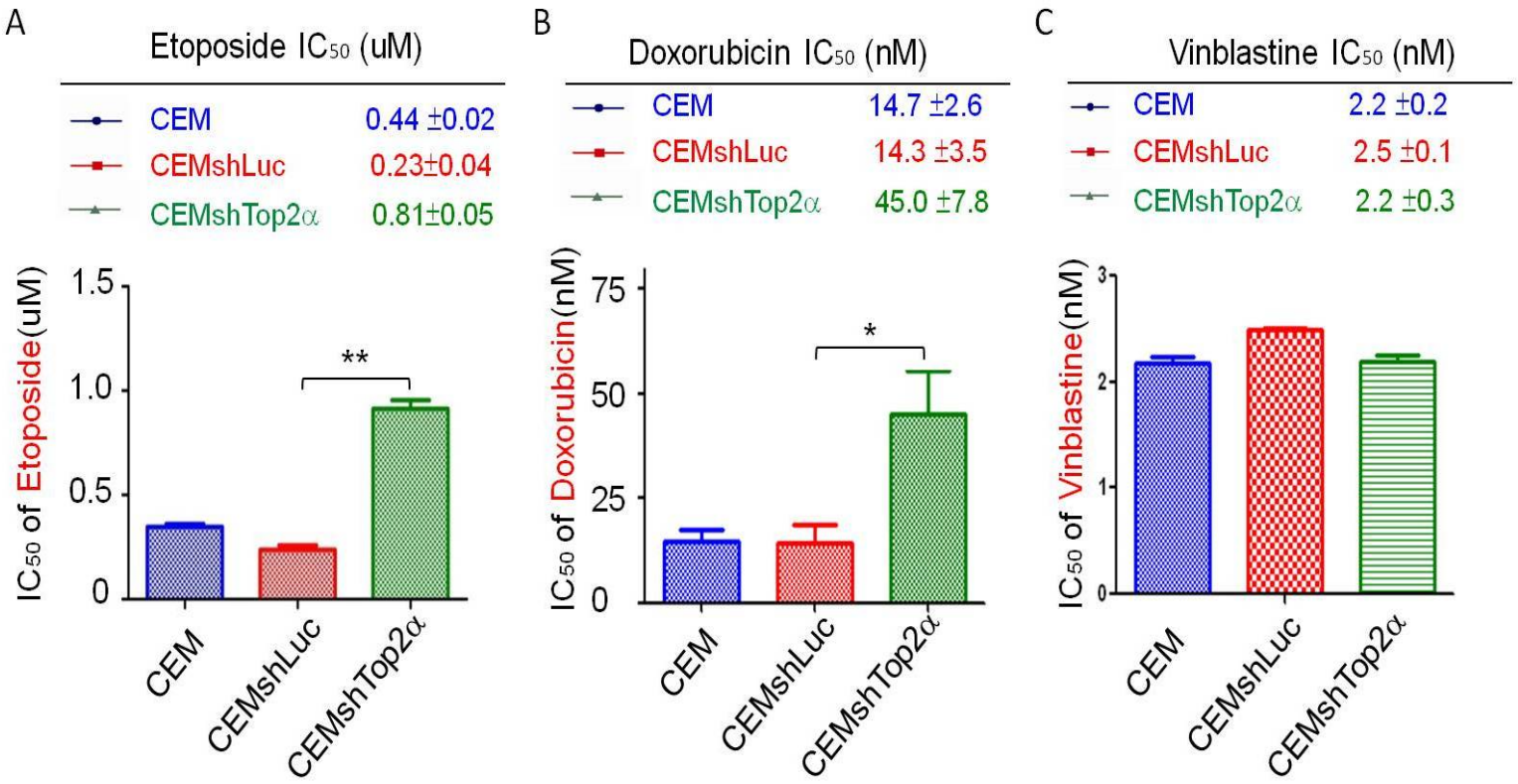


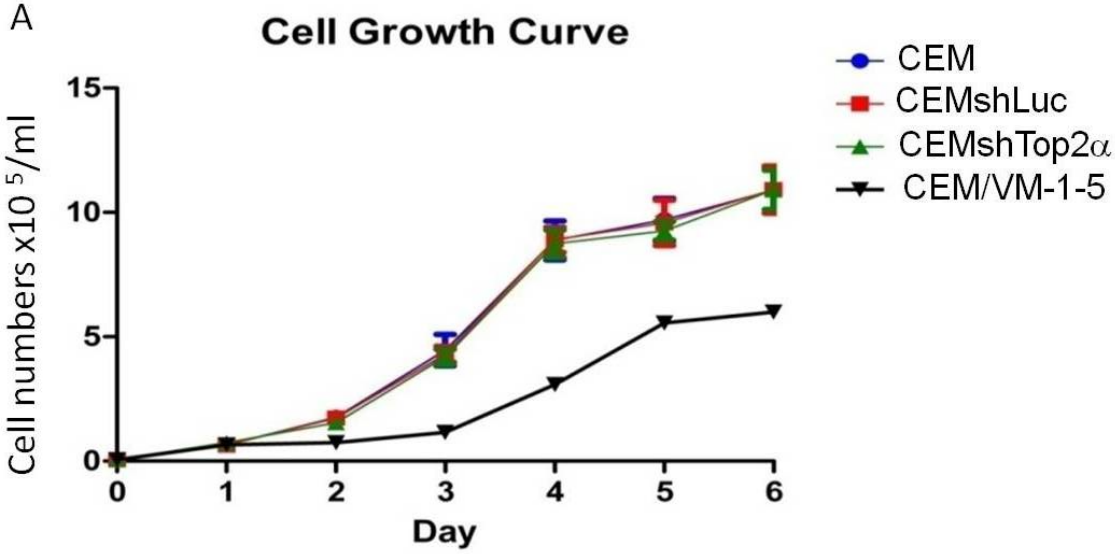
Fig. 7. Suppression of Top2α expression causes resistance to Top2 poisons

CEM, CEMshLuc and CEMshTop2α cells were treated with various concentrations of etoposide (A), doxorubicin (B) and vinblastine (C) for 72 h. IC_{50} values of cells exposed to treatments were calculated from the percentage of viable cells after exposure to treatment obtained from MTT assay. Values are average of three independent experiments done in triplicate ± S.E. *, $p < 0.05$; **, $p < 0.005$.

4.2.4. Cancer Cells were Able to Proliferate With Relatively Low Level of Top2α

While the drug sensitivity data indicate a difference in sensitivity to anti-Top2 agents between Top2α knockdown CEM cells and control CEM cells, interpretation of these data should include cell growth rate or cell cycle distribution in the absence of drug. Cells were seeded at 5,000/200ul in 96-well pates, counted at the indicated times and cell number was determined using a Coulter counter. CEMshLuc and CEMshTop2α were in exponential growth at densities of 4-8x10^5 cells/ml. The doubling time and the cell density at plateaus of CEMshLuc and CEMshTop2α were similar. They were no longer in log growth at 1x10^6 cell/ml (Figure 8A). Knockdown of Top2α had little or no impact on cell growth rate, suggesting that cell proliferation can proceed with relatively low Top2α expression. However, drug resistant CEM/VM-1-5 cells grow much slower, compared with drug sensitive CEM cells, as indicated by cell growth curve and higher doubling time, and had lower cell density at plateau (Figure 8A). Cell cycle distribution of these cell lines as determined by flow cytometry analysis of propidium iodide stained cells didn't show significant differences (Figure 8B).

A



B

Cell Line	Doubling Time (Day)	Distribution of cells in phases of cycle (%±SE)		
		G1	S	G2/M
CEM	1.04	40.10±3.41	47.73±3.05	12.17±0.76
CEMshLuc	0.95	45.03±4.20	42.87±3.86	12.13±0.51
CEMshTop2α	0.91	42.43±0.51	45.00±0.56	12.6±0.62
CEM/VM-1-5	1.18	42.77±7.32	43.87±4.84	13.33±2.63

Fig. 8. Cell growth curve (A), doubling time and cell cycle distribution(B) of CEM, CEMshLuc, CEM Top2α, and CEM/VM-1-5 cells. Values are average of three independent experiments done in triplicate ± S.E.

4.3. Discussion

In this chapter, I showed the downregulation of Top2α in teniposide-selected resistant CEM/VM-1-5 cells compare to parental CEM cells, but not in other anti-Top2 agents selected resistant CEM cells. To determine the role of Top2α in drug response in the cells, I successfully knocked down Top2α protein in parental CEM cells by RNAi technique. CEMshTop2α cells were more resistant (~3 fold) to antI-Top2 agents, etoposide and doxorubicin, but not to the microtubule inhibitor, vinblastine. This indicates that Top2α level determines cell response to anti-Top2 agents. Furthermore, although I was able to knock down Top2α in CEM cells to the similar level as observed in drug resistant CEM/VM-1-5 cells, CEM/VM-1-5 cells were hundreds-fold more resistant to etoposide and doxorubicin, as indicated by their IC_{50} values. I conclude that Top2α expression level in the cells is not proportional to the level of chemosensitivity and these results suggest that there may be other genes or microRNAs changes during the selection process of the drug resistant CEM/VM-1-5 cells.

Top2α has been well-studied for its role in cell proliferation. However, to our surprise, even knockdown of Top2α expression to 40% of the level observed in control cell line, it had little impact on cell proliferation, compared with control cell lines. This suggests that cell can proliferate with relatively low Top2α expression. One possibility is that related proteins, Top1 and Top2β compensate for Top2α expression. As shown in Western blot in Figure 6D, this is not the case and expression of Top1 and Top2β remained unchanged in response to Top2α knockdown. Indeed, other studies also reported no

Top2β compensation for the loss of Top2α in mammalian cells [43, 148]. It is also possible that cells with more potent Top2α knockdown may already die out during the selection process of stable cell lines. Therefore, as the next step, I planned to develop the tetracycline inducible system, where we can turn-on and -off the transcription of Top2α shRNA with and without adding antibiotic, doxycycline, respectively. This part will be discussed in Chapter 7.

Chapter 5

Specific Aim 2

Examine the role of NF-YB in Regulating Top2α

5.1. Background

I next asked what caused the reduced expression of Top2α in drug resistant CEM/VM-1-5 cells. Previous work from our laboratory suggested that the transcription factor NF-YB is a negative regulator of Top2α, working through the inverted CCAAT element 3 (ICE3) in the Top2α promoter (Morgan and Beck 2001). Based on this observation, I first determined the correlation between Top2α and NF-YB expression in drug-sensitive and -resistant cell lines. In addition, I also tested the effect of knock down of NF-YB on the expression of Top2α.

5.2. Results

5.2.1. Expression of Top2α and NF-YB were Inversely Correlated

I found that NF-YB protein levels were 3-fold higher in CEM/VM-1-5 cells compared with CEM cells (Figure 9A and 9B), and there was an inverse correlation between the protein levels of Top2α and NF-YB in CEM and CEM/VM-1-5 cells by Western blot. To determine whether this is a cell line-specific phenomenon, I examined the human rhabdomyosarcoma Rh30 cell line and compared it with its etoposide-resistant subline, Rh30/v1 [142]. I observed a similar result: Top2α was down-regulated in the etoposide-resistant Rh30/v1 cell line, and there was an inverse correlation between the protein levels of Top2α and NF-YB (Figure 9D and 9E). In addition, the expression of NF-YA protein (Figure 9C and 9F) was similar between drug-sensitive and -resistant cells. These data suggest that increased NF-YB may be either related to or the cause of reduced Top2α in drug resistant CEM/VM-1-5 and Rh30/v1 cells.

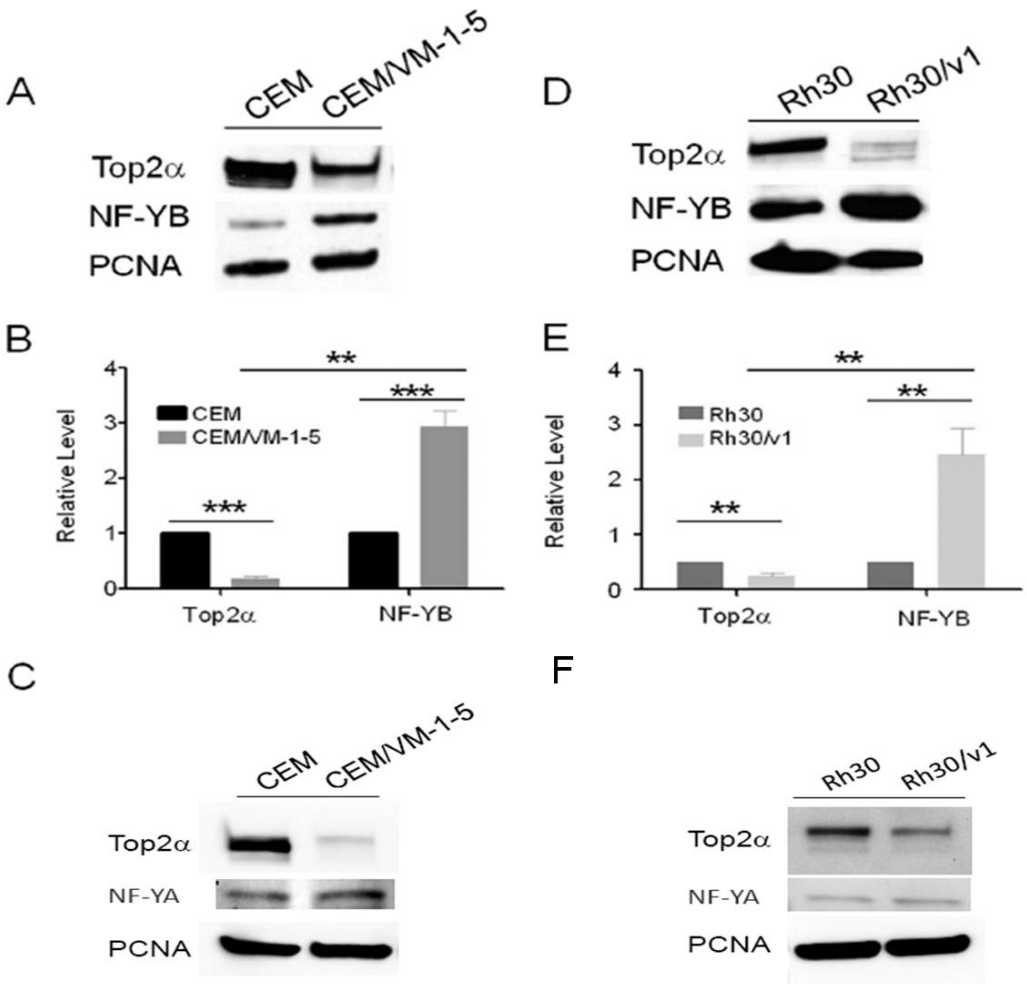


Fig. 9. Inverse correlation between the protein levels of Top2α and NF-YB in CEM and CEM/VM-1-5 cells and Rh30 and Rh30/v1 cells

Western blots of nuclear Top2α and NF-YB expression in CEM and CEM/VM-1-5 cells (A) and Rh30 and Rh30/v1 cells (D). PCNA served as loading control for nuclear proteins. Average of Top2α and NF-YB levels from three independent experiments ± S.D. as determined by densitometric scanning on Western blots and normalized to PCNA signal; either CEM (B) or Rh30 (E) was assigned a value of 1 for comparison. **, p <0.005; ***, p < 0.0001. Western blots of nuclear Top2α and NF-YA expression in CEM and CEM/VM-1-5 cells (C) and Rh30 and Rh30/v1 cells (F).

Furthermore, I found that *NF-YB* mRNA levels were similar in CEM, CEM/VM-1-5 and Rh30, Rh30/v1 cells (Figure 10A and 10B), indicating that the observed differences in NF-YB protein levels were probably a consequence of regulation at the post-transcriptional level. Post-transcriptional repression is a major mechanism by which microRNAs regulate gene expression [120, 149]. Accordingly, we then asked and this will be discussed in Chapter 6.

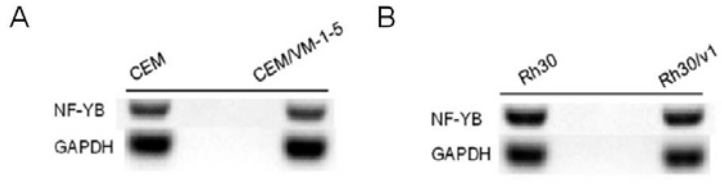


Fig. 10. Similar mRNA level of *NF-YB* in CEM and CEM/VM-1-5 cells (A) and Rh30 and Rh30/v1 cells (B) as determined by RT-PCR.

5.2.2. NF-YB Mediated Top2α Expression Indirectly

To determine the regulatory role of NF-YB in Top2α expression, I further knocked down NF-YB in drug resistant cells, in which NF-YB is highly expressed. However, after knockdown of NF-YB protein, Top2α protein level remained the same, which suggests that NF-YB regulation of Top2α promoter is indirect or NF-YB regulation on the Top2α promoter involves other transcriptional cofactors.

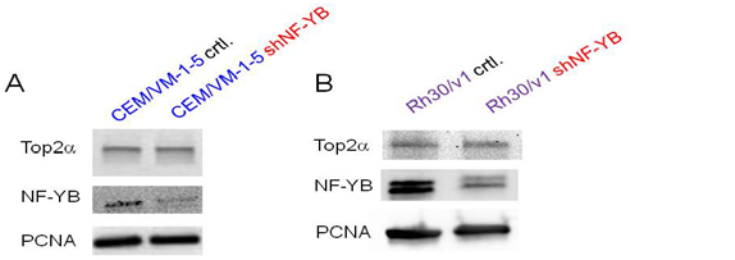


Fig. 11. Knock down of NF-YB does not affect Top2a protein levels

Knockdown of NF-YB in drug resistant CEM/VM-1-5 (A) and Rh30/v1 (B) cells. Western blot of Top2α and NF-YB protein from nuclear extracts in the cells. PCNA served as loading control.

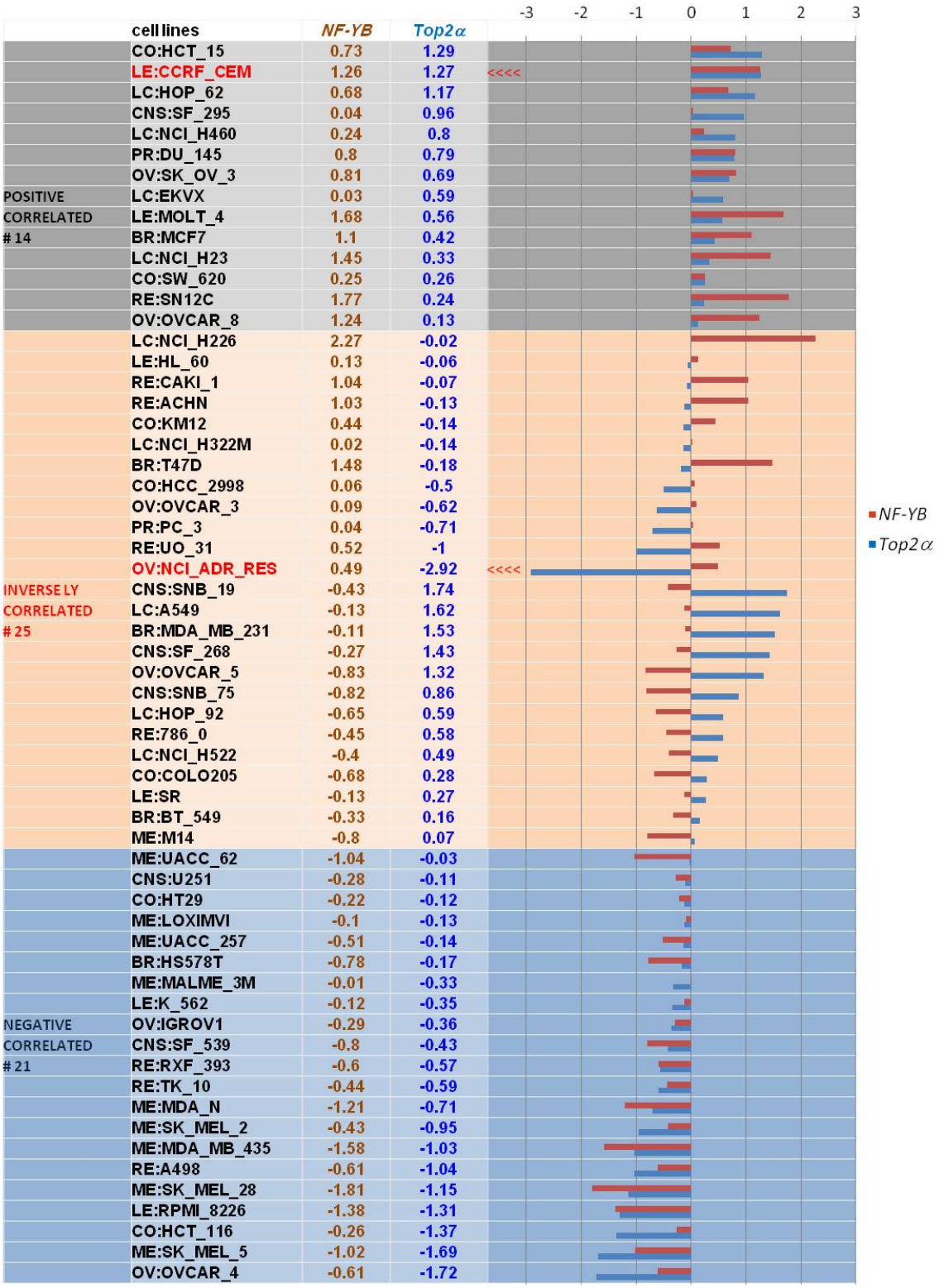
5.3. Discussion

I have now shown that the NF-YB protein is highly expressed in the drug-resistant CEM/VM-1-5 cells, that have decreased level of Top2α compared with the drug-sensitive CEM cells. I observed a similar inverse correlation between Top2α and NF-YB expression in the human rhabdomyosarcoma Rh30 cell line and its etoposide resistant subline, Rh30/v1, where Rh30/v1 cells exihibited downregulation of Top2α and upregulation of NF-YB, compared with parental Rh30 cells. Since NF-Y protein complex is composed of three subunits, NF-YA, NF-YB, and NF-YC, for DNA binding and regulation, I further examined the expression level of NF-YA. NF-YA expression remained similar in both drug-sensitive and –resistant cell lines. However, I was not able to measure the expression level of NF-YC due to lack of good quality of NF-YC antibody. These results suggest that transcription factor NF-YB may function as a negative regulator of Top2α. However, direct knockdown of NF-YB protein didn't rescue Top2α protein expression. One possible mechanism is the involvement of the other transcription cofactors, including p300/CBP, and PCAF which have been reported to mediate NF-Y protein complex regulation on promoters through their interactions [150-152]. Other evidences also revealed that p53 bind to NF-Y protein complex to repress transcription [153-154]. Therefore, it is possible that NF-YB regulates Top2α expression with the binding of other transcription cofactors in the drug-resistant cell lines and that the knockdown of NF-YB alone is not sufficient to recover Top2α expression. Top2α expression may require the net effect of a dynamic and delicate regulation by transcription factor, NF-YB and other unknown cofactors.

Nevertheless, I examined the correlation of Top2α and NF-YB gene expression levels in the National Cancer Institute (NCI)-60 panel. The NCI-60, a panel of 60 diverse human cancer cell lines, has been used by the National Cancer Institute to screen chemical compounds for anticancer drug sensitivity and has been profiled for mRNA and protein expression (http://discover.nci.nih.gov/cellminer/) (Shankavaram et al., 2009). The analyzed result suggests that there is no significant overall correlation, negative or positive, between *Top2α* and *NF-YB* at the mRNA level (Table 10). Some cell lines show a positive relationship between the two genes, others show a negative relationship, and yet others show an inverse relationship, as we have demonstrated here. The CEM cell line in this figure shows that both genes are overexpressed. One explanation for this apparent contradiction with our present results is that CEM cell line in the NCI-60 panel is diploid [155] instead of near tetraploid as are our CEM and CEM/VM-1-5 cell lines [156]. By contrast, some of the cell lines showing an inverse relationship between *Top2α* and *NF-YB* are derived from solid tumors. Of possible relevance to our results, one and the only drug-resistant cancer cell line included in NCI-60 panel, NCI/ADR-RES cell line, the doxorubicin (ADR)-selected OVCAR-8 ovarian cancer cell line shows the inverse correlation between *Top2α* and *NF-YB* mRNA. In accordance with our resistant cell lines, *Top2α* expression is decreased in this NCI/ADR-RES cell line.

Moreover, examination of the *Top2α* and *NF-YB* in cell lines in which both genes are inversely expressed (Table 6, middle group) demonstrated that the ratio of *Top2α* to *NF-YB*, and the differential (opposite) expression of both genes tends to be higher than

in the rest of the cell lines (i.e., the top and bottom groups in Table 10, in which genes are expressed in the same direction). We can speculate that the inverse relationship that we have observed herein and in those NCI-60 cells with inverse expression of *Top2*α and *NF-YB* genes may reflect aspects of the biology of drug resistance. Clearly, examination of the expression of these genes in more pairs of drug-sensitive and drug-resistant cell lines is warranted.



	cell lines	*NF-YB*	*Top2α*	
	CO:HCT_15	0.73	1.29	
	LE:CCRF_CEM	1.26	1.27	<<<<
	LC:HOP_62	0.68	1.17	
	CNS:SF_295	0.04	0.96	
	LC:NCI_H460	0.24	0.8	
	PR:DU_145	0.8	0.79	
	OV:SK_OV_3	0.81	0.69	
POSITIVE	LC:EKVX	0.03	0.59	
CORRELATED	LE:MOLT_4	1.68	0.56	
# 14	BR:MCF7	1.1	0.42	
	LC:NCI_H23	1.45	0.33	
	CO:SW_620	0.25	0.26	
	RE:SN12C	1.77	0.24	
	OV:OVCAR_8	1.24	0.13	
	LC:NCI_H226	2.27	-0.02	
	LE:HL_60	0.13	-0.06	
	RE:CAKI_1	1.04	-0.07	
	RE:ACHN	1.03	-0.13	
	CO:KM12	0.44	-0.14	
	LC:NCI_H322M	0.02	-0.14	
	BR:T47D	1.48	-0.18	
	CO:HCC_2998	0.06	-0.5	
	OV:OVCAR_3	0.09	-0.62	
	PR:PC_3	0.04	-0.71	
	RE:UO_31	0.52	-1	
	OV:NCI_ADR_RES	0.49	-2.92	<<<<
INVERSE LY	CNS:SNB_19	-0.43	1.74	
CORRELATED	LC:A549	-0.13	1.62	
# 25	BR:MDA_MB_231	-0.11	1.53	
	CNS:SF_268	-0.27	1.43	
	OV:OVCAR_5	-0.83	1.32	
	CNS:SNB_75	-0.82	0.86	
	LC:HOP_92	-0.65	0.59	
	RE:786_0	-0.45	0.58	
	LC:NCI_H522	-0.4	0.49	
	CO:COLO205	-0.68	0.28	
	LE:SR	-0.13	0.27	
	BR:BT_549	-0.33	0.16	
	ME:M14	-0.8	0.07	
	ME:UACC_62	-1.04	-0.03	
	CNS:U251	-0.28	-0.11	
	CO:HT29	-0.22	-0.12	
	ME:LOXIMVI	-0.1	-0.13	
	ME:UACC_257	-0.51	-0.14	
	BR:HS578T	-0.78	-0.17	
	ME:MALME_3M	-0.01	-0.33	
	LE:K_562	-0.12	-0.35	
NEGATIVE	OV:IGROV1	-0.29	-0.36	
CORRELATED	CNS:SF_539	-0.8	-0.43	
# 21	RE:RXF_393	-0.6	-0.57	
	RE:TK_10	-0.44	-0.59	
	ME:MDA_N	-1.21	-0.71	
	ME:SK_MEL_2	-0.43	-0.95	
	ME:MDA_MB_435	-1.58	-1.03	
	RE:A498	-0.61	-1.04	
	ME:SK_MEL_28	-1.81	-1.15	
	LE:RPMI_8226	-1.38	-1.31	
	CO:HCT_116	-0.26	-1.37	
	ME:SK_MEL_5	-1.02	-1.69	
	OV:OVCAR_4	-0.61	-1.72	

Table 6. *NF-YB* and *Top2α* mRNA expression levels in the NCI-60 panel of cancer cell lines
Shown is a graphical summary of *NF-YB* and *Top2α* mRNA levels in the form of a mean graph of the NCI-60 cells. Expression above the mean levels is drawn to the right of the centerline, and below the mean is drawn to the left.

Chapter 6

Specific Aim 3

Examine the Role of microRNAs in Top2α-Mediated Drug Resistance

6.1. Background

Many studies have shown that microRNAs are often aberrantly expressed in cancer [reviewed in 135, 136], and are involved in mediating drug sensitivity and resistance [139, summarized in Table 4]. Furthermore, I found that *NF-YB* mRNA levels were similar in CEM, CEM/VM-1-5 and Rh30, Rh30/v1 cells (Fig. 10), indicating that the observed differences in NF-YB protein levels are probably a consequence of regulation at the post-transcriptional level. Post-transcriptional repression is a major mechanism by which microRNAs regulate gene expression [120, 149]. Accordingly, I then asked whether microRNAs are involved in the regulation of NF-YB protein, which might further mediate Top2α expression and drug-responsiveness in CEM and CEM/VM-1-5 cells and in Rh30 and Rh30/v1 cells.

6.2. Results

6.2.1. MicroRNAs Expressed Differentially in Drug-Sensitive CEM and Drug-Resistant CEM/VM-1-5 Cells

We used microRNA profiling by the Applied Biosystems real-time PCR array (TaqMan Human MicroRNA Array v1.0) to analyze microRNA expression in drug-sensitive CEM and drug-resistant CEM/VM-1-5 cells. The array carries unique hairpin-loop RT primer sets that allow for detection of 365 mature human microRNAs in the cells. Compared with CEM cells, microRNA profiling revealed that CEM/VM-1-5 cells with acquired resistance to teniposide exhibit substantial changes in microRNA expression. 32 microRNAs were consistently either up- or down-regulated in CEM/VM-1-5 cells, as determined by C_t (cycle threshold) values from three separate microRNA profiling experiments (X. He, and W. T. Beck, unpublished observations). Because higher C_t values indicate lower expression levels, one of the human (Homo sapiens) microRNAs, *hsa-miR-485-3p*, was found to be consistently substantially lower in CEM/VM-1-5 cells compared with CEM cells after conversion to expression level (Figure 12A and 12B) and this microRNA is focus of my study.

Using miRanda (http://www.microrna.org/microrna/home.do), Target Scan (http://www.targetscan.org/), and MicroCosm (http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl) prediction programs, I found that the 3′-UTR of NF-YB harbors a putative *hsa-miR-485-3p* binding site (Figure 12C), which is conserved in human, rhesus monkeys, chimpanzees, and dogs. It is noteworthy that the decreased expression of *hsa-miR-485-3p* in CEM/VM-1-5 cells is inversely related to the

overexpression of NF-YB protein (Figure 9A and 9B). Moreover, I found by sequencing that there is no mutation in the region of NF-YB 3′-UTR, to which *hsa-miR-485-3p* binds in either CEM or CEM/VM-1-5 cells (data not shown), suggesting that the binding of microRNAs to the NF-YB 3′-UTRs is unlikely to be altered. H*sa-miR-485-3p* is referred to as *miR-485-3p* hereafter.

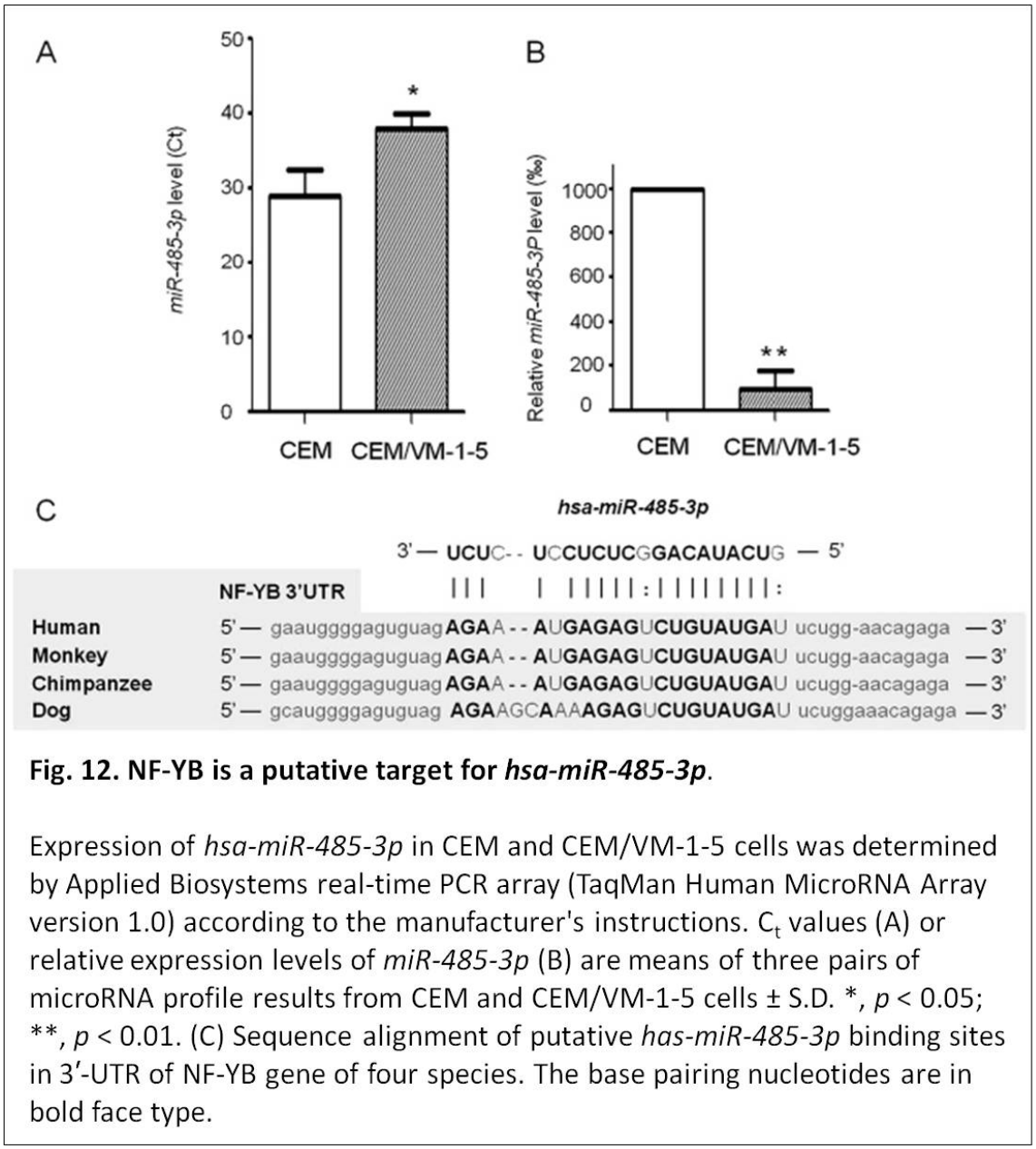


Fig. 12. NF-YB is a putative target for *hsa-miR-485-3p*.

Expression of *hsa-miR-485-3p* in CEM and CEM/VM-1-5 cells was determined by Applied Biosystems real-time PCR array (TaqMan Human MicroRNA Array version 1.0) according to the manufacturer's instructions. C_t values (A) or relative expression levels of *miR-485-3p* (B) are means of three pairs of microRNA profile results from CEM and CEM/VM-1-5 cells ± S.D. *, $p < 0.05$; **, $p < 0.01$. (C) Sequence alignment of putative *has-miR-485-3p* binding sites in 3′-UTR of NF-YB gene of four species. The base pairing nucleotides are in bold face type.

6.2.2. NF-YB is a Direct Target of *miR-485*-3p

To validate that NF-YB is a direct target of *miR-485-3p*, I constructed a luciferase reporter (pGL3-thymidine kinase) carrying the NF-YB 3′-UTR with the putative *miR-485-3p* binding site. I cotransfected either *miR-485-3p* expression vector (pCDH-*miR-485-3p*) or miR-Crtl (pCDH-empty vector) with luciferase reporter (pGL3-NF-YB-3′-UTR) into HEK293T cells. Relative luciferase activity of the NF-YB 3′-UTR with *miR-485-3p* binding site was significantly lower (~46%) in the presence of *miR-485-3p* expression vector (pCDH-*miR-485-3p*) than that of the miR-Crtl (pCDH-empty vector) (Figure 13A). Moreover, the relative luciferase activity of the pGL3-NFYB-3′-UTR with the *miR-485-3p* binding site was also lower (~45%) than that of the pGL3-NF-YB-3′-UTR-d (pGL3-NF-YB 3′-UTR with *miR-485-3p* binding site deleted) in HEK293T cells cotransfected with the *miR-485-3p* expression vector (Figure 13B).

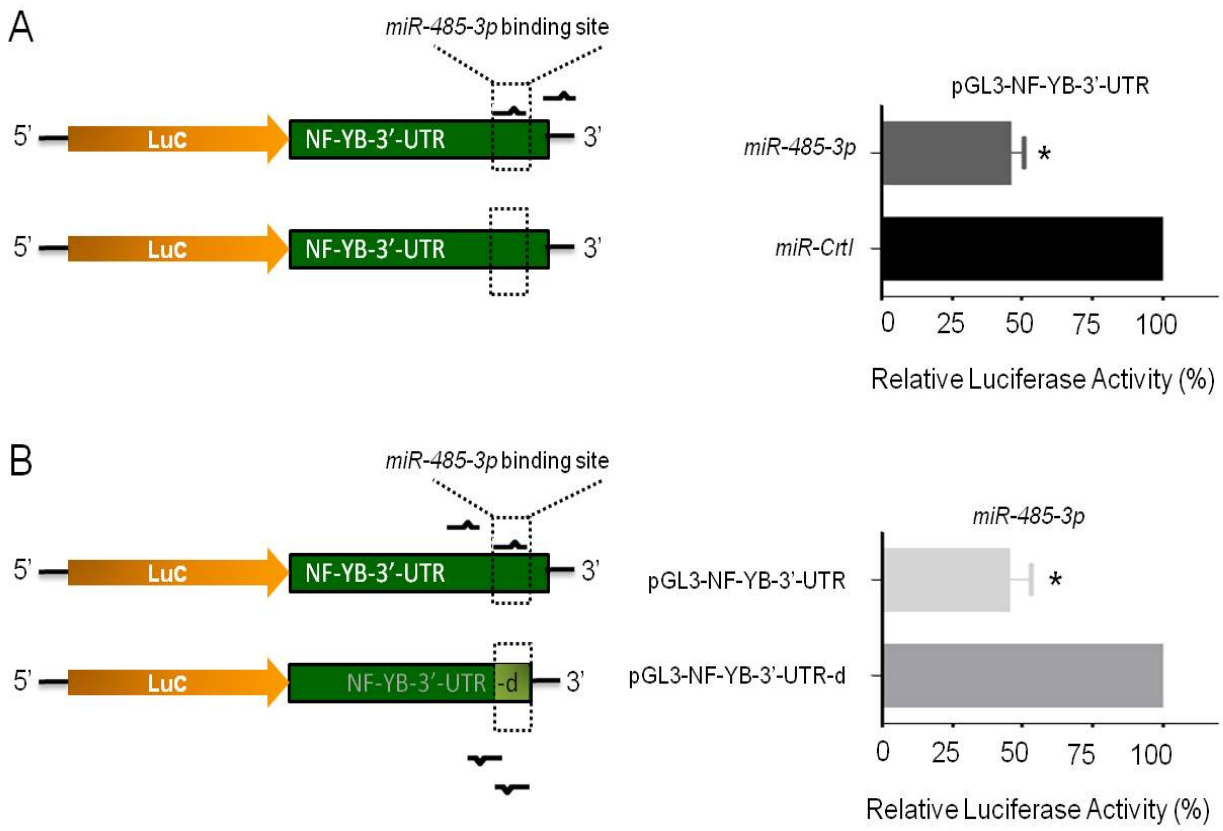


Fig. 13. *MiR-485-3p* targets NF-YB 3′-UTR.

A. Luciferase reporter containing a putative *miR-485-3p* binding site, NF-YB-3′-UTR (pGL3-NF-YB-3′-UTR), was cotransfected with either *miR-Crtl* (pCDH-empty vector) or the *miR-485-3p* expression vector (pCDH-*miR-485-3p*), with schematic diagram on the left.

B. *MiR-485-3p* expression vector (pCDH-*miR-485-3p*) was cotransfected with luciferase reporter containing NF-YB-3′-UTR with or without putative *miR-485-3p* binding site, desinated as pGL3-NF-YB-3′-UTR or pGL3-NF-YB-3′-UTR-d, respectively, with schematic diagram (left). Relative luciferase activities were measured and normalized against β-galactosidase activity. Values are average of three separate experiments performed in triplicate ± S.E.; *, $p < 0.05$.

6.2.3. *MiR-485*-3p Modulated the Sensitivity of CEM/VM-1-5 Cells to Top2 poisons

Drug-resistant CEM/VM-1-5 cells have increased expression of NF-YB protein and reduced endogenous *miR-485-3p* expression. To ascertain whether *miR-485-3p* regulates NF-YB, I examined the effects of overexpressing *miR-485-3p* on NF-YB expression in CEM/VM-1-5 cells. Thus, I transduced CEM/VM-1-5 cells with either the pCDH-*miR-485-3p* or the *miR-Crtl* (pCDH-empty vector) virus particles. Western blot analysis revealed decreased NF-YB protein levels in pCDH-*miR-485-3p* stably transduced CEM/VM-1-5 cells, compared with the *miR-Crtl* transduced CEM/VM-1-5 cells and no difference in NF-YA protein levels were seen in response to *miR-485-3p* overexpression cells (Figure 14A). To determine whether this was a cell line-specific phenomenon, I examined the etoposide-resistant human rhabdomyosarcoma Rh30/v1 cells. Introduction of *miR-485-3p* into these cells also decreased NF-YB protein levels in Rh30/v1 cells (Figure 14B), indicating that the *miR-485-3p*-mediated regulation of NF-YB is not cell line-specific.

Because our previous finding suggests that NF-YB is involved in the negative regulation of Top2α*,* I further analyzed the levels of Top2α in CEM/VM-1-5 *miR-485-3p* cells (Fig.14A). It is noteworthy that I found that the level of Top2α protein was upregulated, whereas the level of NF-YB was downregulated compared with *CEM/VM-1-5 miR-Crtl* cells, suggesting that NF-YB mediates Top2α expression via *miR-485-3p*. A similar effect was observed in the etoposide-resistant Rh30 subline Rh30/v1 (Fig.14B). These results suggest that *miR-485-3p* may regulate drug-responsiveness by increasing NF-

YB expression, which in turn negatively mediates Top2α expression. To answer this question, I examined the effects of *miR-485-3p* on the drug sensitivity of CEM/VM-1-5 cells. MTT assays revealed that CEM/VM-1-5 *miR-485-3p* cells exhibited enhanced sensitivity (2-fold) to etoposide, compared with CEM/VM-1-5 *miR-Crtl* cells, as indicated by decreased IC_{50} values (Figure 14C). In contrast, no significant difference in responsiveness to vinblastine, a microtubule inhibitor, was found in *miR-485-3p* overexpressing CEM/VM-1-5 cells (Fig.14D). Taken together, *miR-485-3p*-mediated down-regulation of NF-YB in CEM/VM-1-5 cells was accompanied by increased sensitivity of CEM/VM-1-5 cells to Top2 poison, etoposide.

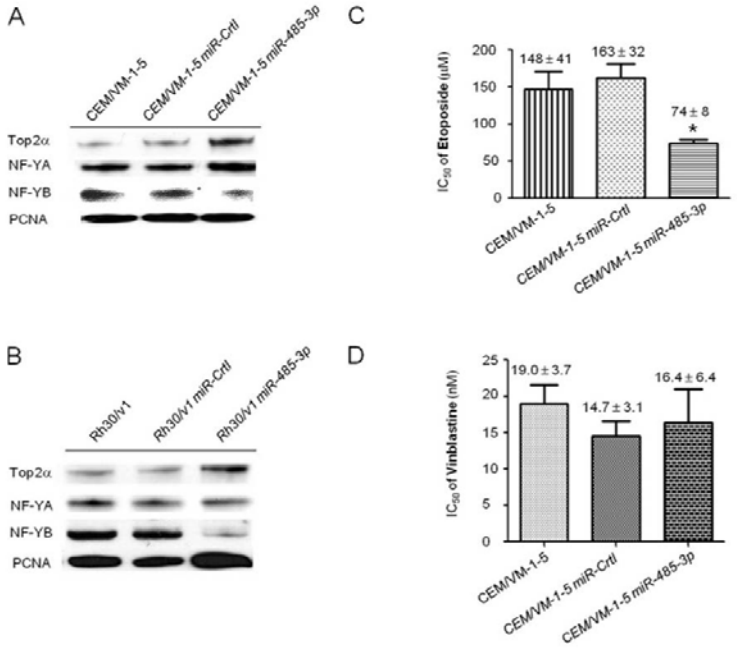


Fig. 14. *MiR-485-3p* inhibited NF-YB expression and mediated drug responsiveness.

Western blots of nuclear Top2α, NF-YB, and NF-YA expression in CEM/VM-1-5 (A) and Rh30/v1 cells (B). CEM/VM-1-5 and Rh30/v1 cells were transduced with either *miR-485-3p* expression virus or control virus (*miR-Crtl*). PCNA served as loading control for nuclear protein. IC_{50} values of cells exposed to etoposide (C) or vinblastine (D) at various concentrations were calculated from the percentage of viable cells after exposure to treatment obtained from MTT assay. Values are average of three independent experiments done in triplicate ± S.E. *, $p < 0.05$.

6.3. Discussion

Recent studies indicate that microRNAs are involved in mediating drug sensitivity and resistance. Therefore, based on microRNA profiling results and computer- based microRNA target prediction programs, we studied the role of *miR-485-3p* in drug resistance. M*iR-485-3* was differentially expressed in drug-sensitive and -resistant cells and has a putative target in the 3′-UTR of NF-YB. I first examined the repression effect of *miR-485-3p* to its target, NF-YB, by ectopically expressing *miR-485-3p* in the cells. I also confirmed, by luciferase assay, the binding of *miR-485-3p* to NF-YB 3′-UTR. Overexpressing *miR-485-3p* in CEM/VM-1-5 cells led to reduced expression of NF-YB, a corresponding up-regulation of Top2α, and increased sensitivity to the Top2 poison, etoposide. This is consistent with the inverse correlation between NF-YB and Top2α expression we observed in Figure 9A and 9D. However, the results in Chapter 5 suggest that knocking-down of NF-YB alone is not sufficient to recover Top2α expression. Based on these findings, it is possible that *miR-485-3p* may regulate other genes or transcription cofactors that interact with NF-YB to regulate Top2α expression. There are more than 5000 potential target genes of *miR-485-3p* based on microRNA.org prediction algorithm, which cover several of the major molecular pathways as predicted and classified by computer based program, DIANA-mirPath. Some are cancer-related, including Notch, MAPK, and VEGF signaling pathways, to name a few. What is the contribution of other *miR-485-3p*-related targets and pathways to Top2α-related resistance is not clear and requires further experimental and functional study to verify.

Furthermore, among the proteins and transcription cofactors that interact with NF-YB, *p53* [153] and *TAFII* (TBP-associated factor II) [157] are potential targets of *miR-485-5p,* the opposite strand of pre-miRNA (miRNA hairpin precursors with stem-loop structures). In the biogenesis of microRNA, the pre-miRNA is processed into a miRNA/miRNA* duplex. One strand of the pre-miRNA, termed as mature miRNA, post-transcriptionally regulate target mRNA. The other strand, known as the inactive strand or miRNA* (miRNA star), is thought to be degraded [158]. The mature microRNAs may be produced by 5' (left) arms or 3' (right) arms of the miRNA precursors. However, accumulating evidence suggests that miRNA* can contribute to regulate mRNAs and miRNA/miRNA* ratios may vary among developmental stages [159-160]. Accordingly, *miR-485-3p* and *miR-485-5* may mediate the expression of NF-YB and its associated transcription cofactors in a dynamic way to further regulate Top2α expression.

Despite the insights into the regulatory function of microRNAs that are beginning to emerge, the mechanism underlying the differential expression of *miR-485-3p* in drug-resistant and -sensitive cells is not clear. Some studies suggest that epigenetic alterations [161], deregulation of microRNA processing factors [162], and chromosomal abnormalities [163] can contribute to down-regulation or up-regulation of microRNAs. *MiR-485-3p* resides on chromosome 14q32.31, a region that includes *miR-127* [164], and *miR-370* [165], which has been suggested to be epigenetically regulated. Furthermore, chromosome 14q32.31 is a region in which allelic deletions [166] and translocations [167] are frequently identified.

Targeting microRNAs for cancer therapy is an emerging field to optimize cancer therapy, which includes inactivation of oncogenic miRNAs, activation of tumor suppressor miRNAs, and targeting specific miRNAs to restore drug sensitivity. Experimental studies have shown that modified anti-sense oligonucleotides can block the function of miRNAs *in vitro* [168-169] or *in vivo* [170]. However, major limitations for optimal use of modified anti-sense oligonucleotides have to be overcome, because they are easily degraded and lack of appropriate *in vivo* delivery systems. Recently, some studies also suggest that serum microRNAs are novel noninvasive biomarkers for cancer, since serum and plasma contain a large amount of stable miRNAs derived from tissues and organs [171-173]. In particular, Anderson et al. identified 15 miRNAs, including *miR-485-3p*, that were upregulated in patients with advanced prostate cancer [173]. Furthermore, there are several clinical trials (www.ClinicalTrials.gov) being conducted to investigate differential microRNA expression profiles after cancer patients receive chemotherapy to find predictors of response to chemotherapy (trial ID: NCT01391351, NCT00864266), or between treatment-sensitive and treatment-resistant cancer (trial ID: NCT01050504). Ranade et al. has reported that higher *miR-92a-2** level in tumor samples from SCLC (small cell lung cancer) is associated with chemoresistance and with decreased survival [174]. By comparison of microRNA profiles from serum or tumor specimens, the specific microRNAs that are critically involved in drug response might be identified as biomarkers to predict treatment outcomes, or as potential drug targets to reverse resistance in cancer cells. It is clear that more than one target or mechanism of drug resistance is activated by certain drugs and each microRNA can target to several genes.

The more drug-related targets or mechanisms that can be mediated by microRNAs could warrant for the better therapeutic potential.

Chapter 7

Specific Aim 4

Examine the Cytotoxic Effect Mediated by Antibiotic, Doxycycline (Doxy) and its Role in DNA Top2α and Top2β Expression

7.1. Background

Top2α has been well-studied for its role in cell proliferation. However, to our surprise, even knockdown of Top2α protein to 40% of levels in control cell line, it had little impact on cell proliferation, compared to control cell lines (see Chapter 4). As discussed in Specific Aim 1, it is also possible that cells with more potent Top2α knockdown may already die out during the selection process of stable cell lines. Therefore, I planned to develop the tetracycline inducible system, in which we can turn-on and -off the transcription of Top2α shRNA with and without adding antibiotic, doxycycline (Doxy), respectively.

7.2. Results

7.2.1. Knockdown of Top2α expression by tetracycline inducible system

The lentiviral vector pLV-THshTop2α used to knockdown Top2α has the tetracycline operon (tetO) and H1 promoter upstream of shTop2α region and the GFP gene as a reporter driven by the EF-1α promoter [147] (Fig.15A). The activities of both the H1 and EF-1α promoters are regulated by antibiotic tetracycline (tet) or it's analog, doxycycline (Doxy) [147]. Tetracycline (tet) is a specific inhibitor of bacterial protein synthesis and has long been used clinically as an antibiotic agent, and doxycycline (Doxy), a member of the tetracycline antibiotic group is commonly used to treat a variety of infections. To generate the tetracycline inducible system, pLV-THshTop2α was co-transduced into the cells with another lentiviral vector, pLV-tTR/KRAB-Red, which expresses a fusion protein of tet repressor (tTR), the silencing domain (KRAB module) and red fluorescent protein driven by the EF-1α promoter [147] (Figure 15A), termed CEM-tTR-shTop2α. After the lentiviral vectors integrated into the chromosome of cells (Figure 15B), in the presence of Doxy, the fusion protein tTR/KRAB will be bound by Doxy and dissociated from the tetO, unblocking the downstream Top2α shRNA transcription, which results in knockdown of Top2α expression (Figure 15B right). On the contrary, without the presence of Doxy, tTR/KRAB bind to the tetO and there will be no transcription of Top2α shRNA (Figure 15B left). I subsequently generated and characterized stable CEM-tTR-shTop2α cells where Top2α expression can be conditionally knocked down to 24% in the presence of 8ug/ml of Doxy for three days, as determined by Western blot. However, in the control CEM-tTR-shLuc cells with the same treatment, I observed

certain level of reduction in Top2α protein. I further examined the Top2α knockdown effect on cell proliferation and to my surprise, 8ug/ml Doxy-treatment dramatically suppressed cell growth in both Top2α-knockdown and control cell lines, which suggests the inhibitory effect of antibiotic, Doxy to human cancer cells.

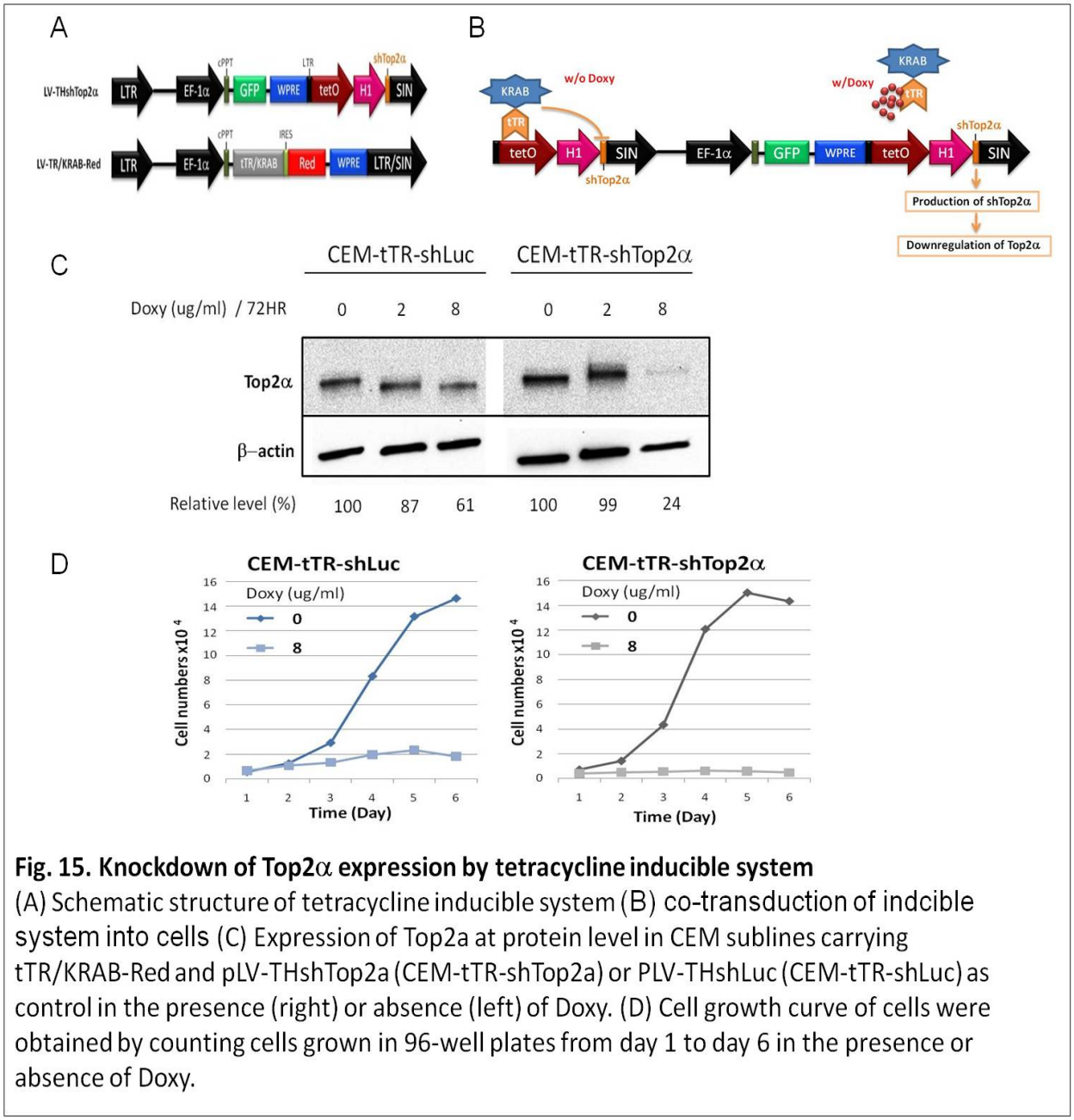


Fig. 15. Knockdown of Top2α expression by tetracycline inducible system
(A) Schematic structure of tetracycline inducible system (B) co-transduction of indcible system into cells (C) Expression of Top2a at protein level in CEM sublines carrying tTR/KRAB-Red and pLV-THshTop2a (CEM-tTR-shTop2a) or PLV-THshLuc (CEM-tTR-shLuc) as control in the presence (right) or absence (left) of Doxy. (D) Cell growth curve of cells were obtained by counting cells grown in 96-well plates from day 1 to day 6 in the presence or absence of Doxy.

7.2.2. Doxycycline Causes Growth Inhibition and Cytotoxicity in Cancer Cells

To better understand the inhibitory role of doxycycline (Doxy) in human cancer cell lines, I measured Doxy's effect on cell proliferation and cytotoxicity in three different human cancer cell lines: lymphoblastic leukemia CEM cells, breast cancer MCF-7 cells and rhabdomyosarcoma Rh30 cells as shown in (Figure.16). Cancer cells were treated with various concentration of Doxy and incubated for indicated time, followed by MTT or MTS assay for measuring cell growth curves and cytotoxicity as detailed in Material and Methods. Among these cancer cell lines, dose of 2ug/ml of Doxy did not inhibit cell proliferation or induce cytotoxicity. However, dose of 8ug/ml of Doxy inhibited cell growth in all three cell lines, which indicates the inhibitory effect to the cancer cells depends on high-dose of Doxy. In accordance with this result, I found the cytotoxicity of Doxy to cancer cells in a dose- and time-dependent manner. Furthermore, cell morphology was monitored by microscopy. Cancer cells treated with 8ug/ml Doxy were dying as visualizing cell rounding, and easy detachment from the dish for the attached Rh30 and MCF-7 cells, while suspension CEM cells were manifested by shrinkage and debris presence. Based on these results, cytotoxic effects of the Doxycycline on the cancer cells need to be taken into consideration when using the tetracycline-inducible system in manipulating gene expression.

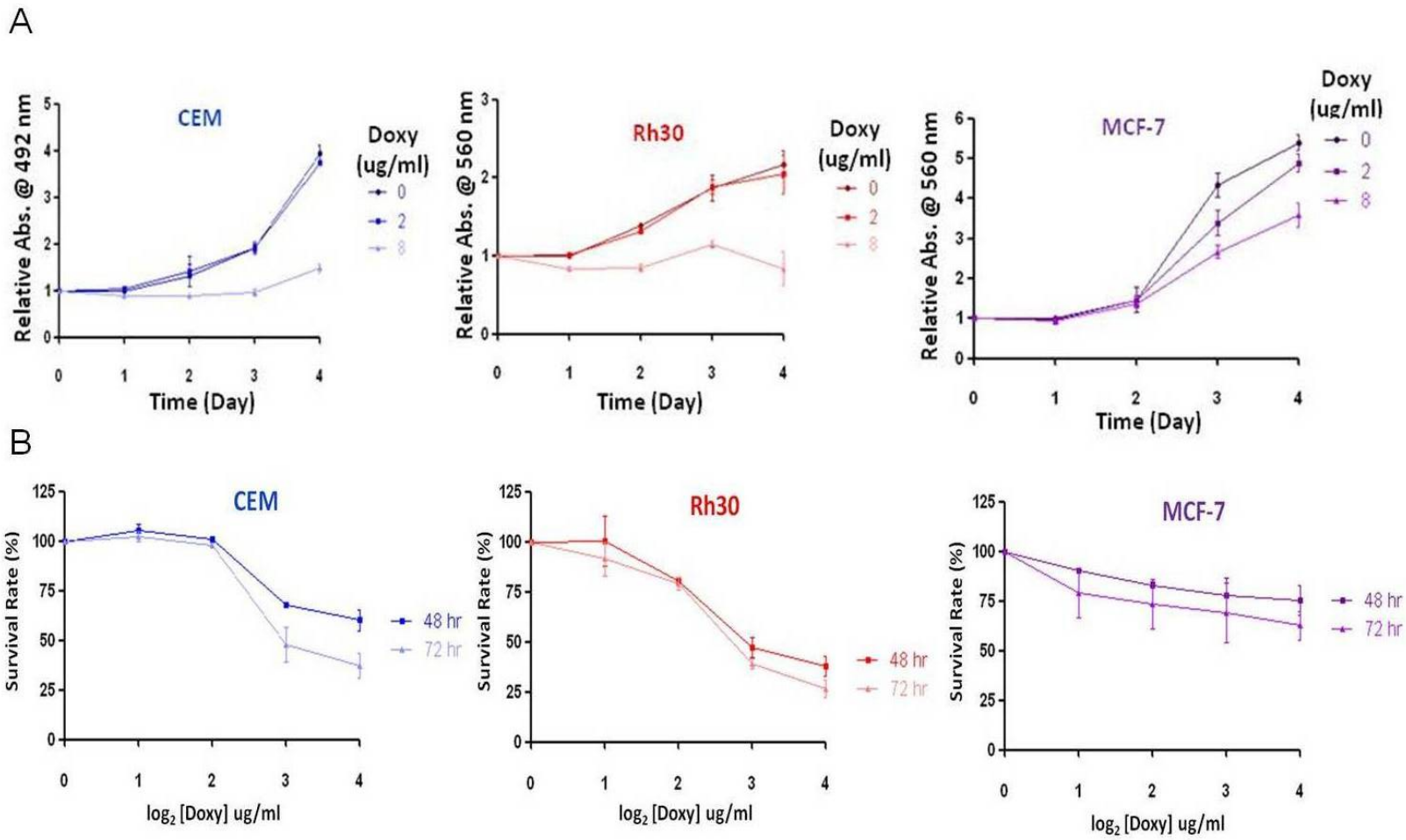


Fig. 16 . Growth inhibition of cancer cell lines after Doxycycline treatment

(A) Cell growth curves were obtained by seeding 2000 cells/ well in the presence of various concentrations of Doxy and measured by MTT or MTS assay at indicated time points. Relative Absorbance (Abs) was normalized to Abs. of cells treated without Doxy at day 1. Values were averagesof two separate experiments performed in triplicate ± SE. (B) Cytotoxicity of Doxy to cells were obtained by treating logarithmically growing cells with Doxy and measuring by MTT or MTS assay. Values were average of two separate experiments performed in triplicate ± SE.

7.2.3. Doxycycline Induces Apoptosis in Cancer Cells

Since apoptosis is one major mechanism leading to cancer cell death, I further analyzed apoptosis by staining Doxy-treated CEM cells with either DNA binding dye, Hoechst 33342 for microscope monitoring or annexin/ propidium iodide (PI) for flow cytometry quantification (Figure 17). Blue fluorescent Hoechst 33342 dimly stains the normal chromatin of live CEM cells without Doxy treatment, however, Hoechst 33342 brightly stains the condensed chromatin of apoptotic cells (Figure 17A, yellow arrows) after treatment with 4ug/ml or 8ug/ml Doxy for three days. In addition, CEM cells were stained with annexin-V and propidium iodide and analyzed by flow cytometry, detailed in Material and Methods. After three days of Doxy-treatment, increasing percentage of cells was in either early stage (annexin-V^+, PI^-) or late stage of apoptosis (annexin-V^+, PI^+)(Figure 17B). These numbers were higher than those seen in untreated control cells.

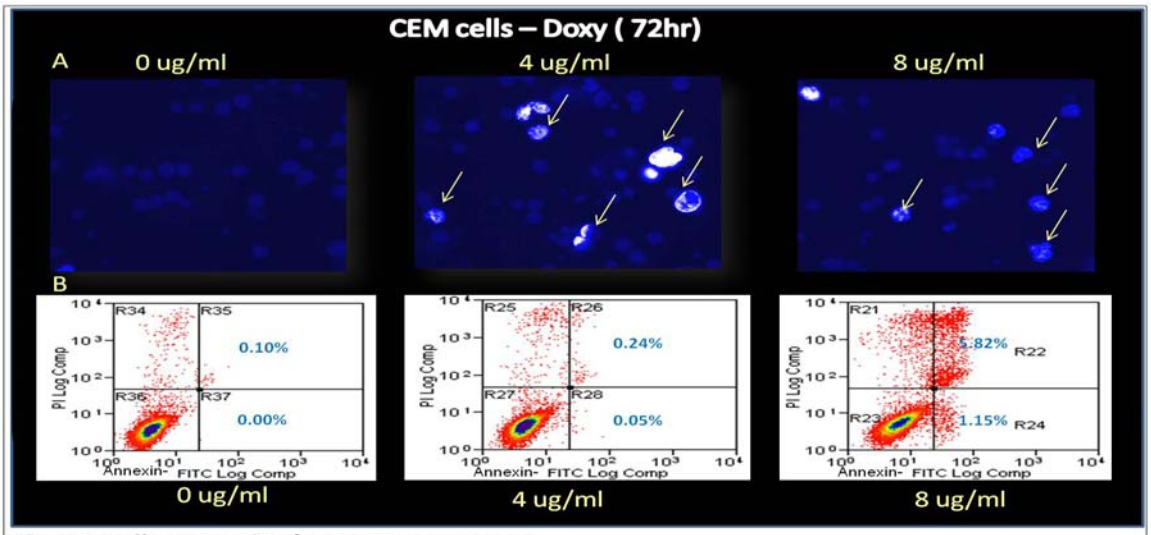


Fig. 17. Cell apoptosis after Doxy-treatment.
Three days after Doxy treatment, CEM cells were fixed and subjected to (A) the DNA-binding dye Hoechst 33342 (blue) staining for apoptosis analysis. Condensation of the nuclear material (arrows) followed by apoptosis. Original magnification: 400x. (B) Annexin V-FITC/PI staining. Flow cytometry profile represents Annexin-V-FITC staining in X axis and PI in Y axis. Annexin-V^-, PI^- cells are live cells, annexin-V^+, PI^- cells are early apoptotic cells, and annexin-V^+, PI^+ cells are late apoptotic. The number represents the percentage cells in each condition.

7.2.4. Doxycycline Dysregulated Top2α and Top2β Expression in Cancer Cells

Cancer cell lines, CEM, CEM/VM-1-5, MCF-7 and Rh30, cultured in the presence of 4ug/ml or 8ug/ml of Doxy for three days, all expressed reduced Top2α protein compared to the respective cells without treatment, as determined by Western blot (Figure18). The reduction of Top2α protein in these cancer cell lines was consistent with our finding on Figure 15C. This result suggests that the dysregulation of Top2α in Doxy-treated cells is not due to the integration of Top2α shRNA lentiviral constructs into chromosomes as shown in Figure 15C. Doxy at different concentrations (4ug/ml and 8ug/ml) reduced Top2α expression in a dose-dependent manner in CEM, CEM/VM-1-5, MCF-7 and Rh30 cell lines. The downregulation of Top2α expression can explain the decreased growth rate of in Doxy-treated cells (Fig.15D). Interestingly, I observed the corresponding upregulation of Top2β protein in CEM, and MCF-7 cells with the Doxy-treatment (Fig.18). However, Top2β protein was not detected in CEM/VM-1-5, and Rh30/v1 cells. It has been documented that in some cancer cell lines [54-58], including CEM/VM-1-5 cells, there is no Top2β protein expression. The mechanism behind this is unknown and apparently, Doxy-treatment cannot recover Top2β protein expression in the cell lines without endogenous Top2β protein expression.

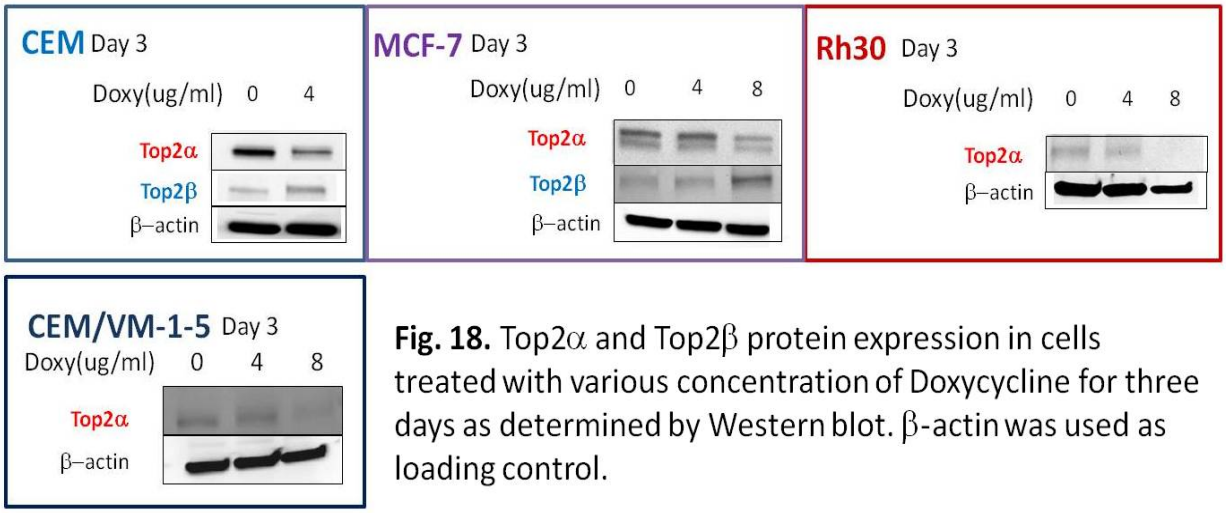


Fig. 18. Top2α and Top2β protein expression in cells treated with various concentration of Doxycycline for three days as determined by Western blot. β-actin was used as loading control.

7.2.5. Differentiating Agents Mediate Top2α and Top2β Expression in Cancer Cells

So far, I have focused our study on the regulation of Top2α based on the evidence that 1) DNA Top2α is more sensitive to etoposide, doxorubicin and merbarone than DNA Top2β [38], 2) DNA Top2α expression is linked to the proliferative status of cells, 3) DNA Top2α is required for cell survival, and 4) DNA Top2β is dispensable for cell survival. However, little is known about the regulation of Top2β. I further examined the possible mechanisms that mediate Top2β expression.

Increased expression of Top2β has been found in non-proliferating, differentiated cells and tissues [59-60, 62-65]. Furthermore, differentiation of CEM cells [83] and promyelocitic leukemia cell line, HL-60 cells (FAB M2) [64, 175] have been reported by treating them with differentiating agents such as retinoid acid (RA), phorbol ester, or

DMSO. This differentiation transition has been reported to correlate with altered expression of Top2β [64, 175]. Accordingly, I asked whether the dysregulation of Top2α and Top2β protein in Doxy-treated cells is associated with differentiation of cancer cells. I treated CEM cells and HL-60 cells with differentiating agents, DMSO (1%) and TPA (100nM) for three days and examined the morphology by microscope. TPA (12-O-tetradecanoylphorbol-13-acetate) is one common phorbol ester. Untreated HL-60 and CEM cells remained spheroid when grown in suspension (Figure 19A and 19E). HL-60 cells treated with Doxy (8ug/ml) underwent apoptosis and showed the characteristic membrane blebbing (bubble formation) seen in apoptotic cells (Figure 19B), which was in consistent with results of the Doxy-induced apoototic CEM cells (Figure 17). On the other hand, suspension cells, HL-60 and CEM, treated with differentiating agents DMSO (1%) and TPA (100 nM) showed the characteristic cell adhesion and spreading onto tissue plates (morphology seen in differentiating cells). The different morphology results observed suggest that Doxy-treated cells may not undergo differentiation.

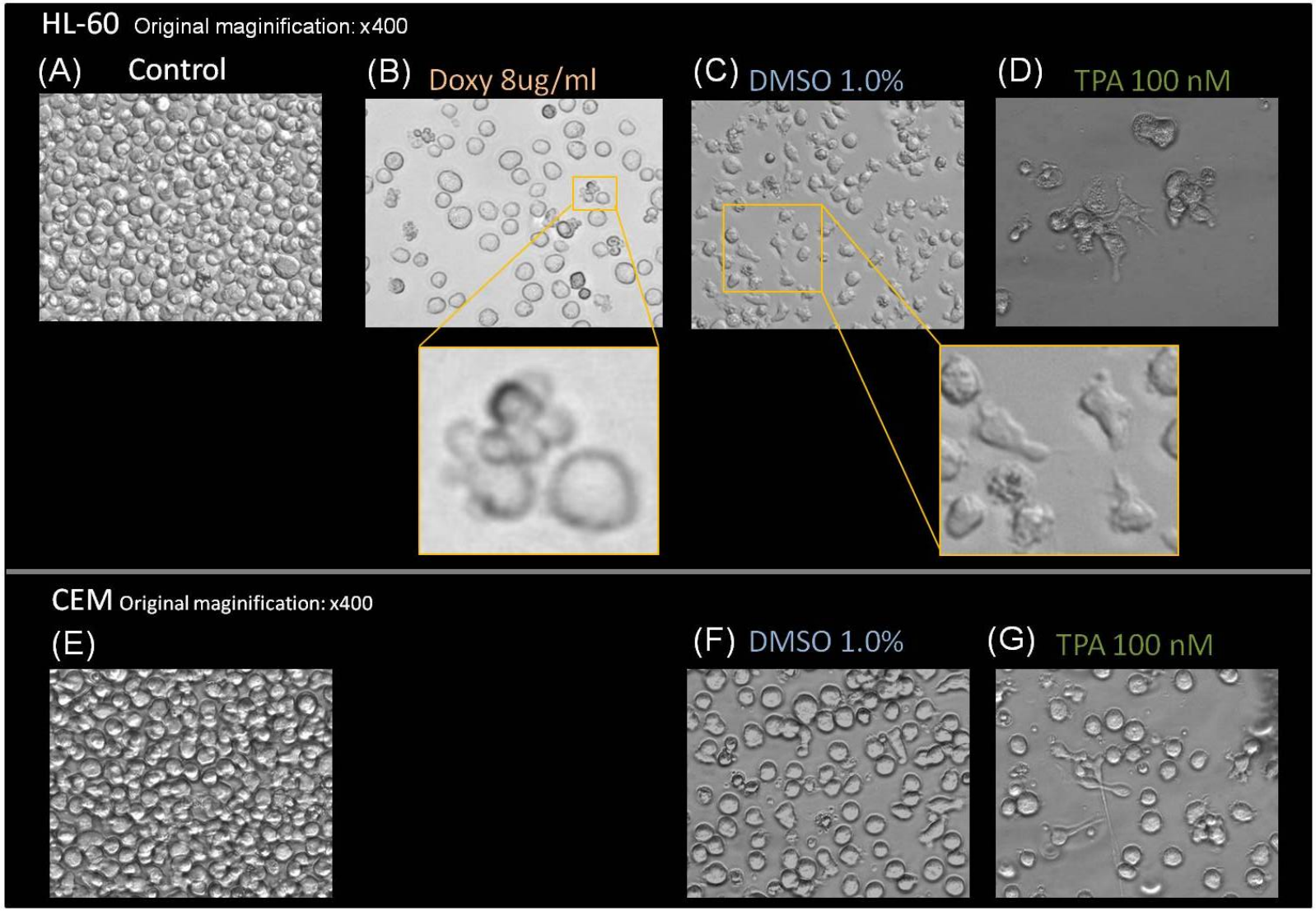


Fig. 19. Effects of Doxy, DMSO and TPA on HL-60 and CEM morphology
Morphology of HL-60 and CEM cells without any treatment (A,E) or HL-60 with 8ug/ml Doxy (B), 1% DMSO (C), 100nM TPA (D); CEM with (F), 1% DMSO (G), 100nM TPA.

Furthermore, I observed the upregulation of Top2β protein in both Doxy- and DMSO-treated HL-60 cells as determined by Western blot. However, the inverse correlation between Top2α and Top2β in Doxy-treated CEM and MCF-7 cells on (Fig.18) was not presented in HL-60 cells (Figure 20A). Moreover, Top2α was also upregulated alone with Top2β in DMSO-treated cells (Figure 20B), while both Top2α and Top2β protein levels were similar in DMSO-treated CEM cells (Figure 20D).

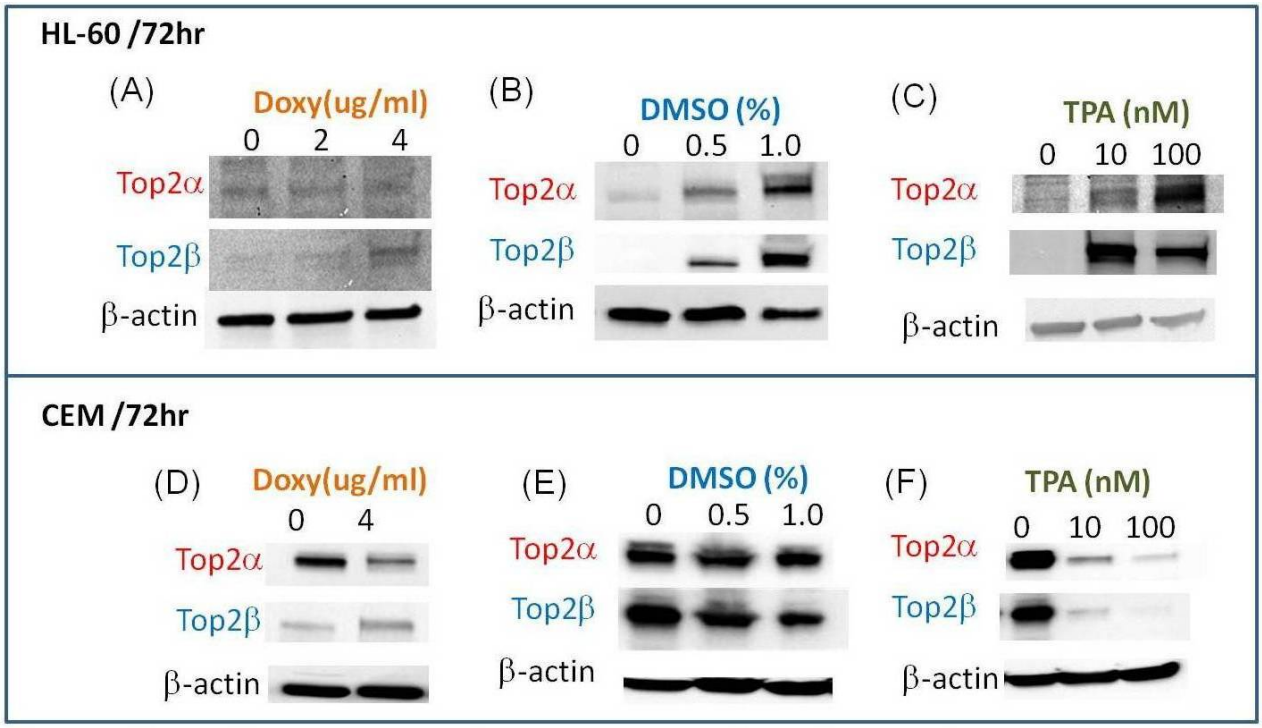


Fig. 20. Effects of Doxy, DMSO and TPA on Top2α and Top2β expression

Western blot of Top2α and Top2β protein from total protein lysates in HL-60 and CEM cells. Cells were treated with various concentrations of Doxy (A,D), DMSO (B,E) and TPA (C,F) for three days. β-actin served as loading control.

7.3. Discussion

The ability to “turn-on” or “turn-off” the expression of genes or shRNA *in vitro* in response to small molecules is an important tool in molecular biology research.

The tetracycline inducible system has been widely used [reviewed in 176], where the expression of shRNA can be regulated by tetracycline or its analogue doxycycline. Antibiotic doxycycline is used in treatment of genitourinary tract infections and is generally regarded as relatively non-toxic drug for human use. It functions by binding to the subunit of microbial ribosomes and inhibits their protein synthesis [177-178].

Here, we established the tetracycline inducible system in order to manipulate Top2α expression conditionally and minimize possible side effects due to long term suppression of Top2α. Our results here revealed that even though we can knockdown Top2α protein to relatively low level (~25%) in the presence of Doxy in the CEM cells, compared to the control cells without Doxy-treatment, high concentration of doxycycline (8ug/ml) can inhibit cell proliferation, induce cytotoxicity and suppress Top2α expression in CEM cells and other cancer cell lines we tested. It is important to avoid the potential side-effects of the agents used in the inducible system. This result indicates that tetracycline-inducible system is not ideal to manipulate Top2α expression since doxycycline alone can perturb Top2α expression. Cytotoxic effects of the doxycycline in cancer cells should be also taken into consideration when using the tetracycline-inducible system in manipulating gene expression. The inhibitory role of doxycycline has been reported in human prostate cancer, and osteosarcoma cells [179-180]. Other study suggested doxycycline may interfere with mitochondrial protein synthesis in

mammalian cells based on the evidence that eukaryotic mitochondria has similar protein synthesis machinery as prokaryotes [181]. Our results suggest one possible Doxy-induced cytotoxic mechanism is mediated by apoptosis in cancer cells (Figure 17). The ability of doxycycline to induce apoptosis in cancer cells makes it a potential chemotherapeutic agent.

In addition to the suppression of Top2α in Doxy-treated cells, I observed the corresponding upregulation of Top2β in CEM, MCF-7 and Rh30 cells (Figure 18). Increased expression of Top2β has been found in non-proliferating, differentiated cells and tissues [59-60, 62-65]. Other studies also reported that differentiating agents, DMSO [175] and ATRA (all-*trans* retinoic acid)[82] lead to granulocytic maturation of human promyeolocitic HL-60 cells and decrease expression of Top2β protein. In contrast, ATRA was also reported to increase Top2β protein expression in HL-60 cells [64]. This contradiction in Top2β expression is unclear. Based on the evidence that differentiating agents, including ATRA (*all-trans*-retinoic acid)[182] and HDACi (Histone deacetylase inhibitors)[reviewed in 183], may constitute a non-cytotoxic approach against cancer cells by inducing cancer cells to mature when many cancer cells have an immature phenotype that allows them to proliferate abnormally.

We attempted to understand the regulation of Top2β in Doxy-treated cells and its possible relationship to differentiation. In order to better understand the molecular mechanisms underlying the inhibitory effect of doxycycline on cancer cells and the

potential of Doxy as therapeutic agent. However, the different morphology between Doxy-treated HL-60 cells, which exhibit apoptosis character, and differentiating agents, DMSO- and TPA-treated HL-60 cells with spreading and adhesion morphology suggests that Doxy-treated cells may not undergo differentiation. Furthermore, Top2α and Top2β protein expression pattern is different between Doxy-treated (inversely correlated) and DMSO-treated (positively correlated) HL-60 cells. Top2α and Top2β protein levels remain similar in DMSO-treated CEM cells. The regulation of Top2 levels in cancer cells appears to be complicated. These observations have led to the suggestion that Top2 levels might be regulated differently in etiologically different cancers in response to differentiating agents.

Chapter 8

8.1. Conclusion and Future Perspective

The results described in this thesis exploit the use of selected drug resistant cell lines as models to study the regulation of Top2α during the development of resistance to Top2 poisons. I showed that changes in Top2α expression alter the sensitivity of cancer cells to the Top2 poisons, etoposide and doxorubicin, and that cancer cells can survive with relatively low level of Top2α, which plays a major role in cell proliferation. I found an inverse correlation between Top2α and the transcription factor NF-YB in Top2 poison-sensitive and –resistant cancer cell lines. NF-YB has been previously suggested to regulate Top2α expression by binding to its promoter region [92]. Recent studies have revealed the role of microRNAs in cancer development and drug resistance by negative regulation of its target genes. Accordingly, we profiled microRNA expression in drug-sensitive CEM cells and teniposide-resistant CEM/VM-1-5 cells, and we found some consistently upregulated or downregulated microRNAs. Among these microRNAs, *miR-485-3p* was consistently highly expressed in drug-resistant CEM/VM-1-5 cells. Interestingly, this *miR-485-3p* has a potential target in the NF-YB 3'-UTR based on computational prediction programs. In support of this prediction, I validated the direct binding of *miR-485-3p* to the NF-YB 3'-UTR by luciferase assay and showed that ectopic expression of *miR-485-3p* suppressed NF-YB expression, which is associated with the recovery of Top2α protein. However, direct knockdown of transcription factor NF-YB by shRNA did not rescue in recovery of Top2α expression as we expected.

One possible mechanism is the involvement of the other transcription cofactors. P300/CBP, and PCAF have been reported to mediate NF-Y protein complex regulation through their interactions on promoters [150-152]. Other studies also revealed that p53 binds to the NF-Y protein complex to repress transcription [153, 184]. Furthermore, it is possible that *miR-485-3p* regulates other genes or transcription cofactors that interact with NF-YB to mediate Top2α expression. There are more than 5000 potential target genes of *miR-485-3p* based on the microRNA.org prediction algorithm, which covers several of the major cancer-related signaling pathways as predicted and classified by the computer based program, DIANA-mirPath (http://diana.cslab.ece.ntua.gr/pathways/). The contribution of other *miR-485-3p*-related targets and pathways to Top2α-related resistance is not clear and requires further experimental and functional studies to verify. It is interesting to know that among the proteins and transcription cofactors that interact with NF-YB, *p53* is a potential target of *miR-485-5p,* the opposite strand of its pre-miRNA, which was proposed to regulate its target gene [159-160]. Therefore, *miR-485-3p* and *miR-485-5p* may mediate not only the expression of NF-YB, but also its associated transcription cofactors in a dynamic way to further regulate Top2α expression. Top2α expression may require the net effect of a dynamic and delicate regulation by the transcription factor, NF-YB and other unknown cofactors.

To address this question, it would be useful to ask whether p53 and NF-YB interact by performing coimmunoprecipitation experiments, employing cell lysates derived from drug-sensitive and -resistant cell lines. To further study the possible role of *miR-485-5p* on its potential target p53, we can examine p53 expression following the ectopic

expression of a chemically modified antagomiR [170] against *miR-485-5p* to abolish *miR-485-5p* expression in the cells. These results can provide insights into the molecular mechanisms of fine tuning of Top2α. Furthermore, it is important to ask whether this work has any clinical relevance. Can our finding of altered expression of *miR-485-3p* between drug-sensitive and -resistant cells be applied to the understanding failure of chemotherapy in patients? It has been reported that microRNA expression profiling can not only classify several cancers [185], but also predict survival and relapse of disease [186].

Targeting microRNAs for cancer therapy is becoming an emerging field in cancer therapy, which includes inactivation of oncogenic miRNAs, activation of tumor suppressor miRNAs, and targeting specific miRNAs to restore drug sensitivity. Experimental studies have shown that modified anti-sense oligonucleotides can block the function of miRNAs *in vitro* [168-169] or *in vivo* [170]. However, major limitations for optimal use of modified anti-sense oligonucleotides have to be overcome, because they are easily degraded and lack of appropriate *in vivo* delivery systems. Recently, some studies also suggest that serum microRNAs are novel noninvasive biomarkers for cancer, since serum and plasma contain a large amount of stable miRNAs derived from tissues and organs [171-173]. In particular, Anderson et al. identified 15 miRNAs, including *miR-485-3p*, that were upregulated in patients with advanced prostate cancer [173]. Furthermore, there are several clinical trials (www.ClinicalTrials.gov) being conducted to investigate differential microRNA expression profiles after cancer patients receive chemotherapy to find predictors of response to chemotherapy (trial ID:

NCT01391351, NCT00864266), or between treatment-sensitive and treatment-resistant cancer (trial ID: NCT01050504). Ranade et al. have reported that higher *miR-92a-2** levels in tumor samples from SCLC (small cell lung cancer) are associated with chemoresistance and with decreased survival [174]. By the same token, comparison of microRNA expression profiles in patients who are sensitive or resistant to treatments with anti-Top2 agents may help to predict treatment outcome or guide chemotherapy. It will be of interest to determine *miR-485-3p* plays similar role in the clinic as we have observed in cultured cancer cell lines. By comparison of microRNA profiles from serum or tumor specimens, the specific microRNAs that are critically involved in drug response might be identified as biomarkers to predict treatment outcomes, or as potential drug targets to reverse resistance in cancer cells. It is clear that more than one target or mechanism of drug resistance is activated by certain drugs and each microRNA can target to several genes. The more drug-related targets or mechanisms that are found to be mediated by microRNAs may aid in the development of novel and better therapies for the treatment of some cancers.

In Chapter 7, I established the tetracycline inducible system to manipulate Top2α expression. I was able to turn-on and -off the transcription of Top2α shRNA with the addition and absence of the tetracycline analogue, antibiotic doxycycline (Doxy), respectively. However, of interest, I observed that Doxy is cytotoxic to human cancer cell lines. Its inhibitory effect on cancer cells appeared to be mediated partly by inhibition of cell proliferation, induction of cytotoxicity and apoptosis, and downregulation of Top2α. I observed an inverse relation of Top2α and Top2β in Doxy-treated cell lines.

Altered expression of Top2β has been related to the differentiation status of cells. To study whether cells undergo differentiation during treatment with Doxy, I treated cells with differentiating agents, DMSO and TPA. I observed the upregulation of Top2β in differentiating agent-treated cells, but the correlation between Top2α and Top2β was inconsistent among cell lines and treatments. Furthermore, the morphology of Doxy-treated cells was different from the differentiating agent-treated cells, suggesting that the altered expression of Top2β in Doxy-treated cells may not be due to cellular differentiation. According to my results in Chapter 4 and other reports [43, 148], Top2β does not compensate for the loss of Top2α in the cells. However, I observed an inverse relation (Doxy-treatment), and positive relation (DMSO-treatment) between Top2α and Top2β in HL-60 cells in response to these treatments. This leads us to speculate whether Top2β expression sensitizes cells to Top2 poisons. To examine the functional role of Top2β in the cells, we can measure the susceptibility of CEM, MCF-7 and HL-60 cells to Top2 poisons in response to Doxy-treatment. As upregulation of Top2β did not relieve the inhibition of cell proliferation in Doxy-treated cells, Top2β level seems to be critical only for maintaining cellular viability. This is consistent with my observation that Top2β–deficient CEM/VM-1-5 cell line has similar cell doubling time as Top2β–expressing CEM cells. In general, these cell lines can be used as model to study the specific roles of Top2α and Top2β at the cellular level.

In conclusion, my results presented in this dissertation have revealed novel regulation of the important enzyme, DNA topoisomerase II, indirectly through microRNA regulation of

a Top2 transcription factor. My work has also revealed a possible involvement of Top2β in the response of cells to differentiating agents, but further work is required to understand this phenomenon. Overall, it is possible that the results presented herein may open novel avenues of investigation of Top2 inhibitors, with ultimate application to the clinic.

8.2. References

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