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암억제 유전자 p53 타겟 유전자 발굴과 p53에 의한 세포사에서 이들 유전자의 역할

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Identification of p53 target genes and their roles in p53-mediated cell death

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CONTENTS

LIST OF TABLES IX

LIST OF FIGURES X

ABSTRACT XIII

CHAPTER 1 INTRODUCTION 1

1.1 Apoptosis 2

1.2 Significance of Apoptosis 2

1.3 Regulation of Apoptosis 4

1.3.1 Extrinsic pathway 5

1.3.2 Intrinsic pathway 6

1.4 Disease as a consequence of dysregulated apoptosis 8

1.5 p53 11

1.5.1 p53 as tumor suppressor 12

1.5.2 Protein description 14

1.5.3 Regulation of p53 activity 15

1.5.4 Loss of p53 in cancer 17

1.5.5 p53 family 19

1.5.6 Fate of cell; life or death 26

1.5.7 Functional significance of p53 26

1.5.7.1 Cell cycle arrest 26

1.5.7.2 Apoptosis 28

1.5.7.3 DNA Repair 31

1.5.7.4 Senescence 31

1.5.7.5 Inhibition of angiogenesis 32

1.5.7.6 p53 as transcriptional regulator 32

1.2 MAP kinase phosphatases 35

1.2.1 Gene organization 36

1.2.2 DUSP6 38

1.2.2.1 Structural features 40

1.2.2.2 Regulation of DUSP6 41

1.2.2.3 Physiological outcome of DUSP6 42

1.3 SSFA2 44

1.4 PHLDA1 44

1.5 A cell killing peptides 45

1.6 Spontaneous tumor regression of tumor 47

1.6.1 Toll like receptors 50

CHAPTER 2 Identification of p53 target genes and their roles in p53-mediated cell death 56

2.1 ABSTRACT 57

2.2 INTRODUCTION 59

2.3 MATERIAL AND METHODS 63

2.3.1 Cell cultures and reagents 63

2.3.2 Screening of p53-upregulated gene 64

2.3.3 Infection of adenovirus 64

2.3.4 Semi-quantitative Reverse transcription-polymerase chain reaction (RT-PCR) and Real Time quantitative RT-PCR (qRT-PCR). 64

2.3.5 Short hairpin RNAs construction and analysis of apoptotic

cell death 65

2.3.6 DUSP6 Overexpression 66

2.3.7 Western blotting 67

2.3.8 Chip analysis 68

2.3.9 ERK dephosphorylation 69

2.3.10 Statistical analysis 70

2.4 RESULTS 76

2.4.1 Identification of DUSP6, PHLDA1 and SSFA2 as p53 target genes 76

2.4.2 DUSP6 is induced by p53 in response to different cellular stresses and Identification of a novel p53 putative binding sites in DUSP6 promoter region 80

2.4.3 DUSP6, PHLDA1 and SSFA2 induced by p53 in response to genotoxic stresses leading to apoptosis 85

2.4.4 DUSP6 over expression enhanced the apoptotic response to death stimuli 98

2.4.5 DUSP6 suppresses ERK phosphorylation and elevates apoptotic response to 5-FU 102

2.5 DISCUSSION 105

CHAPTER 3 A novel cell killing peptide 109

3.1 ABSTRACT 110

3.2 INTRODUCTION 111

3.3 MATERIAL AND METHODS 113

3.3.1 Cell cultures and reagents 113

3.3.2 Mice 113

3.3.3 Peptide synthesis 114

3.3.4 In *vitro* killing activity of MTD peptides 114

3.3.5 Evaluation of MTD peptides against tumor growth suppression 114

3.3.6 Histological examination of tumor 115

3.3.7 Statistical analysis 115

3.4 RESULTS 116

3.4.1 A novel TU:MTD peptides induce cell death vigorously and efficiently than other conventional chemotherapeutic agents *in vitro* 116

3.4.2 Tumor-homing MTD (TU:MTD) peptides suppress tumor growth 122

3.4.3 TU:MTD specifically kills the cancer cell line not primary cell, hepatocytes 130

3.5 DISCUSSION 134

CHAPTER 4 A role of Noxa in LPS-mediated tumor regression 136

4.1 ABSTRACT 137

4.2 INTRODUCTION 138

4.3 MATERIAL AND METHODS 141

4.3.1 Cell cultures and reagents 141

4.3.2 Short hairpin RNA interference of Noxa construction and analysis of cell death in response to LPS 143

4.3.3 Mice 144

4.3.4 Syngeneic tumor model 144

4.3.5 Histological examination of tumor 145

4.3.6 Infection of adenovirus 145

4.3.7 Reverse transcription-polymerase chain reaction (RT-PCR) 145

4.3.8 Plasmid construction and over expression of IRFs 146

4.3.9 Western blotting 147

4.3.10 Chip analysis 148

4.3.11 Statistical analysis 149

4.4. RESULTS 152

4.4.1 LPS sensitize the tumor cell and activate the Bcl-2 family protein, notably Noxa in spontaneous tumor regression 152

4.4.2 LPS regulate the Bcl-2 family protein, notably Noxa in different cancer cell line 161

4.4.3 Interferons also regulate the Bcl-2 family proteins 163

4.4.4 Noxa induction by LPS is independent of p53 168

4.4.5 Noxa is transcriptionally regulated by IRFs in response to

LPS or IFNs 170

4.4.6 Involvement of IRF1, IRF3 and IRF7 to transactivate Noxa in LPS mediated apoptosis 176

4.4.7 IRF4 is repressor of IRF1, IRF3 and IRF7 to transactivate Noxa 181

4.4.8 Noxa is key protein in spontaneous tumor regression by LPS 186

4.5 DISCUSSION 193

CHAPTER 5 CONCLUSION 197

REFERENCES 200

ACKNOWLEDGEMENT 221

LIST OF TABLES

Table 1-1.	List of p53 activated gene involved in apoptosis	29
Table 1-2.	Classification, nomenclature, properties and Physiological function of dual-specificity MKPs	39
Table 2-1A	List of RT PCR and Real time PCR primers	71
Table 2-1B	List of shRNA primers	74
Table 2-1C	Primers for Overexpression of DUSP6	75
Table 2-1D	List of primers for Chip analysis	75
Table 2.4.1	Expression profiles of p53 overexpressed HCT116 cells by cDNA microarray analysis	77
Table 3.4.1	Peptide nomenclature and sequences	117
Table 4.1A	List of primers for shRNA and ScRNA of Noxa	150
Table 4.1B	List of primer for RT PCR	150
Table 4.1C	List of primers for IRFs overexpression	150
Table 4.1D	List of primers for Chip analysis	151

LIST OF FIGURES

Fig.1-1 Hallmarks of the apoptotic and necrotic cell death process 3

Fig.1-2 Regulation of apoptosis 7

Fig.1-3 p53 pathway 13

Fig.1-4 Schematic representation of p53 protein 15

Fig.1-5 Worldwide distribution of cancer related with p53 mutation In Human tumor cell 18

Fig.1-6 Structure of p53 family 22

Fig.1-7 Fate of cell is decided by p53 25

Fig.1-8 p53 as transcriptional regulator 34

Fig.1-9 Regulation of DUSP6 43

Fig.1-10 Signaling pathway of Toll like receptors 54

Fig.2-1 p53 transactivates number of genes 79

Fig.2-2 p53-dependent induction of DUSP6 82

Fig.2-3 The selective p53 binding to the DUSP6 promoter region under genotoxic stress 84

Fig.2-4 The role of DUSP6 in p53-mediated apoptosis 91

Fig.2-5 The shRNA of SSFA2 and PHLDA1 inhibited apoptotic response in the p53-mediated apoptosis 96

Fig.2-6 Ectopic expression of DUSP6 enhanced the apoptotic responses 100

Fig.2-7 DUSP6 suppresses ERK phosphorylation and elevates apoptotic response to 5-FU 104

Fig.2-8 Possible mechanism of DUSP6 in p53-mediated apoptosis 107

Fig.3-1 A novel peptide dramatically and vigorously causes the cell death than conventional chemotherapeutic agent 5FU 121

Fig.3-2. TU:MTD peptides induces suppression of the tumor growth in mice 128

Fig.3-3 TU:MTD peptide relatively specific to cancer cell, not to primary cell 133

Fig.4-1 LPS sensitized the tumor cell to regulate the

	pro-apoptotic, particularly Noxa and anti-apoptotic proteins of BH3 family in spontaneous tumor regression	159
Fig.4-2	LPS regulate the Bcl-2 family protein, notably Noxa in different cell line	162
Fig.4-3	Interferons also regulate the Bcl-2 family proteins	167
Fig.4-4	Noxa induction by IFNs is p53-independent	169
Fig.4-5	IRFs transcriptionally regulated the Noxa in response to LPS or IFNs	174
Fig.4-6	IRF1, IRF3 and IRF7 induced Noxa in LPS induced cell death	180
Fig.4-7	IRF4 is repressor of IRF1, IRF3 and IRF7	184
Fig.4-8	Noxa is the key protein in LPS mediated spontaneous tumor regression	191
Fig.4-9	Bcl-2 family proteins decide the fate of tumor cells	196

ABSTRACT

Identification of p53 target genes and their roles in p53-mediated cell death

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Apoptosis, an essential altruistic behaviour of cell as a whole maintains the integrity of total organism in normal physiological condition. p53 is one of the key player in maintaining the integrity of organism by removing the harmful, unnecessary or damaged cells. A number of genes transactivated by p53 are involved in these phenomena through signaling or transcriptional regulation of apoptotic and pro-apoptotic or non-apoptotic molecules. In this study, I have identified several novel p53 target genes and characterized their

functional roles in p53-induced cell death. Furthermore, I also characterized the role of Noxa, a known p53 target gene, in LPS-mediated tumor regression. Besides conventional cell killing BH3 domain, I proposed a novel killing domain, mitochondrial target domain (MTD) in Noxa that has been known as a responsible domain for mitochondrial translocation of Noxa. BH3 domain and newly explored mitochondrial targeting domain (MTD) of Noxa are essential for the release of Cytochrome C, pro-apoptotic and apoptotic molecules from mitochondria as well as mitochondrial fragmentation ultimately resulting cell death.

p53 has been known to play critical roles in diverse cellular responses such as cell cycle arrest, senescence and apoptosis through transcriptional control of its target genes. Identification of the p53 upregulated genes by DNA chip analysis and characterization of the new p53 transactivated genes will advance our understanding of scene behind the p53 exerted multiple phenomena. Genotoxic stresses stabilize the p53 protein which, in turn, transactivates target genes to cause apoptosis. In this thesis I show that DUSP6, SSFA2 and PHLDA1

are the novel target genes in p53 mediated apoptosis. To explore the p53 transactivated genes and their role in cell death, microarray followed by introduction of short hairpin of RNA and functional experimental approach were adapted. The computational analysis of DUSP6 promoter region revealed the putative binding site for p53. Additionally, the Chromatin immunoprecipitation analysis revealed that p53 transactivates of DUSP6. The functional analysis of DUSP6 shows that it suppresses the ERK phosphorylation and elevates 5-FU induced cell death.

Peptides derived from mitochondrial target domain (MTD) of Noxa are very effective to kill the tumor cells. So, the tumor homing synthetic peptide TU-1 MTD, TU-2 MTD and TU-3 MTD were evaluated for tumor suppression efficacy. Specifically, TU-2 MTD and TU-3 MTD dramatically cause the cell death of CT26.WT cells *in vitro* and in mice. These findings suggest that MTD peptide can be developed as to peptide-based cancer drug.

In contrast to classical p53 transactivation of Noxa, I explored transactivation of Noxa gene in p53-independent manner and its

pivotal role in LPS-induced rejection of the tumor in the syngeneic mice. Herein, architectural signaling of IRFs in Noxa promoter region was dissected to understand the underlying mechanism of LPS-mediated rejection of the tumor in the syngeneic mice. I demonstrate that LPS, a critical molecule signaling to the TLR4/TLR3, and IFNs regulate the Noxa expression by activating IRF1, IRF3, and IRF7. This induction of Noxa by LPS or IFNs in tumor cells is required for tumor regression mediated by LPS.

Collectively, this thesis will contribute to the understanding of p53-transactivated genes and their role in cell death and physiological role of Noxa in spontaneous tumor regression.

CHAPTER 1

INTRODUCTION

1.1. Apoptosis

The word "*Apoptosis*" derived from Greek word meaning "falling down". The term in biological field came to existence in 460-370 BC and Kerr, Wylie and Currie attributed the term apoptosis to Professor James Cormack who suggested the term [1]. Literally apoptosis is a type of programmed cell death characterized by biochemical events and variety of specific morphological changes like membrane blebbing, loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation leading to cell death. In contrast to necrotic cell death, disposal of cell debris do not damage the host [2] (Fig. 1-1.)

1.2. Significance of Apoptosis

An impressive implication of apoptosis is differentiation of digits by massive cell death in interdigital mesenchyamal tissue, development and flushing of immature neurons in later stage of adult brain formation [3].The development of B and T lymphocytes in human body is a complex process that effectively creates a large pool of diverse cells to begin with, then weeds out those potentially damaged cells to the body. Sorting out of ineffective and potentially damaged immature cells leaving the highly efficient and functional T cell is the result of apoptosis [4].

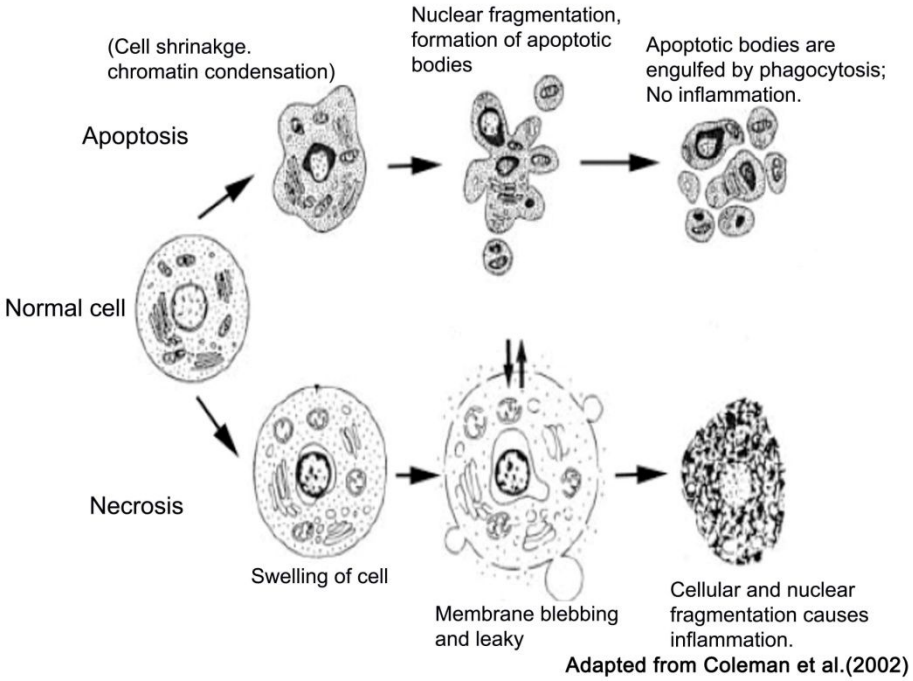


Fig. 1-1: Hallmarks of the apoptotic and necrotic cell death process. The fate of cell in apoptosis includes cell shrinkage, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies containing organelles, cytosol and nuclear fragments that is phagocytosed without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation.

Due to its significant role in various biological processes, apoptosis is omnipresent phenomena in all kinds of metazoans [5] like in mammals, insects

[6], nematodes [7], and hydra [8]. Moreover, apoptosis like phenomena may play a vital role in plant and yeast development [9].

1.3. Regulation of apoptosis

Apoptosis is a consequence of diverse range of cell signals from extrinsic or intrinsic inducers. Toxins, hormones, growth factors, nitric oxide or cytokines cater extracellular signal and transduce through transmembrane and activate downstream signals to complete the phenomena. However, Intracellular apoptotic signaling is result of stresses to cell. The binding of nuclear receptors to glucocorticoids, heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration are the precursor for intracellular apoptotic signals in damaged cell {Mattson, 2003 #346}.

There are several regulatory proteins communicate with the apoptotic, pro-apoptotic or non apoptotic signals to release effective molecules involved in cell death pathway and determine whether the process continues or not. The ultimate molecules in the apoptotic cell death are caspases, a family of highly specific cysteine proteases essential for the proteolytic activity of the cell [10]. Generally, caspases are expressed as inactive precursor form and are activated by apoptotic signals to cleave initiator caspases and activate downstream effector caspases thereby amplifying the proteolysis [11].

In mammalian cells, two routes of regulation leading to apoptosis have been identified; the intrinsic pathway targeting mitochondria and extrinsic pathway directly transducing the signals through adapter proteins to death machineries [12].

1.3.1. Extrinsic pathway

The extrinsic pathway is activated by the ligase of death receptors of tumor necrosis factor (TNF) receptor superfamily Fas or TRAIL receptors by their associate transmembrane ligands. TNF, major extrinsic mediator of apoptosis, is a cytokine produced mainly by T cells and the activated macrophages in response to infection. There are two receptors for TNF: TNF receptor-1 (TNF-R1) and TNF receptor-2 (TNF-R2). The interaction of TNF with TNF-R1 has been known to initiate the pathway that lead to either caspases activation via the intermediate membrane proteins, TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), or indirect activation of transcription factors involved in cell survival and inflammatory responses [13].

Similarly, binding of transmembrane protein Fas ligand (FasL) to its associate receptor, Fas, recruits Fas associated death domain (FADD) to form death inducing signaling complex (DISC) containing caspases-8 and caspases-10. In type I cells, the processed caspases-8 directly activates effector caspases and

triggers the execution of apoptosis, whereas in type II cells, the DISC complex starts feedback loop that spirals into increasing release of pro-apoptotic molecules from mitochondria and amplifies activation of caspases-8.

1.3.2. Intrinsic pathway

Mitochondria are a power house of cell and essential organelles to multicellular life. A number of signal molecules transducing pro-apoptotic signal and pathological stimuli converge on mitochondria to induce mitochondrial outer membrane permeabilization (MOMP). Subsequently, mitochondrial intermembrane proteins are released and the apoptotic molecules, such as Cytochrome C and Smac/DIABLO, are translocated to cytosol. Release of Cytochrome C causes the formation of apoptosome, a multicomponent adaptor complex that acts as a catalyst for caspases 9 oligomerization and activates the effector caspases-3 and -7 that cleave important regulatory and structural proteins resulting in cell death [14]. The mitochondrial release of Smac/DIABLO and serine protease Omi/HtrA2 induces apoptosis by inactivating inhibitor of apoptosis proteins (IAPs) which are caspases inhibitors. Thus, MOMP is the hallmark of intrinsic death pathway [15].

Apoptotic signals, coming from the inside of the cell, are frequently originated from the nucleus, being a consequence of DNA damage induced by

irradiation, drugs or other sorts of stresses. DNA damage in most cases eventually results in the stabilization of the p53 transcription factor which promotes expression of pro-apoptotic Bcl-2 members and suppresses anti-apoptotic Bcl-2 and Bcl-xL. Other organelles besides mitochondria and the nucleus such as the ER and lysosomes also have been implicated in apoptotic signaling pathways and it should be kept in mind that presumably hundreds of proteins are part of an extremely fine-tuned regulatory network consisting of pro- and anti-apoptotic factors.

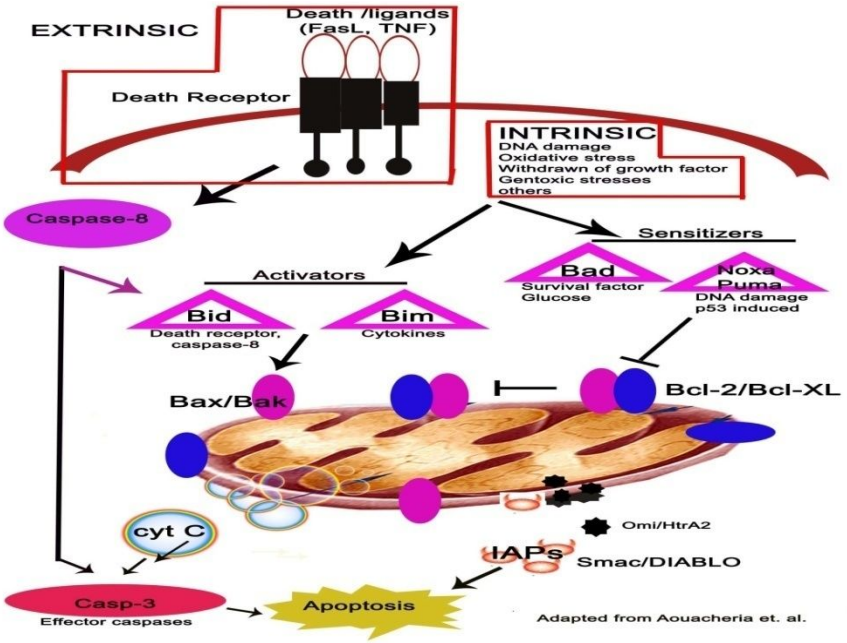


Fig.1.2: Regulation of apoptosis. There are two major pathways responsible for apoptosis: the intrinsic mitochondrial pathway and extrinsic death receptor pathway. Several pro-apoptotic molecules of Bcl-2 family members convey apoptotic signal to mitochondria and Cytochrome C, Smac/DIABLO and Omi/HtrA2 are translocated into cytosol due to the subsequent

disruption of mitochondrial membrane. BH3-only proteins act as upstream guides for cellular stresses or damages which can either activate Bax/Bak ("activators") or inactivate Bcl-2/xL ("sensitizers"). The released Cytochrome C from disrupted mitochondria interacts with Apaf-1 in the presence of dATP to form the apoptosome, which binds and activates procaspase-9. Caspase-9 in turn activates downstream effector caspases-3, -6 and -7 resulting apoptosis. Anti-apoptotic proteins such as Bcl-2 and Bcl-xL are inhibited by and pro-apoptotic proteins Bax and Bak induce Cytochrome C release. However, in extrinsic pathway, the interaction of death ligands like TNF, FasL, and TRAIL to their corresponding transmembrane death receptors induce caspases-8 activation. The activated caspases-8 either processes downstream effector caspases or cleaves cytosolic Bid generating truncated Bid (tBid). tBid translocates to mitochondria and promotes the release of Cytochrome C in association with Bax/Bak.

1.4. Disease as a consequence of dysregulated apoptosis

In the adult human body, several hundred thousand cells are produced every second by mitosis but the number of cell is kept relatively constant through apoptosis for the maintenance of homeostasis and specific tasks such as the regulation of immune cell selection and activity [16]. Imbalance in homeostasis can play a primary or secondary role in various diseases with insufficient apoptosis which leads to cancer as result of cell accumulation, resistance to therapy, defective tumor surveillance by the immune system, failure to eliminate auto reactive lymphocytes and uncontrolled apoptosis induction in specific organs due to autoimmunity and persistent infections (failure to eradicate

infected cells). Whereas excessive apoptosis contributes to neurodegenerative diseases (Alzheimers' disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis), AIDS (depletion of T lymphocytes), and ischemia (stroke, myocardial infarction) [17]. Malfunction of the death machinery results from the mutation of genes that code for factors directly or indirectly involved in the initiation, mediation or execution of apoptosis and several mutations in apoptotic genes have been identified as contributing factor in human diseases [18].

Biologists have special focus on the involvement of defective apoptosis pathways in tumor formation, progression and metastasis as well as the occurrence of multidrug resistance during cancer therapy [19]. Since the exploration of phenomena became more and more clear that tumorigenesis is not simple and is the consequence of excessive proliferation due to the activation of oncogenes but to the same extent depends on the frequently concurrent impairment of apoptosis checkpoints [20]. Interestingly, many of the modifications such as oncogene-driven deregulated proliferation and invasion induce malignant transformation and escape from apoptosis even though sensitizes a cell to apoptosis [21]. A transformed cell can achieve protection against apoptosis by inappropriate activation or expression of anti-apoptotic

proteins (which usually act as oncogenes), or by the inactivation of pro-apoptotic factors (which usually are tumor-suppressors).

Bcl-2 was the first anti-apoptotic gene playing an important role in tumorigenesis that was over-expressed in a variety of cancers, contributing to cancer cell survival through direct inhibition of apoptosis [22]. On the contrary, mutated or down-regulated Bax and Bak are observed in certain cancers [23] and deficiency of those genes promotes tumorigenesis in mice [24]. Additionally, oncogenic Akt/PKB kinase is constitutively active or ectopically expressed in human cancer and negatively regulates pro-apoptotic Bad and pro-caspase 9 [25]. Moreover, Akt/PKB stimulates the NF-kB survival pathway through phosphorylation of IkB kinase α (IKK α) and inhibiting the activation of tumor suppressor protein p53 through phosphorylation of MDM2 [26]. As a whole, archetype for modulator of apoptosis checkpoints is in the defense against malignant transformation; by presenting tumor suppressor p53 that is inactivated in presumably more than 50% of all human cancers [27].

Understanding of mechanisms and alterations by which components of the apoptotic machinery contributes to pathogenic processes would be helpful in crafting the development of more effective, highly specific and better-tolerable therapeutic approaches. Those may include the targeted activation of pro-apoptotic tumor suppressors or alternatively the blockade of anti-apoptotic

oncogenes in the case of cancer, whereas the inhibition of pro-apoptotic key components such as the caspases might be promising for the treatment of premature cell death, for instance, during neurodegeneration [17].

1.5. p53

In the era of 1960s and 70s, many cancer researchers focused on understanding of viruses and oncogene, several groups noted the existence of cellular protein that was overexpressed in many cancers. This protein, with an approximate molecular weight of 53 kDa was designated as p53 and identified in 1979 by Lionel Crawford, David Lane, Arnold Levine and Lloyd Old, working at Imperial Cancer Research Fund (UK), Princeton University, and Sloan-Kettering Memorial Hospital. One of the early studies of SV40-transformed cell had showed that a p53 protein was co-precipitated with the large T-antigen. However, it had been hypothesized to exist before as the target of the SV40 virus, a strain that induced development of tumors [28]. Subsequently, p53 was shown to bind with several other oncoproteins, produced by different tumor viruses, including the human papillomavirus (HPV) E6 protein and the adenovirus E1B 55K protein [29]. In 1984, a group of researchers reported that co-transfection of murine p53 with a plasmid encoding activated c-Ha-ras oncogene could transform rat embryo fibroblasts cells in a manner similar to that observed with proto-oncogenes such as myc or E1A, further supporting the "oncogenic"

property of p53 [30]. All these facts led to the notion that p53 was a novel oncogene. But this story was far from the whole truth, its character as a tumor suppressor gene was finally revealed in 1989 by Bert Vogelstein working at John Hopkins School of medicine [31].The idea of p53 as an oncogene was in transition and the wt p53 was instead considered as a key tumor suppressor. Further study revealed that cellular levels of p53 elevated in cellular stresses such as DNA-damage caused by UV-irradiation or chemicals. Such genotoxic stresses acts in cytostatic manner, deciding the fate of cell either to arrest their proliferation through cell cycle regulation or trigger activation of cell suicide program [32].

1.5.1. p53 as tumor suppressor

p53 is important in multicellular organisms, where it regulates the cell cycle and thus functions as a tumor suppressor that is involved in preventing cancer. The susceptibility of p53 deficient mice to spontaneous tumorigenesis is the direct evidence for p53 function as a tumor suppressor. A continual dissection of the function of protein has equally changed our way of thinking about tumor biology and each added to body of literature that has helped to define p53 as tumor suppressor. As such, p53 has been described as “the guardian of the genome”, “the guardian angel gene”, and the “master watchman” referring to its role in conserving stability by preventing genome mutation. The level of p53 in

normal cells is tightly controlled by its major inhibitor MDM2, which prevents its action and promotes its degradation by acting as ubiquitin ligase [33]. When genomic integrity of a cell is disturbed by various metabolic or genetic stresses like activation of oncogenes, UV radiation, nutrient deprivation, hypoxia and some DNA damaging drugs, latent p53 is transformed into an active form and stabilized. Upon activation, it triggers a wide range of cellular processes like cell cycle arrest, DNA repair, apoptosis, differentiation, senescence, inhibition of angiogenesis and metastasis, depending on the type of cells and stresses (Fig.1-3).

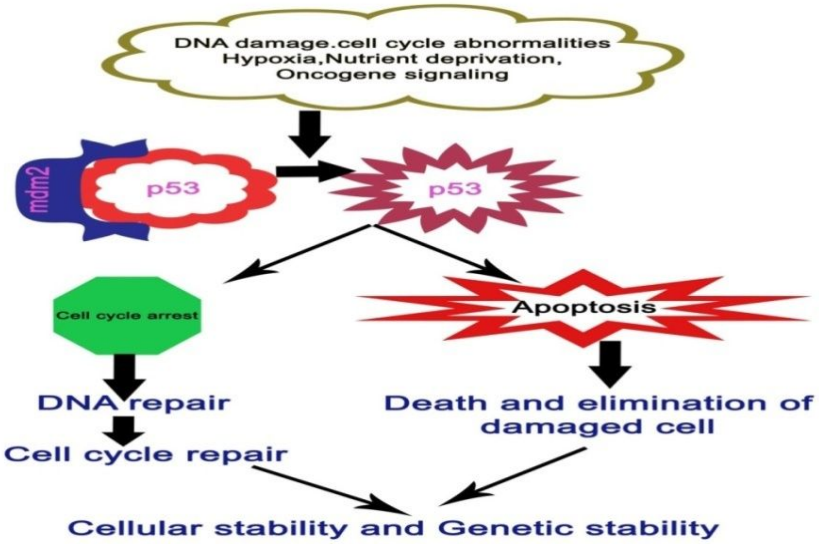


Fig.1-3: p53 pathway. In normal cell, the level of p53 is tightly regulated by its negative regulator MDM2. Upon metabolic or genetic stresses such oncogene activation or DNA damage; various signals will dissociate the p53 from MDM2 and activate. Functionally activated p53 lead to upregulation or down-regulation of numerous target genes, thus, initiating a series of events resulting cell cycle arrest, senescence or apoptosis.

The molecular functions of p53 have been subject of intensive research over the past 25 years. To initiate the chain of event leading either to inhibition of cell cycle progression or programmed cell death, p53, as a transcription factor, must recognize and bind to its recognition elements that are within its target genes. These landmark concepts laid the base for the future characterization of the major p53 function and transcriptional regulation of target genes. Numbers of the transcriptionally regulated genes participate in various cellular processes and activation of these target genes and ultimately decide the fate of cells.

1.5.2. Protein description

Human p53 is phosphoprotein of molecular weight 53 kDa encoded by a 20 kb gene containing 11 exons localized on short arm of chromosome 17(17p13) [34]. The protein contains 393 amino acids and is composed of several functional domains: N-terminal transcription activation domain (TAD), also known as activation domain 1 (AD1) which activates transcription factors: residues 1-42, activation domain 2(AD2) important for apoptotic activity: residues 43-63, proline rich domain responsible for apoptotic activity of p53: residues 80-94, central DNA binding core domain (DBD) containing one zinc and several arginine amino acids: residues 100-300, nuclear localization of signaling domain: residues 316-325, homo oligomerization domain (OD): residues 307-355 and C-terminal

involved in down-regulation of DNA binding of the central domain: residues 356-393[34]. Most p53 mutations identified in tumors occur at sites located on central DNA binding core domain. Most of these mutations destroy the ability of the protein to bind to its target DNA sequence and thus prevents transcriptional activation of these genes. As such, mutations in DBD are recessive loss of function mutation where as a molecule of p53 with mutation in the OD dimerize with wild type p53 and prevent from activating transcription. Therefore OD mutations have dominant negative effect on function of p53.

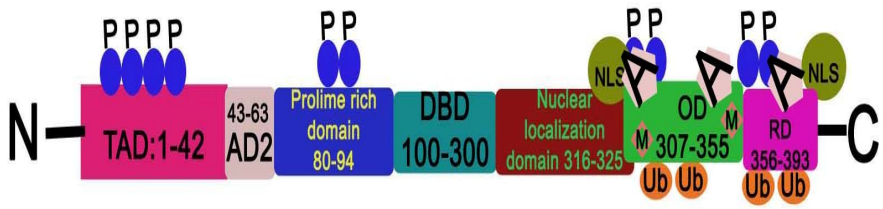


Fig.1-4: Schematic representation of the p53 protein. Ub-ubiquitination, P-phosphorylation, A-acetylation, NLS-nuclear localization signals.

1.5.3. Regulation of p53 activity

Upon the DNA damage, nucleotide depletion, hypoxia, oncogene expression, metabolic stresses or viral infection, p53 undergoes a series of post-translational

modifications and is activated. [35]. The critical event leading to the activation of p53 is the phosphorylation, acetylation, ubiquitination, glycosylation and sumoylation. The N-terminal transcription activation domain contains large number of phosphorylation sites and is considered as primary target for protein kinases transducing stress signals. The protein kinases that are known to target to TAD of p53, to belong to the MAPK family (JNK1-3, ERK1/2, p38 MAPK) and to respond several types of stresses such as membrane damage, oxidative stress, osmotic shock, heat shock and others kinases (ATR, ATM, Chk1, Chk2, DNA-PK, CAK) are sentinel for genome integrity check points.

Under normal condition, p53 is a short lived protein tightly checked and balanced by its inhibitor MDM2. Phosphorylation of the N-terminal of p53 by protein kinases disrupts MDM2 binding. Due to the acetylation of p53 C-terminal domain, by PCAF (p300/CREB-binding protein associated factor) and CBP/p300, the DNA binding domain of p53 is exposed, subsequently p53 activates or represses specific genes [36].Besides these, deacetylation of p53 by Sirt1 and Sirt7 lead to inhibition of apoptosis [37].

Activation of p53 occurs in three stages (a) an increase in p53 protein concentration either by translational activation or increased half life of p53 (b) conformational change of p53 from latent to active and (c) translocation of p53 protein from the cytoplasm to the nucleus. However nuclear import or export of

p53 is tightly regulated process. Nuclear localization is a determining factor for transcriptional regulation of target gene. P53 contains three nuclear localization signals (NLS) that upon specific stimuli is enable to induce its nuclear import whereas nuclear export of p53 is mediated by two nuclear export signals (NES) [38]. The efficient nuclear export of p53 to cytoplasm is required for p53 degradation and mediated by ubiquitin ligase function of MDM2 and mutation of lysine residues in C-terminus of NES abolishes MDM2 directed nuclear export [39]. So, functional importance of p53 is signified once it translocates to nucleus.

1.5.4. Loss of p53 in cancer

Loss of function of p53 is predominant in 50% of human cancer but their frequency of inactivation is highly heterogeneous, as reflected by the diverse remaining transactivation activities that encompass 0% to 100%. Inactivation of p53 function is resulted from various viruses like SV40, HPV or adenovirus encoding proteins that target p53, nuclear exclusion of p53, ectopic accumulation of MDM2, mutation in MDM2 regulator PTEN, deregulation of AKT and mutations in various signals upstream of p53.

Functional inactivation of p53 gene is consequence of mutation missense, nonsense or insertion/deletion of several nucleotides causing expression of

mutant protein (90% of cases) or absence of protein (10% of cases). In fact p53 mutation is the most frequent genetic disorder found in broad range of human tumors throughout the world. Detailed information about p53 mutation can be found on at http://p53.free.fr/p53_info/p53_cancer.html .

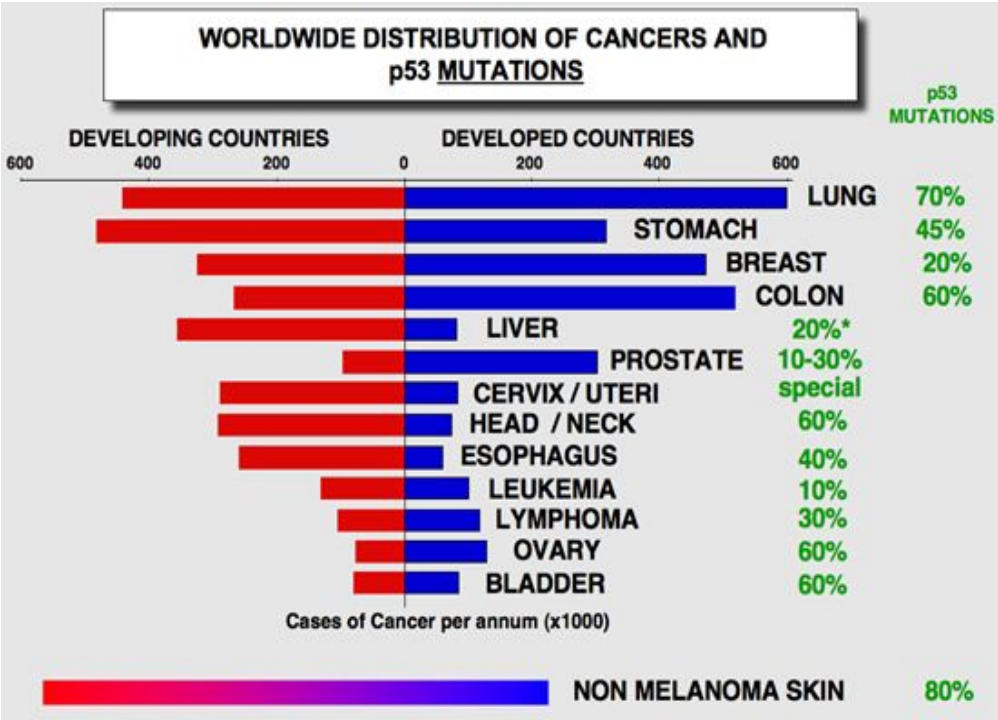


Fig. 1-5: Worldwide distribution of cancer related with p53 mutation in human tumor cell genome as recorded in the UMD_TP53 Mutation database (http://p53.free.fr/index.html).

The p53 degradation by E6 viral protein, expressed by HPV, is observed in lots of cases of cervical cancer [40]. In inflammatory breast cancers or

neuroblastomas accumulate of wild type p53 in cytoplasm of tumor cells lead to functional inactivation of p53 [41].

The stability of p53 protein is regulated by MDM2 coupling ubiquitination and transporting to the proteasome. Overexpression of the p53 negative regulator MDM2 through gene amplification is common strategy that exists in a variety of tumors. Abnormal accumulation of the MDM2 protein is dominant in many tumors, particularly sarcomas [42]. Akt kinase phosphorylates and translocates MDM2 protein to nucleus where it binds and ubiquitinates p53. Growth factor signaling activates MDM2 through Akt activation resulting proper cell growth. However PTEN, one of the p53 regulated gene, down-regulates AKT pathway. So disruption of PTEN results imbalance in the regulation of MDM2 and impair the p53 response. Mutations in the kinases, upstream signaling molecules of p53, such as ATM, DNA damage induced protein kinase in the human disease ataxia telangiectasia (AT), leave p53 in unphosphorylated state at Ser15, Ser20, CHK2 at thr68 and MDM2 at Ser395 abrogate IR induced p53 activation. Such mutations are found in Li-Fraumeni like families [43].

1.5.5. p53 family

After the extensive and progressive research on p53 for nearly about 20 years, scientists granted p53 as the key molecule for the tumor suppression. However,

discovery of several p53 homologous has been disclosed the p53 superfamily of transcription factor that can trigger cell cycle arrest and apoptosis. This exclusive finding deviated from the verdict of the loneliness of p53 in this field. Two new p53 related proteins, p73 and p63 (also called KET, p51, and p40) were cloned and studied in detail to understand the structural, functional similarity with p53 in 1997/98 [44] [45]. The gene structures of p53, p63 and p73 are highly conserved from mollusk to human.

The modular architectures of these proteins shows significant level of similarities with p53 as well as have additional conserved domain at C-terminal which may have regulatory function (fig.1-6).The additional domain; sterile alpha motif (SAM) is believed to involve in protein-protein interaction and play a role in developmental processes. The protein residues 17-27 of p53 (TSFSDLWKLLP) that intermingle with MDM2 are highly conserved in p63 (EVFQHIWDFLE) and p73 (TTFEHLWSSLE). The conservation of bold and underlined sequence is responsible for MDM2 interaction [46]. The sequence identities among the DNA binding domains suggest that p63 and p73 bind to target DNA in similar manner with p53 [47]. Even though p53 has been identified earlier than these two members, there is strong evidence that these two members are ancestors of p53 with original function in development and p53 is evolved later by gene duplication and mutation at a time when tissue renewal and thus risk of cancer was set up in

higher organisms [48]. Moreover, mutations in p63 and p73 are rare phenomena in cancer with exception of p73 LOH in some tumors [44]. Indeed, p63 gene is highly expressed in squamous cells of lung and cervical cancers and deserves as a prognostic marker of lung cancer [49]. In contrast to p53 that has a single promoter, both p63 and p73 have two promoters and as a consequence two diametrically opposing classes of proteins: those containing the TA (TAp63 and TAp73) and those are lacking it (DNp63 and DNp73). DNp63 and DNp73 occur in human and mouse [50].

Although there is striking similarity the ancestors seem to possess developmental function including stem cell biology and neurogenesis as well as their role in tumorigenesis. The functional dissection of p63 and p73 show the transactivation of certain p53 target genes and induction of cell death. There is some evidence of cooperative function of p53 family member in induction of apoptosis related genes e.g. Bax, Noxa and PERP in double knock out mouse embryonic fibroblast [51].

The functional division of p63 and p73 are established for developmental processes and p53 for maintenance of genomic integrity. Even though p53 null mice are more prone to specific cancers without retarding development which suggest that p53 is not important in development. But p56 null mice exhibit developmental defects like hypoplastic upper and lower jaws and have no eyelids,

whisker pads, skin and related appendages including vibrissae, pelage follicles and hair shaft which are prominent feature in wild type mice. Thus p63 is essential for ectodermal differentiation during embryogenesis [52]. On the other hand, p73 null mice show somatic growth defects, chronic infection and inflammation, hydrocephalus, hippocampal dysgenesis and defects in pheromone detection but no increased susceptibility of spontaneous tumorigenesis suggesting their roles in neurogenesis [53]. Overall, p53 family members p63 and p73 are not assigned as conventional tumor suppressor, but can not undermine their roles in tumor suppressive phenomena in cooperation with p53.

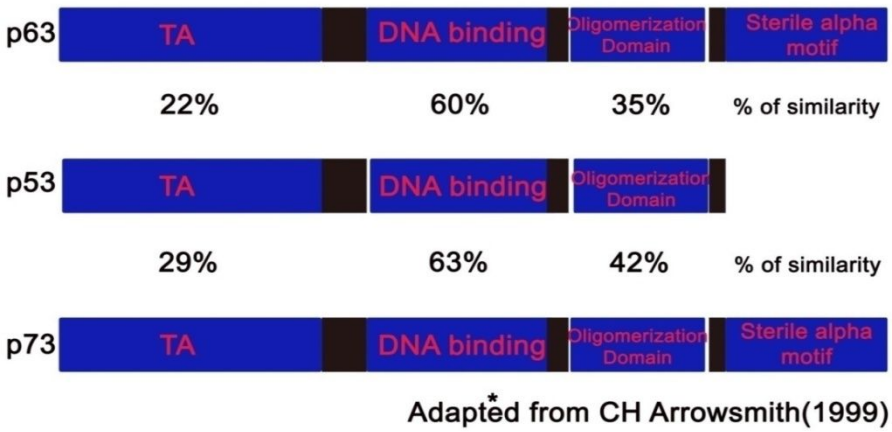


Fig.1-6: Structure of p53 family members. The amino acid similarities relative to p53 are indicated for transactivation domain (TA), DNA binding and oligomerization domain.

1.5.6. Fate of cell; life or death

A lot of literatures are written and discussed about the regulation and modular structure of p53, but an obvious million dollars question is what decides the fate of cell; either to die or live. p53, master watchman, is unaware about its own driving force to decide the fate of cell. Because normal cells often seem to show less response to p53 than their cancer counterparts. The guard of genome is kept in backstage by proto-oncogene MDM2. Moreover, p53 is maintained in very low steady state level and ineffective as transcriptional regulators in the normal cell. The rapidly proliferating cells fascinately response to p53 than resting or normal growing cell. Accepting this fundamental behavior, many drugs are designed for cancer therapy. Interestingly, cytpolasmic and nuclear cellular content of the cell determine the survival or apoptotic signals that are beyond the hand of p53. If the damage is too much, apoptotic response might be frontline and survival signal in back row. Whereas, when there is secretion of high level of growth factors, cell matrix interaction will be tilted toward anti-apoptotic feature and p53 will be activated more likely to be cessation of cell cycle. The intracellular molecular event and degree of abnormality or disruption of genome integrity are impressive factor for p53 activation which dictates whether repairing injury is worthy or not. This is particularly implied on cancer cells, which often

acquire genetic alterations affecting directly or indirectly the functional status of p53 [54].

p53 regulates a number of genes transcriptionally and contributes to biological function such as cell cycle arrest, apoptosis etc. The switch on and off option for particular subsets of genes like cell cycle inhibitory genes or pro-apoptotic genes determines the fate of life between survival or homicide. More specifically, signals that favor the activation of pro-apoptotic genes by p53 will end up in cell death, whereas signals that favor the activation of cell cycle-inhibitory genes will leave the cell growth-arrested but alive. These phenomena are well established in tumor, derived by p53 mutants, in which the mutant p53 is capable of activation of p21/G1 arrest promoter but not the Bax promoter. p21 is proficient in induction of growth arrest but deficient in apoptosis [55]. Besides this, posttranslational modification of p53 affects its target gene, probably through altering its DNA binding specificity. There is a report about the phosphorylation of p53 at multiple sites by transcription factor E2F1, in the absence of p19 ARF. These modifications were important for E2F1-mediated apoptosis. But p300 co-activator JMY is active during stress, interacts with p300 and is recruited to conduct activation of Bax and induction of apoptosis without covalent modification of p53 [56].

Besides pro-apoptotic modulator of p53, some factors like WT-1 tumor suppressor directly stabilize the cell cycle arrest but inhibit p53-mediated apoptosis [57]. Similarly, the BRCA1 tumor suppressor can shift p53-mediated transcriptional regulation for cell cycle arrest and DNA repair. But DNA damage can restore p53-mediated apoptosis by down regulating BRCA1 [58].

The co-repressor mSin3a and histone deacetylases play important role in repression of several p53 targeted genes and in apoptosis. But binding of MDM2 to N-terminal of p53 prevents the repressors from access and makes unavailable for repression factor. The RB tumor suppressor can dissociate MDM2 from p53 and prevent p53 degradation. Formation of this complex restores the repressor function of p53 and increase apoptosis without affecting transcriptional regulation function [59] (Fig1.7).

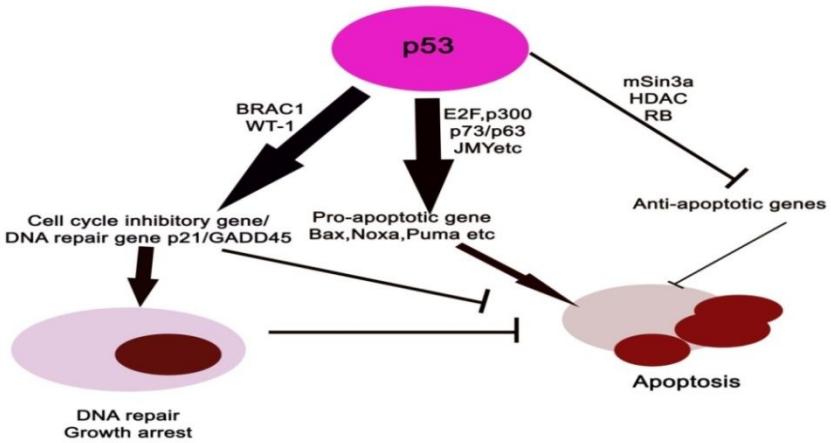


Fig.1-7: Fate of cell is decided by p53. The presences of co-activator and co-repressor as well as post-translational modifications of p53 decide the fate of cell either to die or live.

ER stress enhances the interaction between MDM2 and ribosomal protein inhibiting the MDM2 mediated ubiquitination and degradation of p53 [60]. Evidences suggested that p63 and p53 co-operated to activate apoptotic genes upon DNA damage [51]. In the p63/p73 double knock-outs, p53 is unable to bind the promoters of specific apoptotic genes.

1.5.7. Functional significance of p53

p53 exerts multiple phenomena via transcriptional dependent and independent pathways. The p53 specifically binds to the DNA sequence of target genes. The p53 target genes have functions involved in diverse cellular processes including cell cycle arrest, apoptosis, DNA repair, senescence, angiogenesis and metastasis. p53 is centre of anticancer mechanisms in many cells and is much more discussed about the function in cell cycle arrest and transactivation of target genes and their role in apoptosis. However, exploration of new target gene and identification of its role in p53-mediated apoptosis in unconventional way may be valuable to understand the mechanism of cancer progression due to its negative regulation.

1.5.7.1. Cell cycle arrest

Cell cycle is one of the most dramatic events in eukaryotes and tightly regulated by networks of protein kinases; cyclin-dependent kinases (CDKs) and

CDK inhibitors. Successful completion of cell cycle is checked and controlled by cell cycle checkpoints upon DNA damage recognition. If any damage were found, cell proliferation would be held that allow DNA repair protein to fix the damage. p53 transactivates components of both G1/S and G2/M checkpoints upon physiological stress.

Upon DNA damage, p53 transcriptionally regulates cell cycle inhibitory gene; p21 that can induce both G1 and G2 arrest by inhibiting activity of cyclin-CDK2/cyclin E and cyclin-CDK1/cyclin B, respectively [61]. Additionally, p21 also regulates the RB tumor suppressor pathway. The N-terminal domain of p21 interacts with and inhibits cyclinE/CDK2 halting phosphorylation of pRb protein that binds E2F and abrogates its activity as a transcription factor. Consequently, the cell cycle is held on the G1 phase, providing time for DNA damage repair [62]. p21 masks the function of proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor to increase its activity in both DNA replication and repair. Additionally, p53 regulates the G2/M checkpoints through sequestering 14.3.3σ (sigma) into cytoplasm and inactivating Cdc25 and Cdc2. The induction of 14.3.3σ has been shown to promote translocation of Bax out of cytoplasm and thereby delaying apoptotic signal and allowing cells into G2 arrest[63].

1.5.7.2. Apoptosis

Apoptosis is rhythm of healthy cell that is well synchronized and controls many biological events to maintain the cell integrity. There are a number of genes involved in this phenomena but p53 is above all and well characterized. The first evidence of p53 involvement in apoptosis was established by studies of temperature sensitive mutant p53 that acquires the conformation of wild type p53 when the cell grown at a permissive temperature of 32℃. Upon the temperature shift to 32℃, rapid cell death occurs owing to the role of p53 in apoptosis [64]. Indispensable and physiological roles of p53 in cell death were later supported by the studies of p53 knock-out mice [65]. But abrogation of p53-mediated apoptosis in p53 defective mutated lymphomas do not interfere cell cycle [66]. The mechanisms, by which p53 modulates activation of apoptosis, are still remained as unsolved issues but the functional domain is known to be localized in the C-terminal basic regulation domain between residues 364-393 and the proline rich domain between 64-91 in the p53 protein [67]. There are several sites in the human genome that are target of p53 and a number of genes among them have been reported to play role in p53 dependent apoptosis [21](Table 1-1).

Table 1-1: List of p53 activated gene involved in apoptosis:

MC610	Zhu et al.,2000
Bax	Miyashita et al.,1995
IGF-BP3	Buckbinder et al.,1995
PIG3	Polyak et al.,1997
FAS/CD95	Owen-schaub et al.,1995
P85	Yin et al.,1998
DR5/KILLER	Wu et al.,1997
Apaf-1	Rozenfeld-Granot et al.,2002
Zac-1	Rozenfeld-Granot et al.,2002
PRG3	Ohiro et al.,2002
PAG 608	Israeli et al.,1997
Caspase-1	Gupta et al.,2001
Caspase-6	MacLachlan et al.,2002
PERP	Attardi et al.,2002
P53DINP1	Okamura et al.,2001
PUMA	Nakano et al.,2001
NOXA	Oda et al.,2000
Scotin	Bourdon et al.,2002
PAC1	Yin et al.,2003

Bax is one of the first genes of Bcl-2 family identified as a p53 transactivated gene in response to cellular stress. p53 activation leads to conformational change of Bax that subsequently translocates to mitochondria with concomitant release of Cytochrome C leading to activation caspase 9 [68]. Nakano and his colleagues suggested that the function of Bax is needed in PUMA-mediated apoptosis because Bax deficient cells are resistant to PUMA-mediated apoptosis [69].

Other p53 upregulated pro-apoptotic targets are the BH3 family protein; NOXA and Bid trigger functional activation of Bax and APAF-1, required for apoptosome formation [70]. Interestingly, Bid has been reported to link between the extrinsic and intrinsic pathways since caspase 8 in the death receptor signaling can induce truncation of Bid resulting Bax activation [72]. During apoptosis, p53 can also inhibit the transcription of anti-apoptotic genes such as Bcl-2. However, its anti-apoptotic response is balanced by Bax. p53 can also directly transactivates Caspase-1 and Caspase-6 which play important role in p53 induced neuronal cell death [73]. Besides conventional transcription dependent apoptosis, a fraction of p53 translocates directly to the mitochondria and makes the outer membrane permeabilizes by forming complex with Bcl-XL and Bcl-2 proteins, resulting the release of pro-apoptotic protein such as

truncated BH3-interacting domain death agonist (tBid), BCL-2-associated X (Bax) or BCL-2 homologous antagonist killer (Bak) [74].

p53 transcriptionally up-regulates death receptor Fas/CD95 and death domain containing receptor DR5/killer of the TRAIL family that is involved in extrinsic pathway apoptosis in response to DNA damage.

1.5.7.3. DNA repair

p53 activates genes like GADD45 implicated in both base excision repair (BER) and nucleotide excision repair via PCNA [71]. But exact mechanism of p53 in DNA repair is still unsolved and it is assumed that p53 induce the recruitment of nucleotide excision repair factor XPC (xeroderma pigementosum group C) and TFIIH against the damaged DNA [72]. Moreover, p53 also exhibits 3'-to-5' exonuclease activity suggesting its role in external proofreading [73].

1.5.7.4. Senescence

Cellular senescence is a consequence of telomerase shortening stress, oxidative stress and DNA damage. In senescence, normal cell ceases to continuation of division and undergoes numbers of morphological and metabolic changes. Recent data suggest that p53 may have a role in cell senescence, considered to be a consequence of tumor suppression and aging [74].

Interestingly, p53 induced p21 is known to be important for activation of the RB pathway and for stimulating cellular senescence in response to DNA damage and telomerase uncapping.

1.5.7.5. Inhibition of angiogenesis

Angiogenesis is an indispensable for multiplication of tumor and progression of metastatic phenotype. p53 is involved in the regulation of angiogenesis through several factors. In hypoxic condition, p53 promotes MDM2-mediated ubiquitination and proteasomal degradation of the HIF-1alpha subunit of hypoxia-inducible factor 1 (HIF-1). Loss of p53 function allow the ectopic expression of HIF-1alpha resulting the increased transactivation of hypoxia-induced VEGF expression and augments neovascularization and tumor growth [75]. In human breast cancer, p53 promotes an anti-angiogenic activity by increasing the secretion of angiogenesis inhibitor thrombospondin [76]. Moreover, cell invasiveness is a result of degradation of extracellular matrix by matrix metalloproteinase that is highly expressed in tumor. Both MMP-1 and MMP-13 are transcriptionally repressed by activated p53 [77].

1.5.7.6. p53 as transcriptional regulator

A paradigm for the central importance of apoptosis checkpoints in the defense against malignancy is regulation of functional activity of p53. As

transcription factor, p53 regulates the expression of various apoptotic genes such as p21, Bax, Puma, Noxa, Apaf-1, Fas, and DR5 [21] or represses the expression of anti-apoptotic proteins, e.g. Bcl-2, Bcl-XL or survivin [78] and carries out different p53 dependent cellular tasks.

To initiate chain of negative or positive regulation of apoptosis, p53 must recognize and bind to its recognition elements (REs) within target genes. This consensus p53 DNA RE consists of two pairs (half-sites) of head-to-head arranged pentamers, 5'-PuPuPuCA/TA/TGPyPyPy-3' (Pu is purine, Py is pyrimidine) separated by 0-13 nucleotides [79]. p53 transactivates a number of its target genes by binding in classical consensus sequence within the gene. However, there is still a number of target genes which are up-regulated in response to p53 having other consensus sequence different from conventional consensus sequence. Exploration of such p53 target genes and their roles in apoptosis is the interest of this study.

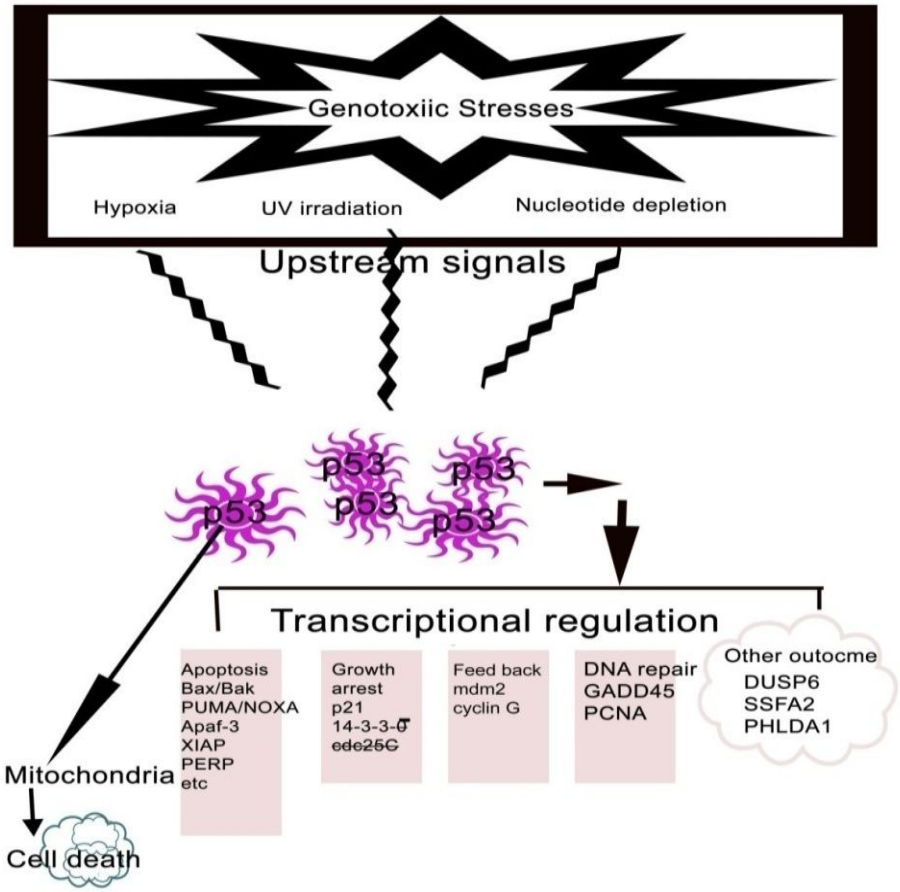


Fig.1-8: p53 as transcriptional regulator. The diverse stresses, like UV irradiation, hypoxia, cytokines, metabolic changes and nucleotide damage, activate p53 that in turn actively participates in transcription of downstream targets. p53 binds in sequence specific manner to response elements in the regulatory region of genes and may result in outcomes that include apoptosis, growth arrest, DNA repair and other outcomes. As the list of p53 transactivated genes continue to explore, the range of functions in which p53 is involved is still increased in conventional way or indirect regulation of intermediate molecule.

1.2. MAP kinase phosphatases

The mitogen-activated protein kinases (MAPK) are highly conserved enzymes that transduce extracellular signal to corresponding effectors molecules and then orchestrating diverse cellular processes including proliferation, differentiation, migration, stress responses, inflammation and apoptosis [80]. The dissection of mammalian MAPKs exhibits three major groups; c-Jun amino terminal kinases (JNK1, JNK2 and JNK3), p38 kinases (p38α, p38β, p38γ and p38δ) and extracellular signal regulated kinases (Erk1 and Erk2). The phosphorylation on both threonine and tyrosine residues in the conserved T-loop motif within catalytic domain of kinases is paradigm for activation. This is mediated by a dual specific MAP Kinase kinase (MAPKK, MKK or MEK), which in turn is regulated by phosphorylation of serine/threonine residues by MAP kinase kinase kinase (MAPKKK, MKKK or MEKK). Hence, the dephosphorylation of the MAP kinase is vital for negative regulation of cellular process in this pathway. Besides classical serine/threonine phosphatase, there are several dual specificity phosphatase (DUSPs) involved in deactivation of MAP kinases isoforms. This double edge phosphatase is functionally grouped into MAP kinase phosphatases (MKPs) [81].

1.2.1. Gene organization

On the basis of structural and functional analysis of genome sequence, the MKPs can be grouped into three subfamilies. The subfamily I: DUSP1, DUSP2, DUSP4 and DUSP5; subfamily II: DUSP6, DUSP7, DUSP9 and DUSP10; and subfamily III: DUSP8 and DUSP16 [81]. The phylogenetic hierarchy and substrate preference also supports this classification (Table 1-2).

Moreover, they all share common features; extended active-site motif with highly homologous sequence with VH-1 protein tyrosine phosphatase of vaccinia virus, N-terminal two short region homologous with Cdc25 phosphatase catalytic site and cluster of basic amino acid residues binding domain for MAP kinases. The cellular localization of MKPs may be cytoplasm, nucleus or both.

The genes of subfamily I are constructed with four exons and exact positions of the introns are highly conserved [81]. The first exon encodes the N-terminal of each of the three proteins and a part of the second Cdc25 homology (CH2) domains. The active-site motif of all four proteins is encoded within exon 4, and length of exon 3 is similar for all these DUSPs, revealing their common ancestral gene.

The subfamily II genes consist of three exons [82]. Similar to subfamily I, exon 1 encodes the N-terminal and the 3'- most exon encodes the active site. In

contrast to subfamily I, the central portion of protein is encoded by a single exon. However, DUSP10 share some feature with subfamily III like MAP kinase docking site motif and similar substrate specificity. Moreover, it has extended N-terminal spliced exon that is not frequent in other DUSPs.

The genes of subfamily III contain six exons. The lengths of exon 2, 3, 4 and 5 are identical. The exon 1 encodes only the first of the CH2 domain that is peculiar features of this family but exon 2 ends within the same region of the second Ch2 domain like in exon 1 of subfamilies I and II. The central backbone of protein is encoded by two exons as in subfamily I. Exon 5 encodes the catalytic site but not carboxyl terminus. The extended carboxyl terminus with introns, between catalytic domain and carboxyl terminal of protein encoded by exon 6 that is the characteristic feature of this subfamily [81]. As a whole highly conserved introns and exons within all three subfamilies of DUSPs suggests the common ancestral gene for each subfamily.

Regulations of MAPKs are highly conserved in a wide variety of phylogenetically distinct eukaryotes ranging from simple organisms, like yeasts, worms, flies, to complex higher organisms, plant and mammals.

Interestingly, MAPKs are vital mediators that convey signal from upstream pathways in response to various environmental stresses. The fundamental

mechanisms behind the activation of MAPKs are phosphorylation on threonine and tyrosine residues. The dual specific MKPS dephosphorylate on phosphothreonine and phosphotyrosine and thereby inhibit MAPKs activity. Some reports suggested that deregulation of MKPs signaling confined cancer cells not only to development but also to resistance against chemotherapeutic agents [83]. The MAPKs regulation is the most common wavering in human cancers. Hence, balance between phosphorylation and dephosphorylation of transcription factors by protein kinases and phosphatases decides the fate of cell. Understanding the MKPs specificity in negative regulation of MAPKs during the development and progression of tumor in response to different stresses can be applied for the treatment of human cancer.

1.2.2. DUSP6

Dual specificity phosphatase 6 is the synonym of mitogen activated protein kinase phosphatase 6. This protein is trivially named as MKP-3/PYST1/rVH and genomic organization shows 3 exons coding 42.3 kDa proteins DUSP6 is expressed in lung, heart, brain, and kidney, but not significantly in skeletal muscle or testis [84]. The DUSP6 is a cytoplasmic enzyme and displays selective substrate specificity toward the classical ERK1/2 MAPKs but has no activity towards either JNK or p38.

Table 1-2: Classification, nomenclature, properties and physiological functions of dual-specificity MKPS

Gene/family	MKP	Trivial name	Chromosomal location	Subcellular localization	Substrate preference	Physiological function
DUSP1/I	MKP-1	CL100, hVH1	5q34	N	JNK, p38, ERK	Negative regulator of immune function Protect mice from lethal endotoxic shock Metabolic homeostasis and mediates resistance to cellular stress in mouse fibroblasts
DUSP4/I	MKP-2	hVH2, Typ1	8q12-p11	N	JNK, p38, ERK	Apoptosis
DUSP2/I		PAC-1	2q11	N	p38, ERK	Positive regulator of inflammatory response Knockout mice are resistant to immune inflammation
DUSP5/I		hVH-3, B23	10q25	N	ERK	
DUSP6/II	MKP-3	pyst1, rVH6	12q22-q23	C	ERK	Negative feedback regulator of ERK2 downstream of FGFR signaling P53-mediated apoptosis
DUSP7/II	MKP-X	Pyst2, B59	3p21	C	ERK	
DUSP9/II	MKP-4	Pyst3	Xq28	C	ERK>p38	Essential for placental development and function (Labyrinth function)
DUSP8/III		hVH5, HB5	11p15.5	C/N	JNK, p38	
DUSP10/II (?)	MKP-5		1q41	C/N	JNK, p38	function in innate and adaptive immunity
DUSP16/III	MKP-7		12p12	C/N	JNK, p38	

C: Cytoplasmic/N: Nuclear

Adapted from Theodosiou et al.(2002)

1.2.2.1. Structural features

The DUSP6 is defined with common structure containing a homologous C-terminal catalytic domain with the DUSP VH1 and N-terminal and no catalytic domain with two homologous regions with the sequence found in the phosphatase cdc25. The architectural backbone of DUSP6 elaborates in catalytic domain, CH2 domain and docking site.

The catalytic domain contains the highly conserved consensus sequence **D**X_{26}(V/L)X(V/I)H**C**XAG(I/V)S**R**SXT(I/V)XXAY(L/I)M, in the single letter amino-acid code where X is any amino acid residues. The three amino acids in bold letters have been known to essential for catalysis. The cysteine is required for nucleophilic attack of the phosphorous of ERK1/2 and the formation of the thiol-phosphate intermediate; the conserved arginine binds the phosphate group of phosphotyrosine or phosphothreonine, enabling transition-state stabilization; whereas the aspartate enhance the catalysis by protonating the leaving group oxygen [85]. The key residues of DUSP6, Arg299 and Asp262, are not interacted in low activity state of normal cells. But upon interaction with ERK, the active sites are rearranged, adopting catalytically active conformation and enabling the dephosphorylation of both Thr183 and Tyr185 in ERK [86].

The CH2 domain, extended in N-terminal region and next to upstream of catalytic sites, shows conserved sequence homology to Cdc25 phosphatase and plays a role in substrate binding [87].

The docking site, located in N-terminal and consisted of a cluster of positively charged amino acid [88], has dual role in determining binding specificity and catalytic activity through its the number of consecutive positively charged residues. Moreover, there is electrostatic interaction between positively and negatively charged residues in docking domain and MAP kinase, respectively.

1.2.2.2. Regulation of DUSP6

The regulation of dephosphorylation of ERK has a vital role in controlling the degree and extent of kinase activation and hence determining the physiological outcome of resulting cellular processes [89]. The substrate specificity of DUSP6 to ERK was first established in 1998 [90] and then several works have been carried out to elucidate the catalytic activation of DUSP6. The region of interaction between DUSP6 and ERK is now well established. The analysis of biochemical and solution structure of the ERK2 binding (EB) domain of DUSP6 revealed that the regions that are essential for ERK2 binding contain positively charged cluster of arginine residues. The interaction between EB domain and highly negatively charged region of ERK2 results a conformational change of the

C-terminal catalytic domain, resulting in the enzymatic activation of MKP-3 [91]. The conformational changes, in combination with the catalytic domain binding to phosphorylated ERK, allosterically prompt the reorganization of DUSP6 active-site residues, resulting in a high-activity state of phosphatase (Fig. 1-9).

1.2.2.3. Physiological outcome of DUSP6

Extensive evidence links both tyrosine and serine/threonine phosphorylation with increased cellular growth, proliferation and differentiation. Removal of the incorporated phosphates must be a necessary event in order to turn off the proliferative signals. This suggests that phosphatases may function as anti-oncogenes or growth suppressor genes. Recently, DUSP6 has shown to enhance the release of Cytochrome C from mitochondria by dephosphorylation of ERK1/2 which was known to regulate protein stability of Bcl-2 maintaining in its phosphorylated status. This ERK dephosphorylation correlates with the role of DUSP6 in apoptosis by regulating B-Raf/MEK/Erk pathway [92].

DUSP6 may be constitutively expressed or induced by growth factor [93] or DNA damaging agent following p53 activation. Several studies signify its functional relation to tumor suppression. Down-regulation of DUSP6 in invasive ductal adenocarcinomas suggests its role as tumor suppressor in pancreatic cancer [94]. A further study revealed that abrogation of DUSP6 in pancreatic

cancer is the result of hypermethylation of the expression control region of DUSP6 [95]. In one of nude mouse experiments, the injection of MKP-3-GFP inducible line that was transformed by Ha-ras shows delay in tumor emergence and growth in response to doxycycline as compared to control, signifying a role of DUSP6 in tumor suppression [96]. Besides its role in pancreatic cancer, DUSP6 has been selected as candidate gene whose expression is closely associated with relapse-free and overall survival in human non-small-cell lung cancer [97]. Hence, following all these informations culminate the role of DUSP6 in regulation of cancer development.

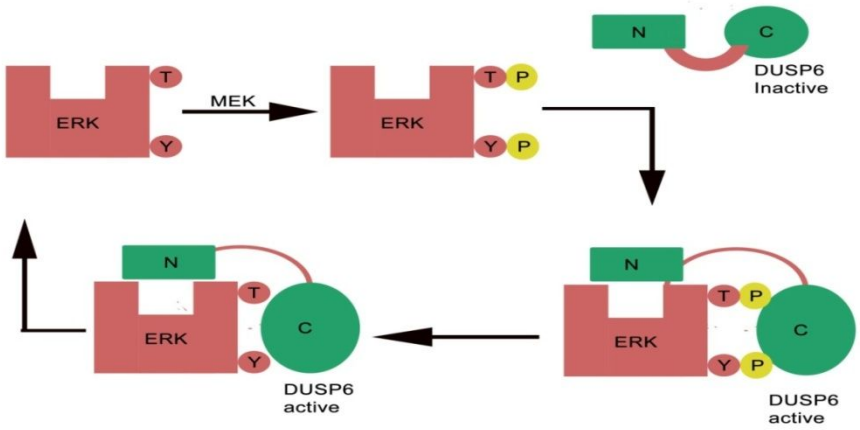


Fig. 1-9: Regulation of DUSP6. ERK binds to DUSP6 and thereby catalytic domain is activated. The carboxy-catalytic domain(C) is represented by circle and the amino-terminal containing the ERK binding domain (N) by rectangle. DUSP6 appears to exist in inactive free-state but binding to ERK through EB domain causes conformational change, resulting activation of phosphatase. This results in ERK dephosphorylation and subsequently dissociations of the complex.

1.3. SSFA2

The Sperm specific antigen 2, also named as Cleavage signal-1 protein (CS-1) or Ki-ras-actin-interacting protein (KRAP) is encoded by 1259 amino-acids with coiled- coil regions and transmembrane region. This cytpolasmic protein is located near plasma membrane and associated with actin filament [98]. Genomic organization shows localization on 2q31.3 and having 3 alternate splicing variants. A 33 kDa peptide corresponding to C-terminus of this protein is found in the testis and seems to be cleaved into 2 peptides of 14kDa and 18kDa found on the surface of mature sperm cell.

The detailed physiological outcome of SSFA2 is still not explored. However, there are some findings of SSFA2 in the regulation of filamentous actin and signals from the outside of the cells [98].

1.4 PHLDA1

The PHLDA1 (pleckstrin homology-like domain, family A, member 1) also known as TDAG51 is located on chromosome 12q15 and encodes protein of 262 amino acid that is a member of pleckstrin homology-related domain family [99]. The PHLDA1 protein contains a series of motif for protein-protein interaction. This includes 16 proline-glutamine and 15 proline-histidine pairs in the carboxy-terminal, which may have a role in transcriptional regulation of apoptosis in T cell

hybridomas and in neuronal and melanoma cells [100] [99]. The expression of this gene was down-regulated in breast cancer and metastatic in compare to primary melanoma cell. Initially this gene was identified in murine T lymphocytes where it is associated with activation-induced apoptosis [99]. Moreover, constitutive PHLDA1 expression significantly enhances the apoptotic response to chemotherapeutic agents in human melanoma cell [100]. In fact, different studies shows that regulation of PHLDA1 expression may lead to disruption of cell survival signaling involved in tumor development and progression but limited information is available in literature about its role and regulation in cancer. So, transcriptional regulation of PHLDA1 in p53-mediated apoptosis will be the important studies to understand the functional relevance of PHLDA1.

1.5 A cell killing peptides

p53 sits at hub of extremely complex network within the cell. Therefore, tight regulation and stability of p53 is important in maintaining the network of apoptotic pathway. Since discovery of functional significance of p53, different chemotherapeutic drugs came to existence in restoration of function of p53 in tumor cell as conventional cancer treatment. But selective and effective elimination of tumor cell by inducing apoptosis is hallmark of cancer therapy. Number of active domains of pro-apoptotic and apoptotic transcript of p53 mimetic peptides were designed and put on trial to treat the cancer.

Cancer is one of the leading causes of the death in the world. The surgical removal of solid tumor limited to benign type, but tumor metastases requires extensive chemotherapy. The limitations of chemotherapy are severe adverse side effects on healthy cell and development of drug resistance. So, different pro-drugs originated from BH3 domain of Bcl-2 family practiced to overcome such limitation. Bcl-2 family proteins including Bax, Bad, Bak, Bid and Noxa are known to stimulate the tumor suppression. Particularly, Noxa protein is a "BH3-only" protein reported to mediate p53-activated apoptosis [70]. Moreover, it was reported that short synthetic peptides of BH3 domain sufficient to regulate Bcl-2 family protein thereby suppressing the cellular defense [101]. Such synthetic peptides homologous to BH3 domain can be adapted to traditional cancer therapy to suppress the drug resistance. To contribute on this field, here we designed novel cell killing mitochondrial targeting domain (MTD) peptides adapted from specific region at the C-terminus of Noxa protein, a "BH3-only" member of Bcl-2 family. Seo et al. reported that MTD domain is essential to mitochondrial translocation and functional significance as tumor suppression lies on this domain [102]. The synthetic peptide coupled with homing vascular domain advanced its application by limiting its specificity to tumor cells. The probable functional behaviour of this peptide is mitochondrial fragmentation and causes necrotic cell death in a BH3-independent manner.

1.6 Spontaneous regression of tumor

In previous paragraphs, a lot of literature and scientific research reviewed regarding the apoptosis and its regulation by different molecule. The synopsis of tumor suppression is the restoration of multiple genes involved in apoptosis and different chemotherapy, ionization radiation, gene therapy and pro-drugs are intended to do that. The idealism of pro-drug, drug and other chemotherapeutic agents is complete tumor suppression. Till date, none such treatments were exists. However, complete regression of tumor occurs spontaneously in some cases. Such spontaneous regression (SR) of tumor is relative term for partial or complete disappearance of tumor without treatment, but not to confuse with permanent cure of tumor [103]. Everson et al. and Boyd reported well documented cases, including a number of cases of colon or rectal cancer [104]. These rare phenomena also reported in cases of retinoblastoma, leukemia and lymphoma [105]. Although conclusive evidence of the factors responsible for spontaneous regression is not well documented, Chalis and Stam suggested the causes for SR. These were cell maturation/differentiation, necrosis, infection and operative trauma [105]. The causative factors in SR are speculative and underlying mechanisms are drawn out from clinical observations. The postulated mechanisms affecting the SR of cancer in general; immunological or hormonal or metabolic, surgical treatment of the primary and postoperative events, Necrosis

and angiogenesis inhibition, oncogenes, growth factors, cytokines, infection and apoptosis etc [104].

The onset of pregnancy [106], severe dietary changes or the termination of oral contraceptives [107] presumably has each corresponded with initiation of spontaneous regression. The endocrine hormonal changes noted in breast and prostate cancer response spontaneous regression. Assumption was made to correlate its immune mediated host response and humoral factors in spontaneous regression of tumors. However, exact immunological mechanisms for spontaneous regression of cancers are not certain and approaches were reported.

The surgical removal or trimming of primary tumor may incline the host immune system toward the remnant tumor mass and causes spontaneous regression [108]. Indeed, removal of advanced cancer can disseminate the tumor cell via the blood or lymphatic vessels. Such dissemination leads to dilution or depletion of cytokines or autocrine growth factors nourishing the primary tumor and load of sufficient tumor antigen stimulate substantial anti-tumor immune responses [109].

The angiogenesis is an augmented to neoplastic process. Vascular insufficient and necrosis are reported as cause for spontaneous tumor regression

[110]. Necrosis itself balances the tumor growth and may limit the stabilization and progression of tumor. A varieties of cytokines including tumor necrosis factor-α (TNF- α) [111], angiostatin (a cleavage product of plasminogen) [112] and tumor growth factor-β (TGF- β) [113] known to modulate vasculation to tumor. The operative procedure and radiation limit the blood and nutrient supply to tumor for progression. But considered to inadequate to cure and considered as spontaneous regression.

Expression of oncogenes and deviation of tumor suppressor genes are determining factors for progression of tumor [114]. Withdrawal of growth factors inhibited this process. Cytokines produced by the tumor or other host tissues was reported to the spontaneous regression of tumor [115]. Cytokine activation is the result of infection and that may increased host immunity reaction which, in turn, orchestrate the individual's defense against a tumor.

Cytokine induced apoptosis has been reported as spontaneous regression of tumor tissues of lymphomas, neuroblastoma and renal cell carcinomas. Even though the tumor necrosis factor α (TNF-α) was used nearly 35 years ago in animal as cytokine, the precise role of TNF-α in tumor regression is not well documented. The well characterized cytokine dependency is the lipopolysaccharides (LPS) induced spontaneous regression of tumor

[116].However, the underlying mechanism and downstream molecules for tumor regression was not well discussed.

Architectural signaling of IRFs in innate immunity dissected to understand the underlying mechanism of LPS mediated rejection of the tumor in the syngeneic mice. Herein, I demonstrate that LPS, a critical molecule signaling to the TLR4/TLR3 and the NOXA, was implicated in the innate immunity system and is required for appropriate TLR4 pathway and responsible for spontaneous tumor regression.

1.6.1 Toll like receptors

A key molecule of the innate defense is the Toll-like receptors (TLRs) that are named for their similarity to Toll receptor on Drosophila, first reported in 1996 to be critical for the fly defense against fungal infection [117]. TLRs are a family of pathogen recognition receptors (PRR) expressed on cells of the innate immune system letting recognition of conserved structural motifs expressed on a diverse array of pathogens referred to as pathogen associated molecular patterns (PAMPs). Individual TLRs are single membrane-spanning non-catalytic receptors that recognize a structurally conserved array of microbial products, collectively allowing the host to detect infection by most types of pathogens [118]. Till date, ten TLRs have been identified in humans (from TLR1-10) [118], [119]. TLRs are structurally composed with two domains: the extracellular ligand-binding domain

characterized by varying numbers of leucine-rich repeats and the intracellular signaling domain. The TLRs signaling domain together with the interlukin-1 (IL-1) family receptors form the receptor superfamily, named Toll/IL-1 receptor (TIR) domain [120]. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are extracellular, while TLR3, TLR7, TLR8, and TLR9 are intracellular. The subcellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. For example, TLR3, TLR7/8, and TLR9 couple with different nucleotide motifs (dsRNA, ssRNA, and unmethylated CpG DNA, respectively) particularly important for recognizing intracellular viral DNAs and RNAs. TLR4 is induced by lipopolysaccharides, a component of Gram-negative bacteria known to induce septic shock. TLR5 couples with flagellin, a component of certain bacteria. TLR1, TLR2, and TLR6 pair with lipoproteins, components of various bacteria and fungi [121].

The toll like receptor signaling pathway follow as shown in figure 1.10 [122]. Briefly, ligand binding stimulates a complex cascade connecting a range of downstream signaling molecules ultimately leading to the induction of several cytokines and chemokines implicated in the propagation of the inflammatory response such as type 1 IFNs, TNFα, IL-1, IL-6, and IL-12. Additionally, TLR activation induces upregulation of co stimulatory molecules such as CD40, CD80, and CD86 [123]. Indeed, upon stimulation, TLR and IL-1R family members

induce inflammatory signaling cascades via cytpolasmic TIR domains through adaptor proteins such as myeloid differentiation factor 88 (Myd88), TIRAP, TRIF, and TRAM [124]. Except TLR3, typically, IL-1R and TLRs signals through MyD88-dependent pathway, which in turn recruits IL-1R-associated kinase (IRAK) complex that includes four subunits [124]: two active kinases (IRAK-1 and IRAK-4) and two non-catalytic subunits (IRAK-2 and IRAK-M). Consequently IRAK-1 is phosphorylated by IRAK-4. Phosphorylated IRAK-1 associates with TNFR-associated factor 6 (TRAF6) and activates both the nuclear factor-κB (NF-κB) and the mitogen-activated protein kinases (MAPKs). In the case of NF-κB, the protein is restrained in the cytoplasm bound to NF-κB-inhibitor (IκB). Phosphorylation of IκB by the IκB-kinase (IKK) complex, which includes the NF-κB essential modulator (NEMO), leads to its degradation, enabling the nuclear translocation of NF-κB. The activation of both NF-κB and MAPKs induces the transcription of various pro-inflammatory genes, including tumor necrosis factor, IL-1, IL-6, IL-8, and IL-12. TLR2 and TRL4 can trigger this pathway via the TIR domain containing adaptor protein (TIRAP) [125]. On the other hand, TLR3 uses a MyD88-independent pathway using the TIR domain-containing adaptor inducing IFN-β (TRIF) [126]. Recruitment of TRIF ultimately activates the transcription factors interferon regulatory factor 3 (IRF3) and the NF-κB. These two transcription factors synergize to activate the transcription of

type 1 IFN genes: IFN-α, IFN-β, and IFN-λ. TLR4 may also trigger this MyD88-independent pathway by association with TRAM [127], which bridges TLR4 and TRIF. TLR7, TLR8, and TLR9 can also trigger production of type 1 IFNs, through the MyD88-dependent pathway, via activation of IRF7 and IRF1 [128], [129].The activated IRF1, IRF7 and IRF3 act as transcription factor for Noxa. The physiological outcome of this LPS mediated activation of Noxa results the spontaneous tumor regression.

In the last few years, a number of genes involved in spontaneous regression of tumors were discovered. Better understanding of those genes and their regulation by different cytokines or in response to infection and their optimal regulation will have significant preventive and therapeutic implication.

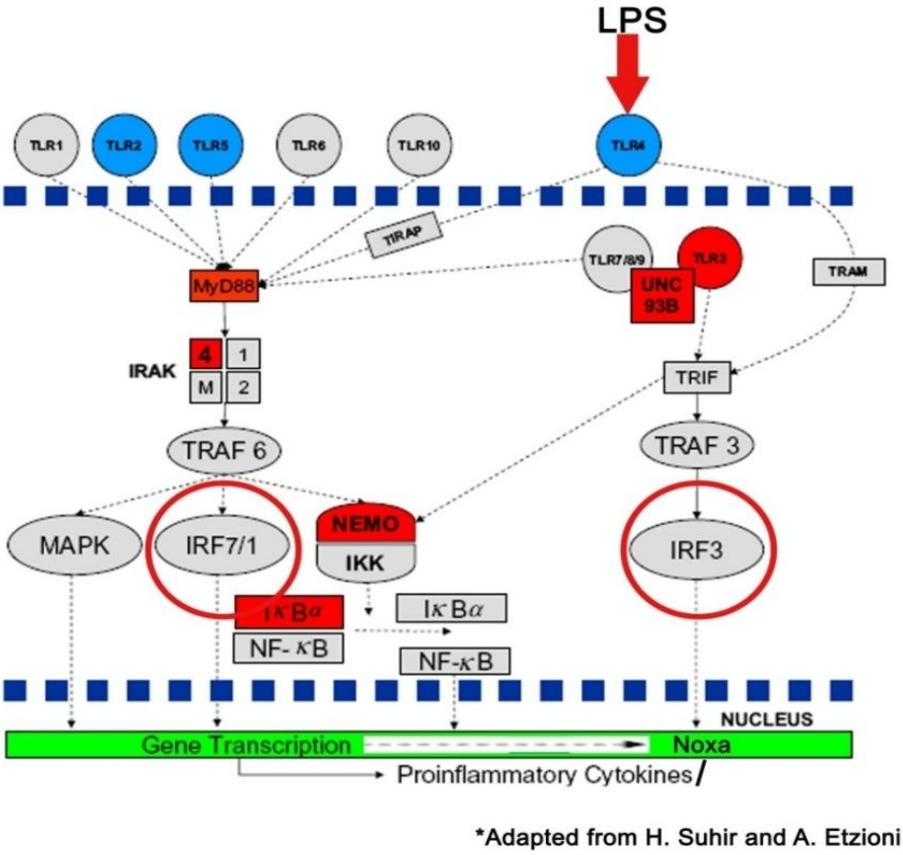


Fig.1.10: Signaling pathway of Toll like receptors. TLRs 1, 2, 4, 5, 6, and 10 are extracellular, while TLR3, 7, 8, and 9 are intracellular. TLRs 1, 2, 5, 6, 7, 8, 9, and 10 trigger the "classical" MyD88- dependent pathway via the TIR-containing cytosolic adaptor MyD88. TLR3 triggers the "alternative" MyD88-independent-TRIF-dependent pathway via the TRIF. TLR4 triggers both the MyD88-dependent pathway via TRAP-MyD88 interaction and the MyD88-independent pathway via TRAM-TRIF interaction. The MyD88-dependent pathway results in the activation of both NF-κB and MAPKs via the IRAK complex, which includes four subunits: two kinases—IRAK 1 and 4—and two non-catalytic units—IRAK 2 and M. In the case of NF-κB, the protein is restrained in the

cytoplasm bound to NF-κB inhibitor (IκB). Phosphorylation of IκB by the IκB-kinase complex (IKK), which includes the NF-κB essential modulator (NEMO), leads to its degradation enabling the nuclear translocation of NF-κB. The activation of both NF-κB and MAPKs induce the transcription of various inflammatory cytokine genes, including TNF, IL-1, IL-6, IL-8, and IL-12. TLR7, TLR8, and TLR9 can also trigger type 1 IFNs production through the MyD88- dependent pathway, via the activation of IRF7 and IRF1. The TLR3/4-MyD88-independent-TRIF-dependent pathway activates the transcription factors IRF3 and NF-κB, both synergize to activate the transcription of type 1 IFNs. The interferon regulatory factor 1, 3 and 7 in turn transactivates the Noxa in LPS mediated pathway.

CHAPTER 2

Identification of p53 target genes and their roles in p53-mediated cell death

2.1. ABSTRACT

The master watchman p53 well-known as a tumor suppressor modulates the cell cycle arrest, senescence and apoptosis through the transcriptional regulation of its target genes. A number of the p53-induced genes were scrutinized by DNA chip analysis to understand the pros and cons of p53-exerted multiple phenomena and their roles in the tumor suppression. Here, we have identified three genes (DUSP6, PHLDA1 and SSFA2) upregulated by ectopic over-expression of p53 in HCT116 cells. 5-FU-treated HCT116 cells showed the increased level of DUSP6, PHLDA1 and SSFA2, indicating that endogenous p53 is able to transactivate these genes. The mitogen activated protein kinase phosphatase 3 (MKP3) or dual specificity phosphatase 6 (DUSP6), PHLDA1 (pleckstrin homology-like domain, family A, member 1) and Sperm specific antigen-2(SSFA2) are the novel transcription targets of p53 in mediating apoptosis. Moreover, introduction of short hairpin RNAs for DUSP6 and PHLDA1 reduced the level of apoptosis induced by 5-FU, Etoposide and TRAIL. Ectopic expression of DUSP6 enhanced the apoptotic response in HCT116 cells. These findings suggest that DUSP6 and PHLDA1 play a role in the p53-mediated apoptosis. Interestingly, the computational analysis of DUSP6 promoter region revealed the new putative binding site for p53 to activate DUSP6. The functional dissection of DUSP6 revealed the dephosphorylation of Extracellular signal- regulated kinase (ERK1/ERK2) in p53 dependent manner.

However, introduction of short hairpin RNA for SSFA2 into HCT116 cells showed the slight reduction in the level of cell death induced by 5-FU, Etoposide and TRAIL. These findings suggest that SSFA2 appear to play a minor role in the p53-mediated apoptosis.

Keyword: *p53, DNA chip analysis, apoptosis*

2.2. INTRODUCTION

p53 (synonym of protein 53 or tumor protein 53) is a transcription factor encoded by the TP53 gene, plays a key role in the regulation of the cell cycle, DNA repair, apoptosis [130] and functions as a tumor suppressor that is involved in preventing cancer [131]. Identification of the p53 target genes by DNA chip analysis and characterization of new p53-transactivated genes will advance our understanding of scene behind the p53 exerted multiple phenomena. Chemotherapeutic and genotoxic agents stabilize the p53 tumor suppressor protein that actively transactivates target genes to cause apoptosis [70]. The p53-induced apoptosis is a consequence of transcriptional regulation of p53 target genes or permeabilization of mitochondria outer membrane to release Cytochrome C [102].

Protein DUSP6 encodes a dual-specificity phosphatase and has very strict specificity for ERK and localization in the cytosol are unusual characteristics that differ greatly from those of other MAPK phosphatases such as MKP-1 and MKP-2 [84]. ERK is a key effector mitogen activated kinase (MAPK) involved in the RAS-GTP signal transduction pathway. Active RAS-GTP mediated by signals from growth factor-receptor tyrosine kinase recruits and stimulates RAF-1, which leads to activation of MAPK/ERK kinase (MEK) and ERK by subsequent phosphorylation [96]. Phosphorylated ERK translocates into the nucleus and

activates various transcription factors, resulting in cell growth and differentiation [90]. DUSP6 can dephosphorylate activated ERK and put an end to the growth-stimulating signals; this is thought to be one of the feedback loops for controlling MAPK signal pathways in normal cell growth [91]. Inactivation of DUSP6 may abrogate the feedback loop and subsequently uncontrolled cell growth, which in turn can lead to carcinogenesis and progression. The sub-cellular cytpolasmic localization of DUSP6 makes it a more effective inactivator for ERK by keeping it in the cytoplasm and preventing it from translocation into the nucleus where the target effectors reside. Most pancreatic cancer cells harbor the gain-of-function mutations in the KRAS2 gene [132]. However, DUSP6 as negative regulator of MAPK signaling has a tumor suppressive function enrolled in pancreatic carcinogenesis and progression [94].

Sperm specific antigen 2 (SSFA2) is also known as a Ki- ras-induced actin-interacting protein(KRAP) or cleavage signal-1 protein (CS1) gene reported to be involved in some step of early cleavage of the fertilized oocyte [133]. Although it has been suggested to influence the cellular events related to attachment, migration, proliferation and apoptosis, the physiological function of SSFA2 remains to be solved.

The PHLDA1 (pleckstrin homology-like domain, family A, member 1), the synonym of TDAG51, is known to down regulate in number of tumor cells

particularly in breast cancer [134]. The determination of PHLDA1 expression may be the prognosis value in breast cancer. PHLDA1 expression was first associated with apoptosis in T cell hybridoma [99] and anoikis in hippocampal neuronal cells [135]. However, the exact mechanism by which PHLDA1 regulate the apoptosis is still unknown, its expression being induced by a variety of stimuli in p53 dependent manner and might acts as mediator of apoptosis.

Transcriptional regulation of new p53 downstream genes and their physiological function on cell death machineries would illustrate how p53 bring out its tumor suppressing effect under different genotoxic and cellular stresses. Exploration of the consensus binding site for p53 has important role in understanding the functional significance of target genes. But, the proper location of this consensus binding site particularly other than conventional consensus sequence in promoter region is difficult. A Microarray-based study indicates that only a small proportion of p53-target genes contain this consensus site in their regulatory region indicating the existence of potential alternative binding site for p53 [136]. In search of such potential p53 target genes that mediate regulation of cell death in p53 dependent manner, I was able to locate a new p53 binding site in promoter region of DUSP6. In this dissertation work, I enlighten the novel p53 dependent transactivation of DUPS6 and its role in p53-mediated apoptosis. The elevation in expression level of DUSP6 occurs in

p53-dependent manner and particularly under genotoxic stresses. The abrogation of DUSP6 by short hairpin results substantial resistance to 5-FU, Etoposide and TRAIL treatment suggesting its role in p53-mediated apoptosis. DUSP6 has a low basal activity but its enzymatic activity is stimulated with the p53 pathway activation. Here, I showed that DUSP6 specifically dephosphorylate ERK1/2 and its possible role in apoptosis in p53 dependent manner.

But, when SSFA2 is knocked down with short hairpin of RNA (shRNA), cells exhibit a limited reduction of apoptosis induced by 5-FU, Etoposide and TRAIL.

2.3. MATERIAL AND METHODS

2.3.1. Cell culture and reagents

A human colorectal carcinoma, HCT116 parental cell and p53-deficient HCT116 cell were maintained in McCoy's 5A media with 10%FBS, supplemented with 2 mM L-glutamate, 100 U/mL penicillin and 100 μg/mL Streptomycin at 37^0C under 5% CO_2 in humidified incubator. Similarly, cervical cancer, HeLa cell and breast cancer, MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI respectively with 10% FBS, supplemented with 2 mM L-glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin. A recombinant human TRAIL was generated as described previously [137]. 5-flurouracil (5-FU) and Etoposide were purchased from Aldrich-Sigma (St. Louis, USA). Blasticidin was purchased from Invitrogen life technologies (California, USA). Anti-actin antibody was purchased from Chemicon international (California, USA). Anti-DUSP6 antibody was purchased from Abnova Corporation (Taipei, Taiwan). Anti-p53 antibody was purchased from Oncogene (USA). Anti-p21 antibody was purchased from BD Bioscience (USA). Anti-pERK1/2 and total anti-ERK antibody was purchased from Santa crutz biotechnology.Inc (USA). Horseradish peroxidase (HRP) - conjugated goat anti-rabbit/-mouse IgGs were purchased from Jackson ImmunoReasearch Lab (West Groove, PA).

2.3.2. Screening of p53-upregulated gene.

A number of genes responsive to p53 were screened using DNA chips microarray (Digital genomics, Seoul, Korea) as described previously [137].

2.3.3. Infection of adenovirus

The recombinant adenoviral vector expressing wild-type p53 (Ad-p53) and wild type adenovirus (Ad-WT) were infected to HCT116 $p53^{-/-}$ cells. Ad-WT was used as negative control. The multiplicity of infection (MOI) was defined as ratio of infectious virus particles to cells. The titer of the virus was calculated using the standard Tissue culture Infectious Dose 50 ($TCID_{50}$) following the manufacturer standard protocol (Ad Easy^{TM} vector system, Qbiogene, Inc. USA).

2.3.4. Semi-quantitative Reverse transcription-polymerase chain reaction (RT-PCR) and Real Time quantitative RT-PCR (qRT-PCR).

HCT116 $p53^{-/-}$ cells were infected with 50 MOI of wild type-adenovirus and p53 expressing adenovirus for 6, 10, and 24 hours, HCT116 $p53^{-/-}$ and HCT116 wild type cells were treated with 5-FU for 6 and 24 hours as well. Total RNA was isolated from cells using TRIzol (Invitrogen, USA) following the standard protocol. 2 μg of total RNA was reverse transcribed with Improm-II reverse transcriptase (Promega, USA) following standard manufacturer protocol. About 200 ng of the

reverse transcribed cDNA was analyzed by semi quantitative RT-PCR. Twenty five cycles of amplification following denaturation at 94^{0}C for 30s, annealing at 60^{0}C for 40s and extension at 72^{0}C for 30s was performed. The PCR products were separated on 1-2% agarose gel and stained with ethidium bromide and band intensities of amplified DNAs were compared after visualization with a UV transilluminator. Furthermore, real time quantitative quantification qRT-PCR reactions were prepared with SYBR green PCR kit (QuantiTectTM, Qiagen, Hilden, Germany) for number of genes. A Corbett cycler and its system interface software (Corbett life science, Sydney, Australia) were used to run samples and analyze data. All samples were analyzed in triplicate and normalized to GAPDH level, and the results are expressed as-fold induction compared with untreated controls. The primer set used to amplification of corresponding genes are listed in table 2-1A.

2.3.5. Short hairpin RNAs construction and analysis of apoptotic cell death.

Vector pC6A:GFP:H1 was designed to express the green fluorescent protein (GFP) and short hairpin interference RNA (shRNA) under the CMV promoter and H1 promoter respectively. A DUSP6 , PHLDA1 and SSFA2 shRNA cassettes were generated by annealing the Oligonucleotides containing respective target sites with overhang BglII and ClaI and then ligation into Bgl II and ClaI site of pC6A:GFP:H1 as pC6A:GFP:H1:shDUSP6, pC6A:GFP:H1:shPHLDA1 and

pC6A:GFP:H1:shSSFA2 respectively . The Oligonucleotides used for generation of shRNA are listed in table 2-1B. HCT116 wild type cells and HCT116 $p53^{-/-}$ cells were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1 shRNA of DUSP6, PHLDA1 and SSFA2 gene with effectene transfection reagent (Qiagen, Chatsorth, CA, USA) and clones were selected with blasticidin (10 μg/mL). Transiently selected clones were subjected to treatment with 5-FU (200 μg/mL), Etoposide (100 μg/mL) and TRAIL (50 ng/mL) as indicated time and cell death rate were measured by counting survived or dead cells of GFP expressing cells with apoptotic morphological examination. More than 300 cells in three separate fields were counted for each measurement. The knock down efficiency of gene was checked by RT-PCR and Western blotting.

2.3.6. DUSP6 Overexpression

To determine the role of DUSP6 in p53-mediated apoptosis, the DUSP6 overexpression vector was constructed. To generate overexpression vector (pC6A:2A:GFP:DUSP6), full length (381 amino acids) fragments of DUSP6 was amplified from cDNA of DUSP6 by the Polymerase chain reaction (PCR) using specific primers containing Kpn1 in 5' and EcoRV in 3' overhang and amplified DNA was cloned into pC6A:2A:GFP over expression vector at the Kpn1 and EcoRV sites. The construction was verified by an automatic DNA sequencer. The primers used for amplification are listed in table 2-1C. HCT116 wild type cells

and HCT116 p53$^{-/-}$ cells were transfected with either pC6A:2A:GFP vector or pC6A:2A:GFP:DUSP6 with effectene transfection reagent (Qiagen, Chatsorth, CA, USA) and clones were selected with blasticidin (10 μg/mL). Transiently selected clones were subjected to treatment with 5-FU (200 μg/mL), Etoposide (100 μg/mL) and TRAIL (50 ng/mL) as indicated time. The percentage of cell death was determined by standard protocol for trypan blue exclusion assay. Briefly, total cells were collected in eppendorf tube and stained with equal volume of 0.4% of trypan blue for 2 minutes and cells were counted using hematocytometer under microscope. The Overexpression of DUSP6 was confirmed by Western blotting.

2.3.7. Western blotting

HCT116 wild type and HCT116 p53$^{-/-}$ (2X10^{5}/well) cells were seeded on 6 well plates (Nunc, Roskilde) and next day treated with 5-FU (200 μg/mL), Etoposide (100 μg/mL) and TRAIL (50 ng/mL) for different time intervals. Cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-Cl pH 7.5 and 150mM NaCl) supplemented with 1mM phenylmethylsulfonylfluoride (PMSF), 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1μg/mL pepstatin A, 2mM sodium fluoride and 1 mM sodium orthovanadate. Soluble lysates were subjected to SDS-PAGE and transferred to a Polyvinylidene Fluoride (PVDF) membrane (BIO-RAD, USA). Then membrane was probed with

specific antibodies, followed by HRP-conjugated anti-IgG antibody. Enhanced chemiluminescence (ECL) was used for the detection of bound antibody.

2.3.8. Chip analysis

To find out the DUSP6 transactivation, we carried out Chromatin Immunoprecipitation (CHIP) assay adapting the standard procedure (www.abcam.com/techinical and the epigenome Network of Excellence). Briefly, HCT116 wild type ($2X10^8$) cells were seeded on 100mm dish (Nunc, Roskilde) and treated with 5-FU (200 μg/mL) for different indicated time points on the following day. For cross-linking the bound proteins to chromatin, the media containing 1% formaldehyde (Sigma, Aldrich) was added drop-wisely directly on dish and then rotate gently at room temperature for 10 minutes. The cross linked cells were resuspended on swelling buffer (25 mM Hepes pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM DTT and freshly added protease inhibitors). Following 10 minute incubation on ice, nuclei were released by dounce 20 times up and down. Nuclei were resuspended in Chip lysis buffer (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8, 1% Triton X-100, 0.1% sodium deoxycholate and freshly added protease inhibitors) and sonicated to shear the chromatins of average fragment size 500-1000bp. This sheared protein-DNA complexes were immunoprecipitated with anti-p53 antibody (Oncogene, USA) and captured on protein A sepharose beads (GE, Healthcare). After

immunoprecipitation, beads were washed with three times with wash buffer and one time with final wash buffer, respectively (wash buffer 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 150 mM NaCl, 20 mM Tris-Cl pH 8 and final wash buffer 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 500 mM NaCl, 20 mM Tris-Cl pH 8). Finally, chromatin complex was eluted with elution buffer (1% SDS, 100 mM $NaHCO_3$) and DNA-protein cross-linking was reversed by addition of proteinase K and heating at 65^0C for overnight and then DNA was purified with phenol extraction followed by ethanol precipitation. PCR amplification of DUSP6 gene was performed using specific primers detailed in table 2-1D.

2.3.9. ERK dephosphorylation

To understand the functional role and its link with p53-mediated apoptosis, ERK dephosphorylation substrate specificity in p53 dependent manner was dissected. HCT116 wild type and HCT116 $p53^{-/-}$ as well as shRNA construct of DUSP6 cells ($2X10^5$/well) were seeded on 6 well plates (Nunc, Roskilde) and followed serum starvation for next 18 hours and then treated with 5-FU (200 μg/mL) for different time intervals. Cells were lysed in RIPA buffer. Soluble lysates were separated on SDS-PAGE and transferred to a Polyvinylidene Fluoride (PVDF) membrane (BIO-RAD, USA). Then membrane was probed with specific antibodies, followed by HRP-conjugated anti-IgG antibody. Enhanced chemiluminescence (ECL) was used for the detection.

2.3.10. Statistical analysis

Values are presented as mean± standard deviation. The paired student's *t-test* was applied to determine whether difference between values were significant. A statistical probability of $p<0.05$ was considered significant.

Table 2-1A. List of RT PCR and Real time PCR primers

List of RT PCR primers:

C3Orf4	5'-GGATTGTGGAGACGGTGTATC-3'
	5'-GGGATATAAGCTTCGGCAAAT-3
CYP24A	5'-TGGTGACATCTACGGCGTAC-3'
	5'-TTTGCGGTAGTCGCGATAG-3'
DUSP4	5'-CTACTCGGCGGTCATCGT-3'
	5'-GAACCAGGAGCTGATGTCG-3'
DUSP6	5'-CGAGTCTGACCTTGACCGA-3'
	5'-AGGCATCGTTCATCGACAG-3'
EREG	5'-GTTTCCATCTTCTACAGG-3'
	5'-CTGAGGTAACTCTCTCAT-3'
GAPDH	5'-CCATCACCATCTTCCAGGAGCGAG-3'
	5'-TGCCAGTGAGCTTCCCGTTCAGCTC-3'
MSX1	5'-CAGAAGATGCGCTCGTCA-3'
	5'-GCCAGAGGCACCGTAGAG-3'
PPM2C	5'-CAACACTGGTGAGTCGACTGA-3'
	5'-AGCCGATCCTGTTTCACG-3'

PHLDA1	5'-CAAGGAGATCGACTTTCGGT-3'
	5'-GGTATTTGGTGCGGATGC-3'
p21	5'-CGACTGTGATGCGCTAAT-3
	5'-CTTGGAGAAGATCAGCCG-3'
p53	5'-GTGCCTTCCCAGAAAACCTA-3'
	5'-TCCGTGCCAGTAGATTACCA-3'
SSFA2	5’-CACAGAAGCGACGACGCA-3’
	5’-GCATTGGCCACAGTAGTA-3’
SOCS6	5'-CAACAACCATCGCTAGCC-3'
	5'-TGGAAATCCTTCCGGACG-3'
WNT16	5'-GTGCAAGAGGAAACCGTA-3
	5'-ACTGACATCAACTTGGCG-3'

List of Real time PCR primers:

C3Orf4	5'-CGATTGAGACGGTGTATC-3'
	5'-GGGATATAAGCTTCGGCAAAT-3'
CYP24A	5'-TCTCAAGAAACAGCACGACA-3'
	5'-TTTGCGGTAGTCGCGATAG-3'
DUSP4	5'-CTACTCGGCGGTCATCGT-3'
	5'-CTCGGAGGAAAACCTCTCAT-3'
DUSP6	5'-CTCTTTGAGAACGCAGGAGA-3'
	5'-AGGCATCGTTCATCGACAG-3'
EREG	5'-GTTTCCATCTTCTACAGG-3'
	5'-CCCACTTCACACCTGCAGTA
GAPDH	5'-CCATCACCATCTTCCAGGAGCGAG-3'
	5'-GTGGAAGGACTCATGACCACAG
MSX1	5'-AGACGCAGGTGAAGATATGG-3'
	5'-GCCAGAGGCACCGTAGAG-3'
PPM2C	5'-GACCTTCATGTGGCCAATAC-3'
	5'-AGCCGATCCTGTTTCACG-3'
P53	5'-CTCAGATAGCGATGGTCTGG-3'
	5'-TCCGTGCCAGTAGATTACCA-3'

SOCS6 5'-ACACCTTCTCCTCCTCCTA-3

5'-TGGAAATCCTTCCGGACG-3'

SSFA2 5'-AATCTTGGATTTGGACGTGA-3'

5'-GCATTGGCCACAGTAGTA-3'

WNT16 5'-GTGTTCCTGTGACACCACCT-3'

5'-ACTGACATCAACTTGGCG-3'

Table 2-1B. List of shRNA primers:

DUSP6	5'-GATCCCC-AAGACGGTGGCGTGGCTCAAC-TTCAAGAGA-GTTGAGCCACGCCACCGTCTT-TTTTTGGAAAT-3' 5'-CGATTTCCAAAAA-AAGACGGTGGCGTGGCTCAAC-TCTCTTGAA-GTTGAGCCACGCCACCGTCTT-GGG-3'
SSFA2	5'-GATCCCC-ATGTGGAGCAAGATGAGTTGC-TTCAAGAGA-GCAACTCATCTTGCTCCACAT- TTTTTGGAAAT-3' 5'-CGATTTCCAAAAA-ATGTGGAGCAAGATGAGTTGC-TCTCTTGAA-GCAACTCATCTTGCTCCACAT-GGG-3'
	5'-GATCCCC- GAGCCTCACTGGATGAACA-TTCAAGAGA- TGTTCATCCAGTGAGGCTC-TTTTTGGAAAT-3' 5'-CGATTTCCAAAAA- GAGCCTCACTGGATGAACA -TCTCTTGAA- TGTTCATCCAGTGAGGCTC-GGG-3'
PHLDA1	5'-GATCCCC-AACGGAGAGGCCGAGCCAAGC-TTCAAGAGA-GCTTGGCTCGGCCTCTCCGTT-TTTTTGGAAAT-3' 5'-CGATTTCCAAAAA- AACGGAGAGGCCGAGCCAAGC-TCTCTTGAA-GCTTGGCTCGGCCTCTCCGTT-GGG-3'

Table 2-1C. Primers for Overexpression of DUSP6

DUSP6	5'-CGGGGTACCATGATAGATACGCTCAGAC-3'
	5'-GGCGATATCCGTAGATTGCAGAGAGTCCAC-3'

Table 2-1D. Primers List for Chip analysis

DUSP6(-1625… -1425)	5'-GCGCTGGACAAAGGGAGGA-3'
	5'-TGAGCGAGGTGTGCTGGGA-3
DUSP6(-2404… -2125)	5'-AGCCTACATTTCCCAAACCAGGGA-3'
	5'-TAATCCGGCCTTCCCTCCCTAAA-3'

2.4. RESULTS

2.4.1. Identification of DUSP6, PHLDA1 and SSFA2 as p53 target genes

To identify the target genes transactivated by p53, I performed the DNA chip analysis using commercial oligonucleotides DNA Chips (Digital Genomics, Seoul, Korea). I found CY24A1, C30rf4, DUSP4, DUSP6, EREG, MSX1, PPM2C, PHLDA1, SOCS6, SSFA2 and WNT16 as p53 upregulated genes through chip analysis (Table 2-1). To confirm p53 transactivates those genes, I infected the adenovirus expressing p53 in HCT116 $p53^{-/-}$ cells and endogenous p53 was stimulated by 5-FU in HCT116 WT cells. The mRNA expression of those genes in both cases verified results. Moreover, mRNA expression elevation independent in Ad-WT infected HCT116 $p53^{-/-}$ cells and 5-FU treated HCT116 $p53^{-/-}$ cells confined the phenomena. The semi quantitative RT PCR showed increased level of CYP24A1, DUSP6, MSX1, PPM2C, PHLDA1, SOCS6 and SSFA2 that were quantified with real time PCR (Fig.2.1, A-B). Out of which DUSP6, PHLDA1 and SSFA2 were further dissected to understand the functional role in apoptosis.

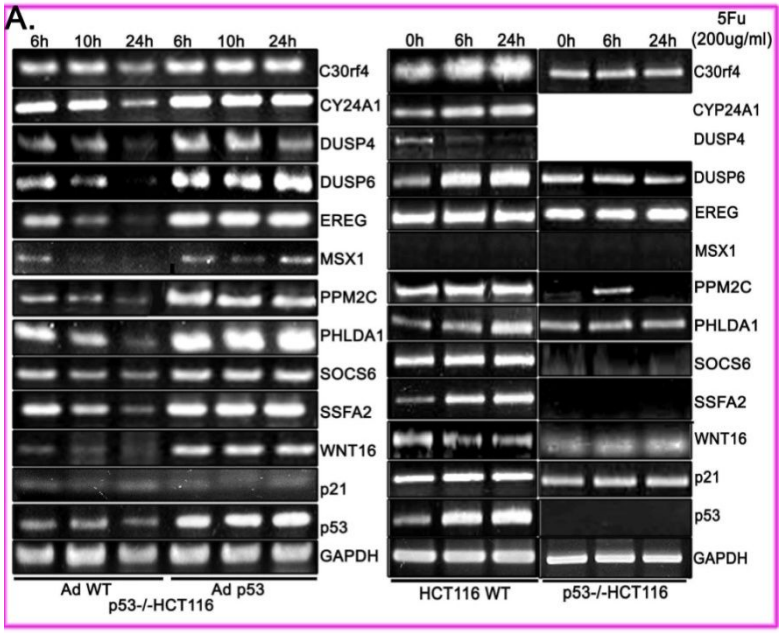
Table 2.4.1: Expression profiles of p53 over expressed HCT116 cells by cDNA microarray analysis

Gene	Title	Global M			Function
		6h	10h	24h	
CYP24A1	Cytochrome P450 family 24 subfamily A, polypeptide 1	0.73	1.95	4.21	Electron transport
DUSP4 DUSP6	Dual specificity phosphatase 4 Dual specificity phosphatase 6	0.84 0.62	0.77 1.22	2.26 4.15	MAPKKK cascade protein amino acid dephosphorylation, regulation of cell cycle, apoptosis
EREG	Epiregulin	0.98	1.85	3.37	Angiogenesis, cell proliferation, cell-cell signaling, EGFR signaling pathway, regulation of cell cycle
MSX1	Msh homeo box homolog 1(Drosophila)	0.74	0.61	2.00	Cell proliferation, cell-cell signaling
PPM2C	Protein phosphatase 2C magnesium dependent catalytic subunit	0.81	1.92	3.12	Protein amino acid dephosphorylation
SOCS6	Suppressor of cytokine signaling 6	0.35	0.79	2.65	JAK-STAT, defense response intracellular signaling cascade regulation of cell growth
WNT16	Wingless type MMTV integration site family member 16	0.96	2.04	3.61	Cell- cell signaling
C3orf4	Chromosome 3 open reading frame 4	-0.10	- 0.08	0.98	-
SSFA2	Sperm specific antigen 2	0.6	1.03	2.7	-
PHLDA1	Pleckstrin homology-like domain, family A, member 1	0.51	1.08	1.99	-

(+) Up-regulation and (−) down-regulation

M value indicate log2(Cy5/Cy3); 1= 2-fold increase of Cy5 signal, -1=2-fold increase of Cy3 signal. Cy3 dye for the controls (Ad-wt virus infected HCT116 p53 -/- cells) or Cy5 dye for the samples (Ad-p53 infected HCT116 p53 -/- cells). 6, 10, 24h means incubation time after adenovirus infection.

Fig.2.1



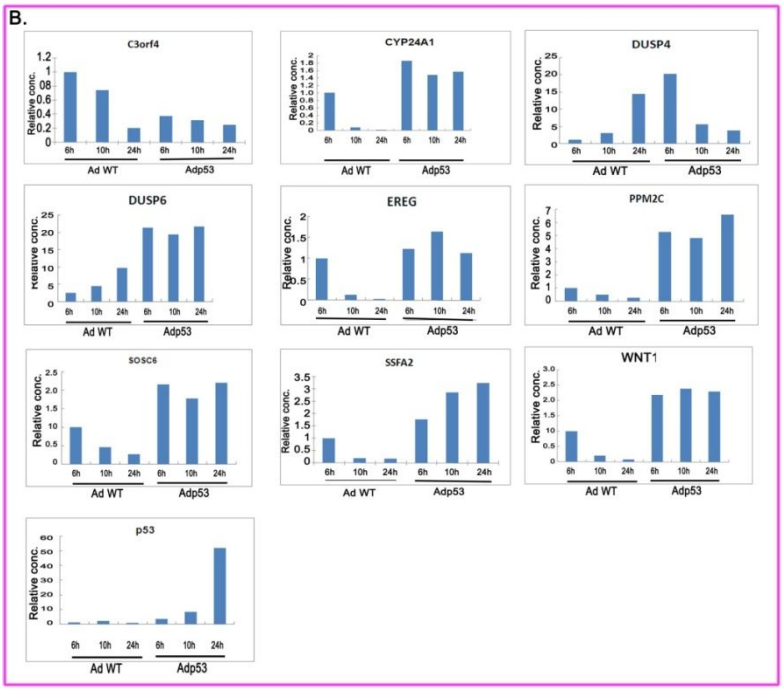


Fig.2.1: p53 transactivates number of genes. A: HCT116 p53-/- cells were infected with wild type-adenovirus or adenovirus expressing p53 for 6, 10 and 24 hours (left panel) and HCT116 wild type and HCT116 p53-/- cells were treated with 5-FU (200 μg/mL) for 0, 6 and 24 hours in order to induce endogenous p53(right panel). Then mRNA levels of number of genes were analyzed by RT-PCR. The PCR products were separated on 1.5-2% agarose gels and stained with ethidium bromide. GAPDH was used as internal control. B: To confirm the expression level of those genes in p53 overexpressed HCT116 cells, HCT116 p53-/- cells were infected with wild type-adenovirus and adenovirus expressing p53 for 6, 10 and 24 hours and the expression level of several genes were analyzed by Real-time PCR. Total RNA from adenovirus infected cells was reverse transcribed. The reverse-transcribed cDNA was amplified by Real-time PCR using SYBR green and indicated primers.

2.4.2. DUSP6 is induced by p53 in response to different cellular stresses and Identification of a novel p53 putative binding sites in DUSP6 promoter region

The increase in mRNA level of DUSP6, PHLDA1 and SSFA2 in response to 5-FU leads to hypothesize that endogenously stimulated p53 regulates these genes. To further determine whether p53 is required for the DUSP6 response to different cellular stresses, HCT116 p53$^{-/-}$ and HCT116 WT cells were treated with 5-FU, Etoposide and TRAIL. In contrast to DUSP6 cellular expression in HCT116 WT, the expression of DUSP6 decreased with time points in HCT116 p53$^{-/-}$ cells in response to stress (Fig.2.2) suggesting that the DUSP6 is p53 transactivated gene. To assess the potential regulation of DUSP6 by p53, I performed a computational analysis of partial 2.5 kb DUSP6 promoter region using the computer software (Genomatix) to find out potential p53 binding sites. This region analysis revealed the 4 putative binding sites at -1551/-1573 (cagttggcagggaggCATGtcag), -1562/-1584 (ggaggccccctctgaCATGcctc), -2315/-2327 (ccccgcacccacgggCATGcctg) and -2326/-2348 (gggggtgttagcaggCATGcccg). The common features of all these putative binding sites are the identical core region CATG. Thus, to confirm the functional activity and distinct association of p53 with DUSP6 promoter *in vivo*, I did chromatin immunoprecipitation with HCT116 WT cells in response to death stimuli 5-FU. Significantly, DUSP6 promoter was detectable only when these cells were

subjected to 5-FU treated cells (lane 7, 8), but not in untreated cells (lane 5, 6) (Fig.2.3). This finding revealed the novel p53 putative binding site within the DUSP6 promoter that is trigger by genotoxic stresses leading to apoptosis.

Fig.2.2

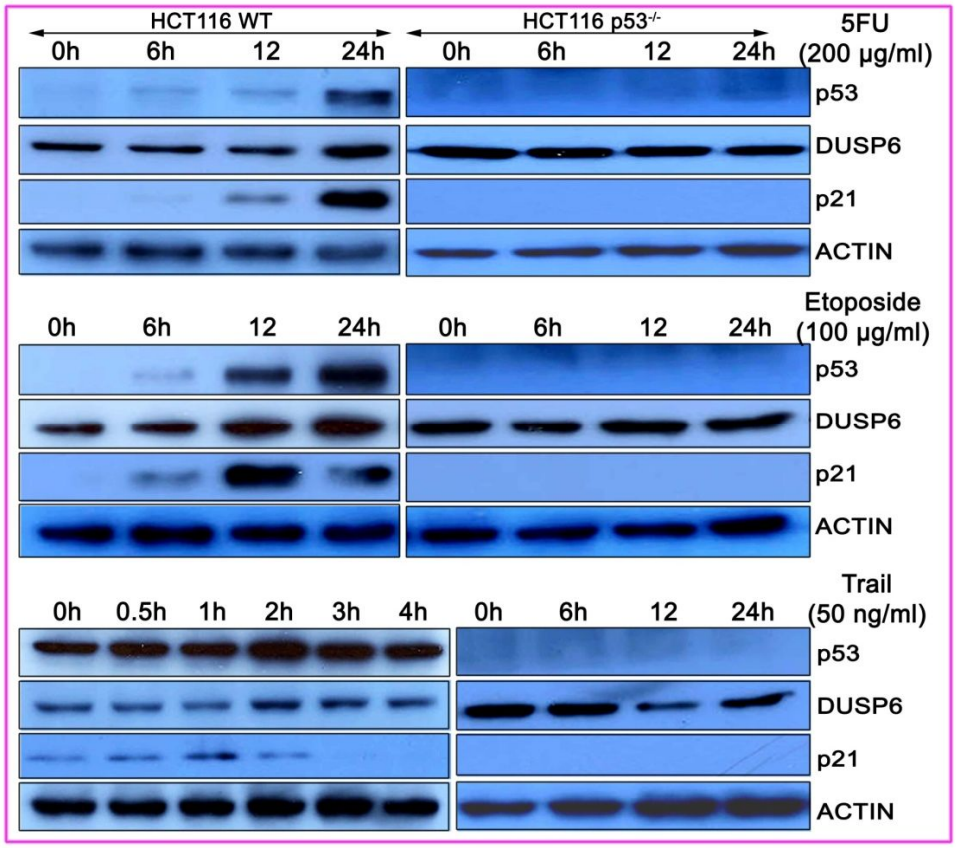


Fig.2.2: p53-dependent induction of DUSP6. HCT116 wild type cells and HCT116 p53-/- cells were treated with 5-FU (200 μg/mL), Etoposide (100 μg/mL) and Trail (50 ng/mL) for different time interval and whole cell lysates were prepared using RIPA buffer. The whole cell lysates proteins were separated on SDS - PAGE and probe with specific antibody. α-actin was used as a loading control.

Fig.2.3

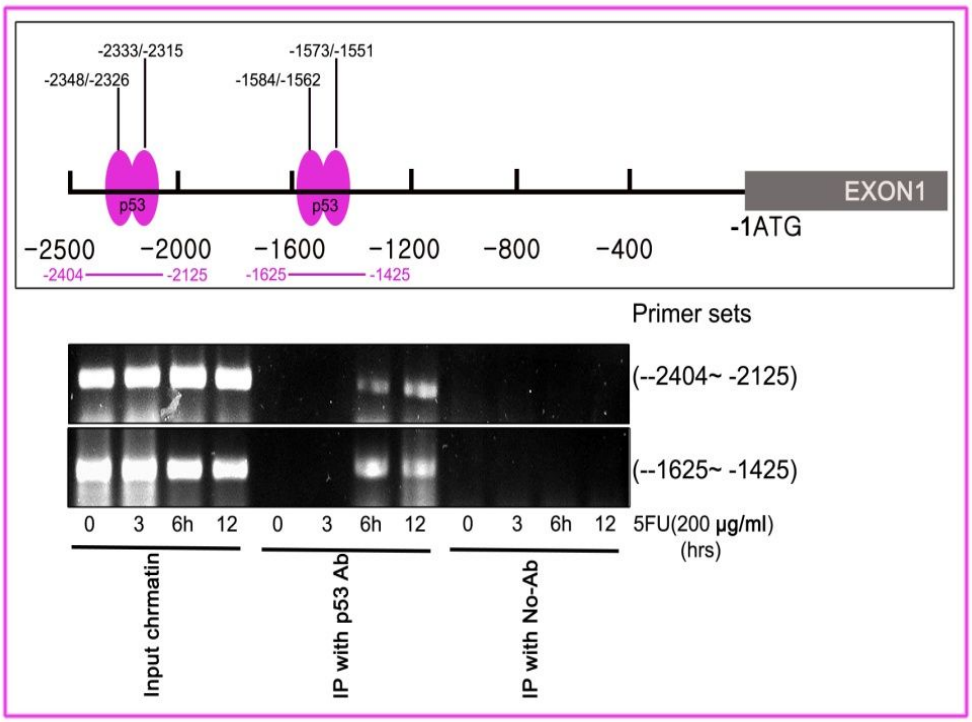


Fig.2.3: The selective p53 binding to the DUSP6 promoter region under genotoxic stress. The schematic representation of novel p53 binding sites in DUSP6 promoter region was shown in upper panel. Chromatin Immunoprecipitation analysis of physical interaction between p53 and DUSP6 promoter was depicted in lower panel. HCT116 cells were treated as indicated and subjected to chromatin immunoprecipitation analysis. A p53-specific antibody (Oncogene, USA) was used to precipitate p53 bound to chromatin. The p53-associated DUSP6 promoter was amplified by PCR using the corresponding primers designed for putative binding sequence. The input DNA from cell lysates before Immunoprecipitation was used as positive control. The elution from beads without antibody was used as negative control. The data is represented as a mean ± 3 separate experiments (significance as compared with the control, $^{*}p<0.05$)

2.4.3. DUSP6, PHLDA1 and SSFA2 induced by p53 in response to genotoxic stresses leading to apoptosis

The transactivation of DUSP6, PHLDA1 and SSFA2 under genotoxic stress is probable features of p53-mediated apoptotic pathway. To dissect the role of DUSP6, PHLDA1 and SSFA2 genes in apoptosis, I examined the apoptotic cell death in HCT116 $p53^{-/-}$ and HCT116 WT in response to various death stimuli such as 5-FU, Etoposide and TRAIL when knocked down with shRNA. For this purpose, I constructed short hairpin of human DUSP6, PHLDA1 and SSFA2 in pC6A:GFP:H1 vector that was then transfected into HCT116 WT cells. The transfected HCT116 cells were transiently selected using blasticidin. The RT-PCR and western blot confirmed the induction of DUSP6 by 5-FU whereas same stimulus did not increase DUSP6 in pC6A:GFP:H1:shDUSP6 clones indicating the knocking down of DUSP6 gene expression (Fig.2.4A). Moreover, HCT116 cell expressing the short hairpin against DUSP6 showed substantial inhibition of the apoptotic cell death induced by 5-FU, Etoposide and TRAIL (Fig.2.4B, C, D). However, HCT116 $p53^{-/-}$ cells expressing the short hairpin against DUSP6 gene showed limited reduction of cell death in response to Etoposide and TRAIL suggesting the role of p53 in activation of DUSP6 and thereby its role in apoptosis (Fig.2.4E). To generalize the functional link of DUSP6 in p53-mediated apoptosis, short hairpin of DUSP6 introduced into other cell lines; HeLa cells and

MCF-7 cells and subjected to treat with death stimuli. Interestingly, short hairpin of DUSP6 expressing HeLa cells showed the significantly reduction in apoptotic response to Etoposide and TRAIL (Fig.2.4F). However, introduction of short hairpin of DUSP6 in MCF-7 cells result the significant reduction in apoptotic response only to TRAIL (Fig.2.4G).

In the same way, RT-PCR confirmed the knock down of SSFA2 in HCT116 WT cells (Fig.2.5A) and that clones limitedly inhibited the apoptotic cell death induced by 5-FU (Fig.2.4B), Etoposide (Fig.2.4C) and TRAIL (2.4D). The apoptotic cell death in HCT116 $p53^{-/-}$ cell was resistant to 5-FU and Etoposide; however, HCT116 $p53^{-/-}$ cells expressing the short hairpin against SSFA2 gene showed the limited inhibition in response to TRAIL (Fig.2.4E).These finding suggest that SSFA2 may play a role in other pathway mediated by p53 rather than in p53-mediated apoptosis.

The RT PCR confirmed the knocked down of PHLDA1 gene in HCT116 cells expressing short hairpin of PHLDA1 (Fig.2.5F) and blasticidin selected clones showed reduction in cell death induced by 5-FU, Etoposide and TRAIL (Fig.2.5G, H, I). These finding suggest the functional link of PHLDA1 in p53-mediated apoptosis.

Fig.2.4 A

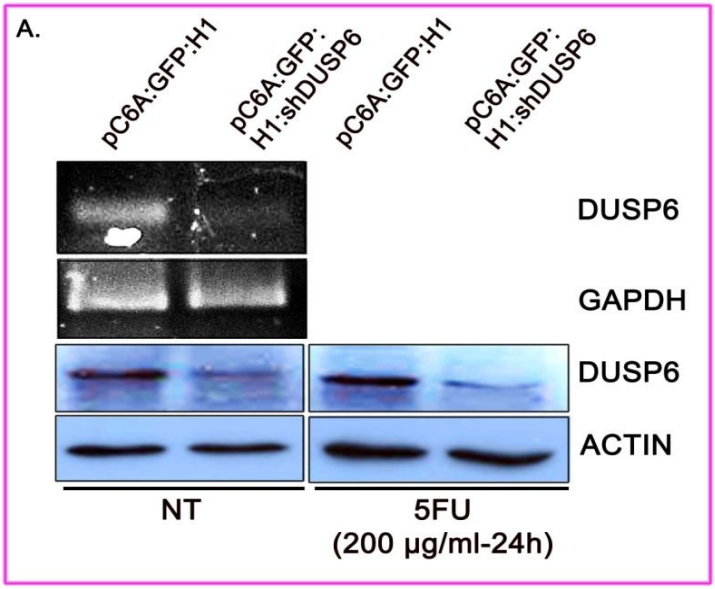


Fig.2.4B

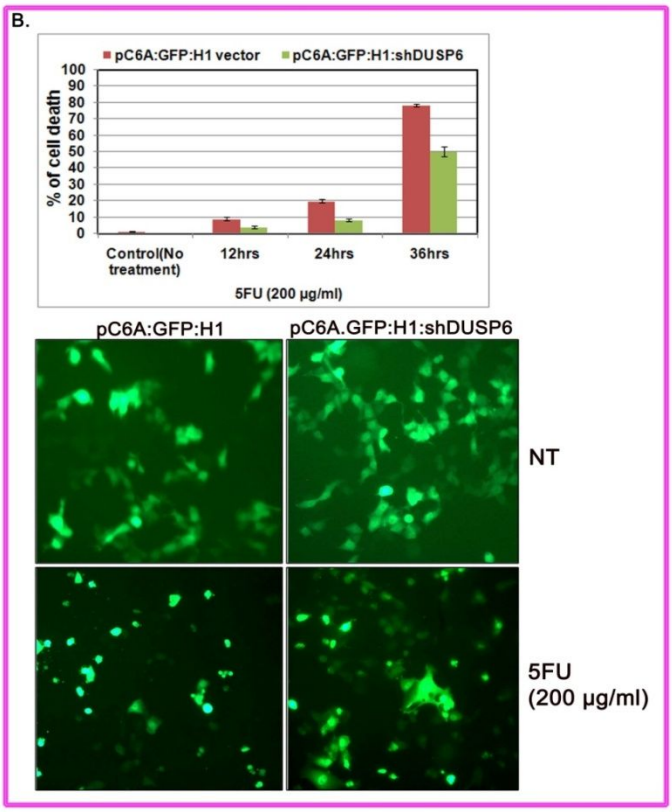


Fig.2.4C

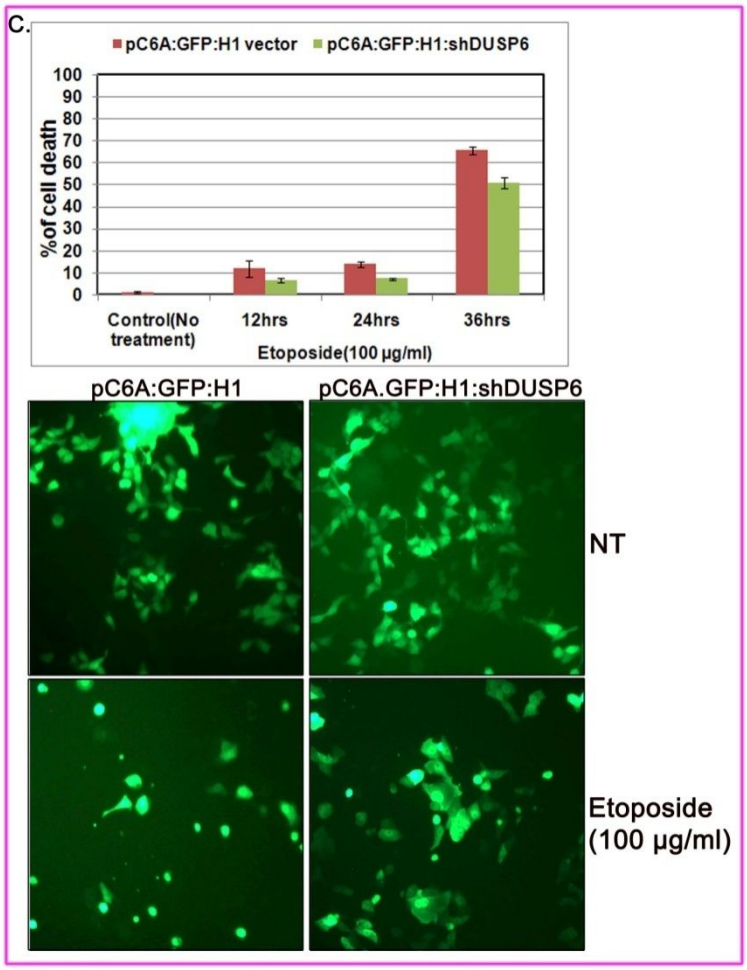


Fig 2.4 D

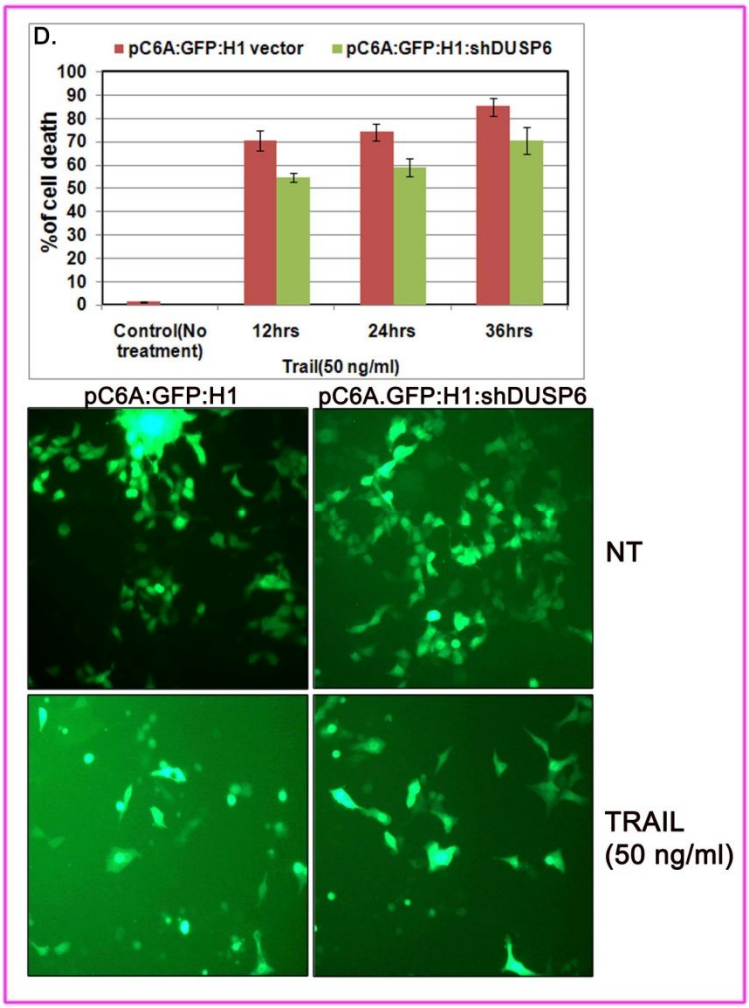


Fig 2.4E

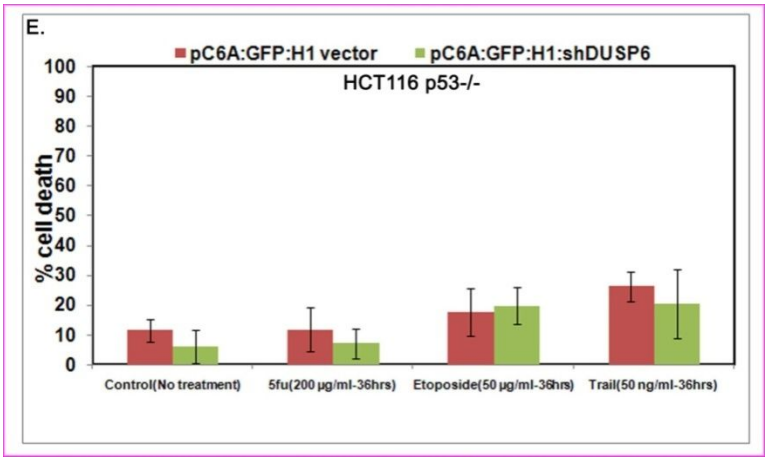


Fig 2.4F

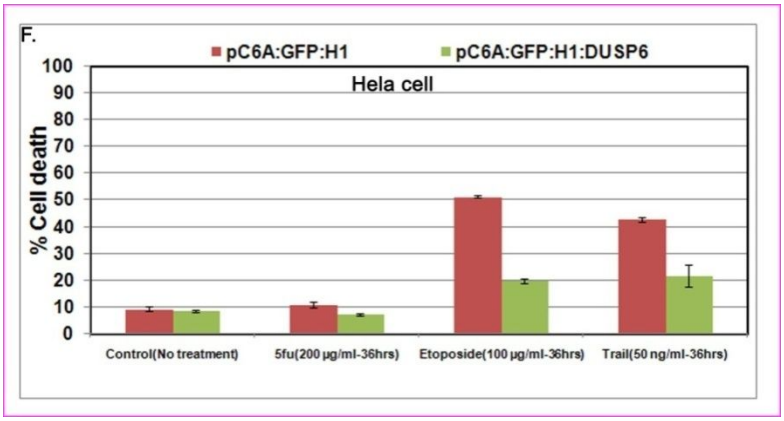


Fig 2.4G

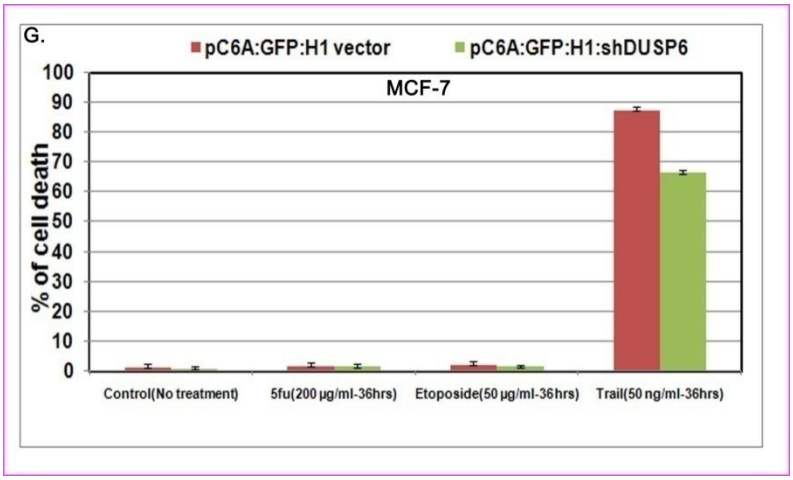


Fig.2.4: The role of DUSP6 in p53-mediated apoptosis. A: HCT116 WT cells and $p53^{-/-}$ HCT116 cells were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shDUSP6 that express shRNA of DUSP6 gene and clones were selected with blasticidin. RT PCR and Immunoblotting carried out to confirm the silencing efficiency in HCT116 cell clones treated with 5-FU (200 µg/mL) for 24h. α-actin in immunoblotting and GAPDH mRNA in RT PCR were used as loading control in each lane. B-D: HCT116 cells that were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shDUSP6 were treated with 5-FU (200 µg/mL)(B), Etoposide (100 µg/mL)(C) and Trail (50 ng/mL)(D) for different time interval and the percentiles of cell death were measured by counting the survived and dead cell of GFP positives clones. Fluorescence microscopic images were

obtained at 24 hours after treatment 5-FU (200 μg/mL) depicted in bottom panel. E: HCT116 p53-/- cells that were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shDUSP6 were treated with different stimuli such as 5-FU (200 μg/mL), Etoposide (100 μg/mL) and Trail (50 ng/mL) for 36hours and the percentiles of cell death were measured by counting the survived and dead cell of GFP positives clones . F-G: HeLa cells (F) and MCF-7 cells (G) were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shDUSP6 were treated with different stimuli such as 5-FU (200 μg/mL), Etoposide (100 μg/mL) and Trail (50 ng/mL) for 36 hours and the percentiles of cell death were measured by counting the survived and dead cell of GFP positives clones. The data is represented as a mean ± 3 separate experiments (significance as compared with the control, $^{*}p<0.05$)

Fig 2.5AB

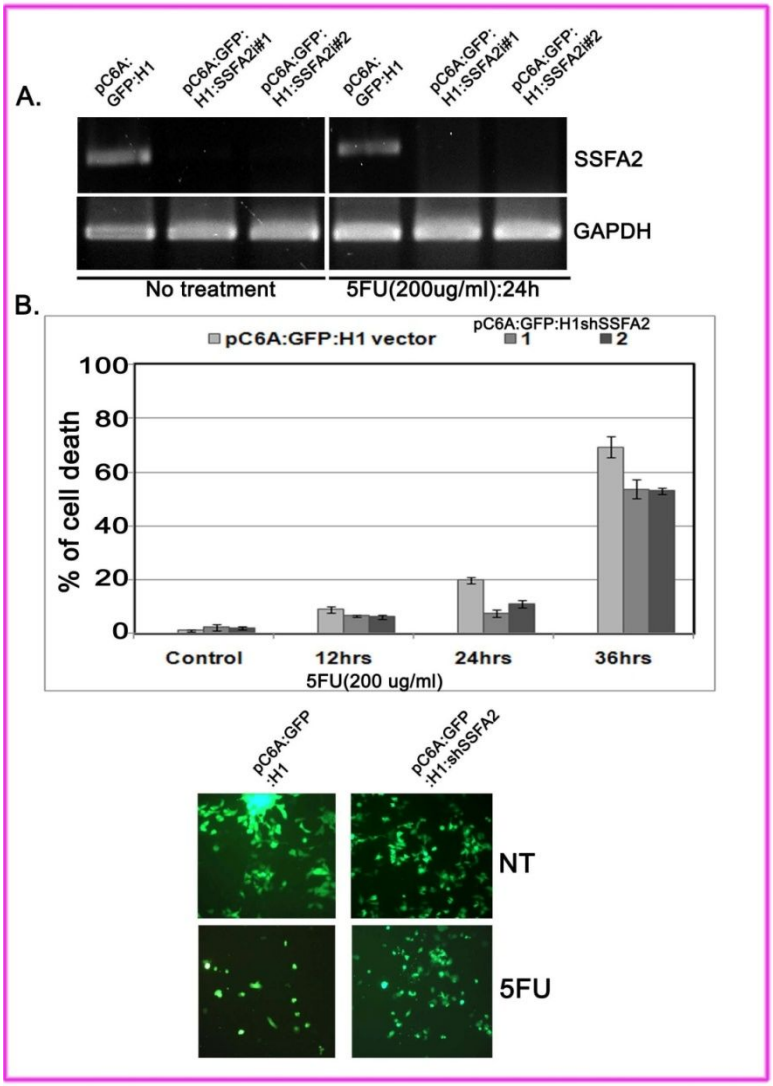


Fig 2.5CDE

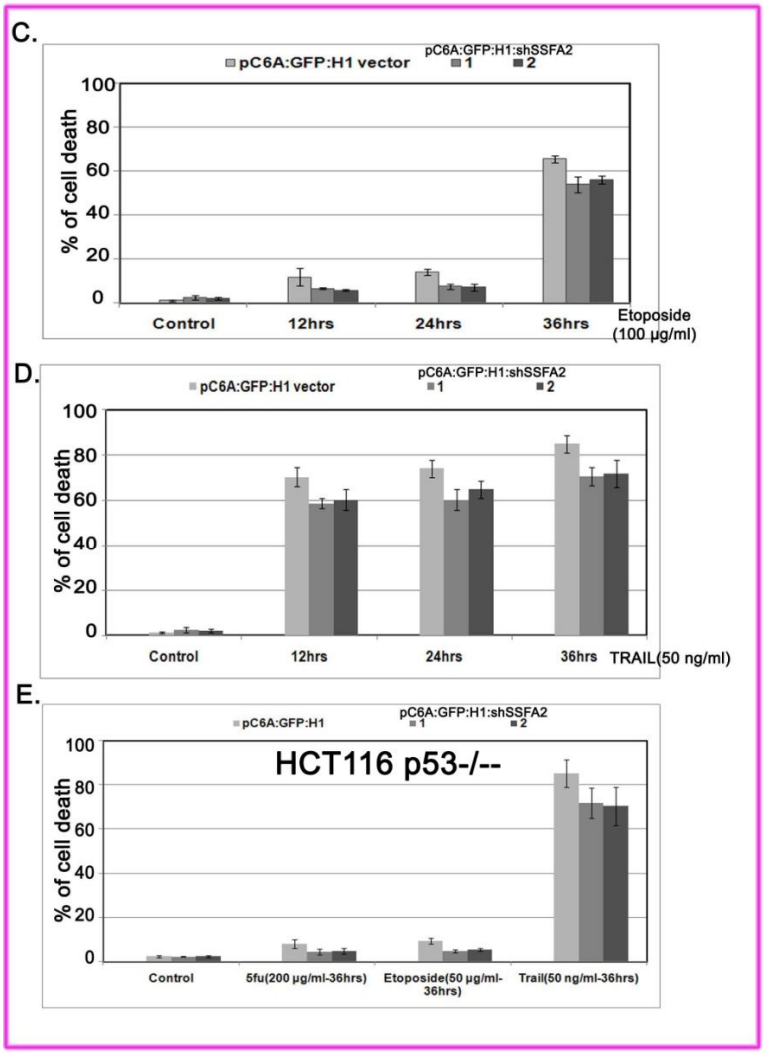
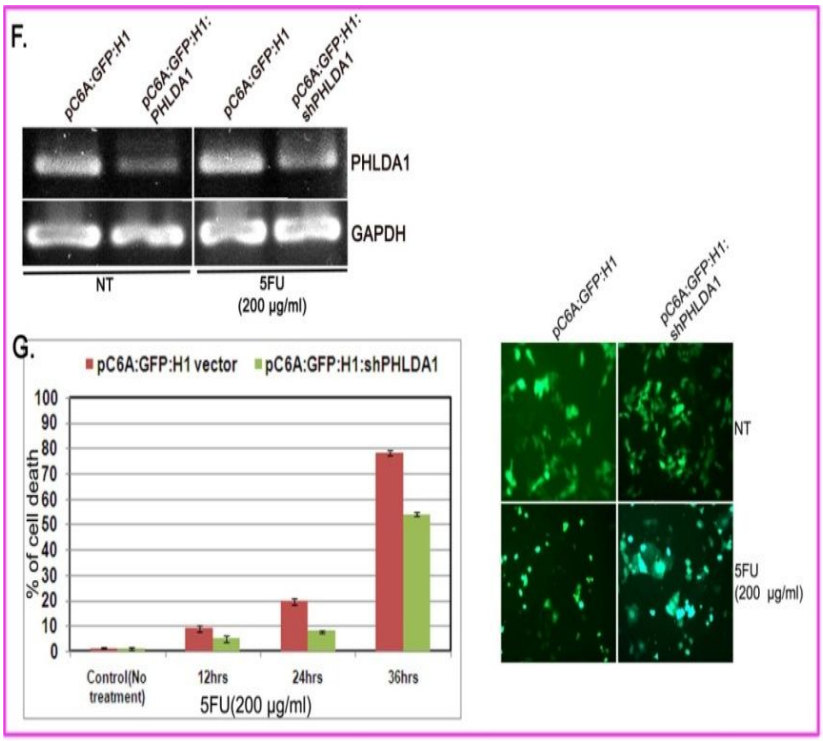
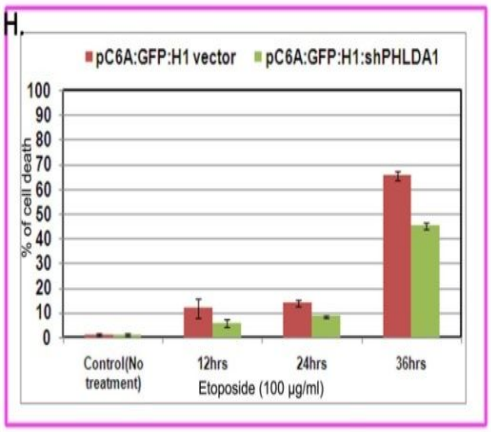


Fig 2.5FGHI





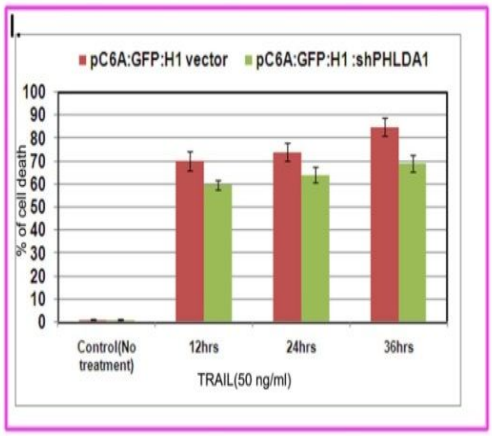


Fig.2.5: The shRNA of SSFA2 and PHLDA1 inhibited apoptotic response in the p53-mediated apoptosis. A: HCT 116 WT cells and HCT116 p53-/- cells were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shSSFA2 that express shRNA of SSFA2 gene and clones were selected with blasticidin. RT PCR carried out to check the silencing efficiency in HCT116 cell clones treated with 5-FU (200 μg/mL) for 24h. RT PCR for GAPDH was used as internal control. B-D: HCT116 cells that were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shSSFA2 were treated with 5-FU (200 μg/mL)(B), Etoposide (100 μg/mL)(C) and Trail (50 ng/mL)(D) for different time interval and the percentiles of cell death were measured by counting the survived and dead cell of GFP positives clones. Fluorescence microscopic images were obtained at 24 hours after treatment 5-FU (200 μg/mL) depicted in bottom panel (B). E: HCT116 p53-/- cells that were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shDUSP6 were treated with different stimuli such as 5-FU (200 μg/mL), Etoposide (100 μg/mL) and Trail (50 ng/mL) for 36hours and the percentiles of cell death were measured by counting the survived and dead cell of GFP positives clones. F: HCT 116 WT cells was transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shPHLDA1 that express shRNA of PHLDA1 gene and clones were selected with blasticidin. RT PCR carried out to makes sure the silencing efficiency in HCT116 cell clones treated with 5-FU

(200 μg/mL) for 24h. RT PCR for GAPDH was used as internal control. G-I: HCT116 cells that were transfected with either pC6A:GFP:H1 vector or pC6A:GFP:H1:shSSFA2 were treated with 5-FU (200 μg/mL)(G), Etoposide (100 μg/mL)(H and Trail (50 ng/mL)(I) for different time interval and the percentiles of cell death were measured by counting the survived and dead cell of GFP positives clones. Fluorescence microscopic images were obtained at 24 hours after treatment 5-FU (200 μg/mL) depicted in right panel (G). Results represent the means ± of three separate experiments (Significant versus the control, $^{*}p<0.05$)

2.4.4. DUSP6 over expression enhanced the apoptotic response to death stimuli

Introduction of the short hairpin of DUSP6 in HCT116 WT and HCT116 p53$^{-/-}$ cells and its response to 5-FU show that its role in cell death is p53 dependent. So, next we examine whether over expression of DUSP6 enhanced the apoptotic response to different death stimuli that was known to induce endogenous p53. For this purpose, human DUSP6 over-expressing HCT116 cells and HCT116 p53$^{-/-}$ cells were incubated with 5-FU, Etoposide and TRAIL. As assumption, ectopic expression of DUSP6 enhanced the response to different death stimuli on HCT116 cell (Fig.2.6B) but slightly in HCT 116 p53$^{-/-}$ cells(Fig.2.6C) suggesting that functional significance in apoptosis is p53 dependent.

Fig 2.6A

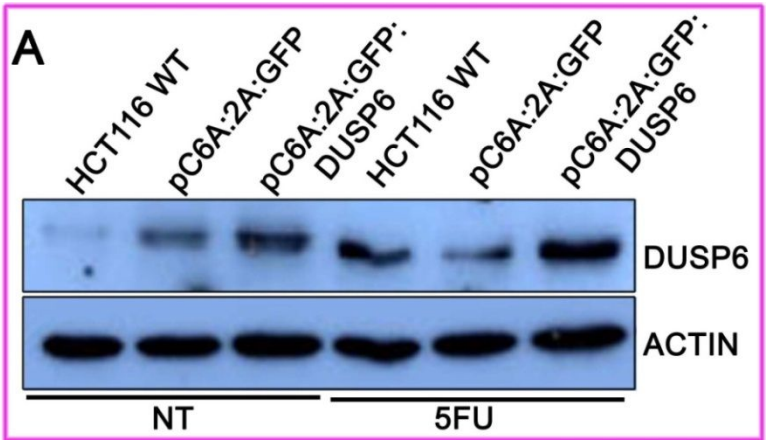


Fig 2.6B

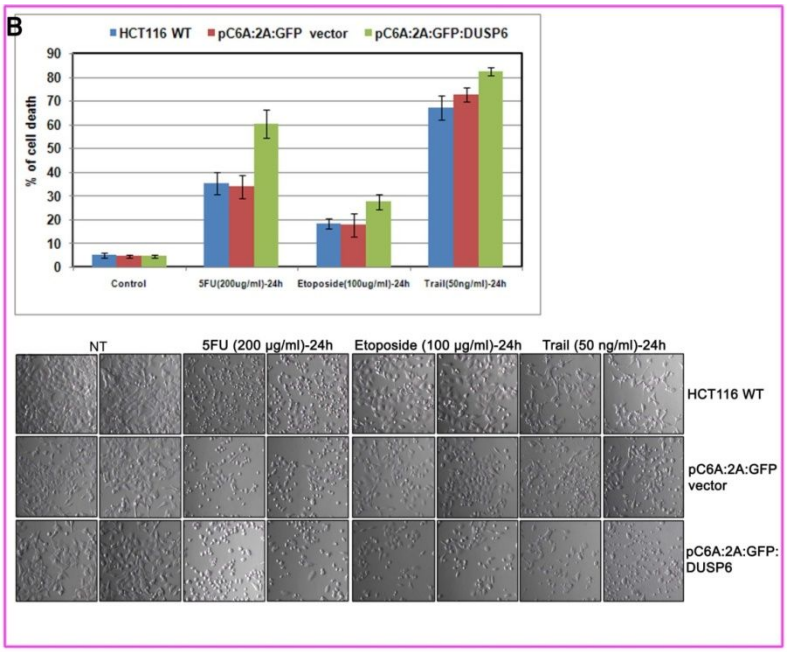


Fig 2.6C

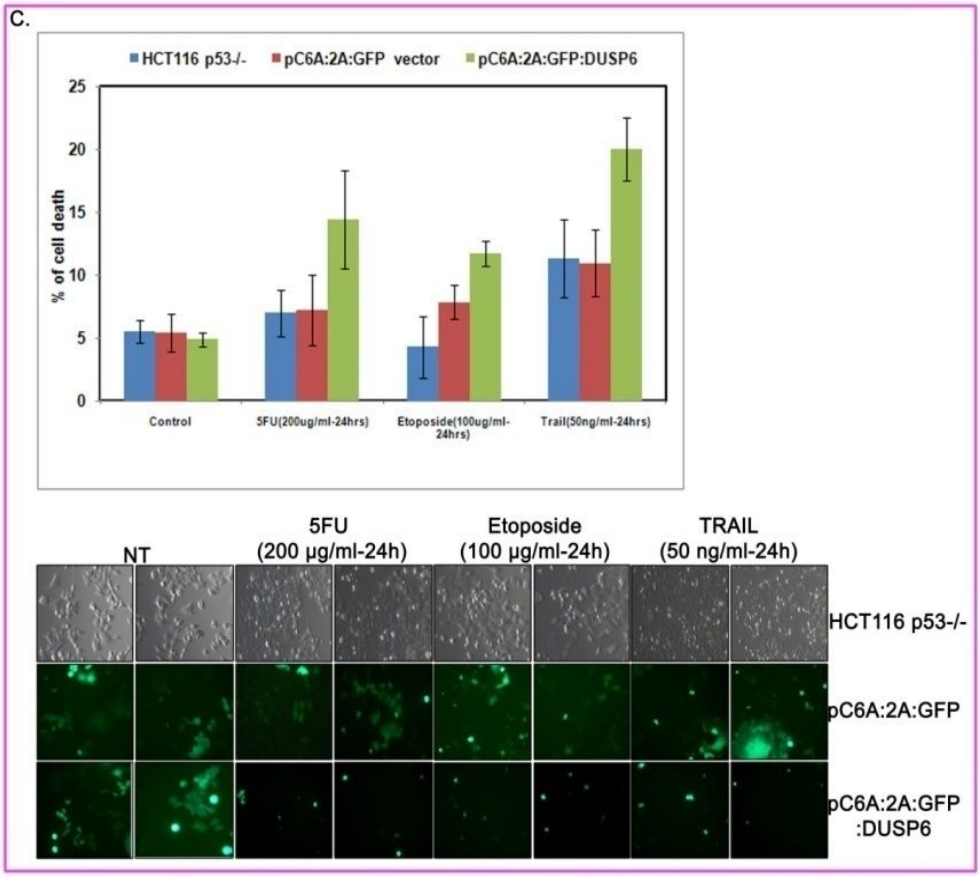


Fig. 2.6: Ectopic expression of DUSP6 enhanced the apoptotic responses. HCT116 WT and HCT116 p53$^{-/-}$ cells were transfected with either human DUSP6 construct pC6A:2A:GFP:DUSP6 or pC6A:2A:GFP vector and then clones were selected with Blasticidin. A: HCT116 cells that were transfected with either pC6A:2A:GFP vector or pC6A:2A:GFP:DUSP6 was treated with 5-FU (200 μg/mL), Etoposide (100 μg/mL) and Trail (50 ng/mL) for 24 hours and DUSP6 expression was analyzed by western blotting. Immunoblotting of α-actin was

used as loading control. The percentiles of cell death were measured by Trypan blue exclusion assay. Phase contrast microscopic images were obtained at 24 hours after treatment depicted in bottom panel (B). C: HCT116 $p53^{-/-}$ cells that were transfected with pC6A:2A:GFP vector or pC6A:2A:GFP:DUSP6 was treated with 5-FU (200 μg/mL), Etoposide (100 μg/mL) and Trail (50 ng/mL) for 24 hours the percentiles of cell death were measured by Trypan blue exclusion assay. Phase contrast and fluorescence microscopic images were obtained at 24 hours after treatment were depicted in bottom panel (C). *Points*, mean of three independent experiments; *bars*, SD.

2.4.5. DUSP6 suppresses ERK phosphorylation and elevates apoptotic response to 5-FU.

DUSP6 is known to be specific for ERK dephosphorylation and magnitude and duration of ERK activity determine the cellular proliferation and growth of tumor cell. To understand the functional link of ERK dephosphorylation to p53-mediated apoptosis, HCT116 WT cells and HCT116 $p53^{-/-}$ cells were subjected to 5-FU and observed for subsequent ERK dephosphorylation. Interestingly, the ERK dephosphorylation was significantly increased with p53 and thereby DUSP6 activation (Fig.2.7A). This dephosphorylation specifically caused by DUSP6 was confirmed by HCT116 cells expressing short hairpin of DUSP6 in response to 5-FU (Fig.2.7B).

Fig 2.7 A

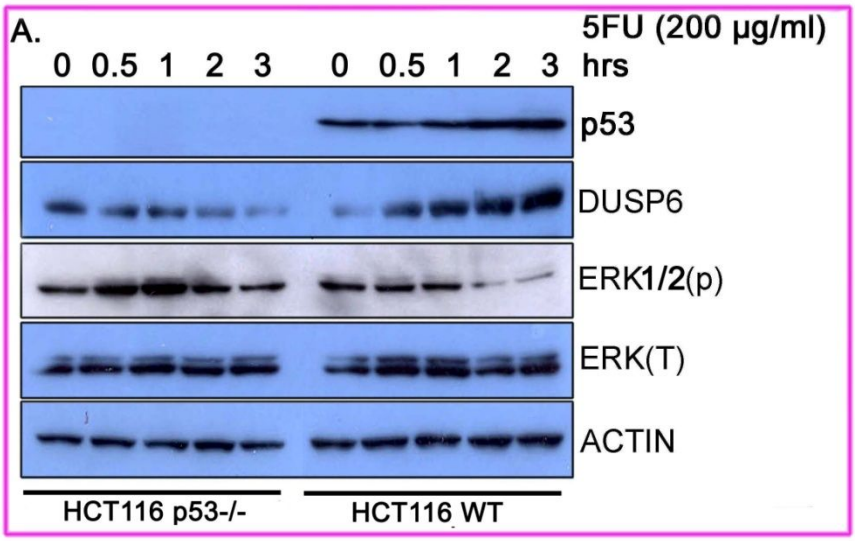


Fig 2.7B

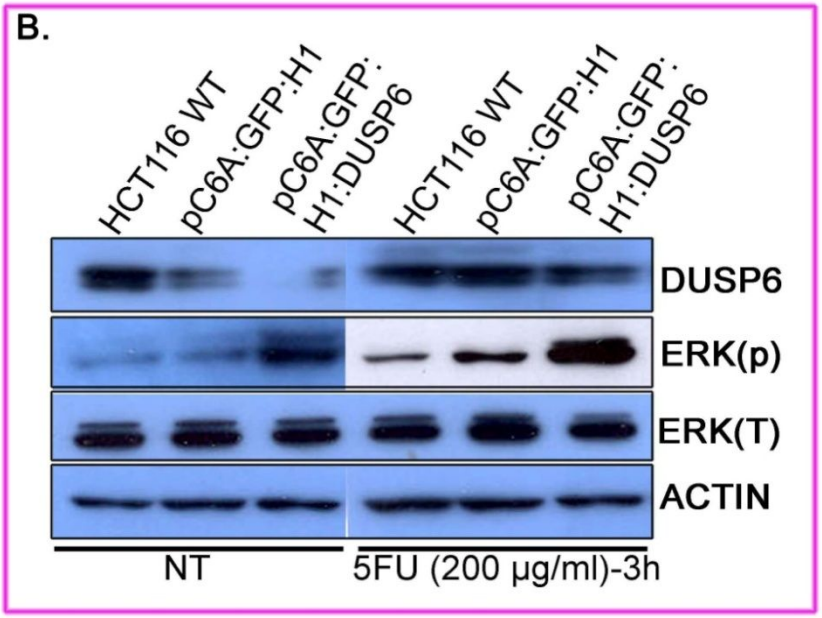


Fig. 2.7: DUSP6 suppresses ERK phosphorylation and elevates apoptotic response to 5-FU. A: Serum starved HCT116 WT and HCT116 $p53^{-/-}$ cells were treated with 5-FU (200 μg/mL) for indicated time interval. p53, DUSP6 and ERK dephosphorylation was detected by western blotting using a p53 antibody (Oncogene, USA), DUSP6 antibody (Abnova, Taiwan) and phosphor-ERK (*p*-ERK) antibody (Santa Crutz, USA) respectively. Total ERK and α-actin used as loading control. B: HCT116 cells expressing short hairpin of DUSP6 gain the function of phosphorylation of ERK in response to 5-FU. ERK phosphorylation and DUSP6 were detected by immunoblotting using specific antibody. Total ERK and α-actin used as loading control.

2.5. DISCUSSION

The fate of chemotherapeutic agent or different DNA damaging stresses are DNA repair, cell cycle arrest or apoptosis [138]. The key mediator in apoptosis is the p53 that actively transactivates certain genes such as pro-apoptotic genes and other associate regulatory genes of cell proliferation. The establishment of p53-mediated apoptotic signaling in regulation of transcript and its downstream cellular response is important for p53-mediated tumor suppression. In this thesis, I tried to identify the different mediators of p53-mediated apoptotic signaling and found that p53 transactivates DUSP6, PHLDA1 and SSFA2 genes.

The functional relation of DUSP6, PHLDA1 and SSFA2 to p53-mediated transactivation is detailed by mRNA expression through endogenous p53 in HCT116 WT cells and ectopic over-expression of p53 in HCT116 $p53^{-/-}$ cells. To facilitate the better understanding of DUSP6 as p53 target gene, I have detailed the functional and structural p53 putative binding site within DUSP6 promoter. The existence of putative binding site other than classical binding site and its physical interaction with p53 (Fig. 2.3) is important for p53-mediated regulation of DUSP6 and its associated role in apoptosis. Moreover, the introduction of short hairpin interfering RNA targeting DUSP6 reduced the level of apoptosis induced by p53-dependent death inducer such as 5-FU and Etoposide or by receptor-mediated death inducer such as TRAIL(Fig.2.4) as well as ectopically

expressed DUSP6 enhanced the apoptotic response to those stimuli (Fig.2.6) suggesting its role in p53-mediated apoptosis.

ERK1/2 is established molecules in the anti-apoptotic defense system [139]. The Raf/MEK/ERK signaling known to be responsible for regulation of BcL-2 family member Bad resulting in the dissociation from Bcl-XL and thereby protecting from apoptosis [140]. Similarly, ERK1/2 dependent phosphorylation of BcL-2 was known to be protective mechanism from proteasomal degradation in endothelial cell [92]. In accordance with survival signaling of phosphorylated ERK, its dephosphorylation of ERK by DUSP6 in p53-mediated apoptosis in response to cellular stress may be additional check points in those cellular signaling. Here I showed that DUSP6 dephosphorylates ERK in p53 dependent manner and abrogation of DUSP6 restores the ERK phosphorylation in response to 5-FU (Fig.2.7) suggesting probable hypothesis in functional link of DUSP6 in p53-mediated apoptosis.

A report showed that constitutive PHLDA1 expression was associated with reduced cell growth and apoptosis in melanoma cell line and down-regulation of PHLDA1 is associated with malignant melanomas [100]. So, transactivation of PHLDA1 might check in balance the cellular fate. Here, I showed that the introduction of short hairpin interfering RNA targeting PHLDA1 reduced the level of apoptosis induced by p53-dependent death inducer such as 5-FU and

Etoposide or by receptor-mediated death inducer such as TRAIL (Fig.2.5). Considering this fact, I could speculate that p53 can upregulate the PHLDA1 to induce apoptosis. However, detailed studies needs to be done to understand the functional importance of PHLDA1 in apoptosis.

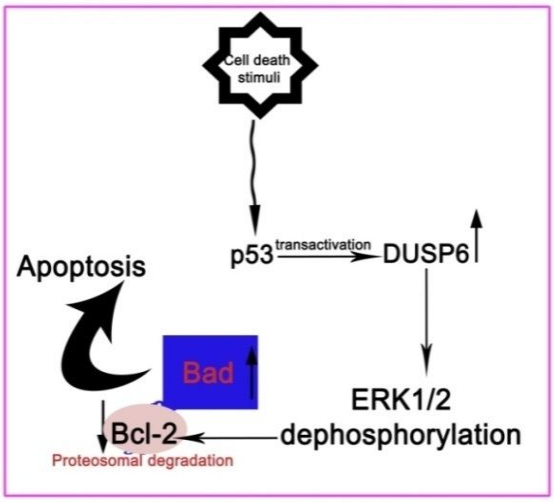


Fig.2.8: Possible mechanism of DUSP6 in p53-mediated apoptosis. Cell death stimuli activate p53 that transactivates DUSP6. The activated DUSP6 dephosphorylate ERK1/2 leaving the Bcl-2 in unphosphorylate condition that is ubibquitinated to proteasomal degradation. Degradation of Bcl-2 dissociates Bad thereby resulting downstream signaling and ultimately apoptosis.

In contrary to DUSP6 and PHLDA1, introduction of short hairpin interfering RNA targeting SSFA2 slightly reduced the level of apoptosis induced by p53-dependent death inducer such as 5-FU and Etoposide or by receptor-mediated death inducer such as TRAIL (Fig.2.5), meaning that SSFA2 induction by p53 is not a key event for p53-mediated apoptosis. Considering the fact that the SSFA2 protein function at the early step of cleavage of fertilized egg, I could speculate that p53 may play a role in securing the genome stability or regulating cell cycle by SSFA2 at the early cleavage step of the fertilized egg. However, further

studies needs to be done in order to reveal the functional significance of SSFA2 induction by p53.

In conclusion, this thesis enlightens how p53 respond to different stresses and regulate the distinct target gene resulting diverse cellular outcomes in tumor suppression.

CHAPTER 3

A novel cell killing peptide

3.1 ABSTRACT

Dysregulation, non-specificity, out of reach of chemotherapeutic drugs to tumor cells and inefficient physiologic cell death process are central dogma of cancer development. In this study, novel peptide approaches for the cancer treatment are proposed to utilize domain found in Bcl-2 family proteins couple with homing domain or protein transduction domain. Herein, we developed synthetic peptides TU1:MTD, TU2:MTD and TU3:MTD that effectively kill the cancer cells. Specifically, TU2:MTD and TU3:MTD dramatically cause the cell death of CT26.WT cells *in vitro* and these peptides efficiently suppressed the tumor growth in mice. Interestingly, the cell killing effect of these peptides is found restricted to tumor cell but not to primary cell hepatocytes. These findings suggest that MTD peptide may provide a new tool to treat the cancer.

Keyword: *MTD, Tumor*

3.2. INTRODUCTION

The balance between apoptosis and cell proliferation architect the human body. Impairment in this interplay of programmed cell death and cell proliferation imbalance the cell integrity resulting cancer. The practice of classical surgical excision of solid tumors is restricted to primary localized one. The treatment of rapidly spreading and malignant tumor requires extensive chemotherapy. But application of chemotherapy is limited by severe adverse effect on healthy organ, lack of tumor specificity resulting dose-limiting toxicity and development of chemo-resistance. Academic and pharmaceutical exploration and application of pro-drug in combination is intended for the treatment of cancer.

Different BH3 mimetic peptide and non-BH3 active peptide adapted from Bcl-2 family protein are reported to be active against cancer [15]. An advantage of those mimetic peptides is that; they are relatively free from side effects, bioavailability to target site and efficient and irreversible sensitivity to the target. The physiological function of those peptides lies on mitochondrial outer membrane permeabilization or fragmentation and subsequent release of intermembrane space protein associate with cell death [141].

The BH3-only protein Noxa and Puma are reported as transcriptional targets of the tumor suppressor protein p53. Recent findings in mice suggest that Noxa

is a major effector of p53-mediated cell death [142]. The sequence KLLNLISKLF (41-50) of carboxy-terminus of NOXA protein has been reported to function as mitochondrial target domain (MTD). This MTD exhibited a cytotoxic effect in a different cancer cell at low concentration and are more efficient than conventional pro-drug TRAIL or Cisplastin [102]. Considering all these facts, MTD sequence fused with homing vascular domain sequence was synthesized and evaluated for its efficiency against cancer cells and tumor. The coupling of homing domain improves the therapeutic index of agents either by increasing the drug concentration inside or by decreasing it in normal cell host tissues. Comparing to conventional chemotherapeutic agents, these synthetic peptides killed several different cancer cells including CT26 WT and HeLa cells vigorously and its specificity is restricted in cancer cells. The application of these peptides might form the basis for a new advanced anticancer drug.

3.3. MATERIAL AND METHODS

3.3.1. Cell cultures and reagents

A mouse colon carcinoma, CT26 WT and cervical cancer, HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin at 37^0C under 5% CO_2 in humidified incubator. Whereas rat hepatocytes were isolated following two step liver perfusion method as described by Seglan (Method cell biology 13:29-83, 1976) and maintained in William's Medium E (WME) (GIBCO, USA) supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin, ITS (1X concentration) and Dexamethason (40 μg/L) at 37^0C under 5% CO_2 in humidified incubator. 5-flurouracil (5-FU) was purchased from Aldrich-Sigma (USA).

3.3.2. Mice

Eight week-old male BALB/c mice were obtained from the SAMATAKO (SAMATAKO, Bio-Korea). The animal use protocol was approved by the Chosun University Animal Experiment Centre Committee. All animals were treated as humanely as possible.

3.3.3. Peptide synthesis

Peptides were synthesized and purified by HPLC to obtain peptides up to 98% purity (OPERON Biotechnologies, Germany). Peptides were suspended in autoclaved 3 times distilled water at 10 mM and stored at -20^0C.

3.3.4 In *vitro* killing activity of MTD peptides

CT26 WT (8×10^4) cells were seeded on 24 wells plate (Nunc, Roskilde) and treated with 5 μM, 10 μM and 25 μM MTD peptides and 5-FU (200 μg/mL) for different time interval. Similarly, HeLa cells (8×10^4) were seeded on 24 well plates (Nunc, Roskilde) and treated with 5 μM peptide for different time interval. The primary cell; hepatocytes ($4 \times 10^{5)}$ were seeded on 24 well plate (Nunc, Roskilde) and treated with different concentration of peptides for different time interval. The percentage of cell death was measured by trypan blue exclusion assay following standard method as described in preceding chapter. The crystal violets staining to survived cell were carried out following standard protocol as described previously. Briefly, cells were stained with 0.4% crystal violet in methanol for 20 minutes at room temperature and then washed with tap water. The morphological changes of cells were examined on phase contrast microscopy.

3.3.5. Evaluation of MTD peptides against tumor growth suppression

Ten BALB/c mice were used for each experiment. The subcutaneous tumor was induced by injection of 1.5×10^5 of CT26 WT cells in 100 μl phosphate buffer saline (PBS) into the subcutaneous tissue of the flank of the mouse with a insulin syringe as described [143]. Tumor cells were grown for 7 to 8 days and TU2:MTD peptide (385 μg/mouse), TU3:MTD peptide (230 μg/mouse) or PBS was intravenously injected through the tail vein every two days until the mice were sacrificed. The tumor size was measured with vernier caliper and the volume of the tumor was calculated as length x width^2 x 0.5. In addition to the tumor growth rate, the body weight and observation of the mice were followed.

3.3.6. Histological examination of tumor

After final dose, the mouse was sacrificed and tumor tissue was excised and weighed out the tumor mass. The excised tumor tissues and internal organs liver and kidney were fixed on 10% neutral buffered formalin solution and embedded in paraffin. Sections of fixed tissue were stained with hematoxylin and eosin following the standard protocol as described [143].

3.3.7. Statistical analysis

Values are presented as mean ± standard deviation. The paired student's *t-test* was applied to determine whether difference between values were significant. A statistical probability of $p<0.05$ was considered as significant.

3.4. RESULTS

3.4.1. A novel TU:MTD peptides induce cell death vigorously and efficiently than other conventional chemotherapeutic agents *in vitro*

In one of the previous study, Seo et al. defined the C-terminal region (41-50) of hNoxa as the MTD and shows potent killing activity against several types of cancer cells [102]. Since MTD peptides used in that experiments fused with the eight arginine (R8) amino acids that can penetrate the cytpolasmic membrane of any types of cells, they must be very toxic to animals if systemically injected. Thus, to develop MTD peptide as a tumor killing agent, it should be redesigned so as to selectively deliver it to tumor cells or tumor blood vessels. Three tumor-homing MTD (TU:MTD) peptides that bear the tumor vasculature-targeting motifs were synthesized (Table 3. 1) [144], [145], and they were subjected for the evaluation of tumor cell killing activities using CT26 mouse colon carcinoma cells and HeLa Cervical cancer cells. As shown in Figure 3.1, TU2:MTD and TU3:MTD peptides showed the vigorous cell killing activities of MTD peptide in CT26 cells and HeLa cells than conventional chemotherapeutic agent 5-flurouracil. However, TU1:MTD peptide didn't showed significant killing activity, indicating that TU2:MTD and TU3:MTD peptide may be the choice of pro-drug for cancer.

Table 3.4.1: Peptide nomenclature and sequences

Peptides	Sequences
R8MTD	RRRRRRRRGRQKLLNLISKLF
TU1:MTD	CNGRCGGKLLNLISKLF
TU2:MTD	CNGRCVSGCAGRCGGKLLNLISKLF
TU3:MTD	CGNKRTRGCGGKLLNLISKLF

R8 indicates 8 arginine amino acid residues.

Fig. 3.1A

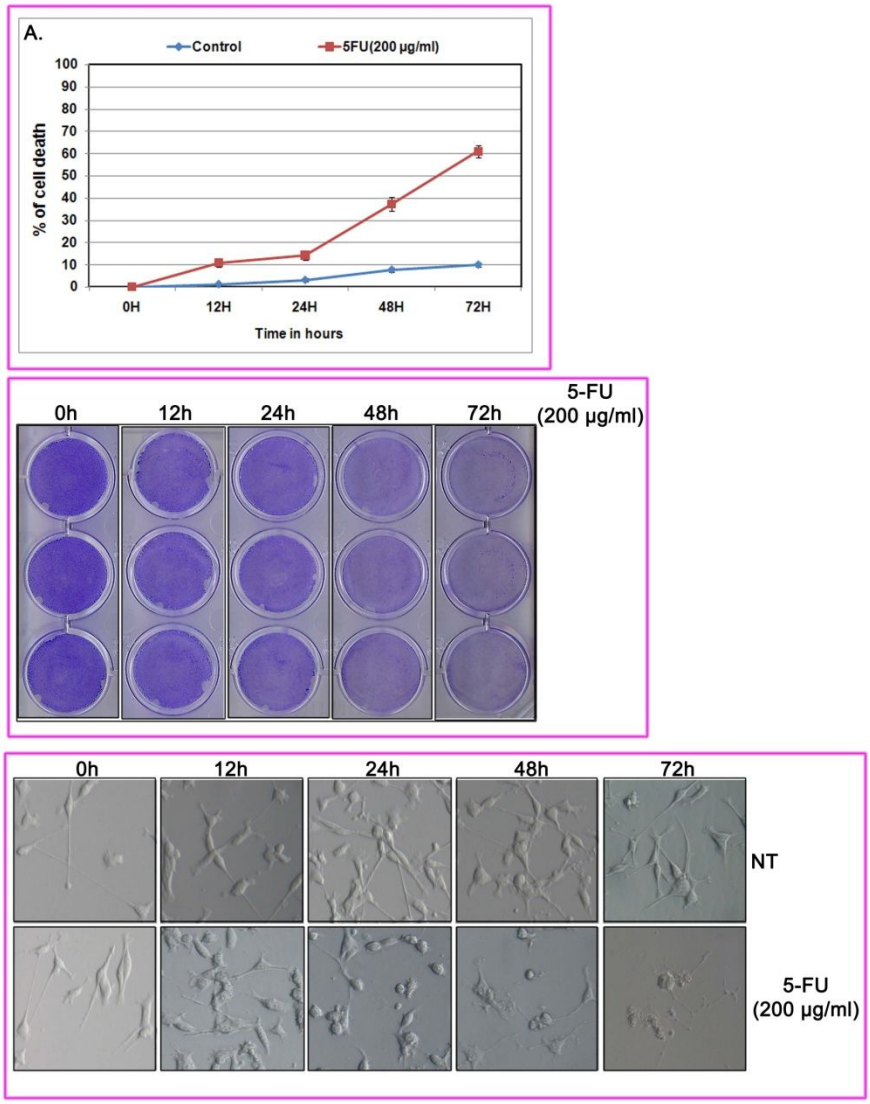
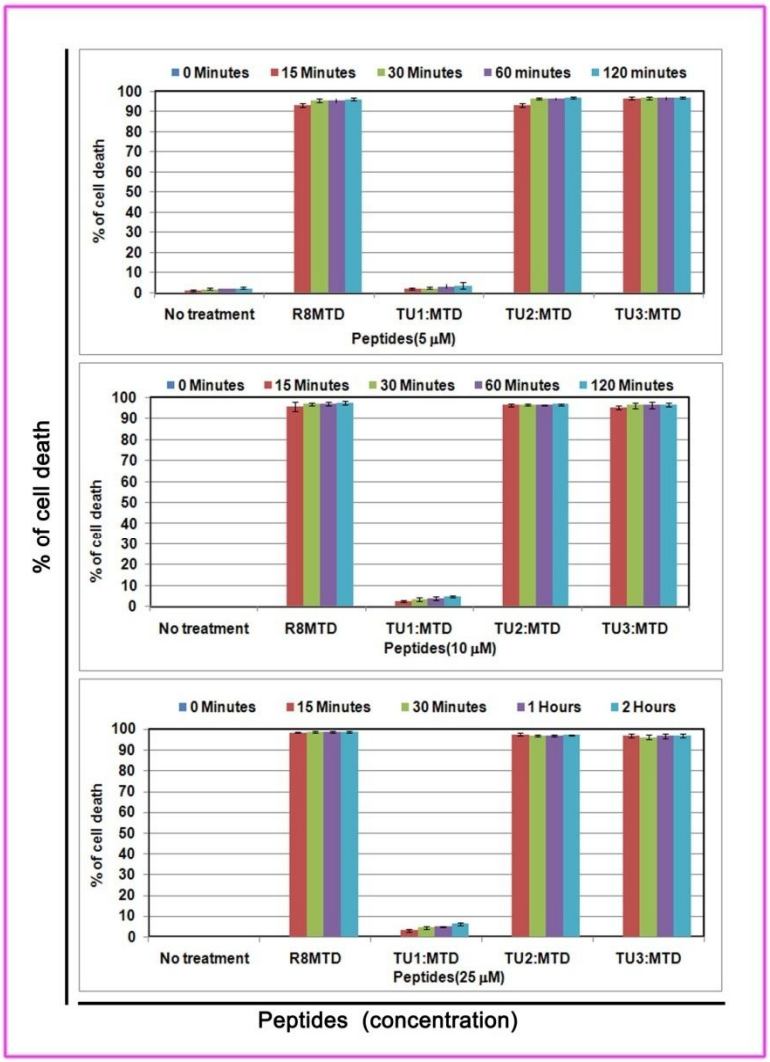


Fig. 3.1B



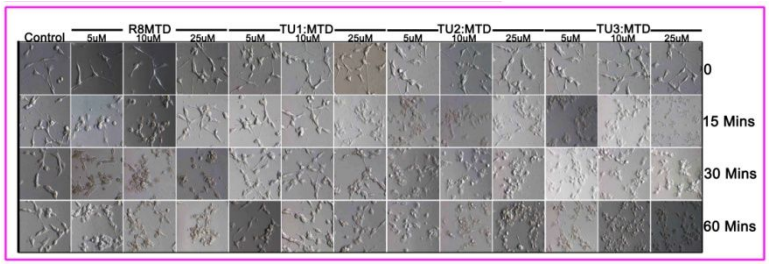


Fig.3.1C

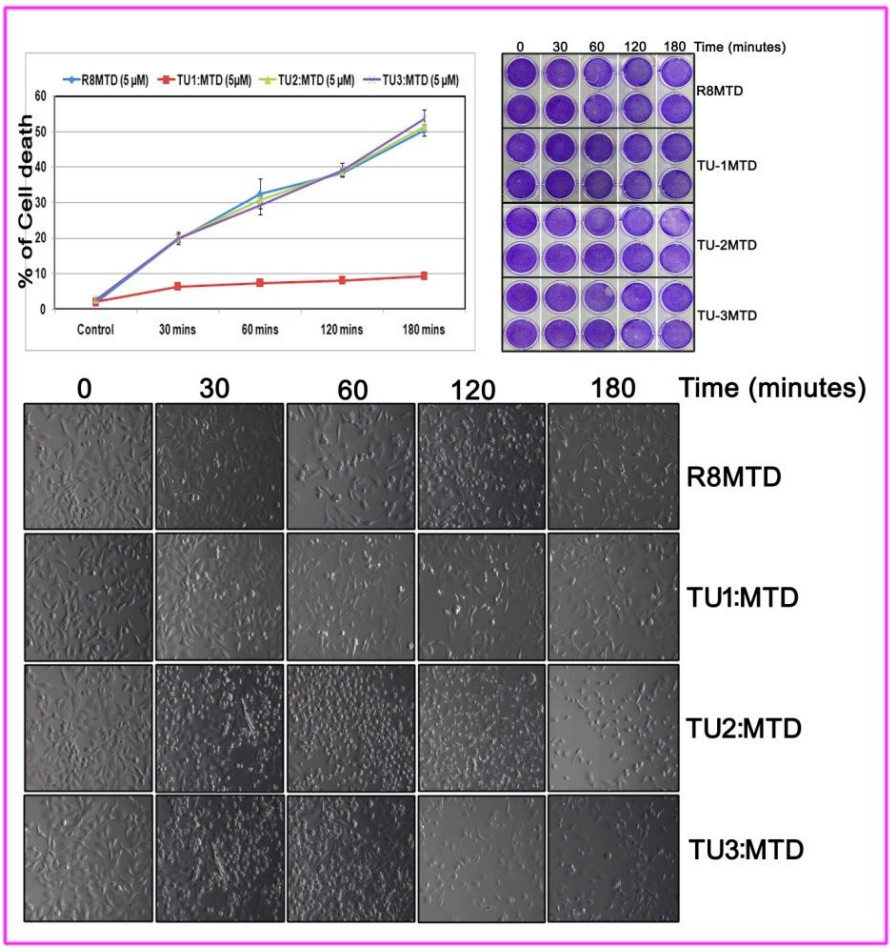


Fig. 3.1: A novel peptide dramatically and vigorously causes the cell death than conventional chemotherapeutic agent 5-FU. A: CT26 WT (80,000) were seeded and next day treated with 5-FU (200 μg/mL) for time interval 12h, 24h, 48h and 72 hours. The percentiles of cell death were calculated by trypan blue exclusion assay. The crystal violet staining of survived cells were depicted on middle panel and phase contrast microscopies of morphological changes of cells observed in different time interval were depicted on lower panel (A). B: Exponentially growing CT26 WT cells were treated with different concentration of R8MTD, TU1:MTD, TU2:MTD and TU3:MTD peptides (5 μM, 10 μM and 25 μm) for different time intervals 15 minutes, 30minutes, 1hour and 2hours. The percentage of cell death was measured by trypan blue exclusion assay. The phase contrast microscopies of morphological changes of cells observed in different time interval were depicted on lower panel (B). C: HeLa cells were seeded and next day treated with 5 μM each peptide for indicated time intervals. The percentiles of cell death were calculated by trypan blue exclusion assay. The crystal violet staining of survived cells were depicted on right panel and phase contrast microscopies of morphological changes of cells observed in different time interval were depicted on lower panel (C).

3.4.2. Tumor-homing MTD (TU:MTD) peptides suppress tumor growth

The efficient and rapid cell death activity against different cancer cell lines lead us to determine whether these peptides have targeting effect on tumor *in vivo* and thereby tumor growth suppression in animal. For this purpose TU2:MTD or TU3:MTD peptides were intravenously injected into Balb/c mice bearing tumors developed by injection of CT26 cells to subcutaneous region. The mice challenged with TU2:MTD or TU3:MTD peptides had significantly smaller tumors than did the mice treated with PBS (Figure 3.2A and 3.2B). Histochemical examination of tumors revealed that massive cell death of tumor cells were observed in mice injected with TU2:MTD or TU3:MTD peptides; however, no cell death of tumor cells was observed in mice injected with PBS (Figure 3.2C). Furthermore, dermis and epidermis regions of PBS-injected mice, TU2:MTD peptide-injected mice, or TU3:MTD peptide-injected mice showed similar structures having no or little cell death in these regions (Figure 3.2D). In addition, microscopic analysis (H/E staining) showed no damages in liver and kidney tissues obtained from the mice injected with TU2:MTD or TU3:MTD peptide seven times every two days (Figure 3.2E), and these peptide-injected mice showed no body weight loss (Figure 3.2F), indicating that TU2:MTD and TU3:MTD peptides do not show apparent toxic effects in mice.

Together, these results indicate that TU2:MTD and TU3:MTD peptides selectively target to the tumor regions and induce massive cell death of tumor cells but not normal cells.

Fig.3.2A

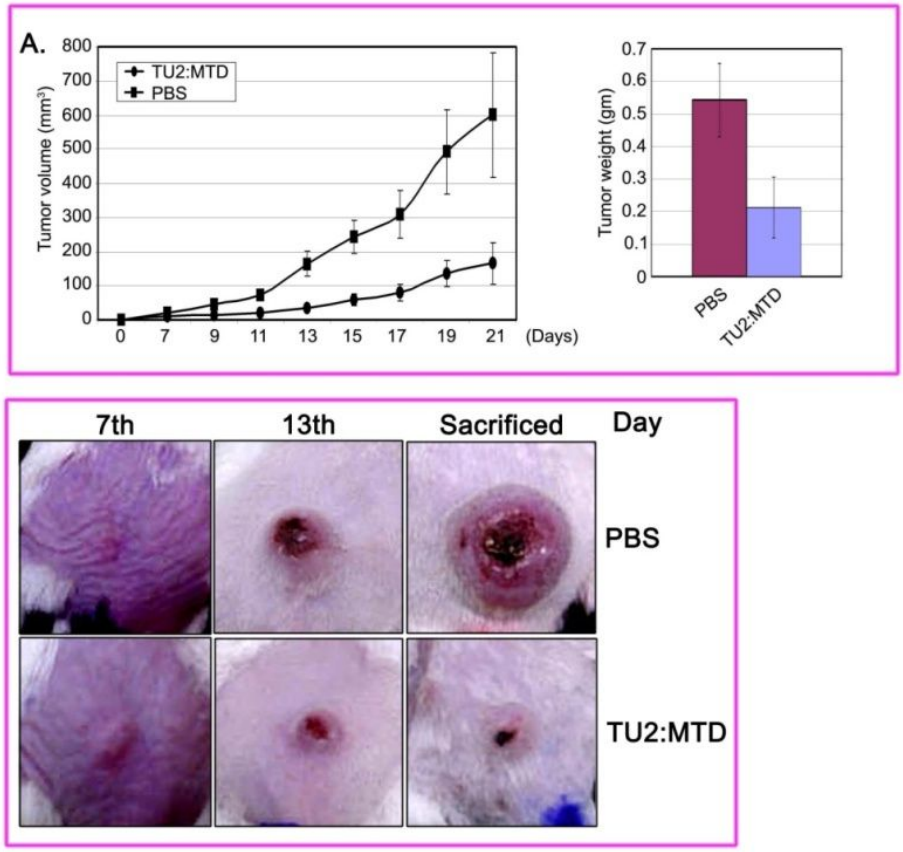


Fig.3.2B

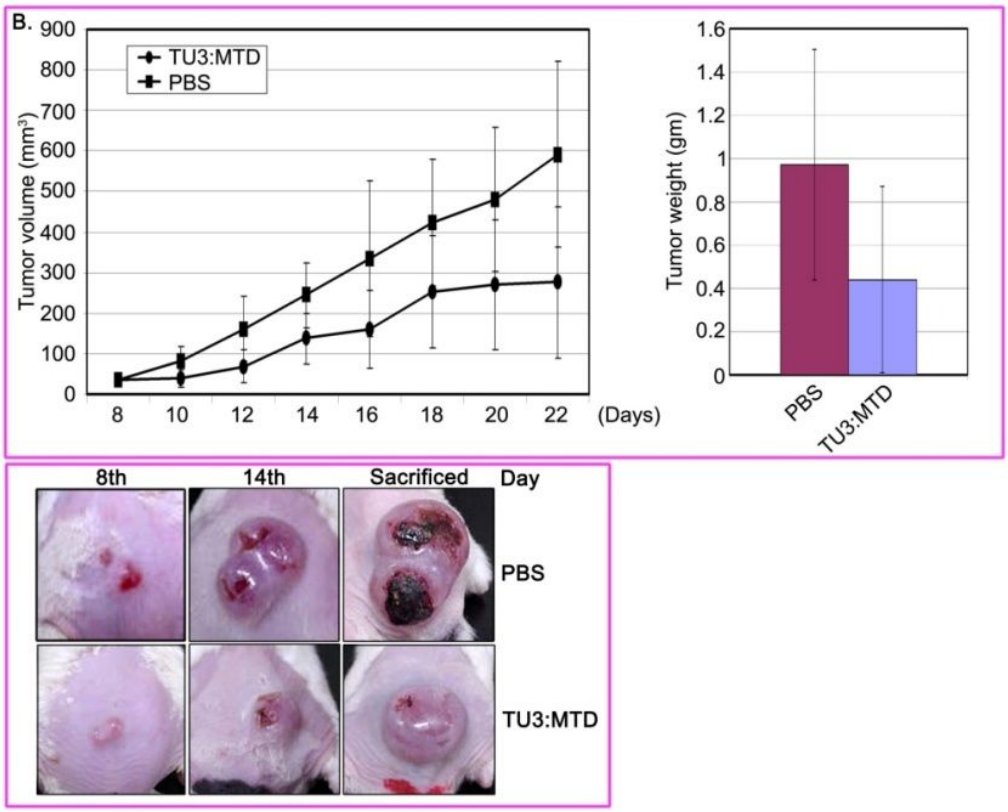


Fig.3.2CD

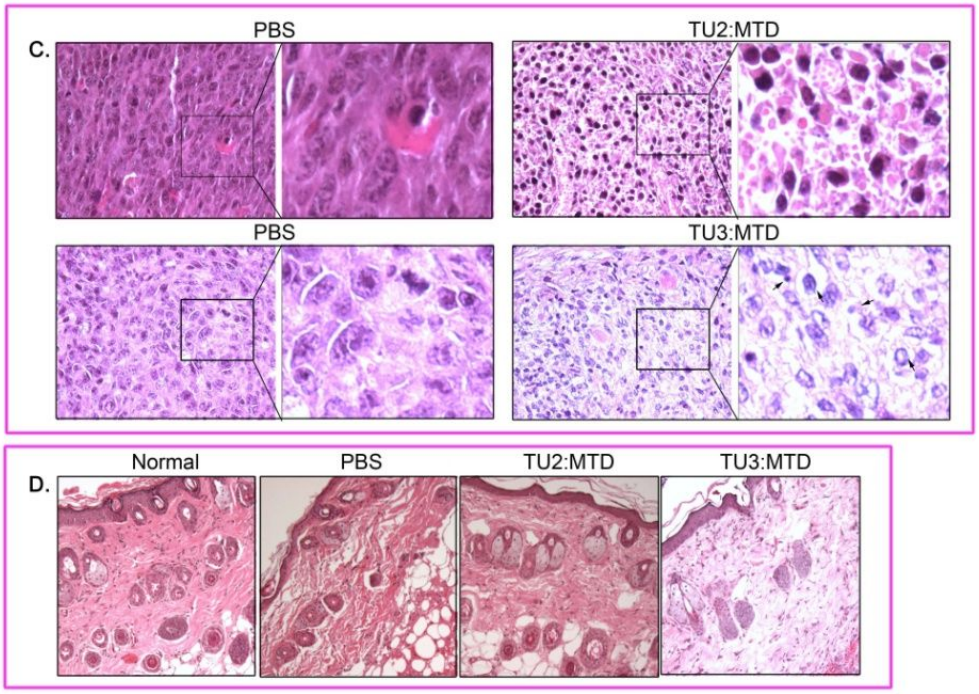


Fig.3.2EF

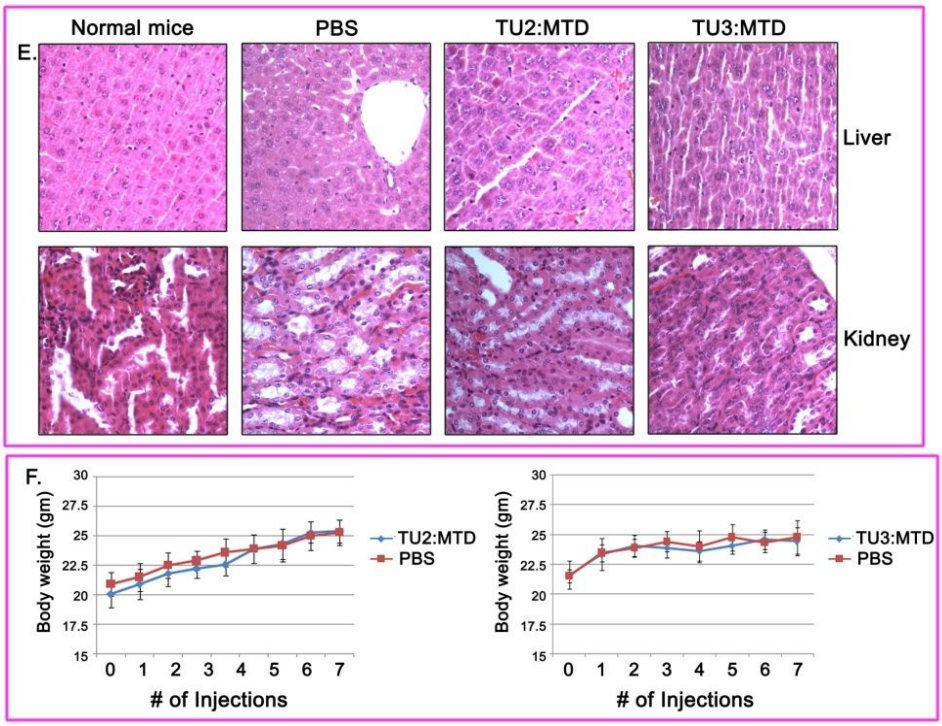


Fig.3.2. TU:MTD peptides induces suppression of the tumor growth in mice. A: CT26 cells were injected subcutaneously into the Balb/c mice. Tumor cells were grown for 7 days and then TU2:MTD peptide (385 μg/mouse) or PBS was intravenously injected through the tail vein (n = 10 animals/group) in one day gap from day 7 to day 19. Tumor volume was calculated as the longest diameter x width^2 x 0.5 and tumor weights were measured after mice were sacrificed. Representative image of tumor in mice was depicted in bottom panel (A).

B: TU3:MTD peptide (230 μg/mouse) or PBS were intravenously injected to the Balb/c mice bearing tumor through the tail vein (n = 5 animals/group) in single day interval from day 8 to day 20. Tumor volume and tumor weights were measured as described above. Representative image of tumor in mice was depicted in bottom panel (B).

C: Tumors were obtained from the mice treated with PBS, TU2:MTD or TU3:MTD upon sacrificing the mice and were stained with hematoxylin and eosin. Images (400 x magnification) at tumor regions were obtained (left), and the boxed region were enlarged (right).

D: Images (100 x magnification) of dermis and epidermis region of normal skin or adjacent to tumor treated with PBS, TU2:MTD or TU3:MTD peptides were obtained after stained with hematoxylin and eosin.

E: Images (400 x magnification) of liver and kidney from normal mice or mice treated with, PBS, TU2:MTD or TU3:MTD peptides for seven doses in every single day interval were obtained after stained with hematoxylin and eosin.

F: TU2:MTD (385 μg/mouse, n=10), TU3:MTD (230 μg/mouse, n=5), or PBS (n=10 for TU2:MTD, n=5 for TU3:MTD) were injected into mice through tail vein seven doses in every single day interval. Body weights were measured throughout the experiments. Points, means ± of three separate experiments; bars, SD.

3.4.3. TU:MTD specifically kills the cancer cell line not primary cell, hepatocytes

As discussed before, tumor homing domain fused to MTD sequence advance its specificity towards tumor cells. To elaborate its peculiar apparent restriction to cancer cell not to primary cell in vitro too, rat hepatocytes were treated with different concentration of peptides for different time intervals and cell death phenomena were suggested observing morphological changes of cells and trypan blue exclusion assay. Interestingly, TU:MTD peptides relatively did not induce cell death even in higher concentration(Fig.3.3A and 3.3B), but R8MTD peptides treated hepatocytes exhibited the concavity elevation of cell indicating the release of cellular contents resembling the cell death (Fig.3.3B) in higher concentration 25 μM, 50 μM and 100 μM which may be due to protein transduction domain; eight arginine (R8) amino acids assisting the penetration of the cytpolasmic membrane of any types of cells. Collectively the fusion of homing domain to MTD peptides may open the way to be considered itself as pro-drug for cancer treatment.

Fig.3.3A

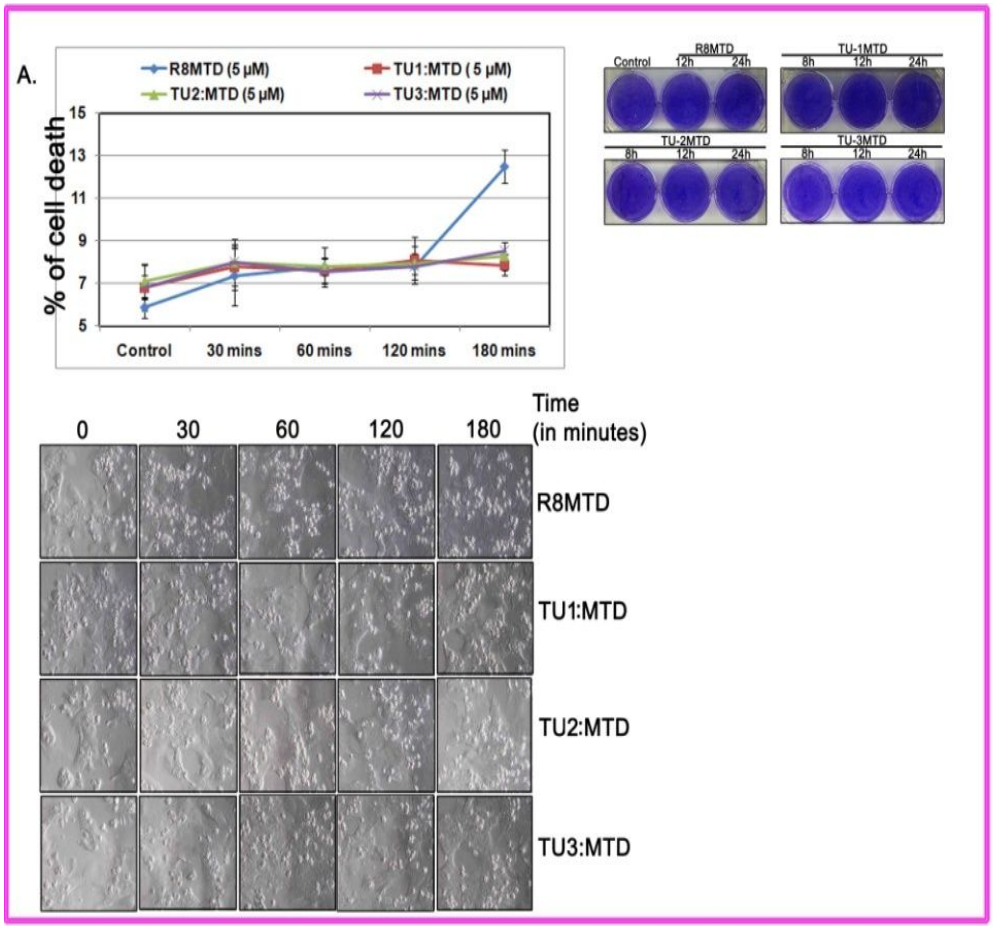


Fig.3.3B

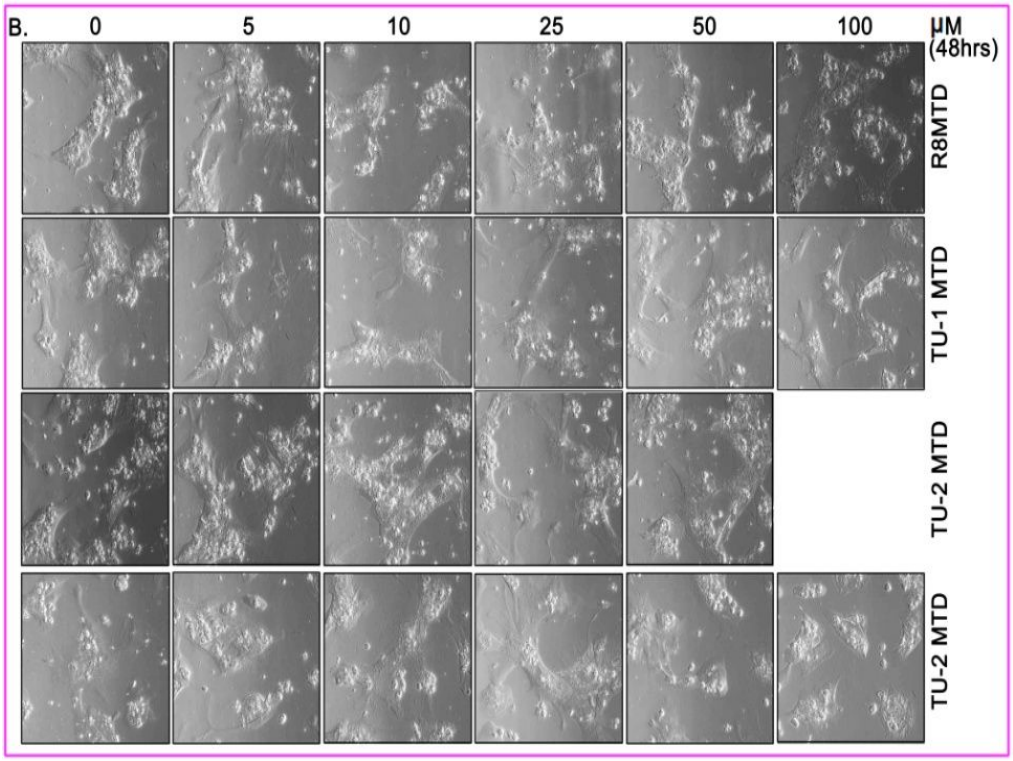


Fig.3.3: TU:MTD peptide relatively specific to cancer cell, not to primary cell. A: Rat hepatocytes were seeded and followed by treatment with R8MTD, TU1:MTD, TU2:MTD and TU3:MTD at concentration 5 μM for time interval 0.5, 1, 2 and 3 hours. The percentiles of cell death were calculated by trypan blue exclusion assay. The crystal violet staining of survived cells after indicted time intervals were depicted on left panel and phase contrast microscopies of morphological changes of cells observed in different time interval were depicted on lower panel (A). B: Rat hepatocytes were seeded and next day treated with R8MTD, TU1:MTD, TU2:MTD and TU3:MTD at concentration 5 μM, 10 μM, 25 μM, 50 μM and 100 μM for 48 hours and morphological changes of cells were observed in phase contrast microscopy (B).

3.5. DISCUSSION

The increasing number of cancer cases represents major health challenges to global development in the new century. Since past to present, cancer treatments cater surgical removal of solid tumor and sensitization of localized tumor to cell suicidal process. The latter sensitization includes chemotherapy, radiation, gene therapy and immunotherapy [146]. The combination of both is widely practiced for complete removal and elimination of tumor cell. However, these process restraint by reoccurrence and resistant to chemotherapy. The pros and cons of relapse and survival of tumor cell was lies on over-expression of pro-survival protein Bcl-2 and Bcl-XL [147]; thereby maintaining the mitochondrial integrity and tight regulation of pro-apoptotic molecules. The tight regulation of mitochondrial apoptotic pathway determines the fate of drugs. So, disabling pro-survival molecules in that pathway removes their restraint of pro-apoptotic molecules and thereby subsequent cascade of downstream mechanism to cell death.

Recent advance in cancer therapy is the development of many BH3 peptides that directly disrupts the mitochondrial outer membrane and subsequent release of death machinery molecules. Conventionally, the cell killing activity of Bcl-2 family protein was believed to lie on the BH3 domain. However, other 41-50 amino acids residues of C-terminus Noxa was reported to involved in

mitochondrial translocation and I assumed that this domain involved in mitochondrial fragmentation resulting cell death. Therefore I established the non BH3 peptides adapted from C-terminus of Noxa as TU:MTD and subjected to evaluation *in vitro* and *in vivo* for effectiveness against tumor cells.

The application of MTD peptide as drug required the delivery into the target site i.e. tumor site. It was expected that fusion of vasculature homing domain in N-terminal would overcome the cytotoxicity of other conventional chemotherapeutic agents by targeted delivery of active MTD peptides to tumor cells; preventing penetration into healthy organ thereby limiting its adverse side effects as exhibited by R8MTD. The functional significance of homing domain in peptides enhanced specificity to the tumor cell and not other cells supported by histological features of tumor mass and healthy internal organs. These finding suggests that TU:MTD peptides may be the new drug for cancer therapy.

CHAPTER 4

A role of Noxa in LPS-mediated tumor regression

4.1 ABSTRACT

The spontaneous regression of tumor is well documented but better understandings of molecular signature of genes involved in it are needed for therapeutic and preventive implications in tumor regression. Lipopolysaccharide (LPS) has been suggested as a key mediator of spontaneous tumor regression. Here, I tried to identify the mechanism of spontaneous tumor regression in the point of the death of tumor cells. One of the possible mechanisms, I assumed, was that the balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins could be the key factor to determine the fate of tumor cells. Here, I show that the architectural signaling of Bcl-2 family proteins including Noxa has been modulated by LPS *in vitro* and *in vivo*. The further Chromatin immunoprecipitation (Chip) analysis reveals that Noxa is a transcriptional target of interferon regulatory factor 1, 3 and 7 in response to LPS. Noxa upregulation in tumor cells is necessary to eliminate tumor cells according to the results in *in vivo* experiment using a syngeneic tumor model in which tumors were generated by subcutaneous injection of CT26 cells containing short hairpin interference RNA of Noxa. Collectively these findings could suggest that LPS induces or sensitizes tumor cells to death stimuli by up-regulating the pro-apoptotic Bcl-2 family proteins notably Noxa.

Key words: *LPS, Noxa, spontaneous tumor regression*

4.2 INTRODUCTION

The term spontaneous regression reflects the significant reduction in tumor size or a reversal in the progression of tumor without treatment but not to be confused with permanent and complete elimination of cancer [104]. Occasional, though rare, cases of spontaneous regression in human cancer have been seen and documented virtually in all types of human cancer. Spontaneous regression of colorectal tumor [104], pulmonary metastasis ([108], melanoma cancer [148], and non-Hodgkins lymphoma [149] etc were well reported through case reports but any satisfactory explanation for this phenomenon has not been put forward. A lot of causes like as immune recognition, virus or bacterial infection of tumor cell, cytokines, high level of stress induced steroids, hypoxic condition, telomeric breaks and gene mutation have been reported as mediators and induction of genes of either differentiation or cell death or immune responses might initiates spontaneous tumor regression [148].

A number of cases of spontaneous regression of hematologic cancer are associated with bacterial infection [150]. The component of bacteria, lipopolysaccharides (LPS) results the activation of mononuclear phagocytes to secrete many factors, including interferons, prostaglandins, tumor necrosis factor (TNF), and interleukin-I (IL-I) [151]. Such cytokines have been known to accelerate of host defense for swift elimination of tumor cell through toll like

receptor signaling [124]. LPS mediated spontaneous regression of tumor is the best example of cytokines dependency in innate immunity [116]. The anti-tumor properties of LPS is complemented by activation of IFNY. However pros and cons of signature molecule in spontaneous regression still remain unclear.

Type I (IFNα/β) and Type II (IFNY) Interferons induce the expression of a number of IFN target genes and exert direct cytotoxic or anti-proliferative function and regulate the vascular supply to newly grown tumor cell. The production of IFNα/β/Y following infection or LPS signaling regulate the Interferon regulatory factor 3 (IRF3), Interferon regulatory factor 7 (IRF7) [152] and Interferon regulatory factor 1 (IRF1) [153]. The functional behaviour of IRF1, IRF3 and IRF7 as a transcription factor is critical role in apoptosis and anti-tumoral activity in adaptive and innate immunity. Perhaps the process spontaneous regression of tumor required the activation of death machinery to eliminate tumor associated with innate immunity. The discovery and location of putative binding site for IRFs in death molecule of spontaneous regression might be the new tool for tumor therapy. Interestingly, the computational analysis of Noxa promoter reveals the putative binding site for IRF1, IRF3, IRF4 and IRF7. Furthermore, Chromatin immunoprecipitation analysis revealed the IRF1/3/4/7 bind to Noxa promoter region in response to LPS.

Noxa is a BH3-only protein, known as adult T-cell leukemia derived phorbol 12-myristate 13-acetate responsive gene. The p53 dependent upregulation of Noxa in response to DNA damaging agents such as Etoposide, UV radiation and 5-flurouracil and its role in p53 dependent apoptosis is well established [70]. As a BH3-only member of Bcl-2 family, Noxa is known to mediate cell death by interacting with other pro- or anti-apoptotic Bcl-2 family members. The balances of Bcl-2 family proteins determine fate of tumor cell. The expression of other pro-apoptotic proteins Bad, Bax, Bak, and Bid and tight regulation of anti-apoptotic molecules Bcl-2 and Bcl-XL are also associated with LPS suggesting probable role in spontaneous tumor regression.

The physiological behaviour of Noxa in response to LPS, at least in part complimented in spontaneous tumor regression was displayed by surveillance of short hairpin interference RNA of Noxa to CT26 tumor in syngeneic BALB/c mice.

4.3. MATERIAL AND METHODS

4.3.1. Cell cultures and reagents

A human colorectal carcinoma, HCT116 parental cells and p53-deficient HCT116 cells were maintained in McCoy's 5A media with 10%FBS, supplemented with 2 mM L-glutamate, 100 U/mL Penicillin and 100 μg/mL Streptomycin at 37^0C under 5% CO_2 in humidified incubator. A mouse colon carcinoma cell line CT 26 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, supplemented with 2 mM L-glutamate, 100 U/mL Penicillin and 100 μg/mL Streptomycin. Similarly, cervical cancer, HeLa cells and acute T cell leukemia, Jurkat cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI respectively with 10% FBS, supplemented with 2 mM L-glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin. WT and Noxa knock-out BMK (Baby mouse kidney) cells were generous gift from John Hiscott (McGill University, Montreal, Que, Canada) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, supplemented with 2 mM L-glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin. A recombinant human Interferon-α (IFN-α), Interferon-β (IFN-β) and human/murine Interferon-Y (IFN-Y) were purchased from Cytolab / Perotech Asia (Hamada, Israel). 5-flurouracil (5-FU) and Lipopolysaccharides (LPS) derived from *Escherichia coli* were purchased from Aldrich-Sigma. Velcade was purchased from Janssen Pharmaceutica N.V.

(Beerse, Belgium). Blasticidin was purchased from Invitrogen life technologies (California, USA). Anti-actin antibody was purchased from Chemicon international (California, USA). Anti-Bad and Anti-Bim antibodies were purchased from Cell signaling Technology (Beverly, MA, USA). Anti-Bak antibody was purchased from Upstate biotechnology (Lake Placid, NY, USA). Anti-Bax antibody was purchased from Santa crutz biotechnology.Inc (California, USA). Anti-Bid antibody was purchased from Prosci Incorporated (Poway, CA, USA). Anti-Bcl-2 and Anti-Bcl-XL antibodies were purchased from Cell signaling Technology (Beverly, MA, USA). Anti-Mcl-1 antibody was purchased from BD Transduction laboratories (Franklin Lakes, NJ USA). Anti-IRF1 and Anti-IRF3 antibodies were purchased from Cell signaling Technology (Beverly, MA, USA). Anti-IRF7 antibody was purchased from Prosci Incorporated (Poway, CA, USA). Anti-IRF4 antibody was purchased from Santa crutz biotechnology.Inc (California, USA). Anti-Noxa antibody was purchased from Oncogene Research Products (San Diego, CA, USA) or Abcam (Cambridge, MA, USA). Anti-p53 antibody was purchased from Oncogene Research Products (San Diego, CA, USA). Flag-tagged antibody was purchased from Cell signaling Technology (Beverly, MA, USA). Anti-GFP antibody was purchased from Santa crutz biotechnology.Inc (California, USA). Horseradish peroxidase (HRP) - conjugated goat anti-rabbit/-

mouse IgGs were purchased from Jackson ImmunoReasearch Lab (West Groove, PA).

4.3.2. Short hairpin RNA interference of Noxa construction and analysis of cell death in response to LPS.

Vector pC6A:GFP:H1 was designed to express the green fluorescent protein (GFP) and short hairpin interference RNA (shRNA) under the CMV promoter and H1 promoter respectively. Each Noxa and Scramble Noxa cassettes were generated by annealing the Oligonucleotides containing the target sites or scrambled nucleotide of the target site with overhang BglII and ClaI and then ligation into Bgl II and ClaI site of pC6A:GFP:H1 named as pSUPER Noxa sh RNA and pSUPER sc Noxa sh RNA respectively . The Oligonucleotides used for generation of shRNA are listed in table 4-1A. CT26 wild type cells were transfected with either pSUPER vector or pSUPER Noxa sh RNA or pSUPER sc Noxa sh RNA gene with effectene transfection reagent (Qiagen, Chatsorth, CA, USA) and the transfected clones were selected with blasticidin (10 μg/mL) for 48 hours. The selected clones were subjected to treatment with LPS (10 μg/mL) as indicated time. The percentage of cell death was measured by trypan blue exclusion assay following standard method as described in preceding chapter. The knock down efficiency of gene was checked by RT-PCR and Western blotting.

4.3.3. Mice

Eight week-old male BALB/c mice were obtained from the SAMATAKO (SAMATAKO, Bio-Korea). The animal use protocol was approved by the Chosun University Animal Experiment Centre Committee. All animals were treated as humanely as possible.

4.3.4. Syngeneic tumor model

Ten BALB/c mice were used for each experiment. The subcutaneous tumor was induced by injection of 1.5×10^5 cells of CT26 WT or corresponding vector transfected CT26 cells in 100 μl phosphate buffer solution (PBS) into the subcutaneous tissue of the flank of the mouse with a insulin syringe as described [143]. Tumor cells were grown for 12 days and tumor bearing mice were challenged by intra-peritoneal injection of LPS (3mg/kg) or PBS. The tumor size was measured with vernier caliper and the volume of the tumor was calculated as length x width^2 x 0.5. In addition to the tumor growth rate, the body weight and observation of the mice were followed. For BH3 profiling tumor tissue were excised at day 4 after LPS and subjected to lysates preparation and total RNA isolation. Briefly, tumor tissues were dounce with dounce homogenizer with RIPA buffer and homogenized tissue incubated in ice for 30 minutes. The total cell lysates were collected after centrifugation at 4^0C in 12000 rpm for 10 minutes.

4.3.5. Histological examination of tumor

The mouse was sacrificed at 4 days after the LPS/PBS injection before complete regression of tumor, the mouse was sacrificed and tumor tissue was excised. The excised tumor tissues and internal organs liver, heart, spleen, lung and kidney were fixed on 10% neutral buffered formalin solution and embedded in paraffin. Sections of fixed tissue were stained with hematoxylin and eosin following the standard protocol as described [143].

4.3.6. Infection of adenovirus

The recombinant adenoviral vector expressing GFP (Ad-GFP), expressing IRF3 (Ad-IRF3) and expressing IRF7 (Ad-IRF7) were generous gift from John Hiscott (McGill University, Montreal, Que, Canada). IRF1 (ad-IRF1) was generous gift from Dr.Seol. These adenoviral vectors were infected to BMK WT and BMK Noxa-/- cells in different MOI for indicated time. Ad-GFP was used as negative control. The multiplicity of infection (MOI) was defined as ratio of infectious virus particles to cells. The titer of the virus was calculated using the standard Tissue culture Infectious Dose 50 ($TCID_{50}$) following the manufacturer standard protocol (Ad Easy^{TM} vector system, Qbiogene, Inc. USA).

4.3.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TRIzol (Invitrogen, USA) following the standard protocol. 2 μg of total RNA was reverse transcribed with Improm-II reverse transcriptase (Promega, USA) following standard manufacturer protocol. About 200 ng of the reverse transcribed cDNA was analyzed by semi quantitative RT-PCR. Twenty five cycles of amplification following denaturation at 94^0C for 30s, annealing at 60^0C for 40s and extension at 72^0C for 30s was performed. The PCR products were separated on 1-2% agarose gel and stained with ethidium bromide and band intensities of amplified DNAs were compared after visualization with a UV transilluminator. The primer set used to amplification of corresponding genes are listed in table 4-1B.

4.3.8. Plasmid construction and over expression of IRFs

The fragment of full length human IRF4 was amplified from cDNA and cloned into N-terminal flag tagged Topo entry vector. The primer used for amplification listed on table 4.1C. Finally the DNA sequences in entry vector were sub clone into N-terminal GFP-tagged destination vector following standard protocol of Gateway^R Technology (Invitrogen, CA, USA).The N-terminal Flag-tagged IRF1/IRF3/IRF7 plasmids were generous gift from Lee Mirha (Chosun University, Kwangju, South Korea). HCT116 wild type cells were transfected with IRFs over expression vector with effectene transfection reagent (Qiagen, Chatsorth, CA, USA) and next day, the percentage of cell death was measured by trypan blue

exclusion assay following standard method as described in preceding chapter. The over expression of IRFs and induction of Noxa genes were checked by Western blotting.

4.3.9. Western blotting

All of the cells used in this experiment were cultured in 6 well plates ($2X10^5$/well) (Nunc, Roskilde) for one day and treated with Velcade (1 μM), LPS (10 μg/mL) and IFN-α/β/γ (10 ng/mL) for different time intervals. Jurkat cells, HeLa cells and CT26 WT Cells ($2X10^5$/well) cells were seeded on 6 well plates (Nunc, Roskilde) and next day treated with LPS (10 μg/mL) and IFN-α/β/γ (10 ng/mL) for different time intervals. Cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-Cl pH 7.5 and 150mM NaCl) supplemented with 1mM phenylmethylsulfonylfluoride (PMSF), 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1μg/mL pepstatin A, 2mM sodium fluoride and 1 mM sodium orthovanadate. Soluble lysates were subjected to SDS-PAGE and transferred to a Polyvinylidene Fluoride (PVDF) membrane (BIO-RAD, USA). Then membrane was probed with specific antibodies, followed by HRP-conjugated anti-IgG antibody. Immobilon™ Western chemiluminescent HRP substrate (ECL) (Millipore, Billerica, MA, USA) was used for the detection of bound antibody.

4.3.10. Chip analysis

To locate the IRFs binding site within Noxa promoter region, Chromatin Immunoprecipitation (CHIP) assay adapting the standard procedure (www.abcam.com/techinical and the epigenome Network of Excellence) was carried out as described in previous chapter. Briefly, CT26 wild type ($2X10^8$) cells were seeded on 100mm dish (Nunc, Roskilde) and treated with LPS (10 μg/mL) or IFNɣ (10 ng/mL) for different indicated time points on the following day. Bound proteins were cross linked to chromatin by adding the media containing 1% formaldehyde (Sigma, Aldrich) drop-wisely directly on dish rotate gently at room temperature for 10 minutes.

After sonication, cross linked protein-DNA complexes were immunoprecipitated form nuclear extracts by using anti-IRFs antibody and followed by captured on protein A sepharose beads (GE, Healthcare). After immunoprecipitation and elution from the beads, DNA-protein cross-linking was reversed by addition of proteinase K and heating at 65^0C for overnight and then DNA was purified with phenol extraction followed by ethanol precipitation. PCR amplification of Noxa promoter was performed using specific primers detailed in table 4-1D.

4.3.11. Statistical analysis

Values are presented as mean ± standard deviation. The paired student's *t-test* was applied to determine whether difference between values were significant. A statistical probability of $p<0.05$ was considered as significant.

Table 4-1A: List of primers for shRNA and ScRNA of Noxa

Noxa	5'-GATCCCC-GTCGGAACGCGCCAGTGAA-TTCAAGAGA-TTCACTGGCGCGTTCCGAC- TTTTTGGAAAT-3' 5'-CGATTTCCAAAAA-GTCGGAACGCGCCAGTGAA-TCTCTTGAA-TTCACTGGCGCGTTCCGAC-GGG-3'
ScNoxa	5'-GATCCCC-GTCGGAACGCGGGTGTGAA-TTCAAGAGA-TTCACACCCGCGTTCCGAC -TTTTTGGAAAT-3' 5'-CGATTTCCAAAAA-GTCGGAACGCGGGTGTGAA-TCTCTTGAA-TTCACACCCGCGTTCCGAC-GGG-3'

Table 4-1B: List of primers for RT PCR

Noxa	5'-AGCTACCACCTGAGTTCGCA-3' 5'-TCACTTTGTCTCCAATCCTCCG-3'

Table 4.1C: List of primers for IRF4 overexpression

IRF4:

5'- AAGGAAATA ACCACCATGGAACCTGGAGGGCGG-3'

5'- TCATTCTTGAATAGAGGAATGGCG-3'

Table 4-1D: List of primers for Chip analysis

IRF1	
(-3453…-3246)	5'-TCTGGAAGCATGGTGGG-3'
	5'-GGGAATCACAGTTATCTTTGGCAG-3'
IRF3	
(-6258…-5957)	5'-ACATACCAGCTATGACACCG-3'
	5'-GAAAGAGAGAGAGAGAGAAAG-3'
(-5113…-4750)	5'-GCTGAGAGTGAATTTGAAAGGG-3'
	5'-CCCAGTGTAGTCTGGGTTTA-3'
IRF4	
(-6583…-6420)	5'-AGGTCTGGGTGTCTGTGGGTTAAA-3'
	5'-AAGGGTATGTGCAACCACAACCAG-3'
(-4755…-4589)	5'-ACTGGGTTACCACTGGGTTCTGTT-3'
	5'-CAACTAATGGATACGAACTGGAGGG-3'
(-3196…-2919)	5'-CCAACTGCCCATGTGGCTTTGTTT-3'
	5'-GCACAGCATAAACAGCTCCGCTTT-3'
(-2208…-2057)	5'-CACACTTGGCTTCTATTTCTGTTCC-3'
	5'-GGAAGGAATTTGATTCGAGTAGACTTTAG-3'
IRF7	
(-5295…-5055)	5'-GCACATGTGACTTCTTTGTTG-3'
	5'-GACAGTTTAAAGAGTCACCA-3'
(-3812…-3633)	5'-CAGGAGTTTGGCTCTGTACTTTCC-3'
	5'-GGTTCCTGAGCACTGACATTACAGGT-3'
(-2208…-2057)	5'-CACACTTGGCTTCTATTTCTGTTCC-3'
	5'-GGAAGGAATTTGATTCGAGTAGACTTTAG-3'
(-1185…-1032)	5'-CTGCTAACTTTACTACGTGGCT-3'
	5'-ACCCTCCAAGTTCAATACAGGCAG-3'
(-640…-416)	5'-TCCTTGGAACGAACCACA-3
	5'-AAGGTGGGAGGGAAACTTCA-3'

4.4. RESULTS

4.4.1. LPS sensitize the tumor cell and activate the Bcl-2 family protein, notably Noxa in spontaneous tumor regression

Schreiber et al. well documented the LPS mediated spontaneous regression of tumor through the activation of immune system. The activated immune system and death machinery may be the possible tool in the spontaneous regression. To further understand the possible mechanism of the spontaneous tumor regression and correlate the underlying proteins in tumor regression in response to LPS, syngeneic BALB/C mice were adapted to generate tumor. For this purpose, CT26 WT ($1.5X10^5$) subcutaneously injected to flank of mice. After 12 days tumor bearing mice were challenged by intraperitoneal injection of LPS (3mg/kg). Administration of LPS exerted spontaneous regression of the tumor and histological examination of tumor showed combination of necrotic and apoptotic cell death but not with PBS (Fig.4.1A).The elimination of tumor reflects the necrosis kind of feature in tumor site which lead to profile the death executioner in tumor. Interestingly, the profiling of pro-apoptotic and anti-apoptotic protein reveals the executioner cleaved caspase-3 as well as elevation of pro-apoptotic proteins Bim, Bad, Bax, Bak, notably Noxa and active form of bid indicated by decreases in total bid whereas pro-survival molecules Bcl-2 and Bcl-XL as well as Mcl-1 were downregulated indicating its role in tumor cell death (Fig.4.1B). To

understand the LPS effect on systemic circulation different internal organs were profiles for Noxa expression. The lung, kidney, heart, spleen and liver were sensitized to expression of Noxa but macroscopic and histological examination of internal organ showed normal physiological appearance (Fig.4.1C).

Next, I am curious to understand the LPS mediated spontaneous regression restricted to particular tumor or generalized one. Therefore, tumors were generated by subcutaneous injection of mouse epidermis and chemically transformed cells T36274 and mouse myeloma cells SP2/0 and then subjected to LPS injection. The T36274 induced tumor regressed spontaneously but not the SP2/0 induced tumor (Fig.4.1E). Interestingly, the BH3 profiling of T36274 in response to LPS showed the same pattern as CT26. In contrast, the BH3 profiling of SP2/0 revealed the elevated expression of anti-apoptotic Mcl-1, Bcl-2 and Bcl-XL and down-regulation of pro-apoptotic BH3 family protein notably Noxa protein with time (Fig.4.1D). Taken together these profiling I can assume that regulation of pro-apoptotic dominantly Noxa and anti-apoptotic of Bcl-2 family proteins are the possible mediator of spontaneous regulation of tumor.

Fig.4.1A

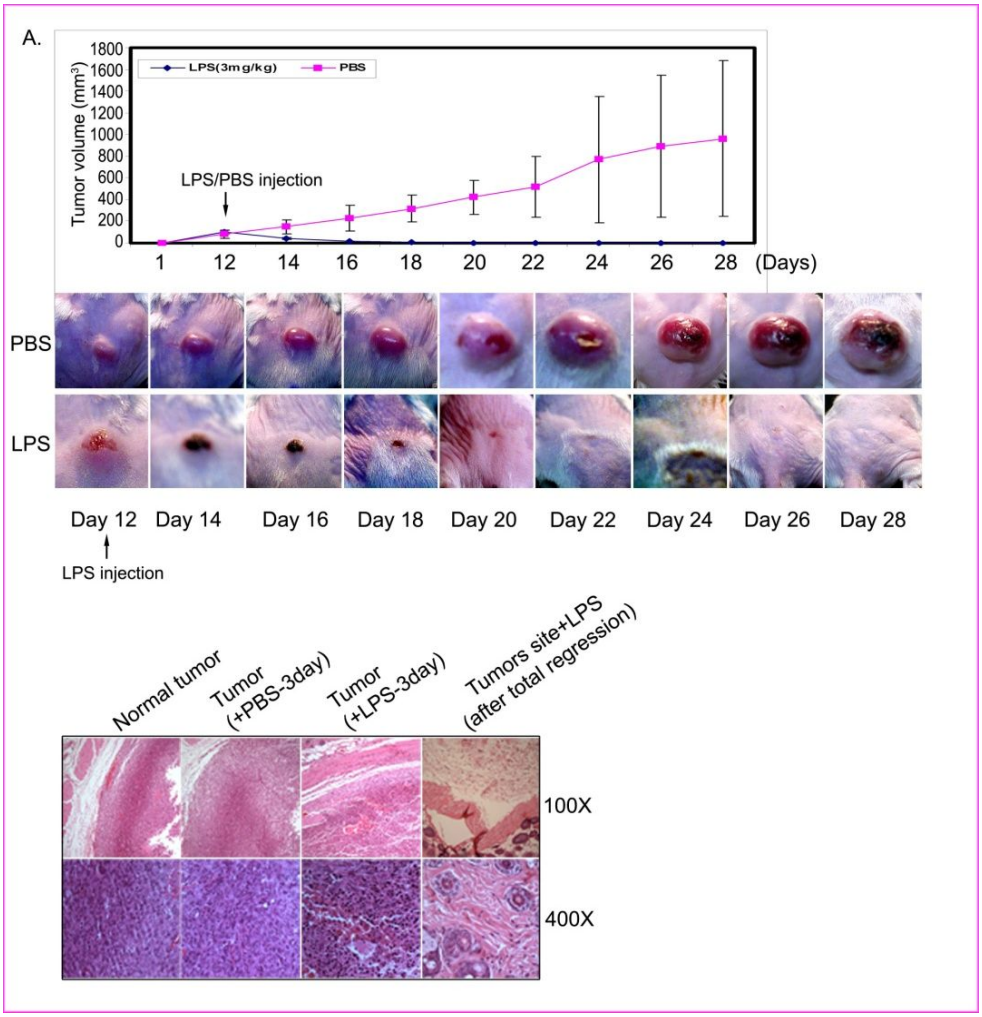


Fig.4.1B

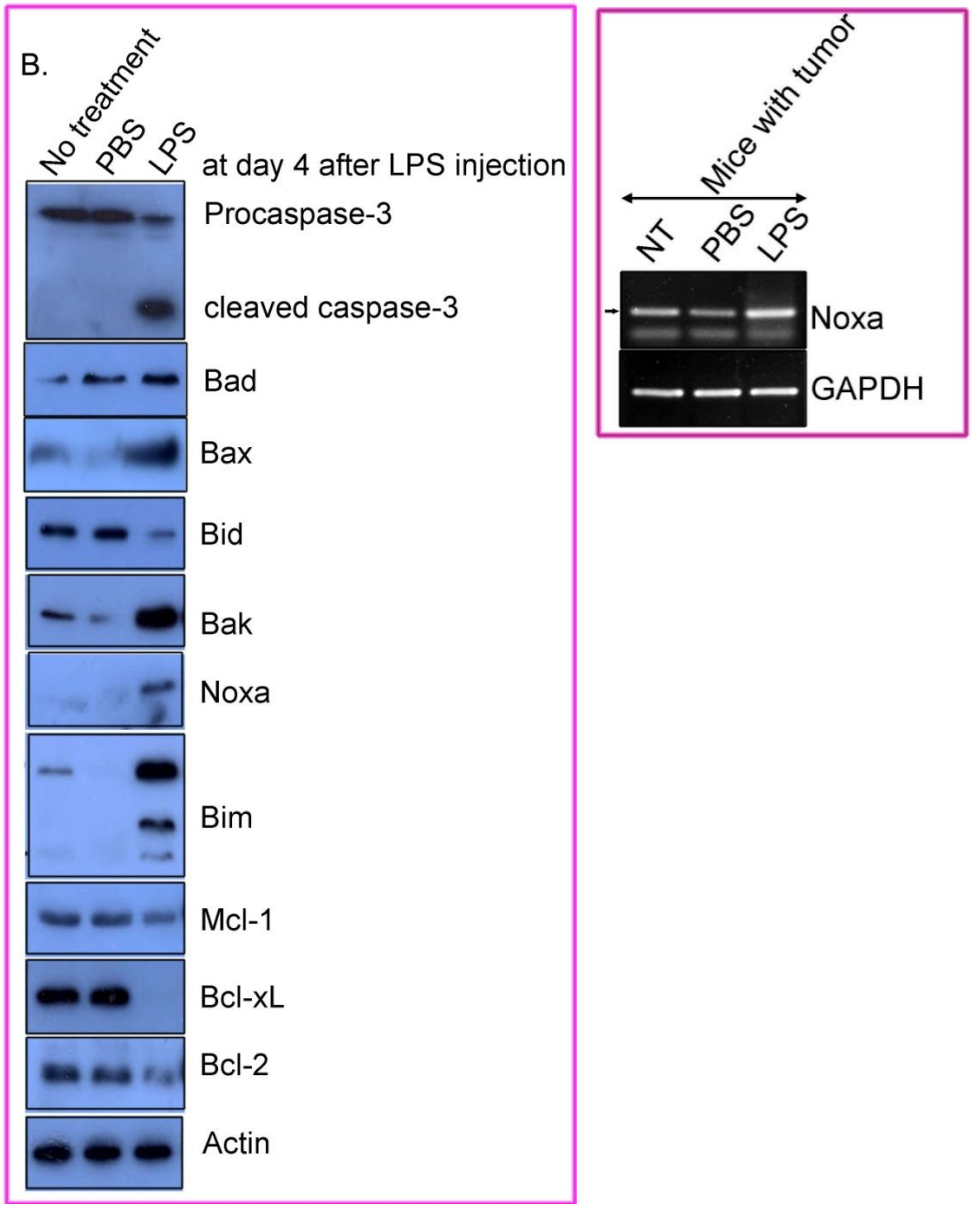
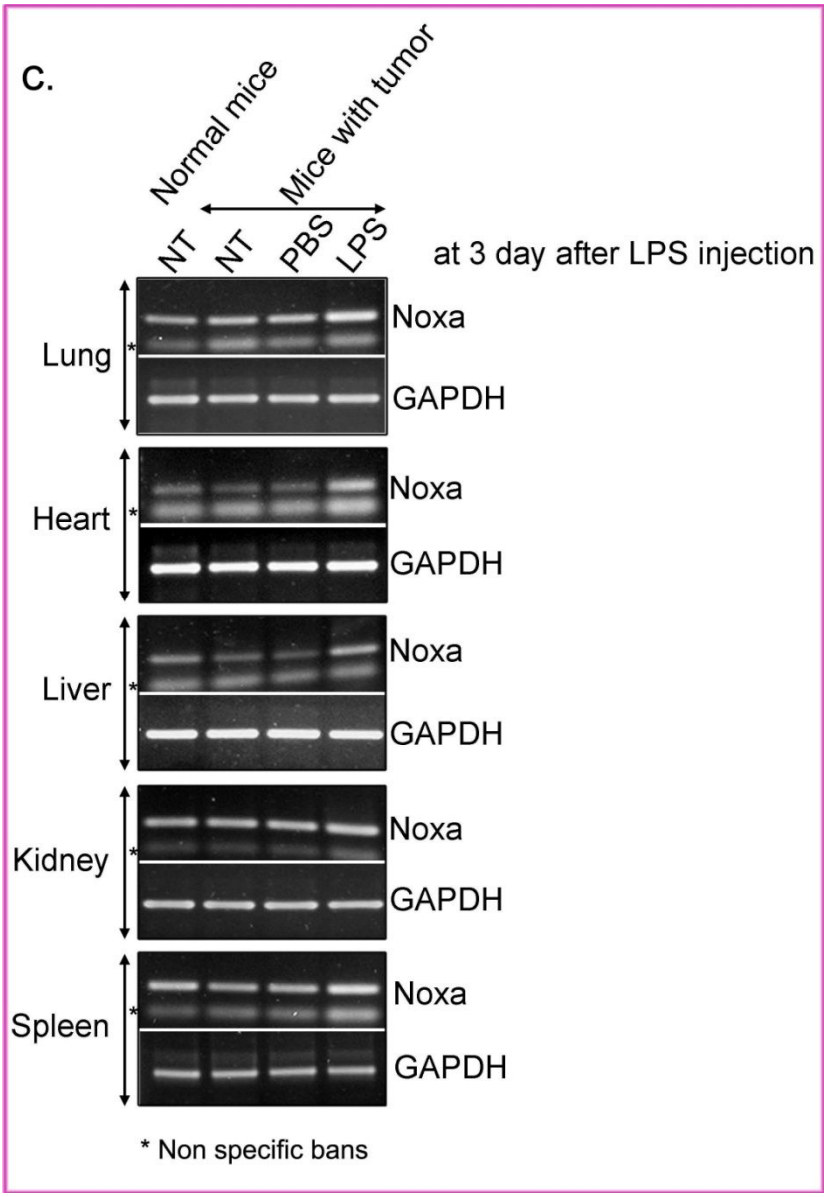


Fig.4.1C



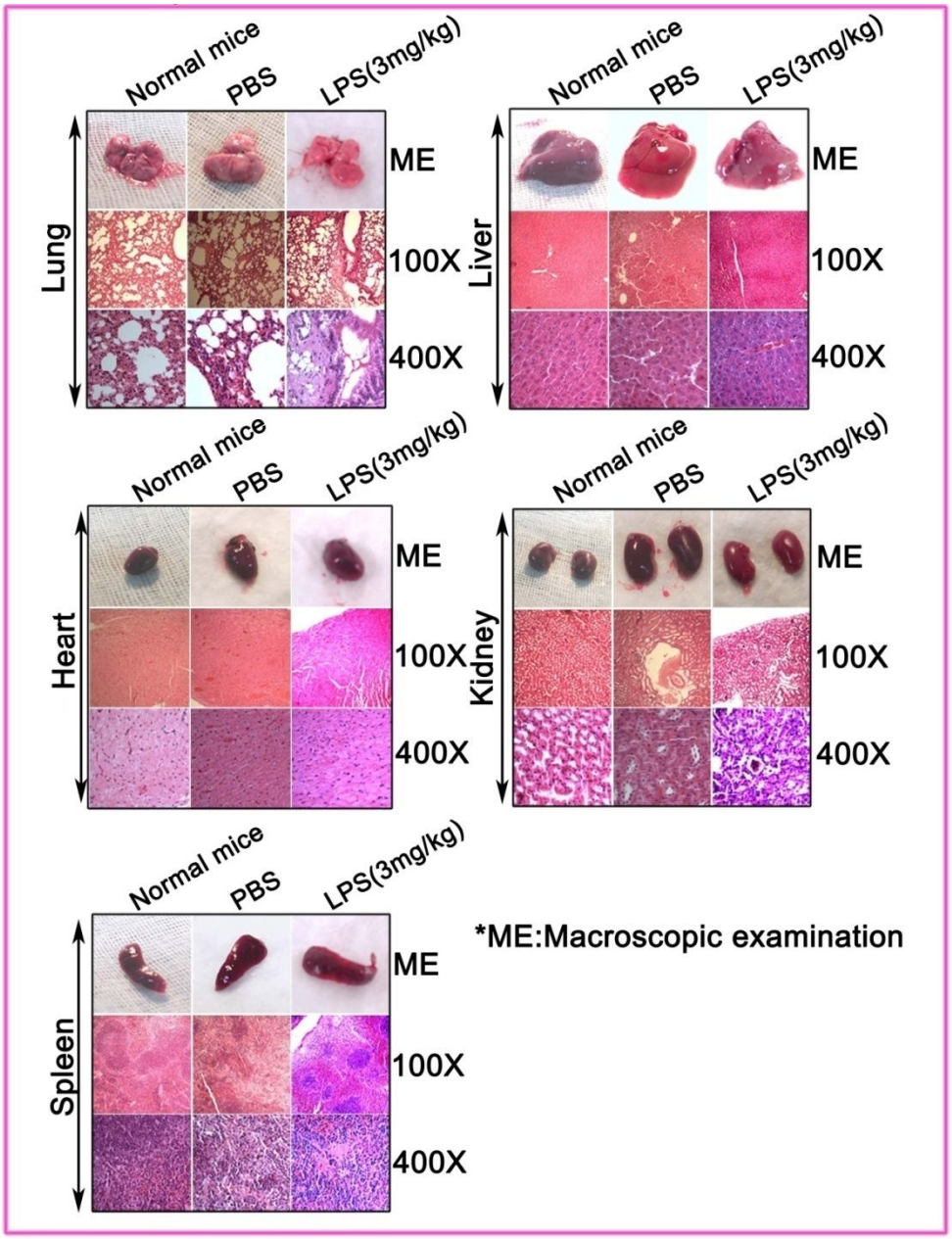


Fig.4.1D

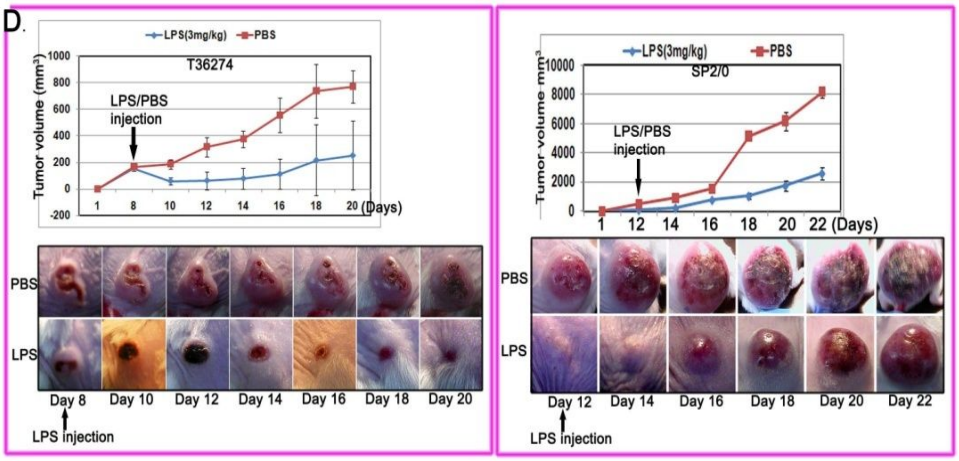


Fig.4.1E

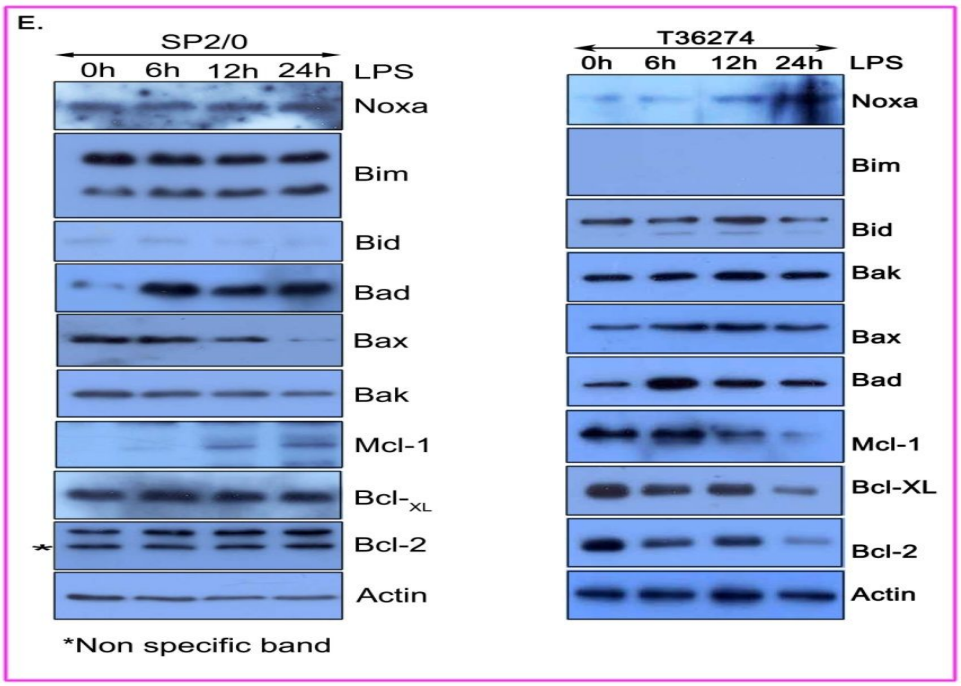


Fig.4.1: LPS sensitized the tumor cell to regulate the pro-apoptotic, particularly Noxa and anti-apoptotic proteins of BH3 family in spontaneous tumor regression.

A: Tumors were developed by subcutaneous injection of CT26.WT ($1.5X10^5$) into Balb/c mice and following tumor development on day 12 LPS (3mg/kg) and PBS as control was administered intraperitoneally(n=10). Tumor volume was calculated as the longest diameter X $width^2$ X 0.5. Representative image of tumor in mice was depicted in middle panel (A).Tumors were obtained from the mice challenged with LPS or PBS upon sacrificing on day 3 and tumor site was obtained after complete regression and were stained with hematoxylin and eosin. Images of (100X and 400 X magnifications) at tumor regions were depicted on bottom panel (A). The data were obtained from 3 separate experiments and tumor size was represented as mean tumor size ± SD of 5 mice per group.

B: Tumors were excised on day 3 or 4 after LPS and PBS injection and dounce with RIPA buffer with homogenizer. Total cell lysates proteins were separated on SDS-PAGE and probe with specific antibody. α-actin was used as a loading control. Total RNA was isolated from the same tissues and Noxa expression was analyzed by RT PCR. GAPDH was used as internal control. The PCR results were depicted on right panel (B).

C: Lung, Heart, Liver, Kidney and Spleen were obtained from LPS or PBS injected mice after day 3. Total RNA was isolated using TRIzol (Invitrogen, USA). RT PCR

carried out to check Noxa expression. GAPDH was used as internal control. Tissues were obtained from the mice challenged with LPS or PBS upon sacrificing on day 3 and were stained with hematoxylin and eosin. Images of (100 X and 400 X magnifications) different internal organs were depicted on bottom panel (C).

D:Tumors were developed by subcutaneous injection of T36274 ($6X10^5$) and SP2/0 ($6X10^5$) into Balb/c mice and following tumor development on day 8 and 12 LPS (3mg/kg) and PBS as control was administered intraperitoneally(n=10) respectively. Tumor volume was calculated as the longest diameter X width2 X 0.5. Representative image of tumor in mice was depicted in bottom panel (D).

E. T36274 and SP2/0 cells were treated with LPS (10 μg/mL) for indicated time intervals. The BH3 profiling of whole cell lysates using α-actin as loading control. Data are representative of three independent experiments.

4.4.2. LPS regulate the Bcl-2 family protein, notably Noxa in different cancer cell line

To understand the LPS exerted regulation of pro-apoptotic and apoptotic molecules *in vitro* too, CT26 WT, HCT116 and HCT116 p53-/- cells were treated with LPS and subjected to BH3 profiling. The expression of Bim, Bad, Bak, Bax and Noxa increased and Bcl-2, Bcl-XL and Mcl-1 decreased as tumor (Fig. 4.2).

Fig.4.2

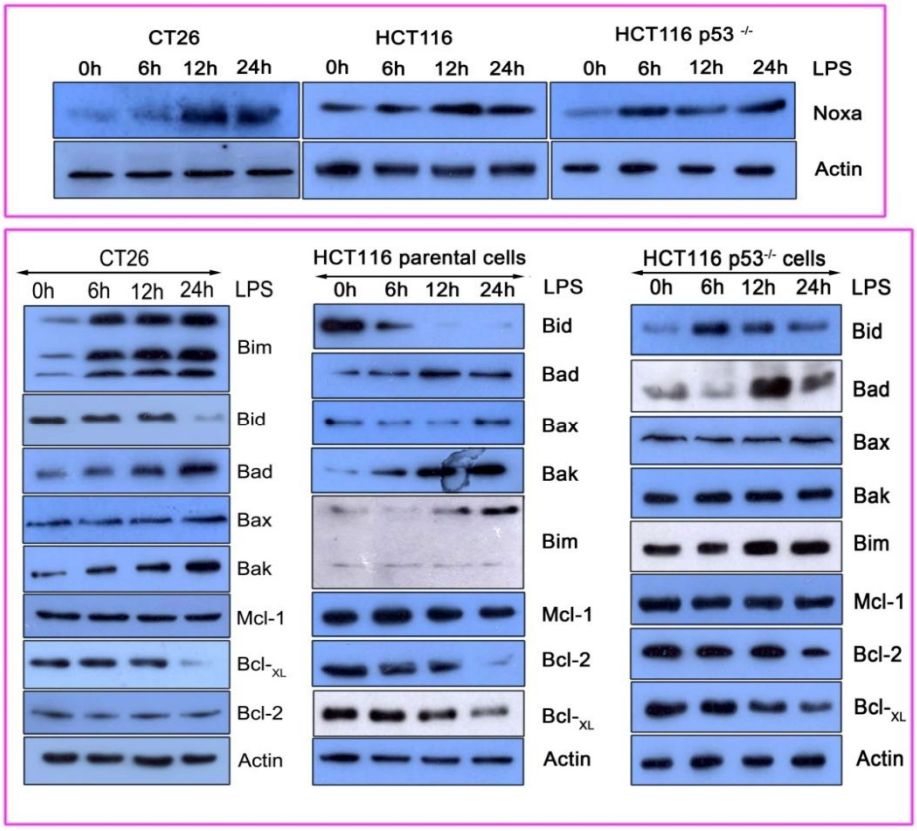


Fig.4.2: LPS regulate the Bcl-2 family protein, notably Noxa in different cell line. CT26, HCT116 and HCT116 p53-/- cells were treated with LPS (10 μg/mL) for 6h, 12h and 24h time intervals. After treatment, whole cell lysates were prepared by using RIPA buffer. The whole cell lysates were separated on SDS-PAGE and probe with specific antibodies. α-actin was used as loading control. Data are representative of three independent experiments

4.4.3. Interferons also regulate the Bcl-2 family proteins

Cellular mechanisms for spontaneous tumor regression include the upregulation of apoptosis related proteins and down-regulation of pro-survival proteins. Since LPS known to activate the Interferons, the BH3 profiling of apoptosis associated proteins were enlightens the possible pathway in LPS exerted multiple phenomena. For this purpose, CT26 cells were treated with Interferons (IFN α, β and Y). The expressions of Bcl-2 family proteins associated with apoptosis: Noxa, Bid, Bim, Bad, Bax, Bak and Noxa were upregulated whereas anti-apoptotic proteins: Mcl-1, Bcl-XL and Bcl-2 were downregulated as in case of LPS treated CT26 cells (Fig.4.3A). Similar expression of BH3 family proteins were observed with all Interferons in HCT116 and HCT116 p53-/- cells (Fig.4.3B). And also Noxa was increased by IFNs in HeLa and Jurkat cells (Fig.4.3C).

Fig.4.3A

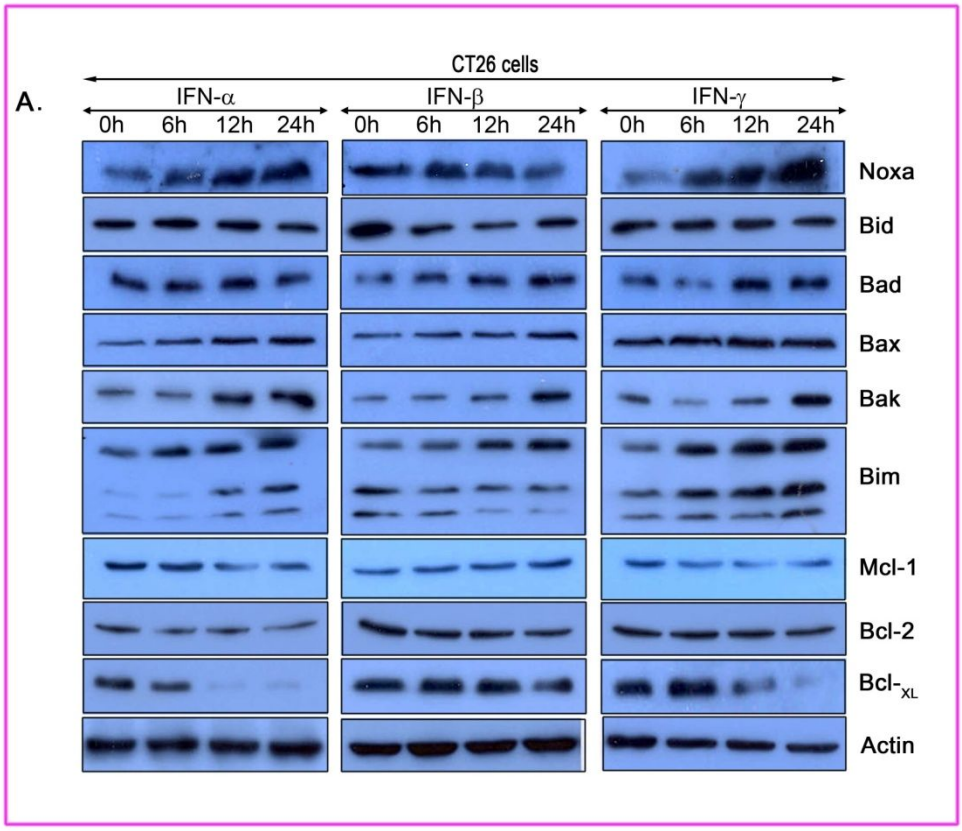
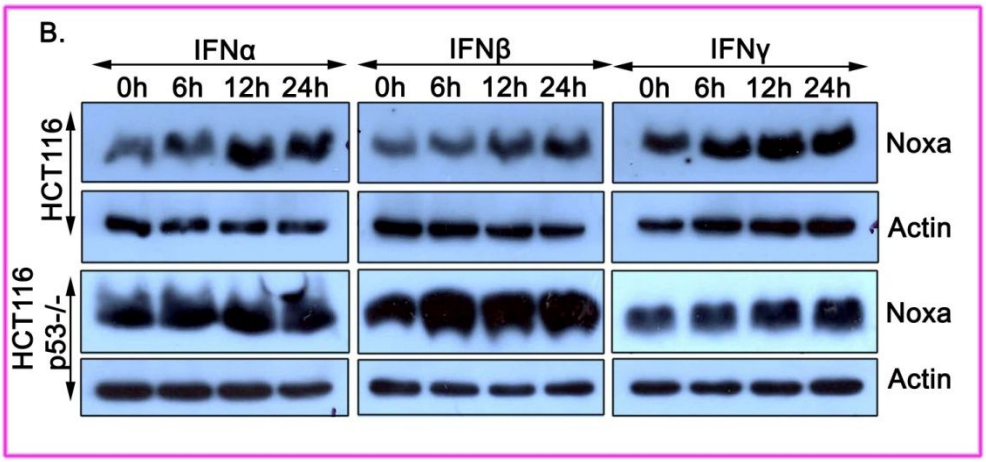
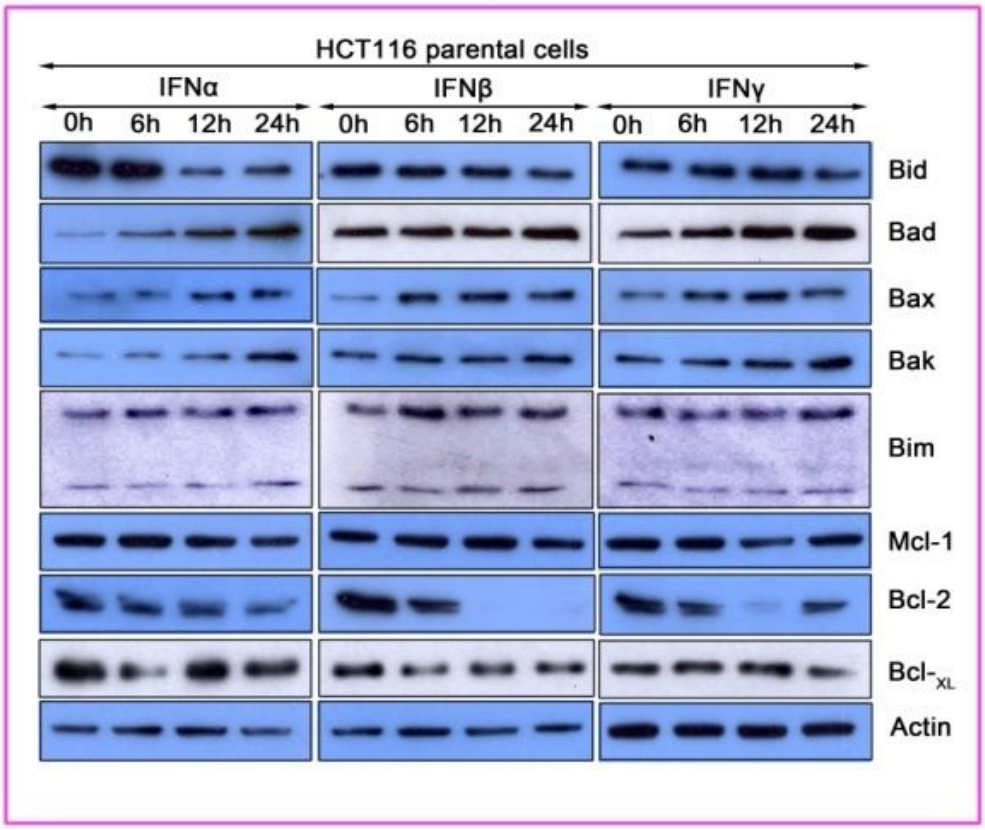


Fig.4.3B





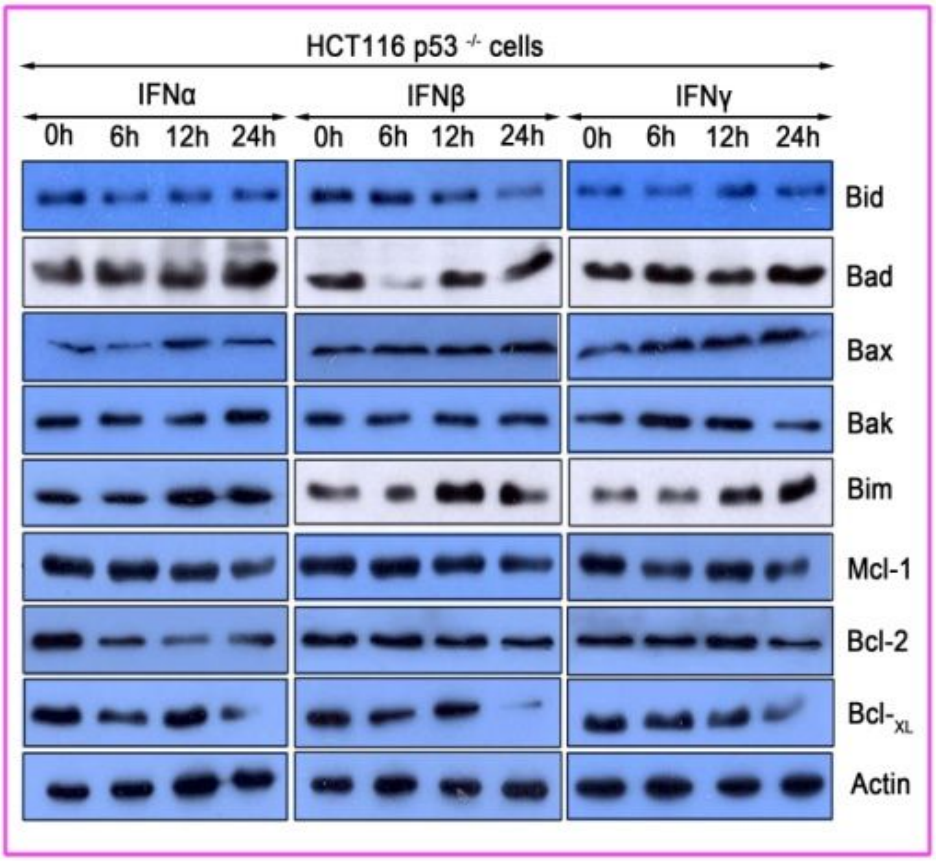


Fig.4.3C

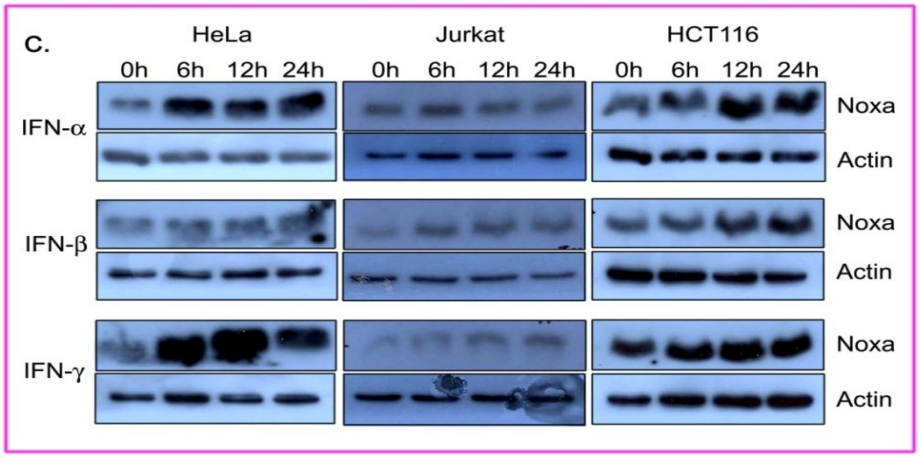
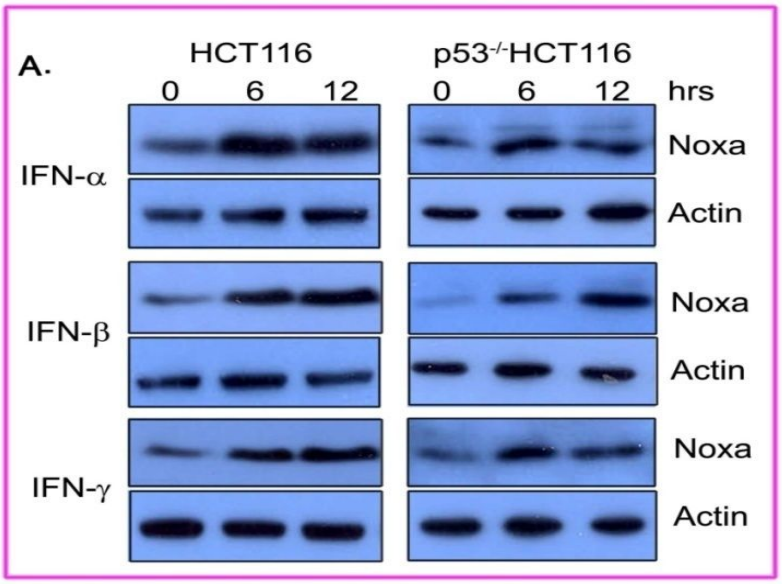


Fig.4.3: Interferons also regulate the Bcl-2 family proteins. CT26, HCT116, HCT116 p53-/-, Jurkat and HeLa cells were treated with IFNα, IFNβ and IFNY for 6h, 12h and 24h. After treatment, whole cell lysates were subjected to immunoblotting to detect the Bcl-2 family proteins in CT26 (A), HCT116/HCT116 p53-/- (B) and only Noxa in Jurkat and HeLa cells(C). Stripping the blot and reprobing for α-actin illustrated equal gel loading.

4.4.4. Noxa induction by LPS is independent of p53

LPS induction of Noxa was observed in different cell line. Since Noxa was known as p53-regulated gene, I sought to determine whether p53 was required LPS dependent induction of Noxa. So, HCT116 p53-/- and HCT116 WT cells were treated with IFNs, 5-FU and Velcade. Noxa was induced in each case even in HCT116 p53-/- cells (Fig.4.4). Together these data suggest that Noxa can be induced by IFN in a p53-independent manner.

Fig.4.4A



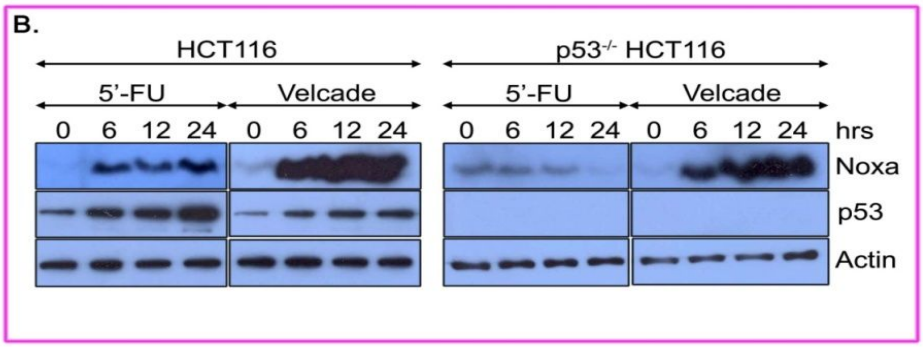


Fig.4.4: Noxa induction by IFNs is p53-independent. HCT116 and HCT116 p53-/- cells were treated with IFNs(α, β and Y), 5-FU (200 μg/mL) and Velcade (1 μM) for indicated time. After treatment, the whole cell lysates probes with specific Noxa and p53 antibodies. α-actin used as loading control.

4.4.5. Noxa is transcriptionally regulated by IRFs in response to LPS or IFNs

LPS or IFNs induction of Noxa in p53-independent manner suggest that other transcriptional factor involved in this pathway rather than conventional existing p53 transcriptional regulation of Noxa. Moreover, the computational analysis of partial 8 kb sequence of Noxa promoter region using the computer software (Genomatix) revealed the putative binding sites for interferon regulatory factors 1, 3, 4 and 7(IRFs). The functional association of IRFs with Noxa promoter *in vivo* was established by chromatin immunoprecipitation with CT26.WT cells in response to LPS and IFNs. Significantly, Noxa promoter was protected only when these cells were subjected to LPS or IFNs treated cells but not in untreated cells (Fig.4.5A). However, Noxa promoter is protected only in untreated cell with IRF4 immunoprecipitated cells suggesting the possible repressor of Noxa transcription. The Chip analysis revealed significant putative binding sites are IRF1:-3424/-3404 (cttgaaaaatGAAAtaaaata), IRF3:-5103/-5083 (atttgaaaggGGAAgagtgag) and -4790/-4770 (atatgaaatgGAGActcgagc), IRF4: -6588/-6568 (cacaGACAcccagaccttgtg) and -4746/-4726 (aaaaAAAAcagaacccagtgg) and IRF7: -3801/-3781 (gataGAAGaggaaagtacaga) and -2207/-2187 (aacaGAAAtagaagccaagtg) within the Noxa partial promoter region. Moreover, the expression level of IRF1, IRF3 and IRF7 were increased by LPS or IFNs in CT26, HCT116 and HCT116 p53-/- as well as in tumor tissue challenged with LPS. But

the expression of IRF4 decreased in LPS challenged tumor (Fig 4.5BC). This finding revealed the transcriptional regulation of Noxa by IRFs in LPS mediated spontaneous tumor regression.

Fig.4.5A

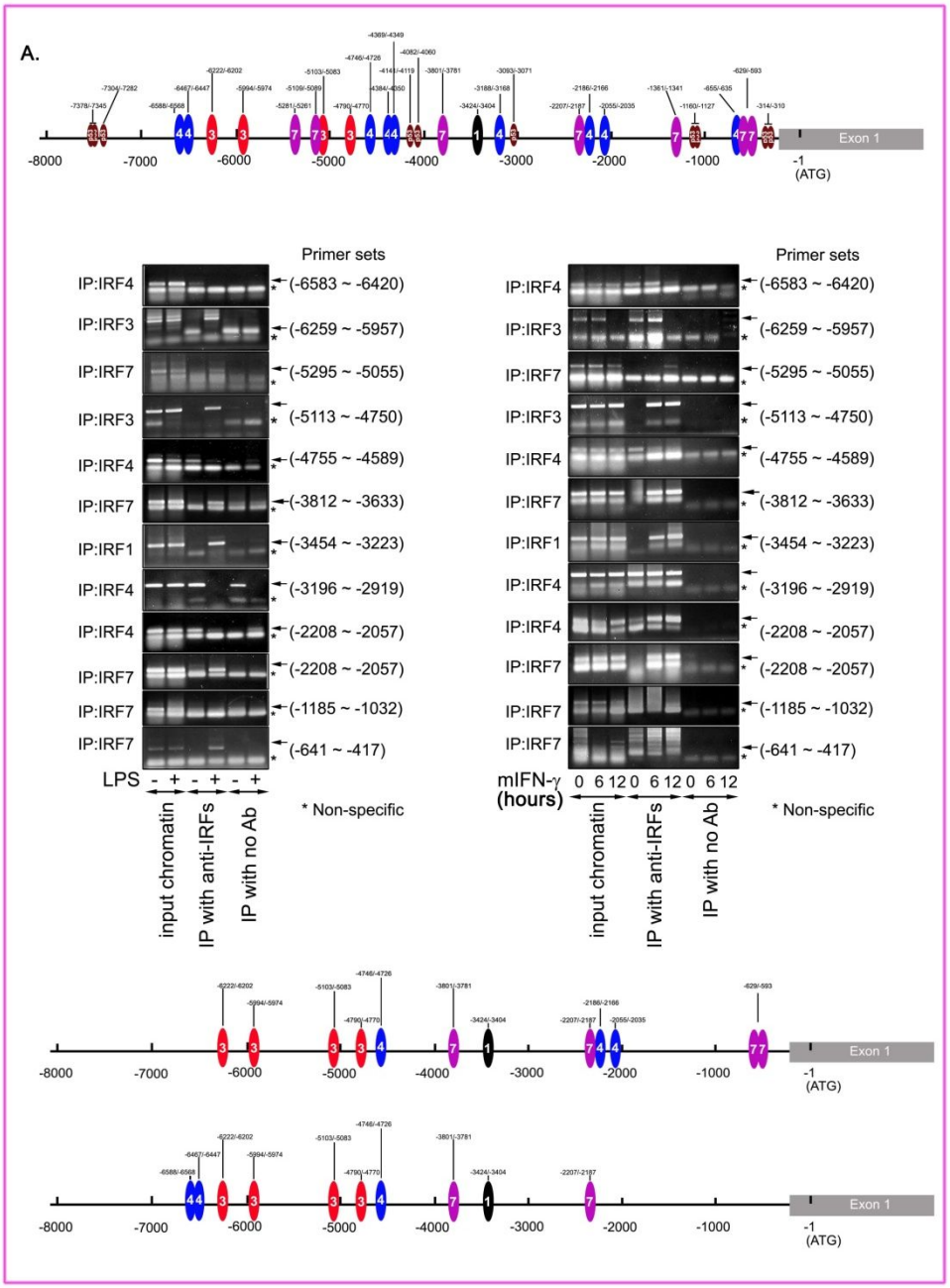


Fig.4.5B

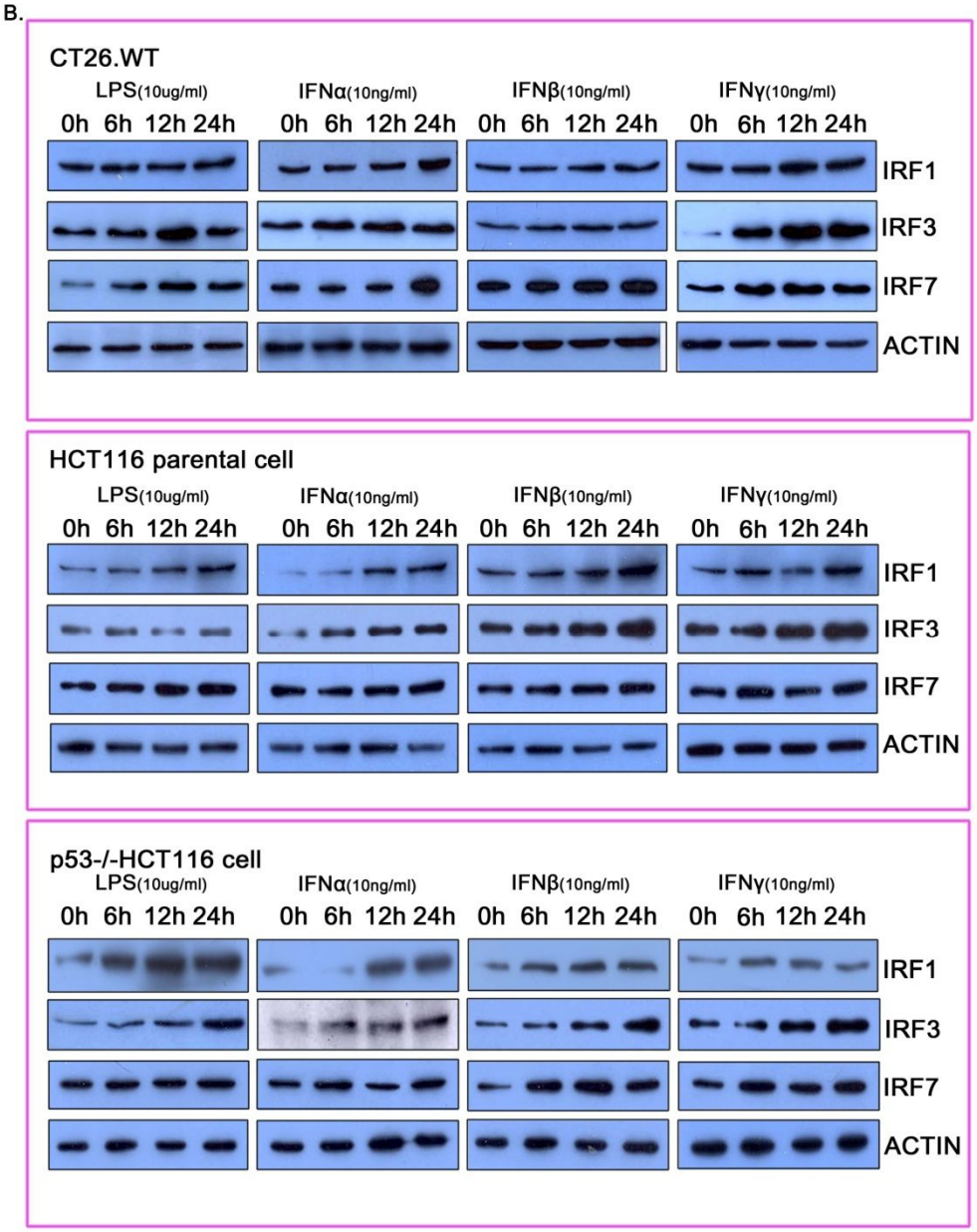


Fig.4.5C

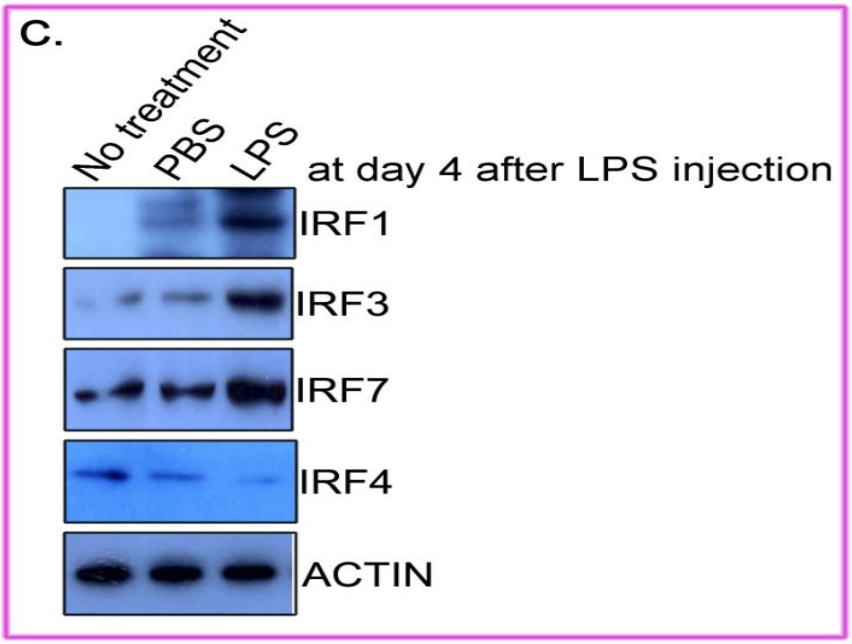


Fig.4.5: IRFs transcriptionally regulated the Noxa in response to LPS or IFNs. A: The schematic representation of IRFs (1, 3, 4 and 7) binding sites in Noxa promoter region was shown in upper panel. Chromatin Immunoprecipitation analysis of IRFs binding to Noxa promoter was depicted in lower panel. CT26 WT cells were treated as indicated and subjected to chromatin immunoprecipitation analysis. IRF1/IRF3/IRF4 and IRF7-specific antibodies were used to precipitate respective IRFs bound to chromatin. The IRFs-associated Noxa promoter was amplified by PCR using the corresponding primers designed for putative binding

sequence. The input DNA from cell lysates before Immunoprecipitation was used as positive control. The elution from beads without antibody was used as negative control. The data is represented as a mean ± 3 separate experiments (significance as compared with the control, $^{*}p<0.05$).

B: CT26 WT, HCT116 WT and HCT116 $p53^{-/-}$ cells were treated with LPS (10 μg/mL) or IFNα/β/Y(10 ng/mL) for 6h, 12h and 24 hours. The whole cell lysates were prepared and subjected to separate on SDS-PAGE. The specific antibody for IRF1/IRF3/IRF4/IRF7 used to probe the separated proteins. α-actin was illustrated as loading control.

C. Tumor tissues were obtained from tumor bearing mice challenged with LPS or PBS at day 4. The whole cell lysates were prepared using RIPA buffer and dounce with homogenizer. The whole cell lysates were probe with specific antibody for IRF1/IRF3/IRF4/IRF7. α-actin was used as loading control.

4.4.6. Involvement of IRF1, IRF3 and IRF7 to transactivate Noxa in LPS mediated apoptosis.

To further detailed the IRFs transactivation of Noxa in LPS or IFNs induced cell death; IRFs (1, 3 and 7) were over-expressed in HCT116 cells. The over-expression of IRFs induced the Noxa resulting significant cell death. The ectopic expression of IRFs and resulting induction of Noxa were confirmed by immunoblotting (Fig.4.6A). Moreover, the direct evidence for IRFs transactivation of Noxa and its role in cell death was assessed with adenovirus expressing IRF1,IRF3 and IRF7 in baby mouse kidney, BMK WT and Noxa knock-out BMK Noxa$^{-/-}$ cells. In BMK WT cells transduction with Ad-IRFs in different MOI, Noxa induction and significant cell death were observed but not in BMK Noxa-/- cells (Fig4.6BC). These observations suggest the direct functional link of Noxa in possible death executioner molecule in spontaneous tumor regression.

Fig.4.6A

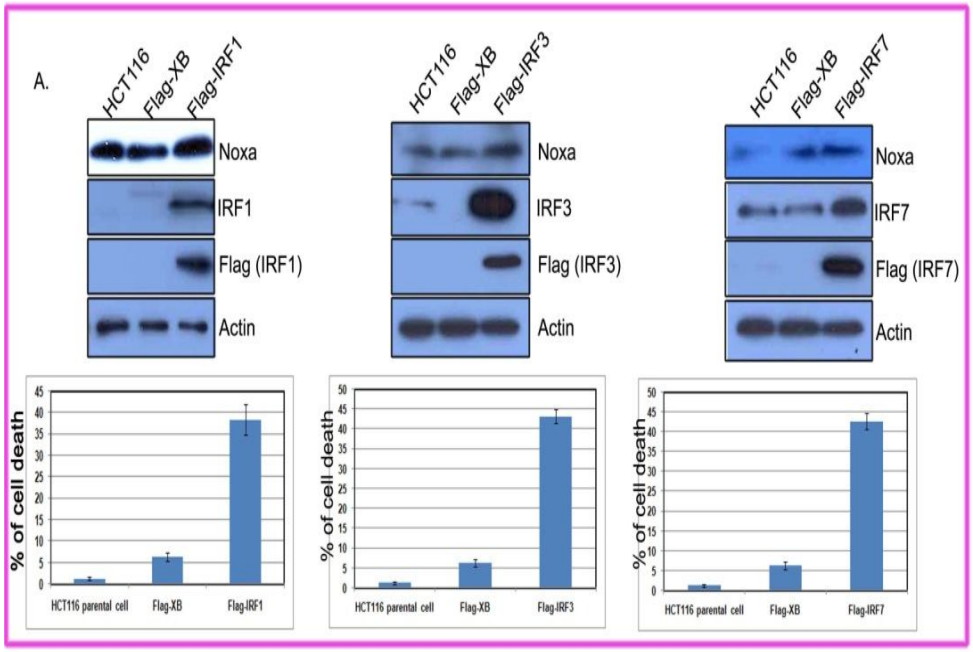


Fig.4.6B

B.

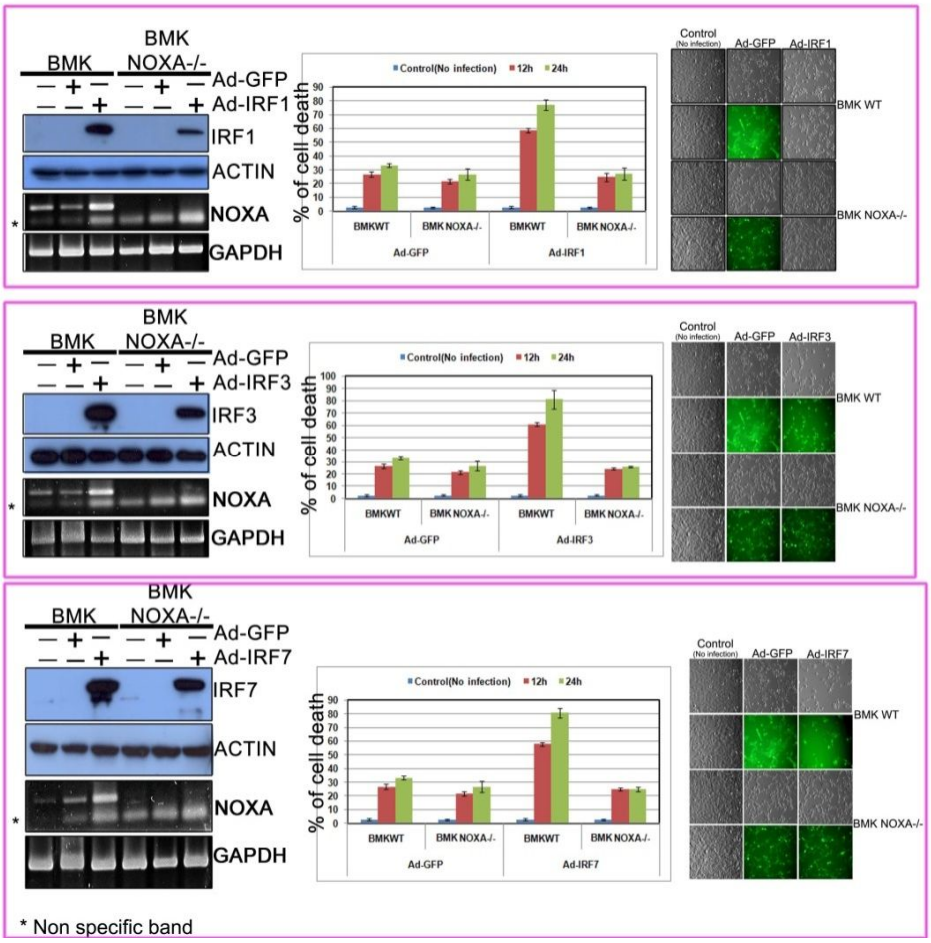


Fig.4.6C

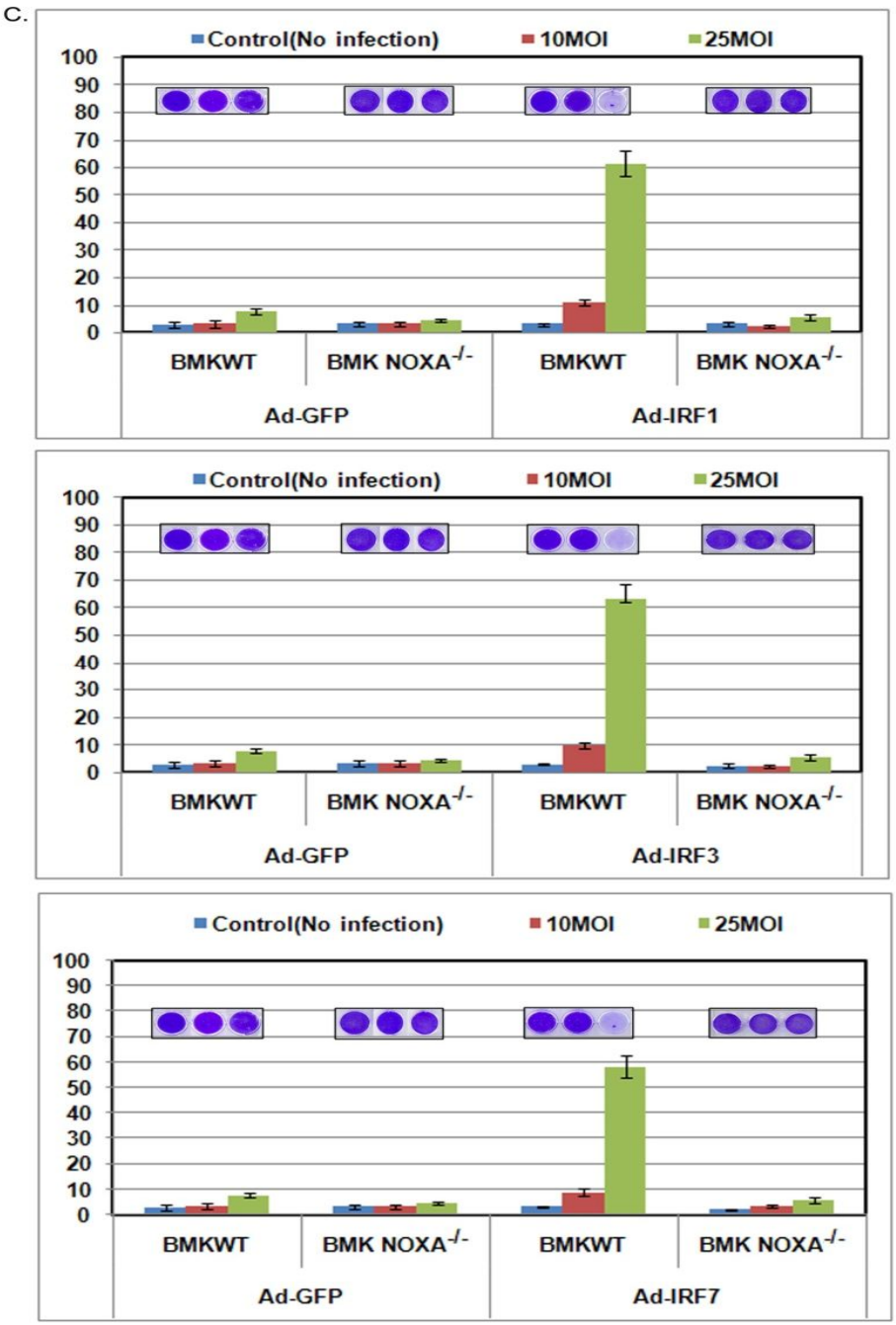


Fig.4.6: IRF1, IRF3 and IRF7 induced Noxa in LPS induced cell death. A: HCT116 WT cells were transfected with Flag-tagged IRF1/IRF3/IRF7 vectors and following day the percentage of cell death was measured by trypan blue exclusion assay adapting standard procedure(Bottom panel). The Whole cell lysates prepared at 24h and subjected to immunoblot analysis for IRF1/IRF3/IRF7, Noxa expression and α-actin as loading control(Top panel).

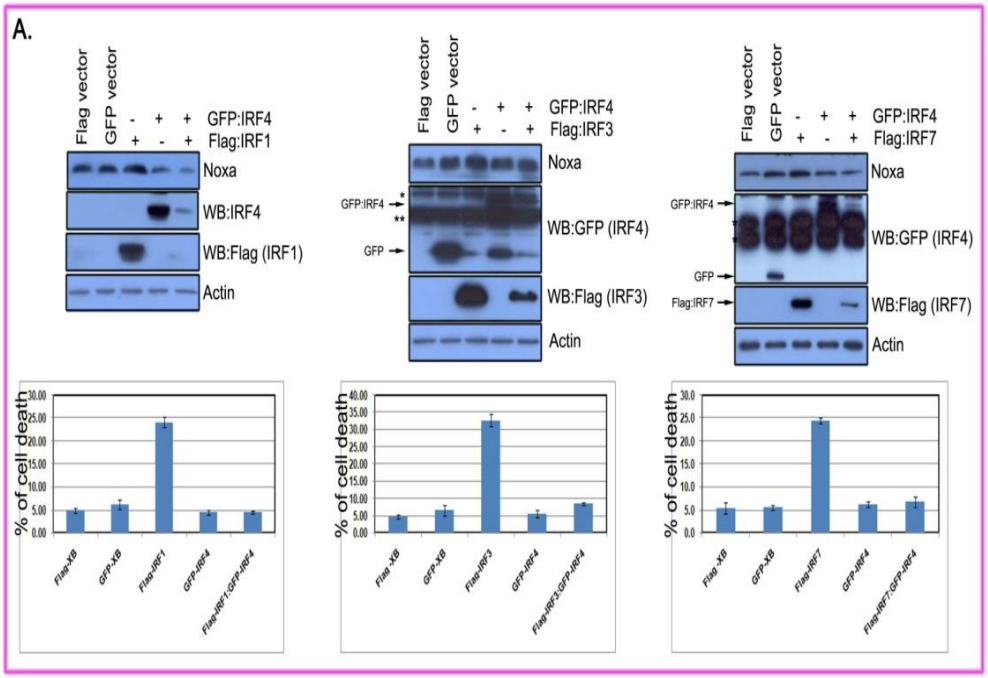
B: WT and Noxa $^{-/-}$ BMK cells were transduced with 50 MOI of Ad-GFP, Ad-IRF1, Ad-IRF3 and Ad-IRF7. Cells were harvested at 24h analyzed for IRF1/IRF3/IRF7, and α-actin as loading control(Right panel). The expression of Noxa was assayed by RT PCR.GAPDH was used as an internal control (Right panel). The percentage of cell death at time points 12h and 24h were calculated following the standard protocol for trypan blue exclusion assay (Middle panel) and Light and fluorescence morphological image of cells were obtained at 24 hours with 100X magnification (Left panel)

C: WT and Noxa $^{-/-}$ BMK cells were transduced with 10 and 25 MOI of Ad-GFP, Ad-IRF1, Ad-IRF3 and Ad-IRF7. The percentage of cell death at time 48h was calculated following the standard protocol for trypan blue exclusion assay and crystal violet cells survival assay was shown in inset.

4.4.7. IRF4 is repressor of IRF1, IRF3 and IRF7 to transactivate Noxa

The Chip analysis result of IRF4 gave hint to be the negative regulator which could protect its binding site only in normal, LPS-untreated state and could not protect in LPS-treated state. To further understand this regulation of IRF1/3/7 by IRF4, the co-expression in HCT116 cells were carried out. Noxa was significantly increased by the transfection of IRF1, 3 or 7 and significant cell death was observed that was probably the consequence of induction of Noxa. Interestingly, the expression of Noxa was reduced and cell viability was recovered upon the IRF4 co-transfection (Fig.4.7A). These finding suggest that the stability and functional activity of IRF1, IRF3 and IRF7 are somehow regulated by IRF4. Therefore, to understand these phenomena the IRF1, IRF3, IRF7 and IRF4 co-transfected in concentration dependent manner. The protein expression after the IRF 4 co-transfection with IRF 1, 3 or 7 result the instability of IRF4 and vice versa (Fig.4.7BC). Moreover, the IRF4 is downregulated in tumor tissue (previous data), and cumulative activation of IRF1/3/7 results the induction of Noxa that is beyond the transcriptional regulation by IRF4 (Fig.4.7D). These cumulative transactivations are responsible for flux of Noxa in spontaneous tumor regression to determine the fate of tumor.

Fig.4.7A



*Non specific band

Fig.4.7B

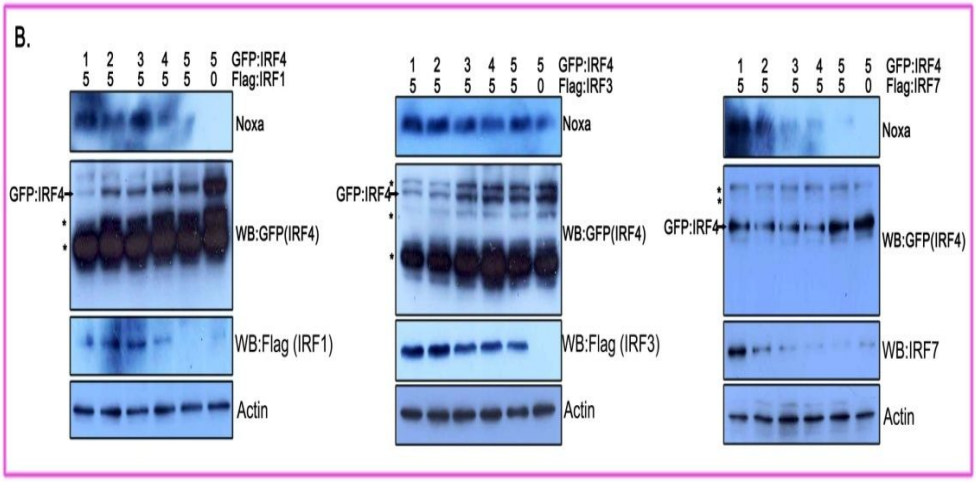
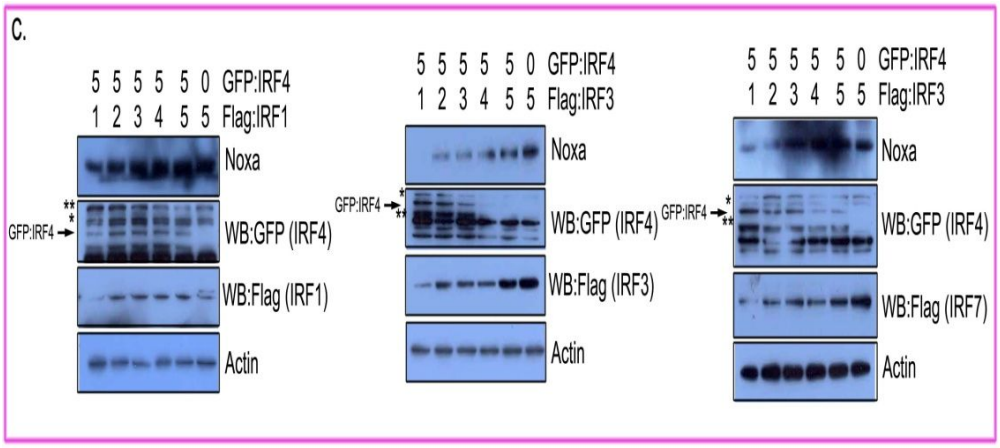


Fig.4.7C



*Non specific band

Fig.4.7D

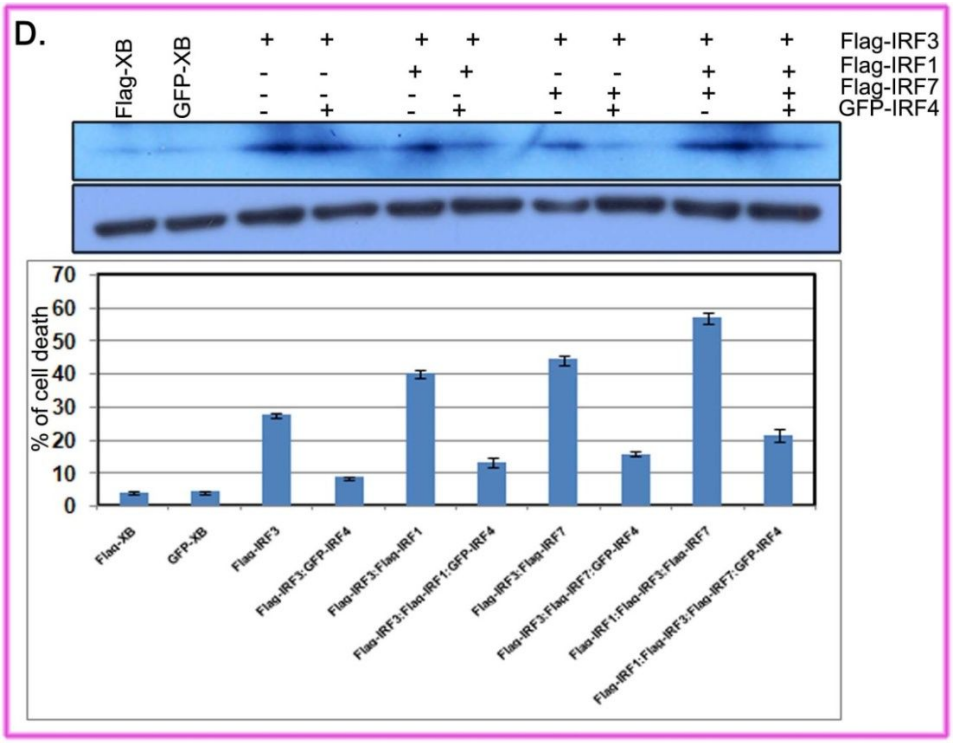


Fig.4.7: IRF4 is repressor of IRF1, IRF3 and IRF7. A: HCT116 cells were transfected or co-transfected with Flag-tagged vector or GFP-vector or flag-tagged IRF1, IRF3, IRF7 or GFP-tagged IRF4 expressing vector. The percentage of cell death at 24hours was calculated following standard procedure of Trypan blue exclusion assay (Bottom panel). The total cell lysates were prepared at 24h using RIPA buffer and subjected to immunoblot analysis for Noxa, GFP-IRF4, and Flag-IRFs with specific antibody. α-actin was used as loading control (Upper panel). Data are representative of three independent experiments.

B: HCT116 cells were transfected with fixed amounts of IRF1, IRF3, IRF7 (500 ng) or no IRF1/3/7 with increasing amounts of GFP-IRF4 (100, 200, 300, 400, 500, 500 ng). The whole cell lysates were prepared at 24h and separated on SDS-PAGE. The separated proteins were probe with specific antibody of Noxa, GFP (IRF4) and Flag (IRF1/3/7). Stripping the blot and reprobing for α-actin illustrated equal loading.

C: HCT116 cells were transfected with fixed amounts of GFP-IRF4 (500 ng) or no GFP-IRF4 with increasing amounts of IRF1, IRF3, IRF7 (100, 200, 300, 400, 500, 500 ng). The whole cell lysates were prepared at 24h and subjected to immunoblotting to detect Noxa, GFP (IRF4) and Flag (IRF1/3/7). Stripping the blot and reprobing for α-actin illustrated equal loading.

D: HCT116 cells were transfected with different combination of Flag tagged IRF3, IRF1, IRF7 and GFP-IRF4 as indicated in figure. The percentage of cell death was calculated following standard protocol of Trypan blue exclusion assay (Bottom panel). The whole cell lysates were prepared on 24h and separated on SDS-PAGE and then probe with specific antibody for Noxa. α-actin was used as loading control (Top panel).

4.4.8. Noxa is key protein in spontaneous tumor regression by LPS

To understand the Noxa induced cell death and its physiological role in LPS mediated spontaneous tumor regression, knock-down expression system of Noxa was adapted. For this purpose, the short hairpin RNA interference of Noxa was introduced to CT26 cells and treated with LPS. The silencing of Noxa enhances the resistance to LPS mediated cell death (Fig.4.8 A). These finding strengthen the possible physiological link in LPS mediated tumor elimination. Therefore, LPS was intraperitoneally challenged to mice bearing tumor generated by Noxa knocked-down CT 26 cells with pSUPER shRNA Noxa vector, expressing shRNA for Noxa or CT26 cell with pSUPER ScNoxa shRNA or pSUPER vector construction. In fig. 4.8B, the tumor generated by control pSUPER vector or pSUPER ScNoxa short hairpin of Noxa containing CT 26 cells was spontaneously regressed by LPS but the tumor generated by pSUPER shRNA Noxa vector containing CT 26 cells was not. Moreover, the Noxa expression was insufficient in pSUPER short hairpin of Noxa containing CT 26 cells generated tumor in contrast to pSUPER vector containing CT26 generated tumor even in sacrificed days (Fig.4.8C). The histological examination of tumor revealed the combination apoptotic and necrotic cell death in ScNoxa shRNA containing CT26 generated tumor and normal state of cell in pSUPER short hairpin of Noxa containing CT 26

cells generated tumor cells (Fig.4.8D). These finding suggest that Noxa is key protein in LPS mediated spontaneous tumor regression.

Fig 4.8A

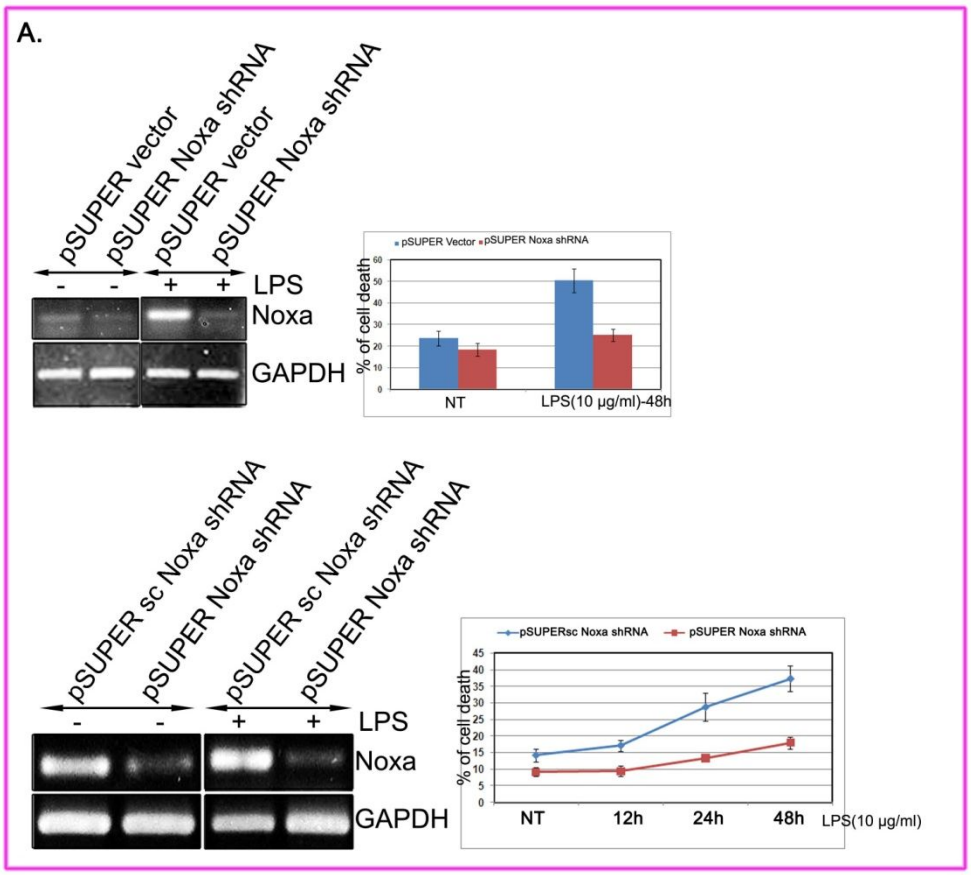
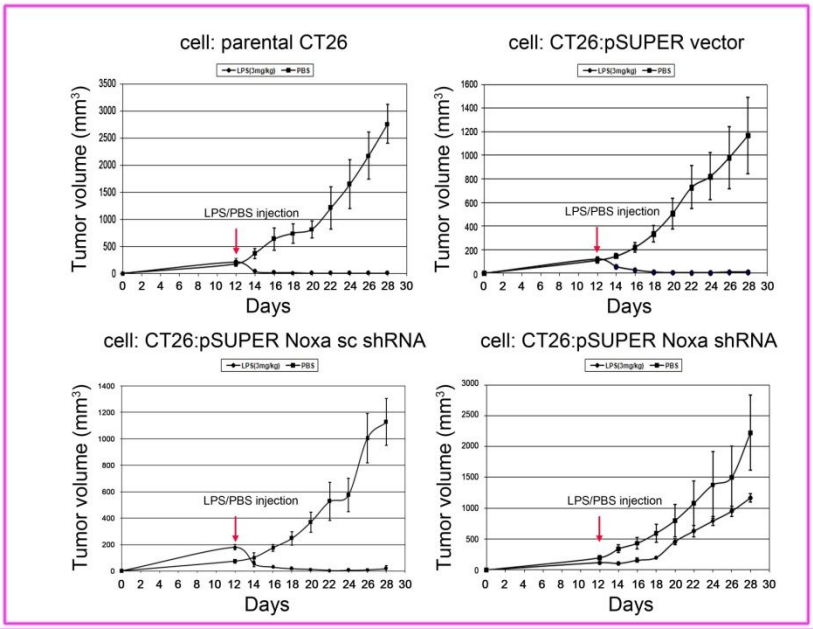


Fig.4.8B

B.



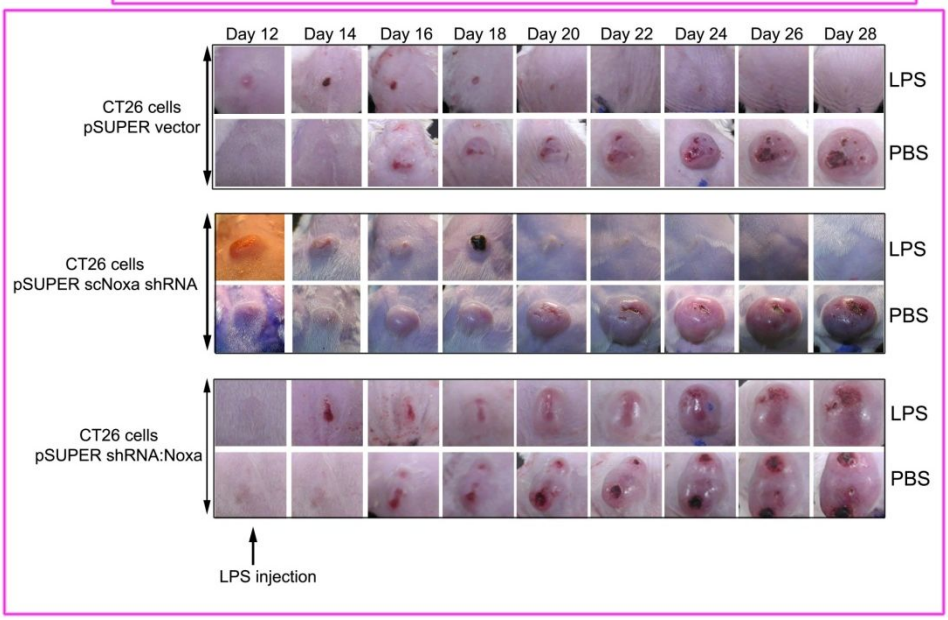


Fig.4.8C

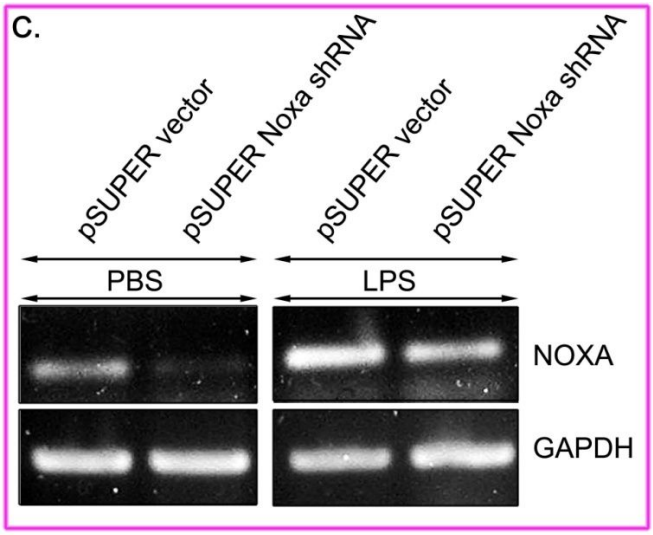


Fig.4.8D

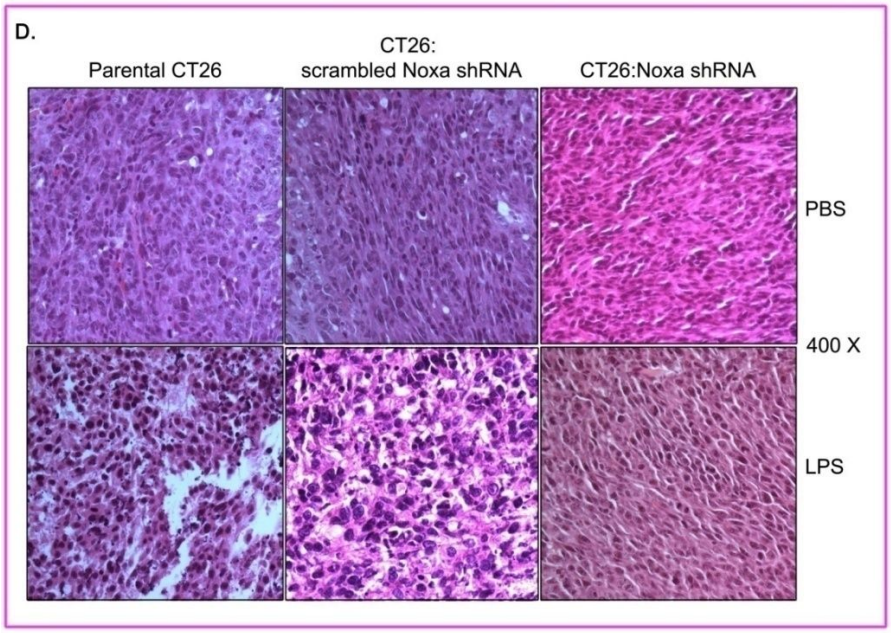


Fig.4.8: Noxa is the key protein in LPS mediated spontaneous tumor regression. A: CT26 Cells were transfected with pSUPER vector or pSUPER sc Noxa shRNA or pSUPER Noxa shRNA. Following day, treated with LPS (10 μg/mL) for 12h, 24h and 48h and the percentage cell death was calculated following standard protocol of trypan blue exclusion assay. The silencing efficiency of Noxa was confirmed at 48hours by RT PCR. GAPDH was used as internal control.

B: Mice were subcutaneously injected on day 0 with $1.5X10^5$ cells of either CT26 WT or pSUPER vector or pSUPER Noxa sc shRNA or pSUPER Noxa shRNA. On day 12 LPS (3mg/kg) and PBS as control were administered intraperitoneally. Tumor volume was calculated as the longest diameter X width2 X 0.5. Representative image of tumor in mice was depicted in bottom panel (B). The data were obtained from 3 separate experiments and tumor size was represented as mean tumor size ± SD of 5 mice per group.

C: Total RNA was isolated from tissue obtained from mice challenged with LPS or PBS at day 4. The expression of Noxa was assayed by RT PCR. GAPDH was used as an internal control.

D: Tumors were obtained from the mice challenged with LPS or PBS upon sacrificing on day 4 and fixed on 10% neutral phosphate buffer formalin solution.

The thin sections of tumor were stained with hematoxylin and eosin. Images of (400 X magnifications) at tumor regions were depicted on figure.

4.5. DISCUSSION

Extensive studies have been continuing on spontaneous tumor regression to understand the causes and its possible role in therapeutic intervention since 19th century. Till date, various researchers find out some possible causes of spontaneous tumor regression through case studies on severe infections by viruses and bacteria or tumor inhibition by growth factors and/or cytokines [154]. But detailed information about the key molecules responsible for execution of tumor cell still remains unclear. Therefore, correlating some death executioner molecules in cases of spontaneous tumor regression by the infections might help enlightening the total phenomena. Adapting this assumption, LPS, the component of gram negative bacteria, is used to induce spontaneous tumor regression.

The difference between cell death phenomena in cultured cells and the regression of tumor in mouse by LPS lead to assume that the systemic activation of immune system associated with death machinery must be needed to execute the tumor cell for spontaneous tumor regression. Simply, LPS might sensitize the immune system and then tumor region alert the death machinery to eliminate the tumor.

The Bcl-2 family proteins are known as key regulatory molecules to decide fate of cancer cells [24]. The BH3 profiling in the different cell lines and tumor region showed upregulation of activators: Bim, Bak, sensitizers: Noxa as well as mitochondrial pro-apoptotic molecules Bax and Bad and down regulation of pro-survival molecules Bcl-XL, Bcl-2 and anti-apoptotic Mcl-1 in response to LPS or IFNs. This Bcl-2 family regulation may be the novel component for spontaneous tumor regression and is involved in dictating the immune system to response the infection.

Noxa, pro-apoptotic BH3-only Bcl-2 family member, was known to mediate apoptosis by interacting and inhibiting counterpart pro-survival Bcl-2 family member, Mcl-1 which leads to displacement and activation of Bax and Bak. This activated Bax/Bak permeabilizes the mitochondrial membrane ultimately resulting cell death [155],[156]. The significant upregulation of Noxa and down regulation or unchanged counterpart Mcl-1 expression probably associated with increased expression of Bax and Bak in LPS-susceptible tumorogenic CT 26 cells and T36274 cells but down regulation of Noxa and upregulation of anti-apoptotic Mcl-1 in the LPS-resistant tumorogenic Sp2/0 cells clues the Noxa as the possible key molecule in LPS mediated tumor regression in CT26 and T36274 generated tumor and not in Sp2/0 generated tumor.

Although Noxa had been known to p53 target gene and mediated apoptosis in response to genotoxic stresses [70], the current studies demonstrate the binding of IRF1, IRF3 and IRF7 to Noxa promoter region and induction of Noxa is p53-independent in response to LPS and IFNs. These results are consistent with protein expression of IRF1/3/7 in response to LPS or IFN. Moreover, increased expressions of IRF1/3/7 induced the Noxa that may accumulate in the cell death. Interestingly, BMK Noxa-/- cells exhibited resistance to cell death even after transduction with Ad-IRF1/3/7 suggesting that IRFs are transcriptional regulator of Noxa.

The appropriate signaling of IRF1, IRF3 and IRF7 constitutes a crucial aspect in the immune system, wherein the selective use of negative regulator TLR signaling might contribute to specificity, magnitude and longevity of apoptotic, immune and anti-tumor responses [152],[153],[157]. In our present study, the evidence of the stability of IRF1, IRF3 and IRF7 proteins somehow depend upon the IRF4 expression and vice-versa suggest the probable role IRF4 as repressor of other IRFs.

Perhaps the most significant observation made in the study is that the expressions of Noxa following the activation of IRF1, IRF3 and IRF7 cumulatively tip the balance among Bcl-2 family members toward a pro-apoptotic response in LPS exerted spontaneous tumor regression. The consequences of Noxa

induction by IRFs is the elimination of tumor cells in association with immune activation. Therefore, silencing of Noxa in CT26 cells fails to spontaneous tumor regression in response to LPS. Considering all these facts, assumption was drawn out for spontaneous tumor regression as the tumor cell systematically recollect immune cell following infection and sensitize the death machinery to accelerate BH3 molecules notably Noxa to execute the tumor cells.

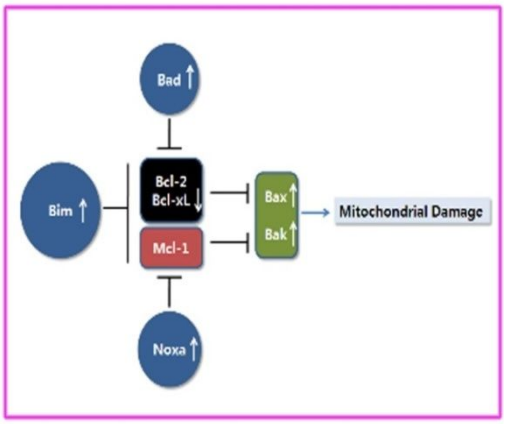


Fig.4.9: Bcl-2 family proteins decide the fate of tumor cells. LPS exerted Noxa displace the Mcl-1 from Bax/Bak that permeabilizes the mitochondrial membrane. The upregulation of other pro-apoptotic molecules Bim, Bad regulate the pro-survival molecule Bcl-XL and Bcl-2. The balance of these molecules converges in mitochondrial damage ultimately leading to cell death.

CHAPTER 5

CONCLUSION

p53, the guardian of the genome, exerts its tumor suppression activity by regulating the transcription of several genes that are involved in the regulation of processes such as cell growth and apoptosis [158]. However, p53 is frequently inactivated in cancer. So, extensive research has been continuing since it was described to restoration of its function [159]. The selective and swift elimination of cancer cell by inducing apoptosis is central dogma of cancer therapy [160]. Additionally, adaptation of active killing domain of pro-apoptotic molecules particularly BH3-family in development of new agents may be another option in case of chemo-resistant [15]. In contrary to these, swift regression of tumor harmonized by innate or adapting immunity associating with death machinery.

The functional relevance of p53 as transcription factor is vital for its role as tumor suppressor. The search of potential p53 target gene that mediates such distinct biological function established a novel SSFA2, PHLDA1 and DUSP6 genes. The expressions of these genes are associated with genotoxic and cellular stresses to enhance the p53-mediated apoptosis. The location of p53 putative binding site within DUSP6 is the novelty in transactivation. The substrate specificity of DUSP6 toward ERK1/2 destabilizes the pro-survival Bcl-2, to proteasomal degradation thereby setting free Bad for its apoptotic function.

Besides restoration of function of pro-apoptotic and apoptotic molecules in tumor suppression with application of chemotherapeutic or genotoxic material,

application of BH3/Non BH3 synthetic peptides may be the novel in concept for cancer therapy. The synthesis of peptide adapted from mitochondrial target domain (MTD) of C-terminus of Noxa decorated with tumor homing domain is the new one. The beauty of such peptides TU2:MTD and TU3:MTD lies on its specificity towards tumor cell.

LPS mediated spontaneous regression is augmented with activation of immune system and dictate the tumor region to alert death machinery. The activation of pro-apoptotic molecules and regulation of anti-apoptotic molecule cumulatively eliminate the tumor cells. The immunoregulatory modulators: IRF1, IRF3 and IRF7 transcriptionally upregulate the Noxa in response to LPS. The physiological significance of Noxa on tumor cell is spontaneous tumor regression mediated by infection.

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