Cytotoxic drug induced JNK activation is DR5-dependent and vital for apoptosis and provides a new target for MSC-mediated delivery of DR5-specific sTRAIL variants

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Summary

Over the past two decades, cancer research has produced tremendous advances in our understanding of the pathogenesis of cancer. Among the most important of these advances is the realization that apoptosis and the proteins involved in this process of it have a profound effect on the development of malignancies. This study is composed of two parts, in part I we found JNK activation is DR5-dependent and it can regulate apoptosis via Bid phosphorylation in HCT116 colorectal cancer cells after treatment with the chemotherapeutic agent 5-FU (5-fluorouracil). In part II we designed a form of soluble TRAIL (TNF-related apoptosis-inducing ligand), which can be delivered by MSCs (mesenchymal stem cells) and showed enhanced apoptosis-inducing activity in combination with 5-FU or silencing of XIAP (X-linked inhibitor of apoptosis protein). All these findings provide more insights into the mechanism of apoptosis and may be helpful to design better therapeutic approaches.

List of Abbreviations

5-FU	5-fluorouracil
Ad	Adenovirus
AIF	apotosis inducing factor
AP-1	activator protein 1
Apaf-1	apoptotic protease activating factor 1
ASK1	apoptosis signal-regulating kinase 1
A-T	ataxia-telangiectasia
ATF	activating transcription factor
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3-related protein
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist
B-ALL	acute B-lymphoblastic leukemia
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma-2
B-CLL	chronic B-lymphocytic leukemia
Bcl-xl	B-cell lymphoma-extra large
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-like protein 11
Bik	Bcl-2-interacting killer
BIR	baculoviral repeat
BH	Bcl-2 homology domain
BM	bone marrow
Bmf	Bcl-2-modifying factor
BRCA1	breast cancer type 1 susceptibility protein
BSA	bovine serum albumin
CAD	caspase-activated DNAse
cDNA	complementary DNA
c-FLIP	cellular FLICE-inhibitory protein
Chk	checkpoint homolog
CK	Casein kinase
CLAMI	cellular lysis and mitochondrial intact
CLL	chronic lymphocytic leukemia
CMML	chronic myelomonocytic leukemia
CNS	central nervous system
CRC	colorectal carcinoma
CRD	cysteine-rich extracellular domain
CRAD	caspase-recruitment domain
C-terminus	carboxy-terminus
Cyto c	cytochrome c

DAXX	death domain associated protein
DcR	decoy receptor
DD	death domain
DED	death effector domain
DsRed	red fluorescent protein
dH_2O	distilled water
DISC	death-inducing-signaling-complex
DMSO	dimethyl sulfoxide
DN	dominant-negative
DNA	deoxyribonucleic acid
DR	death receptor
DSB	double strand breaks
E1A	early region 1A
EGFP	enhanced green fluorecence protein
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERBB2	erythroblastic leukemia viral oncogene homolog 2
ERK	extracellular signal-regulated protein kinase
FACS	fluorescence activated cell sorting
FADD	Fas-associated death domain
FASL	Fas ligand
FBS	foetal bovine serum
FLIP	FLICE-like inhibitory protein
Furin CS	Furin cleavage site
GSE	grape seed extract
GSK	germinal center kinases
GVHD	Graft-Versus-Host disease
H2AX	histone 2AX
hAAT	human alpha anti-trypsin protein
HAUSP	herpesvirus-associated ubiquitin-specific protease
HCC	human hepatocellular carcinoma
HDACi	histone-deacetylase inhibitors
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
IAP	inhibitors of apoptosis protein
ICAD	inhibitor of caspase-activated DNAse
IκB	Inhibitor of kappa B
IKK	IκB kinase
ILZ	isoleucine zipper
IP	immunoprecipitation
IR	ionizing radiation
IRE1a	inositol-requiring kinase 1
IRF	interferon regulatory factor
jBid	JNK-dependent processing of Bid

JNK	c-Jun N-terminal kinases
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence-1
MDM2	murine double minute 2
MEF	mouse embryonic fibroblast cells
MEKK1	MAP kinase kinase kinase
MEM	modified Eagle's medium
miRNA	micro RNA
MKK	mitogen-activated protein kinase kinase
MLK1	mixed lineage kinases 1
MOMP	mitochondrial outer membrane permeabilization
MPC	myeloid progenitor cell
mRNA	messenger RNA
MSC	mesenchymal stem cell
NF-κB	nuclear factor kappa B
NK cells	nature killer cells
NMR	nuclear magnetic resonance
NTP	nucleoside triphosphate
OMM	outer mitochondrial membrane
OPG	osteoprotegerin
ORF	open reading frame
PAK2	p21-activated protein kinase 2
PAT1	proton-coupled amino acid transporter 1
PBS	phosphate buffered saline
PCAF	P300/CBP-associated factor
PCR	polymerase chain reaction
PERK	PRKR-like endoplasmic reticulum kinase
PI	propidium iodide
PIDD	p53-induced protein with a death domain
PIGs	p53-induced genes
PIKK	phosphoinositide 3-kinase-related protein kinase
PKC	protein kinase C
PLZF	promyelocytic leukemia zinc finger
PS	phosphatidyl serine
PTEN	phosphatase and tensin homolog
PPM1D	protein phosphatase 1D
PUMA	p53 upregulated modulator of apoptosis
RIP1	receptor-interacting protein 1
RNA	ribonucleic acid
RNAi	RNA interefence
ROS	reactive oxygen species
qRT-PCR	quantitive real-time PCR
scFv	single chain variable antibody fragment
SCLC	small cell lung cancer

S.D	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrilamide gel electrophoresis
SDF	stromal cell-derived factor
shRNA	Short hairpin RNA
siRNA	small inhibitory RNA
SIMP	soluble mitochondria intramembrane proteins
siRNA	small inhibitor RNA
Smac	second mitochondrial-derived activator of caspase
STAT	signal transducer and activator of transcription 1
UPR	unfolded protein response
tBid	truncated Bid
TEMED	N, N, N', N'-tetramethylethylenediamine
TIMP3	tissue inhibitor of metalloproteinase 3
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR-associated death domain protein
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
TREs	TPA-responsive elements
TS	thymidylate synthase
UV	ultra-violate
VDAC	voltage-dependent anion channel)
VEGF	vascular endothelial growth factor
Wip1	wild-type p53-induced phosphatase 1
wt	wild type
XIAP	X-linked inhibitor of apoptosis protein
z-VAD	benzyloxycarbonil-Val-Ala-DL-Asp-fluoromethylketone

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CHAPTER 1 INTRODUCTION

1.1 Brief introduction to apoptosis

"Apoptosis", originally came from an ancient Greek word that means "leaves falling from a tree". It is one form of programmed cell death (Kerr and Searle, 1972). The principal morphological features of apoptosis are cell shrinkage, chromatin condensation, nuclear fragmentation and the formation of plasma-membrane blebs (reviewed by Hotchkiss et al., 2009). Different from other forms of cell death, apoptosis does not rupture the plasma membrane, thereby avoiding release of inflammatory cytokines and the induction of autoimmunity (reviewed by Nagata et al., 2010). The molecular features of apoptosis include internucleosomal DNA cleavage and random distribution of PS (phosphatidyl serine) between the inner and outer leaflets of the plasma membrane (Fadok et al., 1998). In multi-cellular animals, apoptosis plays an important role in the process of development and aging through controlling the number of cells. Without apoptosis, the human body would be composed of about 10^{14} cells, 2 tons of bone marrow and lymph nodes and a 16-km intestine by the age of 80 (reviewed by Melino, 2001). Therefore the process of apoptosis should be regulated precisely, otherwise it can cause disease pathogensis. For example, excessive apoptosis can cause neuro-degenerative disorders, such as Alzheimer's disease and Huntington's disease, cardiac ischaemia and renal damage (reviewed by Prasad and Prabhakar, 2003; Thompson, 1995). In contrast, deficient apoptosis can contribute pathologically to the development of auto-immune disease and cancer (reviewed by Gerl and Vaux, 2005). Conditions that can induce cell apoptosis include certain developmental signals, like a change of the local concentration of a critical growth factor or tissue morphogen (reviewed by Ashkenazi and Herbst, 2008). Other stimuli like ionizing radiation, heat shock, toxins, bacterial or viral infection can also lead to apoptosis through severe stress or damage to vital cellular components such as DNA or the cytoskeleton (reviewed by Gulbins et al., 2000).

The mechanisms of apoptosis were firstly studied from the investigation of programmed cell death that occurs during the development of *C.elegans* (Horvitz,

1999). In this organism, 131 of the 1090 somatic cells generated during development, are finally eliminated by apoptosis. The execution of apoptosis relies on the function of three proteins: the caspase Ced-3, the adaptor protein Ced-4, and a BH3-only pro-apoptotic member of the Bcl-2 family named Egl-1. In a healthy cell, the Bcl-2 homolog Ced-9 is required for survival (reviewed by Hengartner and Horvitz, 1994). However, when Egl-1 binds to Ced-9 it can lead to the activation of Ced-3 then induce apoptosis (Liu and Hengartner, 1999). This mechanism of activation and regulation of apoptosis appears to be conserved in higher organisms (Estoppey et al., 1997).

1.2 Two pathways lead to apoptosis

In vertebrates, there are two distinct, yet interlinked signaling pathways that control apoptosis activation: the extrinsic pathway and the intrinsic pathway (Figure 1.1 reproduced with permission from Murakami et al., 2011). The intrinsic pathway is primarily caused by intracellular cues, such as DNA damage. The extrinsic pathway is mainly caused by extracellular signals like cytokines which usually generated by cyototoxic cells of the immune system. In both pathways, the pro-apoptotic caspases are activated through a process called ‘the caspase cascade’.

Caspases are a family of cysteine proteases that are highly conserved in multicellular organisms. All caspases are synthesized as zymogens also called procaspases which share a common domain structure consisting of a large (p10) and a small (p20) catalytic subunit (reviewed by Sprick and Walczak, 2004). To date, 14 caspases have been found in mammals, among them only 7 caspases were shown to participate in apoptosis (reviewed by Chowdhury et al., 2008; Petrilli et al., 2007). The apoptotic caspases can be classified as initiator caspases or effector caspases, based on their position in the caspase cascade. Initiator caspases including caspase 8, 9 and 10 are stimulated first by apoptotic signals. Initiator caspases can be activated through being recruited into specific signaling complexes that promote their dimerization. In turn, activation of downstream effector caspases, such as caspase-3, 7

and 6 occurs by their cleavage at specific internal aspartic acid residues by initiator caspases (reviewed by Riedl and Shi, 2004). The effector caspases then cleave a variety of cellular proteins like structural proteins and enzymes, ultimately leading to the characteristic events of apoptosis (reviewed by Fan et al., 2005; Thornberry, 1999).

For example, caspase-3 can remove the negative regulatory domain from the kinase PAK2 (p21-activated protein kinase 2), this can trigger plasma membrane blebbing (Rudel and Bokoch, 1997). Caspase-3 can also cleave the ICAD (inhibitor of caspase-activated DNAse), which allows caspase-activated DNAse (CAD) to move into the nucleus and fragment the DNA (Nagata, 2000). Caspase-mediated proteolysis of cytoskeletal proteins such as actin, fodrin and lamin can result in loss of overall cell shape and nuclear shrinking (reviewed by Degterev et al., 2003).

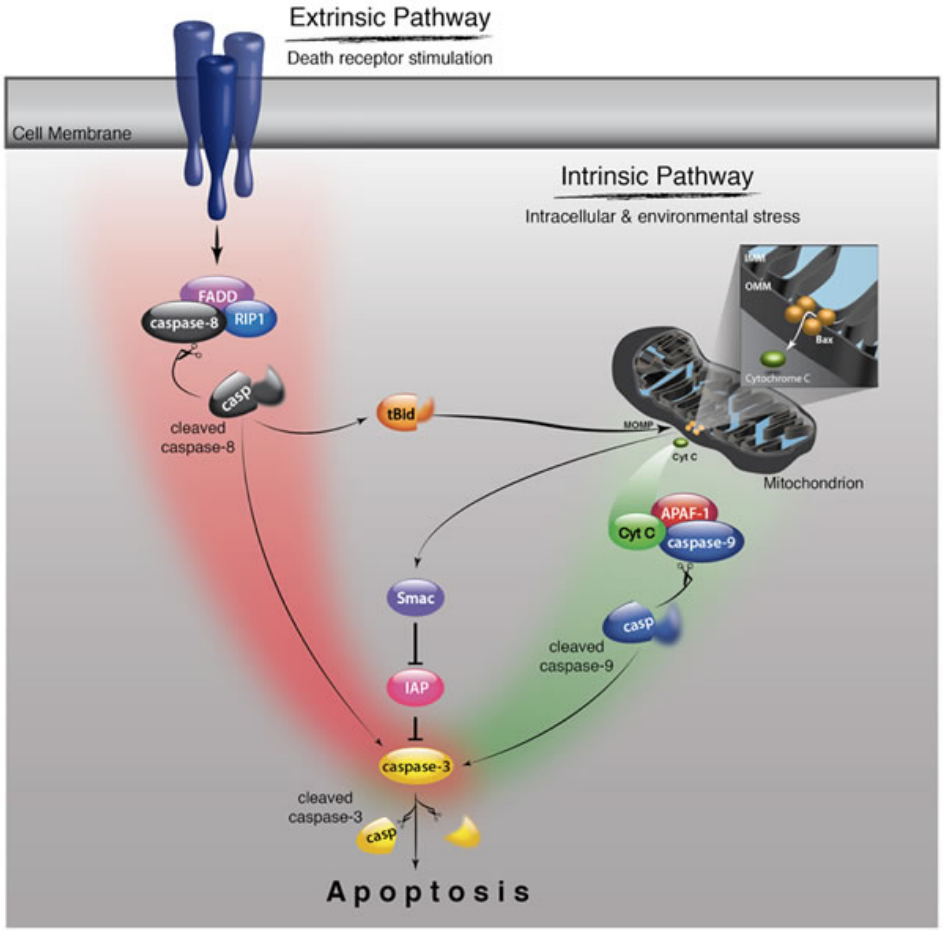


Figure 1.1 Schematic of extrinsic and intrinsic signaling pathways. Reproduced with permission from Murakami et al. 2011.

1.3 Extrinsic pathway

The extrinsic pathway, also named the death receptor pathway, is activated by ligation of death receptor ligands to specific death receptors on the cell membrane. Death receptor ligands are a group of type II transmembrane proteins arranged in stable homotrimers. Death receptor ligands can be generated by specialized immune cells such as NK (nature killer) cells or cytotoxic cells. Death receptors belong to the TNF-R (tumour necrosis factor receptor) superfamily, which has more than 20 members with a broad range of biological functions. Among the members of the death receptor family, the best characterized so far are CD95 (APO-1/Fas), TNF receptor I (TNFR1/CD120), TNF receptor II (TNFR2), TRAIL-R1 (DR4), and TRAIL-R2 (DR5). There is still little known about the function of DR3 (TRAMP/Apo-3) and DR6. Besides apoptosis, death receptors are also involved in the process of cell differentiation and immune regulation (reviewed by Ashkenazi, 2002; Walczak and Krammer, 2000). Death receptors are characterized by CRDs (cysteine-rich extracellular domains) that mediate binding between ligands and corresponding receptors. In addition, death receptors have a cytoplasmic domain of about 80 amino acids named ‘death domain’ (DD) which is essential for the transduction of the apoptotic signal (Tartaglia et al., 1993).

The crucial point of extrinsic pathway is the ligation of death receptors such as CD95 or DR4 and DR5 to their cognate ligands or agonistic antibodies resulting in the formation of a multimeric complex of proteins named DISC (Death-Inducing-Signaling-Complex) (reviewed by Mahmood and Shukla, 2010). The DISC is mainly composed of trimerised death receptors, adaptor proteins like FADD (Fas-associated death domain), TRADD (TNFR-associated death domain protein) or DAXX (death domain associated protein), caspase-8 or caspase-10 (reviewed by Mahmood and Shukla, 2010; Schulze-Bergkamen et al., 2009). Activation of caspase-8 is probably driven by an induced proximity, in which a high local concentration of procaspase-8 at the DISC can lead to its self-cleavage (Schulze-Bergkamen et al., 2009). Procaspase-10 can also be activated in the DISC.

However, whether caspase-10 can still trigger apoptosis without caspase-8 in response to death ligands is controversial. Thus the exact role of caspase-10 is still elusive (reviewed by Lavrik et al., 2005). Activated caspase-8 is a heterotetramer composed of two large subunits (p18) and two small subunits (p10). This is then released into the cytosol to propagate the apoptotic signal by activating downstream effector caspases.

There are two distinct cell types that have been identified (Scaffidi et al., 1998). Type I cells are characterized by caspase-8 activation at the DISC sufficient to directly activate downstream effector caspases such as caspase-3. However, in type II cells the level of activated caspase-8 at the DISC is insufficient to activate caspase-3, therefore an additional pathway is needed to amplify the apoptotic signal to fully activate effector caspases (Scaffidi et al., 1998).

The extrinsic apoptosis pathway is triggered by the binding of death ligands of the tumor necrosis factor (TNF) family to their corresponding death receptors (DRs) on the cell surface. Among these death ligands, TRAIL currently presents the most promising candidate for clinical use.

1.3.1 TRAIL pathway

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an apoptosis-inducing protein that belongs to the TNF super-family (reviewed by Griffith and Lynch, 1998; Wiley et al., 1995). In humans the *TRAIL* gene is located on chromosome 3q26 and its size is about 20 Kb and consists of five exons and four introns (Wiley et al., 1995). *TRAIL* can be transcriptionally up-regulated by several transcription factors such as p53, NF-κB, IRF (Interferon regulatory factor) etc, and its transcripts can be widely detected in different human tissues, most predominantly in spleen, lung and prostate (Kuribayashi et al., 2008).

As a type II transmembrane protein that is composed of 281 and 291 amino acids in human and mice, respectively, TRAIL has an N-terminal domain different from other members of TNF family, while the C-terminal domain is highly conserved. After the cleavage of extracellular C-terminal portion of the TRAIL from the cell surface,

TRAIL can be released as a soluble molecule (reviewed by Ashkenazi, 2002). The physiological role of TRAIL is still not fully understood. TRAIL is not expressed in freshly isolated human T-cells, B-cells, dendritic cells (DCs) and natural killer (NK) cells, while only a small portion of mice NK cells expresses TRAIL (Wang, 2008). *TRAIL* knockout mice are viable and display no apparent symptoms (Cretney et al., 2002). TRAIL might be involved in tumour immune-surveillance, because *TRAIL* knockout mice are more susceptible to experimental and spontaneous tumour metastasis (Cretney et al., 2002). *In vivo* studies using neutralizing antibodies to block the function of TRAIL implicate it in interferon-γ-dependent anti-tumour functions of NK cells (Takeda et al., 2002). In addition to tumour immune-surveillance, TRAIL might also be involved in the regulation of autoimmunity, since it has been found that the serum level of soluble TRAIL were increased in patients with systemic lupus erythematosus (Lub-de Hooge et al., 2005). The levels of TRAIL may also serve as a potential marker in determining the sensitivity to Interferon-ß treatment for human multiple sclerosis patients (Wandinger et al., 2003).

TRAIL has several receptors: two functional receptors, DR4 and DR5 which are also named TRAIL-R1 and TRAIL-R2, and three decoy receptors, TRAIL-R3, TRAIL-R4 and OPG (also named DcR1, DcR2 and osteoprotegerin respectively) (reviewed by Ashkenazi, 2002). Since the affinity of OPG for the TRAIL at physiological temperatures is very low, the physiological role of OPG as a receptor for TRAIL is unclear (Truneh et al., 2000). DR4 and DR5 contain a conserved cytoplasmic death domain motif that can undergo a conformational change upon binding to TRAIL (Bodmer et al., 2000). The binding of TRAIL to DR4 and DR5 results in the recruitment of the adaptor molecular FADD (Fas Associated protein with Death Domain) and caspase-8, which activates caspase-8 and the apoptotic machinery (Kischkel et al., 2000; Sprick et al., 2000). In addition, recruitment of RIP (receptor-interacting protein) to the receptor complex can also result in a caspase-8-independent form of cell death with necrotic morphology (Holler et al., 2000). DcR1, DcR2 and OPG because a lack of a functional cytoplasmic death domain can inhibit apoptosis by sequestering available TRAIL or by interfering with

the formation of a functional signaling complex (reviewed by Kimberley and Screaton, 2004; LeBlanc and Ashkenazi, 2003).

Since being discovered, TRAIL has received great attention because it can induce apoptosis in tumour cells while sparing normal cells (reviewed by Ashkenazi and Herbst, 2008). Additionally, TRAIL can induce apoptosis regardless of p53 status thereby offering an approach to target p53-deficient tumour cells (Mahalingam et al., 2009). Ligation of TRAIL to its receptors does not only lead to the activation of caspases and initiation of apoptosis, but also can induce other signaling pathways, like NF-κB and MAPK (Mitogen-Activated Protein Kinase) pathways (reviewed by Wang, 2008).

Although TRAIL is promising in tumour therapy, the resistance of many primary tumours to TRAIL-induced apoptosis is still a significant problem. Chronic lymphocytic leukemia (CLL), astrocytoma, meningioma and medulloblastoma are relatively resistant to TRAIL despite the expression of the death receptors on the surface of the cancer cells (Dyer et al., 2007). Therefore, great efforts have been made to find the mechanisms behind TRAIL resistance and several have been proposed. These mechanisms that contribute to TRAIL resistance may vary in different tumour cells. As mentioned above, it has been postulated that the decoy receptors can contribute to TRAIL resistance. Over-expression of DcR1 and/or DcR2 can protect cells from TRAIL-induced apoptosis (LeBlanc and Ashkenazi, 2003; Pan et al., 1997; Sheridan et al., 1997). A correlation between TRAIL resistance and the expression of decoy receptors have been found in myeloid leukemia (Riccioni et al., 2005). However, other studies failed to find any correlations between the levels of decoy receptors and TRAIL sensitivity (Zhang et al., 1999). This could be explained by a localization change of DcRs, as DcRs sometimes localize in the cytosol of the cell rather than on the cell surface (Olsson et al., 2001).

In addition, a large number of studies have researched the intracellular mechanisms of TRAIL resistance and several anti-apoptotic factors have been found that could contribute to TRAIL-resistance. Among them, the best characterized inhibitor of TRAIL-induced apoptosis is c-FLIP (cellular FLICE-inhibitory protein).

C-FLIP could interfere with the binding of caspase-8 to FADD thus blocking the activation of the caspase cascade (Irmler et al., 1997). Other inhibitors acting downstream of receptors have also been identified. For example, anti-apoptotic Bcl-2 (B-cell lymphoma 2) family members can inhibit TRAIL-induced apoptosis in type II cells. Over-expression of Bcl-xl can confer TRAIL resistance in pancreatic tumour cells (Hinz et al., 2000). This effect can also be observed in neuroblastoma, glioblastoma and breast carcinoma cells (reviewed by Fulda et al., 2002a). Mcl-1 expression in hepatocellular carcinomas and cholangiocarcinomas can also cause resistance to TRAIL-induced apoptosis (Wirth et al., 2005). Activation of caspases can also be inhibited by members of the IAP (inhibitor of apoptosis proteins) family especially XIAP (X-linked inhibitor of apoptosis protein). High expression levels of XIAP have been found to contribute to TRAIL resistance in a number of tumour cell lines (Schimmer et al., 2004). To this end, many chemotherapeutic agents have been shown be able to down-regulate these anti-apoptotic factors, therefore, combination use of these agents with TRAIL may be a more effective way to cure cancer.

Aside from the well-established TRAIL-resistance mechanisms described above, some novel factors have been discovered that could influence TRAIL sensitivity. For example, post-translational modifications of DR4 and DR5 by glycosylation or palmitoylation were able to affect the initiating events in TRAIL signaling. Reduced expression of peptidyl O-glycosyltransferase GALNT14, which is a specific O-glycosylation enzyme, can confer resistance to TRAIL in cancer cells (Wagner et al., 2007). A decrease of the palmitoylation of DR4 could affect the ability of DR4 to transduce apoptotic signals (Rossin et al., 2009). In addition, microRNAs have been reported to be involved in the sensitivity to TRAIL too. In this context, miR-221 and miR-222 were able to induce TRAIL resistance through their effects on the cell cycle regulator protein p27kip1, phosphatase and tension homologue (PTEN) and tissue inhibitor of metalloproteinase 3 (TIMP3) (Garofalo et al., 2008).

Many cellular signaling pathways can also influence the cells' sensitivity to TRAIL. For example, NF-κB can induce up-regulation of FLIP, Bcl-xl and XIAP, thereby exerting anti-apoptotic effects through these molecules. It has been found that

specific down-regulation of NF-κB through inactivation of I-κB kinase can significantly increase sensitivity to TRAIL (Ravi and Bedi, 2002). Other signaling pathways like protein kinase C, MAPK and Akt have also been shown to affect TRAIL activity, although the mechanisms are still not completely understood (reviewed by LeBlanc and Ashkenazi, 2003).

1.3.2 TRAIL-based tumour therapy

Since TRAIL is promising in the treatment of cancer, several versions of TRAIL and agonistic antibodies targeting TRAIL receptors have been developed and each has their own advantages and drawbacks.

Agonistic monoclonal antibodies that specifically target DR4 or DR5 but not decoy receptors have been developed. Among them the most prominent are Mapatumumab (anti-DR4, Human Genome Sciences-HGS), Lexatumumab (anti-DR5, HGS), AMG-655 (anti-DR5, Amgen) and Apomab (anti-DR5, Genentech). Generally, results to date have shown that these antibodies have promising anti-tumour activity and are relatively safe with no hepatotoxicity, linear pharmacokinetics, and no generation of anti-human antibodies (reviewed by Oldenhuis et al., 2008).

Different from agonistic monoclonal antibodies, recombinant TRAIL can activate DR4 and DR5 at the same time. So far, several versions of soluble TRAIL have been generated, each encoding the extracellular domain of TRAIL (aa119-aa289). The amino terminal of TRAIL was fused to different tags such as a poly-histidine tag or a FLAG epitope or leucine zipper or isoleucine zipper (ILZ) trimerisation domains (Ganten et al., 2006; Pitti et al., 1996; Schneider, 2000; Walczak et al., 1999). These tags can improve receptor oligomerisation, which is important for the transduction of apoptotic signals. However, the toxicity of recombinant TRAIL is a controversial issue. Some reports pointed out that recombinant TRAIL can kill even normal human cells *in vitro* including primary hepatocytes, keratinocytes and astrocytes (Gores and Kaufmann, 2001; Koschny et al., 2007a; Lawrence et al., 2001). Yet other studies have found that primary hepatocytes are resistant to TRAIL in *in vitro* culture (Ganten et al., 2006). *In vivo* studies using mice, cynomolgus monkeys or chimpanzees and

repeated recombinant TRAIL treatments also did not find any toxic effects on normal tissues (Ashkenazi et al., 1999; Walczak et al., 1999). The discrepancy might be due to cell culture artifacts or the specificity of the recombinant proteins used. For example, some versions of TRAIL might form higher-order complexes and thus increase their toxicity (Koschny et al., 2007b; Lawrence et al., 2001).

Currently, a non-tagged TRAIL is probably the preferred version for clinical applications. Without any added exogenous sequences, such TRAIL is also the least likely to be immunogenic in human patients (Ashkenazi and Dixit, 1999). This type of TRAIL is currently in phase I/II clinical trials (Genentech, San Francisco, CA, USA) and has been optimized by the addition of Zn and reducing agent to the cell culture media and extraction buffers as well as by formulation of the purified protein at neutral pH (Kelley et al., 2001; Lawrence et al., 2001). Initial studies in non-human primates, such as, cynomolgous monkeys and chimpanzees, show that short-term intravenous application of TRAIL is well tolerated even at very high doses (Kelley et al., 2001). Results from the phase I studies are also promising. No dose-limiting toxicities or adverse events have been reported so far. Among the five patients eligible for response evaluation, two had a complete remission, two experienced partial remission and one had a stable disease (Ashkenazi and Herbst, 2008). However, many factors could severely reduce the efficacy of TRAIL, such as its short half-life time *in vivo* and the ubiquitous expression of death receptors on the surface of non-transformed somatic cells. In order to increase the specificity of TRAIL to tumour cells, genetic fusion of soluble TRAIL to a single chain variable antibody fragment (scFv) has been generated (Bremer et al., 2004). For example, a TRAIL fusion protein named scFvEGP:sTRAIL, in which TRAIL is genetically fused to the pan-carcinoma associated cell surface antigen EGP (Epithelial Glycoprotein) that is highly expressed on a variety of human tumours including breast, small cell lung cancer (SCLC) and colorectal carcinoma (CRC) (Balzar et al., 1999). TRAIL has also been fused to a tumour-specific CD19 single-chain Fv antibody fragment, and this scFvCD19:sTRAIL can selectively bind to and induce apoptosis in CD19 positive tumour cells as well as patient-derived acute B-lymphoblastic leukemia (B-ALL) and

chronic B-lymphocytic leukemia (B-CLL) cells while normal blood cells remain unaffected (Stieglmaier et al., 2008). Similar TRAIL named scFvCD7: sTRAIL was generated by genetically linking it to a scFV specific for the T-cell surface antigen CD7. Although CD7 is also located on resting leukocytes, activated T-cells and vascular endothelial cells, scFvCD7:sTRAIL can specifically induce apoptosis in malignantly transformed T cell lines and cells freshly isolated from acute lymphoblastic leukemia patients (Bremer et al., 2005).

So far, all these fusion TRAIL forms have been shown to possess potent anti-tumour activity without any severe side effects, making fusion proteins a safe alternative to conventional TRAIL (Ashkenazi et al., 1999; Fulda and Debatin, 2004). Similarly, TRAIL variants capable of selectively targeting DR4 or DR5 but not the decoy receptors could avoid decoy receptor-interference in apoptosis induction and thus be more efficient. Using a computational design strategy, a mutation of aspartate at position 269 to a histidine and glutamate at position 195 to an arginine (D269H/E195R) in TRAIL generated a mutant with a 70-150-fold higher preference for DR5 over DR4 compared to wild type TRAIL (van der Sloot et al., 2006). This DR5-specific TRAIL mutant is more potent than wild type TRAIL due to more effective activation of DR5 and reduced binding to the decoy receptors. Other TRAIL variants are also being evaluated for anti-tumour activity. A DR5-selective TRAIL variant has been found to possess significant anti-tumour activity and a DR4-specific variant with a somewhat lower activity (Kelley et al., 2005). One TRAIL variant with six amino acid mutations named TRAIL.R1-6 was found to be selective for DR4 (MacFarlane et al., 2005). Based on this version, another DR4-specific variant generated by omitting one of the six amino acid substitutions was found to have efficacy that was greatly increased in DR4-sensitive CLL cells (MacFarlane et al., 2005). Another form of DR4-specific variant has also been generated, which is highly selective for DR4 and has a higher activity that is more comparable to wild type TRAIL (Tur et al., 2008). Pre-clinical and clinical research is needed to further elucidate the advantage of these receptor-selective TRAIL variants over wild type TRAIL.

1.3.3 Sensitization to TRAIL-induced apoptosis

Although the anti-tumour activity of TRAIL is promising, TRAIL as a single agent might not be sufficient to induce apoptosis in most primary tumour cells. Fortunately, the combinational use of TRAIL with other anti-cancer drugs or ionizing radiation can increase the effects of TRAIL and has yielded encouraging result (Anan and Gores, 2005; Ganten et al., 2005). This synergistic effect of anti-cancer drugs and TRAIL is mainly due to changes of the transcriptional levels of proteins important for the TRAIL pathway (Cretney et al., 2006; Wajant et al., 2002). In this respect, anti-cancer drugs generally can induce the expression of pro-apoptotic proteins including death receptors, caspase-8, FADD, Bax or Bak and down-regulate the levels of anti-apoptotic molecules like c-FLIP, IAPs, Bcl-xl Mcl-1 and/or Bcl-2 (reviewed by Held and Schulze-Osthoff, 2001; Kelley and Ashkenazi, 2004; Mitsiades et al., 2002).

TRAIL in combination with agents that elevate death receptor levels have been evaluated in several tumour models. For example, the combination use of TRAIL with HDACi (histone-deacetylase inhibitors) results in synergistic effects in tumour cells through up-regulation of DR4 and DR5 (Singh et al., 2005). Pretreatment of *Bax* -/- colon tumour cells that are resistant to TRAIL with 5-FU can restore the TRAIL sensitivity through p53-dependent transactivation of DR5 (Wang and El-Deiry, 2003b). Targeting the anti-apoptotic proteins in the intrinsic pathway such as Bcl-2 or IAPs can also increase TRAIL sensitivity. A small molecular Smac mimetic can enhance TRAIL-induced apoptosis by increasing the activation of caspases (Fulda et al., 2002b; Li et al., 2004). BH31-2 is an inhibitor of Bcl-xl, which in combination with TRAIL showed synergistic effects of apoptosis induction (Ray et al., 2005). Another small molecule Bcl-2 inhibitor HA14-1 was able to restore TRAIL sensitivity in Bcl-2 over-expressing colon cancer cells (Sinicrope et al., 2004).

As mentioned above, the cellular signaling pathways could also influence TRAIL sensitivity, therefore strategies targeting these signaling pathways have also been developed. The multikinase inhibitor sorafenib was reported to enhance TRAIL sensitivity (Meng et al., 2007). Furthermore, sorafenib treatment could restore the

TRAIL sensitivity of *Bax*-/- colon cancer cells by inhibiting the NF-κB pathway thus reducing the levels of Mcl-1 and c-IAP2 (Ricci et al., 2007). Inhibition of NF-κB signaling by specific inhibitors, PS-1145 or AS602868, has also been shown to overcome TRAIL resistance in different tumour cells such as pancreatic cancer and myeloma lines (Khanbolooki et al., 2006; Romagnoli et al., 2007). In addition, inhibition of pro-survival pathways such as PI3K/AKT and ERK (Extracellular Regulated protein Kinase) has also been shown to sensitise tumour cells to TRAIL-induced apoptosis. For example, combinational use of TRAIL with 17-AAG, which is a heat-shock protein (HSP90) inhibitor, has a synergistic effect on apoptosis by down-regulation of AKT and inactivation of ERK (Georgakis et al., 2006). Additionally, pretreatment of tumour cells with rapamycin, an inhibitor of the mTOR (mammalian target of rapamycin) pathway, can greatly increase TRAIL-induced apoptosis by negatively regulating the translation of c-FLIP (Panner et al., 2005). It has also been reported that microtubule-targeting drugs like nocodazole or paclitaxel (Taxol) can also enhance the apoptosis-inducing activity of TRAIL (Kim et al., 2008).

1.3.4 Targeted delivery of TRAIL

Although TRAIL is a promising candidate for tumour therapy, the use of TRAIL as therapeutic agent is limited by several factors like the potential cytotoxicity and its short half-life after systemic administration *in vivo* (Kelley et al., 2001). Gene therapy may enable continuous production of TRAIL in substantial amounts for a relatively long period of time.

For this purpose, a recombinant replication-deficient adenoviral vector encoding TRAIL (Ad5-TRAIL) was engineered (Griffith and Broghammer, 2001). Adenovirus type 5 (Ad5) is a potent gene therapy vector due to its ease of production of high titre virus and high levels of transgene expression (reviewed by Lusky, 2005). There are several advantages of using Ad-TRAIL compared to the recombinant TRAIL-based therapy. First of all, Ad-TRAIL can induce apoptosis in TRAIL-sensitive cells as efficiently as the soluble TRAIL, but does not require consecutive applications

(Griffith and Broghammer, 2001). Secondly, different from the short half-time of recombinant TRAIL, the Ad-TRAIL can express TRAIL over a longer period of time. Ad-TRAIL application is also safe and results from a phase I clinical study using Ad-TRAIL gene therapy so far looked promising as the treatment was well tolerated in three prostate cancer patients (Cordier et al., 2009).

However, the systemic application of adenovirus may be limited by innate and adaptive immune responses (Bramson et al., 1997; Worgall et al., 1997). Repeat application of the virus into donors can result in high levels of vector-specific antibodies, which can impair transgene delivery (Nunes et al., 1999). Furthermore, Ad-TRAIL acts only locally at the tumour site, and it is unlikely that it can travel to other locations within the human body. In order to overcome this limitation, mesenchymal stem cell (MSC)-based gene delivery has been developed.

There are two different types of adult stem cells in the bone marrow, the hematopoietic stem cells and mesenchymal stem cells (MSCs). MSCs have the capacity to differentiate into a variety of cell lineages, including adipocytes, osteocytes, chondrocytes, muscles, and stromal cells (Pittenger et al., 1999). MSCs show promise as a potential delivery vector for many reasons. Firstly, MSCs can be readily transduced with adenoviral and lentiviral vectors (McMahon et al., 2006). Secondly, MSCs can be easily isolated from adult mesodermal tissues such as bone marrow (BM) and expanded *in vitro* and a single lot of expanded MSCs from one healthy donor can be utilized for treatment for many patients. Thirdly, MSCs have the ability to accumulate at different specific sites such as tissue damage, inflammation and cancer in response to chemokines secreted by tumours such as stromal cell-derived factor (SDF)-1, hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (Ozawa et al., 2008; Schichor et al., 2006). Moreover, due to lack of expression of co-stimulatory molecules such as B7-1, B7-2, CD40 and CD40 ligand, MSCs show little or low immunogenicity. This property of MSCs makes it possible to administrate MSCs without HLA (Human Leukocyte Antigens) matching in cell therapy application. Our group reported that MSCs transduced with adenoviral vectors expressing full-length, membrane-bound TRAIL could induce apoptosis of

A549 cells *in vitro* and inhibit tumour growth in an A549 xenograft mice model (Mohr et al., 2008). Moreover, we also found that MSCs expressing soluble TRAIL combined with XIAP silencing could resulted in suppression of metastatic growth of pancreatic carcinoma *in vivo* (Mohr et al., 2010). In the clinic, the application of MSCs has already been tested for the treatment of severe acute GVHD (Graft-Versus-Host Disease) as allogeneic stem cell transplantation (LeBlanc et al., 2004).

Besides the extrinsic pathway, another pathway is also involved in the process of apoptosis called intrinsic pathway.

1.4 Intrinsic/mitochondrial pathway

The intrinsic pathway also called the mitochondrial pathway can generally be activated by "intrinsic stresses" such as DNA damage, hypoxia, heat shock, oncoprotein and survival factor deprivation (reviewed by Tait and Green, 2010). Engagement of the intrinsic pathway usually needs p53 function. When p53 is inactivated, tumour cells often evade apoptosis and can continue to proliferate. The pivotal event of the intrinsic pathway is MOMP (Mitochondrial Outer Membrane Permeabilization). MOMP results in the cell death mainly through two mechanisms. One is the disruption of essential mitochondrial functions (reviewed by Green, 2004). The other is the release of SIMPs (Soluble Mitochondria Intramembrane Proteins), such as cytochrome-c, Smac/DIABLO, endonuclease G, and the serine protease HtrA2/Omi from the mitochondrial intermembrane space (Du et al., 2004; Garrido and Kroemer, 2004). Once in the cytosol, these proteins can trigger apoptosis by promoting caspase activation or acting as death effectors independent of caspase activity (Saelens et al., 2004). The release of cytochrome-c can form a complex named apoptosome with Apaf-1 (Apoptotic protease activating factor 1) as its core component in the presence of ATP/dATP. Apaf-1 is a cytosolic protein with an N-terminal CRAD (caspase-recruitment domain). Association of cytochrome-c and dATP with Apaf-1 can facilitate it exposing the N-terminal CARD, which can

oligomerize and form a platform. The apoptosome can recruit procaspase-9 and thereby activate it which then leads to the activation of executioner caspase-3, -6 and -7 (Li et al., 1997; Zou et al., 1997). Mice model studies showed that *Apaf-1*, *caspase-9* or *caspase-3* knock-out MEFs are resistant to various apoptotic stimuli (Cecconi et al., 1998; Kuida et al., 1996; Yoshida et al., 1998). Another group of proteins released from mitochondria are IAP (Inhibitors of Apoptosis) antagonists including Smac/DIABLO, HtrA2/Omi, and GSPT1/eRF3. IAPs can neutralize activated caspases thereby inhibit apoptosis. Smac/DIABLO can bind IAPs in a manner similar to caspases, therefore reversing their grip on caspases (reviewed by Danson et al., 2007). Mitochondria can also release the pro-apoptotic protein AIF (Apotosis-Inducing-Factor) independent of caspase activation (Susin et al., 2000b). HtrA2/OMI can also contribute to cell death in a caspase-independent manner. For example, *in vitro* study shows that HtrA2/OMI can degrade XIAP, cIAP1 and cIAP2 (Suzuki et al., 2004).

1.5 Regulation of Intrinsic pathway

The key event of the intrinsic pathway is the mitochondria outer membrane permeabilisation (MOMP) which can result in the release of pro-apoptotic proteins from the mitochondrial intermembrane space and this is considered as “point of no return” (reviewed by Tait and Green, 2010). MOMP can also lead to the degeneration of the electron transport chain that is required for most functions of the organelle. In addition, the breakdown of electron transport can promote the generation of reactive oxygen species which can also promote cell death (Green and Evan, 2002).

The MOMP is tightly controlled by the evolutionarily conserved Bcl-2 (B-cell lymphoma-2) family members (reviewed by Youle and Strasser, 2008). The Bcl-2 family was named after Bcl-2 (B-cell lymphoma 2) which was first described in 1984, when it was found in chromosomal translocations involving chromosomes 14 and 18 in follicular lymphomas (Pegoraro et al., 1984). The Bcl-2 family can be classified into pro-apoptotic and anti-apoptotic based on their role in the process of apoptosis.

The pro-apoptotic Bcl-2 family members can be generally divided into two

subgroups based on their structures. The first group is composed of Bax (Bcl-2-associated X protein) and Bak (Bcl-2 homologous antagonist) which contain three BH domains (BH1-3) and can form the proteolipid pore responsible for cytochrome-c release (Leber et al., 2007). Overexpression of them can induce apoptosis. However, *in vivo* studies show that deficiency in Bax and Bak alone have almost no affects on apoptosis (Knudson et al., 1995; Lindsten et al., 2000). Double genes targeting of both *Bax* and *Bak* caused perinatal lethality and defects in haematopoiesis and the CNS (central nervous system) (Lindsten et al., 2000).

The second group members are BH3-only proteins which function by protein-protein interactions with other Bcl-2 family members. The BH3-only proteins include Bad (Bcl-2-associated death promoter), Bid (BH3 interacting-domain death agonist), Bik/Nbk (Bcl-2-interacting killer), Bim, Blk, Bmf (Bcl-2-modifying factor), Noxa and Puma (p53 upregulated modulator of apoptosis) (reviewed by Marsden and Strasser, 2003). These proteins are regulators rather than executioners of apoptosis, because they cannot induce apoptosis in cells without both Bax and Bak (Cheng et al., 2001; Zong et al., 2001). Structural studies reveal that the BH3 domain is an amphipathic α-helix of 16-20 amino acids that can bind a hydrophobic pocket on the surface of anti-apoptotic Bcl-2 family proteins. Based on mimicking BH3 peptides, several chemical inhibitors of anti-apoptotic Bcl-2 family proteins have been developed (Kitada et al., 2008; Kutzki et al., 2002; Wang et al., 2000). Once expressed or activated, the BH3-only proteins can translocate to the outer mitochondrial membrane and exert their pro-apoptotic functions. How the BH3-only proteins activate Bax and Bak and hence produce MOMP is still controversial. Till now, two models have been proposed: one is called the ‘direct activation’ in which BH3-only proteins like Bim, tBid (truncated Bid) and probably Puma can directly bind and activate Bax and Bak. In the other model, Bax and Bak are inhibited by pro-survival Bcl-2 family members. When the BH3-only proteins bind to and neutralize the pro-survival proteins, they liberate Bax and Bak to oligomerise and cause MOMP.

In vivo studies have revealed that different BH3-only proteins have distinctive physiological roles. For example, Bim is a regulator of lymphoid homeostasis, and loss of one allele of Bim can contribute to acceleration of B-cell lymphomagenesis induced by over-expression of c-myc (Egle et al., 2004; Strasser, 2005). Knock-down of *Puma* by RNAi (RNA interefence) can accelerate Myc-induced lymphomagenesis (Hemann et al., 2004). Loss of Puma can also render T-cells resistant to phorbol ester, γ-irradiation, etoposide and cytokine deprivation induced apoptosis (Jeffers et al., 2003; Villunger et al., 2003). *Bid* knock-out mice spontaneously develops a malignancy resembling CMML (chronic myelomonocytic leukemia) (Zinkel et al., 2003). Moreover, mammary epithelial cells deficient in Bad show resistance to withdrawal of epidermal growth factor (Ranger et al., 2003). Depletion of *Noxa* in transformed fibroblasts can partially confer resistance to DNA damage-induced apoptosis (Shibue et al., 2003).

The anti-apoptotic Bcl-2 subfamily members are Bcl-2, Bcl-xl (B-cell lymphoma-extra large), Bcl-w, Mcl-1(myeloid cell leukemia sequence-1), Bfl1/A1 and Bcl-b proteins, which all have the BH1-4 domains and can suppress apoptosis. A number of studies in different tumours confirmed the role of anti-apoptotic Bcl-2 family members in apoptosis resistance. In mice, over-expression of Bcl-2 can promote neoplastic transformation of B- and T- lymphocytes and myeloid cells (McDonnell and Korsmeyer, 1991; Traver et al., 1998). Depletion of *Bcl-xl* in mice leads to death around E14-15 due to severe anaemia and neuronal degeneration (Motoyama et al., 1995). Targeting of Bcl-w in epithelial cells in the small intestine causes increased sensitivity to DNA damage (Pritchard et al., 2000). The *Mcl-1* gene has been mapped to the 1q21 chromosome, a region that is frequently altered in preneoplastic and neoplastic disease (Craig et al., 1994). Mcl-1 over-expression mice show a high incidence of myeloid or B-cell lymphomas depending on the cell types (Zhou et al., 2001).

Among all these Bcl-2 family members, a prominent one is the Bid protein which acts between the extrinsic pathway and the intrinsic pathway.

1.5.1 Bid

Bid is a BH3-only protein which was firstly described in 1996 (Wang et al., 1996b), and then re-cloned at a later stage (Li et al., 1998; Luo et al., 1998). In both studies, Bid was identified as a substrate of caspase-8 and after been cleaved, it could transfer from the cytosol to mitochondria and induce the release of mitochondrial proteins. Bid is phylogenetically conserved, human BID shares 72.3% homology with murine Bid at the amino acid level (Wang et al., 1996b). The human *BID* gene is located on chromosome 22q11.2 while the mice *Bid* gene is located on chromosome 6 (Yin et al., 1999). The mice *Bid* gene has 5 coding exons, spanning a region of around 8 Kb and a non-coding exon, located about 15 Kb upstream of the first coding exon (Sax et al., 2002; Wang et al., 1996b). The human *BID* gene is more complicated, and is composed of 9 exons that span an area of about 39 Kb (Renshaw et al., 2004; Sax et al., 2002). The human *BID* can give rise to at least eight alternatively spliced transcripts, corresponding to 4 different protein variants (Renshaw et al., 2004). In both human and mice, the major protein product of *BID/Bid* is a transcript from the conserved 5 exons with 195 amino acids about 22 Kd in size (Wang et al., 1996b; Yin et al., 1999). Alternative splice variants in human *BID* could yield three different variants: a 241 amino-acid (26.8 Kd) Bid_{EL}, a 137 amino-acid (14.6 Kd) Bid_S and a 99 amino-acid (11.3 Kd) Bid_{ES} (Renshaw et al., 2004). Bid_{EL} and normal Bid both have similar pro-apoptotic ability in *in vitro* assays. Bid_S and Bid_{ES} do not have the BH3 domain and exhibit a dominant negative effect. Until now, there has been little knowledge about the physiological function of these transcripts *in vivo*. *Bid*-deficient mice are resistant to a Fas agnostic monoclonal antibody, which can induce significant hepatocyte apoptosis and severe liver injury (Ogasawara et al., 1993). Still in *Bid*-deficient mice, the activation of caspase-8 is not affected but the downstream effector caspase-3 activation is inhibited (Li et al., 2002c; Yin et al., 1999). Myeloid progenitors from *Bid* null mice show resistance to death receptor-induced apoptosis. The intrinsic pathway is also inhibited in these *Bid* deficient mice. Thus Bid plays an important role in the connection of the extrinsic and intrinsic pathways.

Bid can be widely detected in different tissues, with the highest level in the kidney (Wang et al., 1996b). The sub-cellular distribution of Bid has been mainly in the cytosol. In liver, endogenous Bid is approximately 50% soluble in the cytosol and 50% associated to membranes (Degli Esposti et al., 2003). Currently, there is little knowledge about the transcriptional regulation of Bid. It was found that Bid can be up-regulated by p53 (Sax et al., 2002). Over-expression of p53 could induce the up-regulation of both human and mice Bid mRNA levels *in vivo* and *in vitro*. This induction of Bid might be important for p53-induced DNA damage-induced apoptosis, because *Bid*-deficient fibroblasts were more resistant to DNA damaging agents such as adriamycin and 5-FU induced apoptosis (Sax et al., 2002). Transcriptional repression of Bid has also been reported. It has been found that the PLZF (promyelocytic leukemia zinc finger) protein could down-regulate the expression of Bid (Parrado et al., 2004). The PLZF protein could affect myeloid cell growth and is involved in some acute promyelocytic leukemia. Therefore, the repression of Bid might account for the anti-apoptotic activity of PLZF.

Using NMR (nuclear magnetic resonance) technology, the structure of Bid has been revealed (Chou et al., 1997). Bid is composed of 8 alpha helices and the central two hydrophobic helices (alpha 6 and 7) are surrounded by the amphipathic helices. This structure arrangement is conserved among Bcl-2 family members, such as Bcl-2, Bcl-xl, and Bax (Muchmore et al., 1996). The alpha 6 and 7 helices can form a hairpin structure and facilitate the insertion into the membrane (Garcia-Saez et al., 2004). Depletion of these alpha helices can keep Bid from interacting with mitochondria (Kim et al., 2004b; Lutter et al., 2000). Bid does not possess a transmembrane domain. The BH3 domain is important for the interaction with other Bcl-2 family members and for its pro-apoptotic activity and this domain shares sequence homology with other Bcl-2 family members (Wang et al., 1996b). Although Bid can bind to both Bcl-2 and Bax, the ability to interact with Bax seems more correlated with apoptosis-inducing activity. Bid mutants (M97A, D98A) that are unable to bind Bcl-2 but not Bax, still retain the ability to induce apoptosis (Kim et al., 2004b). Bcl-2 and Bcl-xl both can bind and suppress Bid. Such interaction can prevent Bid from

interacting with Bax or Bak. A Bcl-xl mutant that fails to interact with Bax but is still able to bind to Bid can inhibit Bid-mediated cell death (Cheng et al., 2001). It has been found that the native Bid contains another BH3-like domain at its N-terminus, which could bind to the canonical BH3 domain and suppress its pro-apoptotic function (Tan et al., 1999). Early study showed that over-expression of full length Bid could induce apoptosis (Wang et al., 1996b). In most cases it seems that Bid needs to function in a truncated form (tBid).

The processing of Bid to tBid was found to mainly depend on caspase-8. The proteolytic cleavage of Bid is specific and limited. It usually happens at the connection loop region between the alpha 2 and 3 helices. After the cleavage, the conformation of Bid undergoes a change and releases the N-terminal BH3-like domain thereby exposing the canonical BH3 domain that can now effectively bind to Bax (Oh et al., 2005). As a result, truncated Bid is much more potent than the full length and in many circumstances is required for the activation of Bax, leading to the activation of the intrinsic pathway (Li et al., 1998; Luo et al., 1998). Apart from Bax, Bid can also interact with cardiolipin in the mitochondria which could lead to disturbed oxidative phosphorylation, as many respiratory proteins are bound to cardiolipin (Gonzalvez et al., 2008; Liu et al., 2004a). Furthermore, Bid also has been found to have lipid transfer activity. However, whether this activity has a direct effect on the apoptosis process is still unclear. Bid cannot just induce the release of mitochondrial proteins but also several other prominent mitochondrial dysfunctions associated with apoptosis, like the induction of mitochondrial permeability transition, mitochondrial depolarization, mitochondrial cristae reorganization, and the generation of ROS (reactive oxygen species) (Scorrano et al., 2002; Zamzami et al., 2000).

Besides cleavage, Bid also subject to other modifications after translation. For example, Bid cleavage by caspase-8 can expose a Gly60 site at the newly formed N-terminal of tBid, which is subjected to N-myristoylation shortly after (Zha et al., 2000). This post-translational modification can significantly increase the ability of tBid to translocate to and interact with the mitochondrial membrane. As a result, myristoylated tBid is much more efficient than the normal tBid in inducing the release

of proteins from mitochondria (Zha et al., 2000). Moreover, it has been found that Bid can be phosphorylated. Phosphorylation is a common and important mechanism of acute and reversible regulation of protein function. Most proteins are found to be phosphorylated at serine or threonine sites, and many proteins involved in signal transduction are also phosphorylated at tyrosine sites. The phosphorylation sites of Bid locate at the loop region. It has been shown that murine Bid can be phosphorylated by Casein Kinase I and II at Ser61 and Ser64 residues, which then inhibits the cleavage of Bid (Desagher et al., 2001). Human Bid can also be phosphorylated by Casein Kinase II at Thr59 site which also negatively regulate the cleavage (Degli Esposti et al., 2003). The residues Ser61 and Ser78 of Bid can also be phosphorylated by ATM (Ataxia-Telangiectasia, Mutated) kinase and/or ATR (Ataxia-Telangiectasia and Rad3-related protein) kinase in response to DNA damage. This phosphorylation can provide a protective effect against DNA-damaging drugs (Kamer et al., 2005).

Although DNA-damaging anti-cancer drugs are complex signaling inputs, mounting evidence suggests that the apoptosis signaling pathways initiated by these agents emerge from the DNA damage response. The DNA damage can be sensed by ATM and the homologous ATR protein, both of which can be activated by DSBs (double strand breaks) (Ismail et al., 2005). ATM is one of six members of the PIKK (phosphoinositide 3-kinase-related protein kinase) family. ATM defects in humans can cause a rare autosomal recessive syndrome named A-T (ataxia-telangiectasia) syndrome characterized by progressive neuro-degeneration, radiosensitivity, immune dysfunction, cell-cycle checkpoint defects, genomic instability and an increased predisposition to cancer (Chun et al., 2004). *Atm* null mice are prone to developing T-cell lymphomas and usually die between 3-6 months of age. Additionally, these mice are infertile, hypersensitive to radiation, show motor coordination defects, reduced body size and have defects in their immune system (Barlow et al., 1996; Elson et al., 1996; Herzog et al., 1998; Rotman and Shiloh, 1998). Once activated, ATM can phosphorylate various downstream substrates such as NBS1 (nibrin). Phosphorylated NBS1 acts as an adaptor protein for ATM-mediated phosphorylation

of Chk2 (checkpoint homolog 2). Chk2 can further activate the kinase cdc25 and is responsible for S-phase checkpoint control (Lavin et al., 2005). Other substrates of ATM include p53, the E3 ubiquitin ligase MDM2 (murine double minute 2), Chk1 (checkpoint homolog 1), H2AX (histone 2AX) and the BRCA1 (breast cancer type 1 susceptibility protein), which is involved in breast cancer development (Ahn et al., 2000; Cortez et al., 1999; Fernandez-Capetillo et al., 2002; Matsuoka et al., 1998). As mentioned above, Bid phosphorylation can provide protection against DNA damaging agents. Additionally, Bid phosphorylation is also involved in S phase arrest following DNA damage, although the mechanism is still unknown. Until now, there are still no reports about the reverse process namely Bid dephosphorylation. In the present study we found that phosphatase Wip1 can dephosphorylate Bid. Wip1 is regulated by p53 as well as other transcription factors.

1.5.2 p53

The p53 protein is involved in the control of several biological functions like the cell cycle, cell senescence, DNA metabolism, cellular differentiation and apoptosis. p53 is a widely known tumour suppressor, which was first identified as a protein interacting with the SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). p53 belongs to a family which has three members: p53, p63 and p73. The other two members: p63 and p73 also act as tumour suppressors (reviewed by Slee et al., 2004). *TP53* is often mutated in human cancers, where around half of all cancers have dysfunctional p53 (reviewed by Soussi and Beroud, 2001). Germline mutations of *TP53* can lead to Li-Fraumeni syndrome, which is a familial cancer syndrome including different types of cancer (Malkin et al., 1990). *p53* knockout mice (*p53*-/- and *p53*+/-) develop normally but die prematurely due to cancers (Donehower et al., 1992). The *p53*-null MEFs (mice embryonic fibroblast cells) are more resistant to apoptosis induced by oncogene overexpression and chemotherapeutic agents (Clarke et al., 1993; Lowe et al., 1993b). *p53*-null thymocytes and intestinal stem cells are also more resistant to radiation-induced apoptosis than their wild-type counterparts (Lowe et al., 1993b).

Using knock-in mice models, it was found that the ability of p53 to induce apoptosis and inhibit tumour development mainly depends on its role as a transcriptional factor (Chao et al., 2000; Jimenez et al., 2000). p53 can bind to a consensus response element (p53RE) located proximal to the transcription start site at the gene promoter, the first intron or further downstream of the gene (Menendez et al., 2009). The ability of p53 to induce apoptosis is conserved, even in *Drosophila* and *C.elegans* (Brodsky et al., 2004; Jin et al., 2000; Schumacher et al., 2001).

P53 can promote apoptosis through activation of the extrinsic pathway. CD95, DR4 and DR5 all have been shown to be regulated by p53. Therefore, p53 can enhance apoptosis induced by TRAIL (Liu et al., 2004b; Sheikh et al., 1998). Moreover, p53 can up-regulate the CD95 thereby enhance the interaction between CD95L and CD95 which could in turn augment the apoptosis induced by chemotherapeutic agents (Muller et al., 1998). At the same time, it has been suggested that p53-mediated apoptosis mainly utilises the intrinsic pathway, while the extrinsic pathway is only to augment the apoptosis response (reviewed by Fridman and Lowe, 2003). The first hint suggesting that the intrinsic pathway is involved in p53-mediated apoptosis is that Bcl-2 can inhibit p53-mediated apoptosis (Gottlieb et al., 1994). Later, several Bcl-2 family members were implicated in p53-dependent apoptosis. Bax was the first identified p53-induced pro-apoptotic Bcl-2 family member (Miyashita and Reed, 1995). In a brain tumour model, loss of Bax accounts for nearly half of the accelerated tumour growth which is caused by the loss of p53 (Yin et al., 1997). Moreover, p53 can regulate several other BH3-only Bcl-2 proteins like Puma, Bid and Noxa (Han et al., 2001; Nakano and Vousden, 2001; Oda et al., 2000; Sax et al., 2002; Yu et al., 2003). Several other components of the apoptotic machinery can also be activated by p53, like Apaf-1 and caspase-6 (Kannan et al., 2001; Moroni et al., 2001). p53 can also activate the *redox* genes which encode redox-regulating enzymes such as ferrodoxin reductase and the PIGs (p53-induced genes) (Donald et al., 2001; Hwang et al., 2001). PIGs can produce ROS subsequently cause damage to the mitochondria and initiate apoptosis (Polyak et al., 1997).

Depletion of ferrodoxin reductase in colorectal cancer cells can confer resistance to 5-FU (Hwang et al., 2001). p53 can also induce apoptosis independent of its transcriptional activity. It was found that expression of a temperature-sensitive *p53* allele in somatotropic progenitor cells can cause UV-induced apoptosis even in the presence of inhibitors of transcription and translation (Caelles et al., 1994). Later, several studies suggested that p53-induced cell death independent of its transcriptional activity might be a general phenomenon. For example, Interleukin-3 withdrawal caused apoptosis required functional p53 but not transcriptional activity in DA-1 lymphoma cells (Gottlieb et al., 1996). Furthermore, ionizing or UVB radiation-induced apoptosis in several transformed human cell lines still occurs even in absence of p53-regulated gene expression (Bissonnette et al., 1997). The mechanism behind this might depend on mitochondria. In some tissues, p53 can accumulate at the mitochondrial membrane in response to DNA damage (Erster and Moll, 2004). At the mitochondria, p53 can activate Bax by interacting with anti-apoptotic Bcl-2 family member Mcl-1 (Leu et al., 2004). Moreover, using immunofluorescence and immunoelectron microscopy technologies, it was found that localization of p53 to the mitochondria occurred rapidly, and this preceded release of cytochrome-c and procaspase-3 cleavage (Chipuk and Green, 2003).

P53 itself is subjected to many post-translational modifications like phosphorylation, ubiquitination, sumoylation and acetylation (reviewed by El-Deiry, 2003). p53 can be phosphorylated by ATM and Chk2 on serines 15 and 20, respectively (Dumaz and Meek, 1999; Hirao et al., 2002). MDM2 can also be phosphorylated by ATM following DNA damage, and this can decrease the interaction between MDM2 and p53 and lead to subsequent p53 protein stabilization (Maya et al., 2001). MDM2 has E3 ubiquitin ligase activity towards p53 which can lead to the proteasomal degradation of p53. A protein named Pirh2 has been found to also have ubiquitin ligase activity towards p53 (Leng et al., 2003). The p300 protein also has ubiquitin ligase activity toward p53 (Grossman et al., 2003). The enzyme HAUSP (herpesvirus-associated ubiquitin-specific protease) was found to have deubiquitinating activity on p53 (Li et al., 2002b). p53 can also be acetylated by

PCAF (P300/CBP-associated factor) and deacetylated by Sir2 (El-Deiry, 2003).

Among the many genes whose expression can be positively regulated by p53, one is a phosphatase called Wip1. We found that the expression of Wip1 is regulated by both p53 and the JNK pathway. Up-regulation of Wip1 can in turn lead to the dephosphorylation of Bid.

1.5.3 Wip1

Currently, four main classes of serine/threonine phosphatases have been identified: Type 1 (PP1), Type 2A (PP2A), Type 2B (PP2B), and Type 2C (PP2C a) (reviewed by Mumby and Walter, 1993). Wild-type p53-induced phosphatase 1 (Wip1) also named PP2C δ or PPM1D (protein phosphatase 1D), was originally identified in a screen for ionizing radiation (IR)-induced transcripts in Burkitt lymphoma cells (Fiscella et al., 1997). Wip1 is a monomeric enzyme requires divalent cations, mainly Mg^{2+} or Mn^{2+}, for its catalytic activity. Different from other PP2C family members, Wip1 is insensitive to okadaic acid (reviewed by Barford, 1996; Barford et al., 1998; Jackson and Denu, 2001). The human *WIP1* gene is located on chromosome 17q22/q23 while the mice *Wip1* have been mapped to chromosome 11 (Bulavin et al., 2002; Choi et al., 2000). In mice, *Wip1* mRNA is widely expressed in embryonic and adult tissues, with especially high level in the testis (Choi et al., 2000).

The Wip1 protein size is 61 kDa, and the protein is composed of 605 amino acids that can be classified into two major domains (Choi et al., 2000). One is a highly conserved N-terminal phosphatase domain from amino acids 1-375 whereas the C-terminal domain is a less conserved non-catalytic domain extending from amino acids 376-605. The C-terminal domain of Wip1 contains two putative nuclear localization signals, which might facilitate its nuclear localization. This C-terminal domain is highly conserved among mammalian Wip1s. However, mutation of these motifs failed to prevent nuclear localization (Yoda et al., 2006). Wip1 can be transcribed in a p53-dependent manner *in vitro*, and later *in vivo* studies also confirmed that ionizing radiation treated *p53* wild-type mice tissues exhibited Wip1

RNA induction in their tissues, while in the *p53*-/- mice tissue there is no *Wip1* RNA induction (Choi et al., 2000). Later studies demonstrated that Wip1 could also be induced by UV (ultra-violet), IR, methyl methane sulfonate, anisomycin, and H_2O_2 (Takekawa et al., 2000).

Many studies indicate that Wip1 have oncogene functions. For example, using the whole-genome DNA copy number analysis technique it was found that human breast cancer samples harbour a novel recurring region of DNA amplification on chromosomal region 17q22-q24, which contains the *WIP1* gene (Kallioniemi et al., 1994). Later using the higher-resolution DNA microarray methodology along with very high-resolution mapping of DNA copy number by real-time PCR it was found that just two genes, *PAT1* (Proton-coupled amino acid transporter 1) and *WIP1*, are located in this region. Both *PAT1* and *WIP1* were found to be amplified and over-expressed in 15% of breast tumours (Li et al., 2002a). In addition to mammary gland tumours, *WIP1* is also amplified and over-expressed in neuroblastomas, pancreatic adenocarcinomas, and medulloblastomas as well as in ovarian and gastric carcinomas (Castellino et al., 2008; Fuku et al., 2007; Hirasawa et al., 2003; Saito-Ohara et al., 2003; Tan et al., 2009).

Wip1 null mice were firstly reported in 2004 (Bulavin et al., 2004). The *Wip1* knock-out mice appears largely normal except exhibiting some minor reproductive and immune defects (Bulavin et al., 2004). This study also showed that *Wip1*-/- MEFs were completely resistant to transformation by RAS (rat sarcoma), ERBB2 (erythroblastic leukemia viral oncogene homolog 2) and Myc. Only the combination of RAS and E1A had limited ability to induce tumourigenicity. Interestingly, although *Wip1* is a target gene of p53, the p53 status made no difference to the requirement for *Wip1* in oncogene transformation. Deletion of the *p16Ink4A* and *p19Arf* genes can completely alleviate the requirement for Wip1 in oncogenic transformation. Based on these findings, it seems that Wip1 enables RAS and other oncogenes to transform cells is through by suppression of both p16Ink4A and p19Arf. Oncogene-induced mammary tumours in *Wip1* null mice occurred with a delayed latency compared to their counterparts with wild type *Wip1* (Bulavin et al., 2004). Delayed tumourigenesis

was associated with higher p16Ink4A levels. All these studies indicate that oncogene function of *Wip1* might depend on other proteins.

Under certain circumstances, Wip1 can also promote apoptosis. For example, overexpression of Wip1 in 293 cells can block cell cycle progression and arrested cells in early S phase, resulting in inhibition of DNA synthesis and leading to apoptosis (Tong et al., 1998). Recently, it has also been reported that overexpression of Wip1 can sensitize tumour cells with inactive p53 to chemotherapeutic agents (Goloudina et al., 2012).

Since Wip1 was shown to be a serine/threonine phosphatase, its functions might be understood better by identifying Wip1 targets. To date, at least eight Wip1 dephosphorylation targets have been found: p38 MAPK, UNG2, Chk1, p53, Chk2, ATM, MDM2, NF-κB. Among these target proteins, two distinct motifs appear to be specifically dephosphorylated by Wip1, pTXpY and pS/pTQ. One thing these proteins have in common is their importance in the cellular DNA damage/repair response. Obviously, the functions of Wip1 may be better understood by identifying new target proteins and characterizing the biological results of such dephosphorylation.

1.5.4 JNK

While the initiation and execution of apoptosis depends on the activation of the extrinsic and/or intrinsic pathway, the apoptosis process is also affected by many other signaling pathways like MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated protein kinase), NF-κB (nuclear factor kappa B) and JNK which are activated by treatment of cells with cytokines or by exposure of cells to a variety of stresses (reviewed by Davis, 2000; Karin and Lin, 2002; Kyriakis et al., 1994). Among them, a prominent one is the JNK (c-Jun N-terminal protein kinase) pathway.

JNK also named stress-activated MAPK (SAPK) was firstly described by its activation in response to cycloheximide-induced inhibition of protein synthesis (Kyriakis and Avruch, 1990). To date, multiple splice variants of JNK encoded by three different genes, namely JNK1, JNK1 and JNK3, have been identified (reviewed

by Davis, 2000; Johnson and Nakamura, 2007). Through alternative splicing, at least ten isoforms can be formed. JNK1 and JNK2 are expressed in almost every cell, whereas JNK3 is mainly found in the brain (reviewed by Bode and Dong, 2007; Cuevas et al., 2007). Activated JNK is able to phosphorylate the N-terminal transactivation domain of the c-Jun transcription factor and augment the AP-1 (activator protein 1) transcriptional activity. This phosphorylation enhances c-Jun dependent transcriptional events in mammalian cells (Derijard et al., 1994; Pulverer et al., 1991). The JNK pathway is involved in many physiological processes, including proliferation, apoptosis, and differentiation. The outcomes of JNK activation are dependent on the stimuli and on cell type specific (reviewed by Lin and Dibling, 2002; Liu and Lin, 2005).

JNK belongs to the wider MAPKs (mitogen-activated protein kinases) family. MAPKs are commonly activated via three-tiered phosphorylation cascades, consisting of a MAP kinase kinase kinase (MAP3K), a MAP kinase kinase (MAP2K) and a MAP kinase (MAPK) respectively (reviewed by Dhanasekaran and Johnson, 2007; Dhanasekaran and Premkumar Reddy, 1998). After the activation of MAP3Ks, which then activate the downstream MAP2K proteins MKK4 and MKK7, which in turn phosphorylate and activate JNK (reviewed by Davis, 2000; Weston and Davis, 2002). Until now, over 20 MAP3Ks have been found and 14 of them have been observed to be able to activate JNK through MKK4 or MKK7 (reviewed by Fanger et al., 1997; Johnson and Nakamura, 2007; Raman et al., 2007). Activation of SAPK/JNK by kinases MKK4 and MKK7 requires the dual phosphorylation of Tyrosine (Tyr) and Threonine (Thr) residues, which located in a Thr-Pro-Tyr motif in the activation loop between two kinase domains (Nagai et al., 2007). Because of the large number of MAP3Ks that can activate the JNK cascade, it has made the identification of individual family members responsible for JNK activation by distinct stimuli extremely difficult. So far, the mechanisms by which ASK1 (apoptosis signal-regulating kinase 1) and MLK1 (mixed lineage kinases 1) link diverse apoptotic stimuli to JNK are well characterized (Matsuzawa and Ichijo, 2001; Nagai et al., 2007; Sathyanarayana et al., 2002). ASK1 and MLK1 can be activated by

diverse apoptotic stimuli such as TNF-α, reactive oxygen species, lipopolysaccharide and endoplasmic reticulum stress. However, genetic studies have shown that deletion of the *Ask1* gene in mice resulted in only a weak effect on JNK activation (Matsuzawa et al., 2002). *In vitro* studies with *Ask1*-/- embryonic fibroblasts have indicated that ASK1 is not required for the transient activation of JNK but is required for the persistent activation of JNK and apoptosis induced by TNF-α (Tobiume et al., 2001). In cisplatin-induced apoptosis of ovarian carcinoma cells, similar patterns of ASK1 and JNK activation have been also reported (Chen and Davis, 1999).

All three JNKs have been shown to be involved in apoptosis and their role in the process is well established (reviewed by Davis, 2000; Weston and Davis, 2002). However, the mechanisms behind this are still controversial and appear to be stimulus- and cell type-specific (reviewed by Liu and Lin, 2005). The first evidence that the activation of JNK1 can induce apoptosis came from the initial studies investigating γ-ray induced apoptosis (Chen et al., 1996). The JNK pathway can also promote apoptosis in neurons. Neurons from mice in which the JNK pathway is interrupted are resistant to UV induced apoptosis (Yamamoto et al., 1999). Later, genetic studies using embryonic fibroblasts derived from *JNK1*-/- *JNK2*-/- mice further confirmed that JNK1 and JNK2 are involved in apoptotic signaling (Tournier et al., 2000). MEFs derived from those *JNK*-/- mice showed resistance to apoptosis in response to UV radiation, DNA-alkylating agent methyl methanesulfate and translation inhibitor anisomycin. The role of JNK3 in apoptosis was also established using *JNK3*-/- mice (Yang et al., 1997). Biochemical methods also confirmed the apoptosis induction ability of JNK. In rat PC-12 pheochromocytoma cells, the apoptosis induced by nerve growth factor (NGF) withdrawal can be suppressed by inhibiting the JNK pathway, whereas expressing a constitutively active MEKK1, thus activating the JNK pathway, can induce apoptosis in PC-12 cells (Le-Niculescu et al., 1999; Minden et al., 1994). Studies using JNK-specific inhibitors have also proved the pro-apoptotic role of JNKs. For example, JNK-specific inhibitors can inhibit the apoptosis of hepatocytes and sinusoidal endothelial cells during hepatic I/R injury (Uehara et al., 2005). Similarly, JNK inhibitor has also been shown to inhibit the

apoptosis of cardiomyocytes using a rat cardiac I/R animal model (Ferrandi et al., 2004).

However, as for the mechanism of how JNK activation contributes to apoptosis is still incompletely understood. Currently, one hypothesis is that JNK is an intrinsic component of the mitochondria-dependent death pathway during stress-induced apoptosis, because diminishment of JNK can inhibit the release of cytochrome-c and depolarization of mitochondria membrane potential (Davis, 2000). Another explanation is that JNK may function as a modulator to "break the brake" on apoptosis, rather than a genetic component of the apoptotic machinery to induce apoptosis. The best example is JNK activation can regulate the TNF-α-induced apoptosis. TNF-α induced apoptosis can be suppressed by NK-κB through inhibiting caspases activation and preventing prolonged JNK activation (Karin and Lin, 2002; reviewed by Lin and Dibling, 2002).

Since altered expression of JNK proteins in human tumours and cancer cell lines is often observed, JNK may play a role in oncogenic transformation. However, its role in tumour development still remains controversial. Initially, JNK activation and c-Jun phosphorylation were found to be required for transformation induced by Ras, an oncogene that is mutationally activated in almost 30% of human cancers (reviewed by Adjei, 2001). It might be because c-Jun can transcriptionally repress the gene that encodes the p53 tumour suppressor (Schreiber et al., 1999). The role of JNK in tumourigenesis is also supported by the finding that JNK pathway is constitutively activated in several tumour cell lines (reviewed by Ip and Davis, 1998). In HCC (human hepatocellular carcinoma) cells, JNK was observed with increased kinase activity and was found to be associated with altered histone H3 methylation (Chang et al., 2009). Additionally, several members of the JNK pathway were found up-regulated in prostate cancer (Ouyang et al., 2008). This might due to tumour suppressor PTEN (phosphatase and tensin homolog), which can lead to increased JNK activity, is frequently lost in prostate cancer.

JNK might also act as a tumour suppressor, since *JNK3* mutations were found in 10 of 19 human brain tumours examined (Yoshida et al., 2001). Another study found

that JNK can exert its tumour suppression ability through the immune system involving CD8 T-cells (Gao et al., 2005b). Taken together, JNK in tumour development may depend on the genetic background or tissue origin. Thus further research into the molecular framework of the JNK pathway may help us design strategies for tumour therapy.

The outcome of the JNK pathway activation largely depends on the substrates of the JNK pathway. JNK can phosphorylate the c-Jun N-terminal domain at serines 63/73 and threonines 91/93 (Bannister et al., 1995; Nateri et al., 2005; Vinciguerra et al., 2008). Together with Fos family members (c-Fos, FosB, Fra-1 and Fra-2) they can form a group of proteins called AP-1 (Activator Protein-1). Thus the AP-1 complex can be formed by different combinations of homodimers and hetrodimers, and this combination affect the activity of AP-1 (reviewed by Chinenov and Kerppola, 2001; Vogt, 2002). As a transcription factor AP-1 can control both basal and inducible transcription of several genes containing AP-1 sites (consensus sequence 5'-TGAG/CTCA-3'), also known as TPA-responsive elements (TREs) (reviewed by Angel and Karin, 1991). In a given cell, the AP-1 activity is regulated by a broad range of physiological and pathological stimuli, like cytokines, growth factors, stress signals and infections. AP-1 activation is induced by cis-elements in the promoters of AP-1 encoding genes. The post-transcriptional control of AP-1 subunits could also affect AP-1 activity. It is well documented that AP-1 can exert apoptotic or anti-apoptotic effects.

The decision as to whether AP-1 is pro or anti-apoptotic might depend on not only the antagonistic activity of different subunits but also the tumour cell types, and the genetic background of tumour cell types. For example, early *in vitro* studies indicated that increased AP-1 activity can lead to apoptosis in several different cell lines (Bossy-Wetzel et al., 1997; Ham et al., 1995; Preston et al., 1996). However, at the same time AP-1 can antagonize apoptosis in liver tumours (reviewed by Eferl and Wagner, 2003). The different consequences of AP-1 activity for apoptosis are probably due to differential regulation of target genes. For example, c-Jun can regulate the expression of Bim, a member of the Bcl-2 family, which is crucial for

neuronal apoptosis (Whitfield et al., 2001). In T-cells, c-Jun could also regulate the gene that encodes FasL (Fas ligand), thus promoting apoptosis through FasL binding to the Fas receptor (Kasibhatla et al., 1998). Also in T-cells, c-Jun can induce the expression of the anti-apoptotic *Bcl-2* and *Bcl-xl* genes thus leading to reduced apoptosis (Passegue et al., 2001). *C-Jun* and *JunD*-deficient hepatocytes show increased sensitivity to TNF-α, which implies that AP-1 might regulated genes that protect cells from TNF-α induced cell death (Eferl and Wagner, 2003; Weitzman, 2000).

To date, the targets of AP-1 have been identified so far include p53, Bcl-2 family members, TNF receptor family members, and recently there is a new report that found that c-Jun is involved in the expression of the phosphatase Wip1, which might affect cells response to different stimuli (Song et al., 2010). The fact that JNK can activate the intrinsic pathway has been established many years ago. However, the mechanisms behind this are still clear. In the present study, we found that JNK-dependent Wip1 induction is critical for the dephosphorylation of Bid, thus promoting Bid cleavage and activation of the intrinsic apoptosis pathway.

1.6 Aim of this study

Induction of apoptosis is an important component of anti-tumour therapies, including chemotherapeutic drugs and radiotherapy (reviewed by Kaufmann and Vaux, 2003; Mollinedo and Gajate, 2003). Cancer cells have acquired resistance to apoptosis but are constantly prone to initiate it by different factors (reviewed by Ashkenazi and Herbst, 2008). Therefore, it is important to elucidate the molecular events involved in the acquisition of resistance to apoptosis induced by anti-cancer therapies. Anti-cancer agents that target DNA are effective in clinical use especially when used in combination with drugs that have different mechanisms of action (reviewed by Hurley, 2002). For example, 5-Fluorouracil (5-FU) has been a key drug in the treatment of cancer for more than 40 years. The topoisomerase-1 inhibitor irinotecan (CPT-11) and oxaliplatin (OXA) are now being used in conjunction with 5-FU for the treatment of metastatic colorectal cancer, and have demonstrated promising results (Douillard, 2000; Giacchetti et al., 2000). Despite these improvements, tumour cell resistance still remains a significant limitation to the clinical use of 5-FU (reviewed by Longley et al., 2003).

Many factors could affect cellular responses to 5-FU. For example, it has been reported that depletion of DR5 can confer resistance to 5-FU, however there is still little known about the details (Wang and El-Deiry, 2004). Moreover, other death receptors like CD95 have also been found involved in cellular response to chemotherapeutic agents and ultraviolet light (Iijima et al., 1997; Micheau et al., 1999; Muller et al., 1998; Rehemtulla et al., 1997). Therefore, elucidate the molecular mechanism behind the 5-FU resistance caused by the depletion of DR5 may be helpful to understand the mechanism of resistance to chemotherapeutic agents and/or design better therapeutic approaches overcome chemo-resistance.

In addition, we generated soluble wild-type, DR4-selective and DR5-selective TRAIL expression cassettes. We also created mesenchymal stem cells (MSCs), termed MSC.sTRAIL which can express and secrete these soluble TRAILs, named MSC.sTRAIL-wt, MSC.sTRAIL-DR4 and MSC.sTRAIL-DR5 respectively.

MSC.sTRAILs showed apoptosis-inducing ability in different cells lines. To this end, MSC.sTRAIL-DR5 and MSC.sTRAIL-DR4 showed superior tumour cell-killing properties compared to MSC.sTRAIL-wt in certain cases. For example, MSC.sTRAIL-DR5 was more effective in Colo205 colorectal cancer cells and Hela cervical cancer cells responded better to MSC.sTRAIL-DR4.

Furthermore, in order to increase the apoptosis-inducing potency of MSC.sTRAIL, we developed two sensitization strategies. One is the combinational use of MSC.sTRAILs with silencing of XIAP. In this case, we found that silencing of XIAP in combination with MSC.sTRAIL-DR4 or MSC.sTRAIL-DR5 showed significantly increased apoptosis-inducing activity as compared to MSC.sTRAIL-wt in two different pancreatic carcinoma cell lines. The other sensitization approach is in combination with 5-FU. We found that pre-treatment with low 5-FU dose followed by MSC.sTRAIL-DR5 can lead to higher apoptosis levels in HCT116 human colon cancer cells. Therefore, MSC-mediated delivery of optimised sTRAIL variants in combination with inhibition of XIAP or 5-FU could become a novel treatment strategy for various cancers.

CHAPTER2 RESULTS

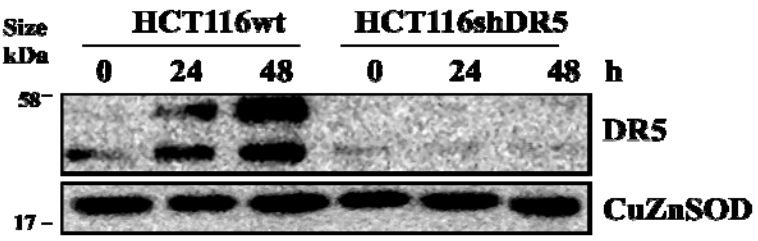
Part I

Cytotoxic drug induced JNK activation is DR5-dependent and regulates apoptosis via Bid phosphorylation

2.1.1 Diminished DR5 protected HCT116 cells against 5-FU induced Apoptosis

5-FU is one of the most effective chemotherapeutic agents and has been used for gastrointestinal tumours for more than 40 years. As for many other anti-tumour therapies, chemo-resistance is still a major clinical problem. DR5, also referred to as KILLER/TRICK, belongs to the TNFR super-family. The majority of studies on DR5 were focused on its role in TRAIL-induced apoptosis. In humans, TRAIL has two receptors: TRAIL-R1 and TRAIL-R2 also named DR4 and DR5, respectively. In mice there is only one death receptor homologous to DR5 called TRAIL-R (Schaefer et al., 2007). It has been reported that *TRAIL-R−/−* mice present impaired apoptotic response to irradiation (Finnberg et al., 2005). It also been found that knock-down of DR5 by RNAi can confer resistance to 5-FU in colorectal cells (Wang and El-Deiry, 2004). These studies provide some insights into the role of DR5 in chemotherapeutic agent-induced apoptosis. In order to investigate the mechanisms underlying the significance of DR5 in chemotherapeutic drug induced cell death, we established one clone of HCT116 cells that shows stable down-regulated expression of DR5, named HCT116shDR5. The DR5 protein level of HCT116shDR5 in comparison to HCT116wt cells (Ctrl) was shown in a Western Blot (Figure 2.1.1 A). HCT116wt and HCT116shDR5 cells were incubated with 5-FU (200 μM) for 24 h, 48 h at 37^{o} C. Cells were then collected and flow cytometric analysis was conducted according to the protocol of Nicoletti, the apoptotic fraction was recognized as sub-G1 population of cells (Nicoletti et al., 1991). As in Figure 2.1.1 B, HCT116wt cells showed around 40% apoptosis after 48 h, at the same time point the percentage of apoptosis in HCT116shDR5 was around 20% ($P < 0.01$). Our results show that the HCT116shDR5 cells were more resistant to 5-FU when compared to HCT116wt cells. Moreover, we measured the growth of HCT116wt and HCT116shDR5 cells over 5 days treated with 5-FU (200 μM). The HCT116wt and HCT116shDR5 cell numbers begin to decline after 48 h and 72 h respectively (Figure 2.1.1 C). We concluded that HCT116shDR5 cells were more resistant to 5-FU when compared with HCT116wt cells. In addition, DR5 can at least partially mediate 5-FU induced apoptosis.

A



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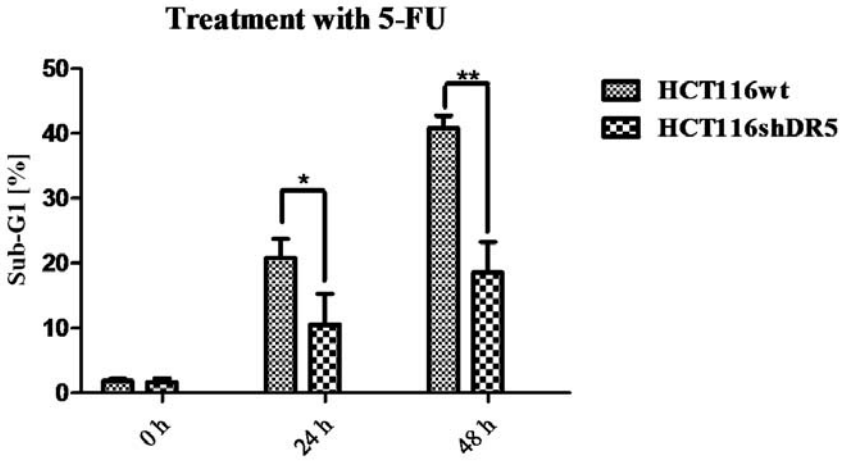

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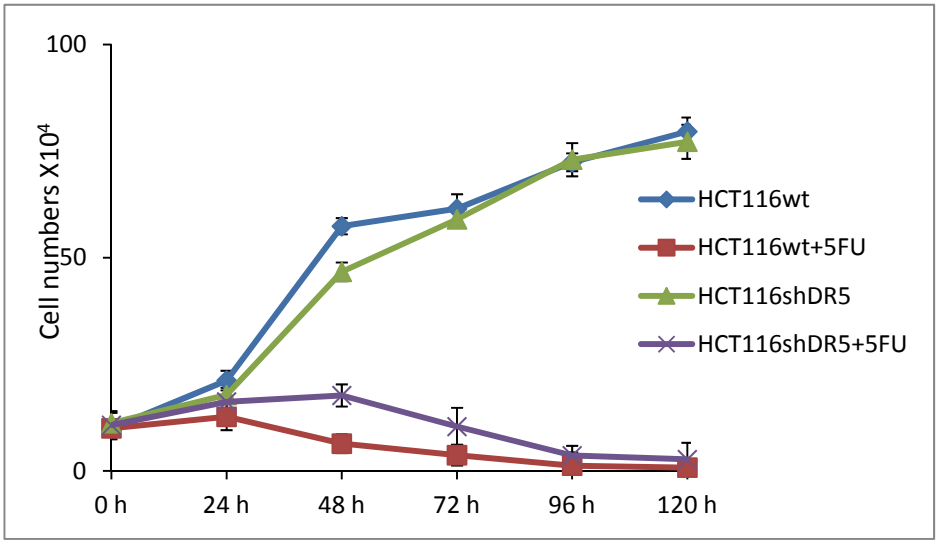

Figure 2.1.1 DR5 silencing can confer resistance to 5-FU in HCT116 cells. A. HCT116wt and HCT116shDR5 cells were treated with 5-FU (200 μM) for indicated time, then the cells were collected and the DR5 protein levels were measured by Western Blot. **B.** Cells were treated as in A, cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells measured by flow cytometry. **C.** The growth curve of HCT116wt and HCT116shDR5 cells over 5 days after 5-FU treatment. The mean of three experiments is shown in each column, bars correspond to S.E. * $P<0.05$, ** $P<0.01$.

2.1.2 The function of DR5 was lost in HCT116shDR5 cells

To analyze the functionality of DR5 in both cell lines, we treated HCT116wt and HCT116shDR5 cells with two different soluble TRAILs (5 ng/ml): wild-type TRAIL and DR5-specific TRAIL which specifically binds to DR5. Although the DR5-specific TRAIL activity was diminished in the HCT116shDR5 cells, the efficacy of the wild-type sTRAIL was similar in both HCT116wt and HCT116shDR5 cells (Figure 2.1.2 A). After treatment with wild-type sTRAIL for 24 h, the apoptosis rates were around 30% in both cells. This might be because DR4 can compensate for the function of DR5 by binding to TRAIL. However, in the case of DR5-specific sTRAIL, the apoptosis-inducing ability was greatly reduced in the HCT116shDR5 cells. After treatment with DR5-specific sTRAIL (5 ng/ml) for 24 h, the apoptosis rate of HCT116shDR5 cells was less than half of HCT116wt cells (Figure 2.1.2 B). These data demonstrate that the function of DR5 was indeed lost in HCT116shDR5 cells.

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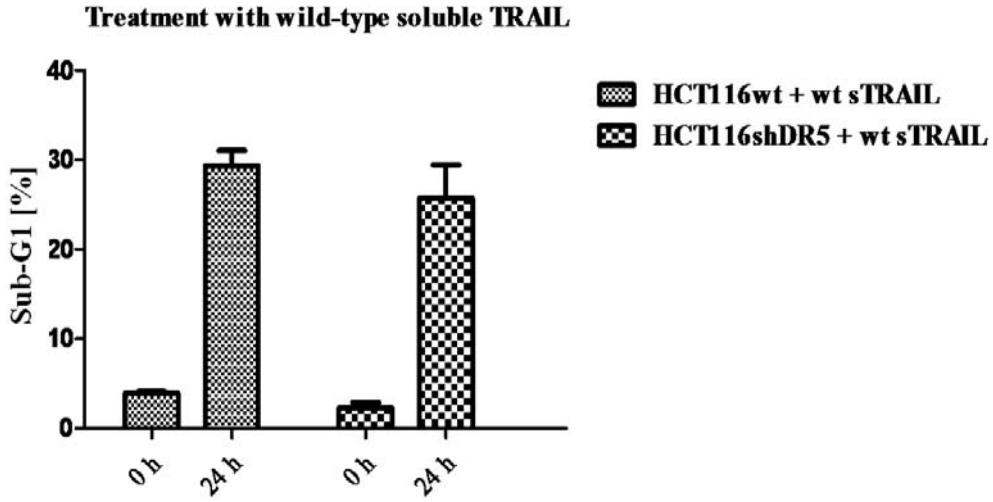

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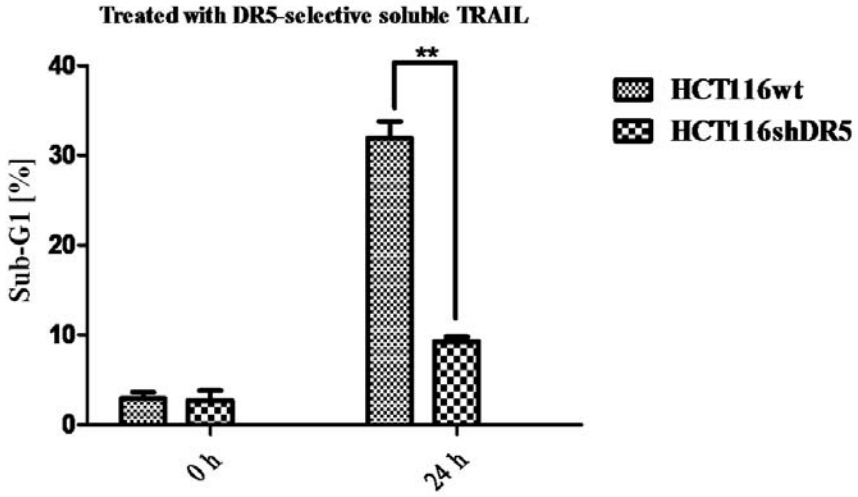

Figure 2.1.2 Sensitivity of HCT116wt and HCT116shDR5 cells to wild-type and DR5-selective sTRAILs. A and **B**. Cells were treated with wild-type sTRAIL or DR5-specific sTRAIL (5 ng/ml) for 24 h. Then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The mean of three experiments is shown in each column, and bars correspond with the S.E. * $P<0.05$, ** $P<0.01$.

2.1.3. Caspase-8 activation is needed for cell death induced by 5-FU

The activation of caspase is a key regulatory point in the commitment of the cell to apoptosis. Therefore, we checked caspase activation after 5-FU treatment in HCT116wt and HCT116shDR5 cells. Some reports indicated that cytotoxic drugs function mainly through the mitochondrial pathway to activate caspase-3 (reviewed by Fulda et al., 2001). In contrast, there are also some studies suggesting that the 5-FU-induces apoptosis possibly via the extrinsic pathway which is initiated by caspase-8 (Backus et al., 2001; Yamamoto et al., 2004). Activated caspase-8 then further activate the downstream caspase-3 to amplify the apoptotic signal. In the first model, caspase-8 might be dispensable for apoptosis. In order to test this, we created a stable HCT116 cell line which is deficient in caspase-8 named HCT116shcaspase-8 (Figure 2.1.3 A). HCT116shcaspase-8 cells were compared with HCT116wt cells for the induction of cell death following exposure to 5-FU. HCT116shcaspase-8 cells were completely resistant to 5-FU induced cell death (Figure 2.1.3 B). These results confirm that 5-FU induced cell death is a caspase-8-dependent process.

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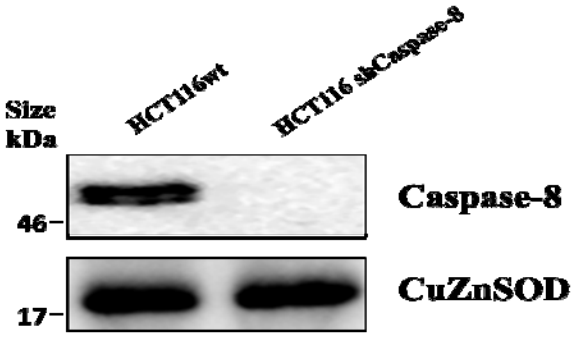

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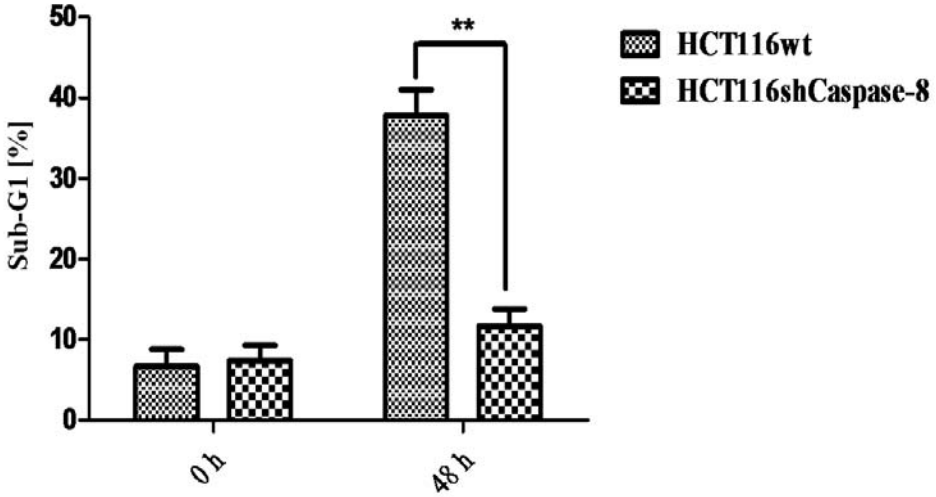
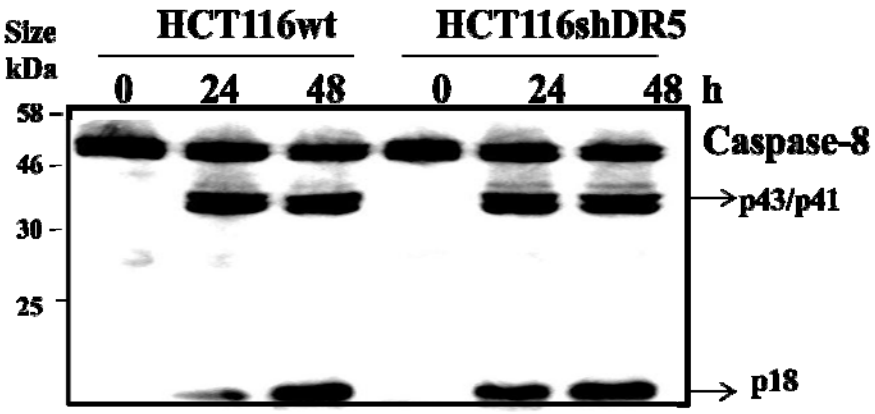


Figure 2.1.3 Caspase-8 is required for 5-FU induced apoptosis in HCT116 cells. **A.** Total cell extracts of HCT116wt and HCT116shCaspase-8 cells were subjected to Western Blot analysis with anti-caspase-8 antibodies, CuZnSOD was used as a loading control. **B.** HCT116wt and HCT116shCaspase-8 cells were treated with 5-FU for the indicated time, then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The mean of three experiments is shown in each column, and bars correspond to S.E. * $P<0.05$, ** $P<0.01$.

2.1.4 Caspase-8 activation was not affected but caspase-3 activation was blocked in HCT116shDR5 cells in response to 5-FU

Since we found that caspase-8 activation is required for apoptosis in response to 5-FU, we checked the caspase-8 activation in HCT116shDR5 cells after 5-FU treatment. To our surprise, the active (cleaved) caspase-8 levels (p43/41 and p18) of HCT116shDR5 cells were similar to HCT116wt cells (Figure 2.1.4 A). In contrast, we found that caspase-3 was activated in HCT116wt cells but its activation was attenuated in HCT116shDR5 cells (Figure 2.1.4 B).

A



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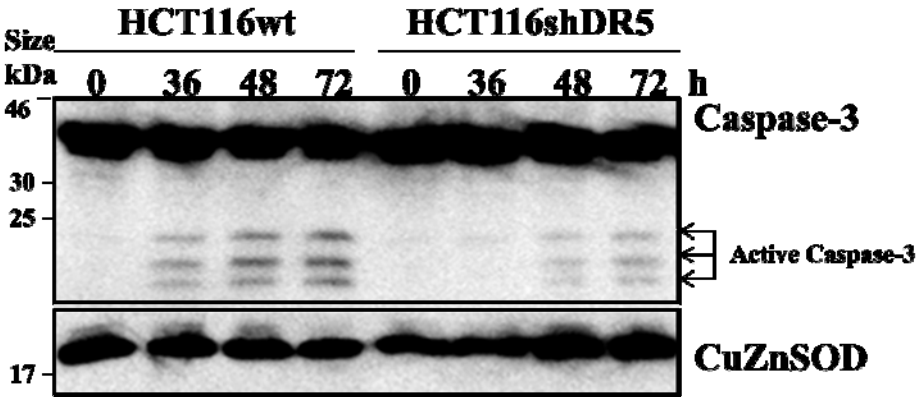


Figure 2.1.4 Caspase-3 but not caspase-8 activation was diminished in HCT116shDR5 cells after 5-FU treatment. A and **B**. HCT116wt and HCT116shDR5 cells were treated with 5-FU for indicated time, then the whole cells were lysed and subjected to Western Blot analysis with indicated antibodies. CuZnSOD was used as a loading control.

2.1.5 Mitochondrial proteins release is inhibited in HCT116shDR5 cells

After found that caspase-3 activation was blocked in HCT116shDR5 cells while the caspase-8 activation was not affected, we examined the steps between the caspase-8 and caspase-3 activation. There are two types of cell based on the requirements for apoptosis and caspase-3 activation. In type I cells, caspase-8 activation is enough to directly activate downstream caspase-3. In type II cells, caspase-8 activation is not enough for the activation of caspase-3, and the mitochondrial/intrinsic pathway is required to amplify the signal. Therefore, we studied the mitochondrial pathway activation in response to 5-FU.

One hallmark of the mitochondrial/intrinsic apoptosis pathway is the release of proteins such as cytochrome-c (cyto-c) and Smac/DIABLO (second mitochondrial-derived activator of caspase) from the intermembrane space into the cytosol (Arnoult et al., 2002; Susin et al., 2000a). We fractionated 5-FU-treated cells using CLAMI (Cellular Lysis And Mitochondrial Intact) buffer. After treatment with 5-FU for 12 h, the cytoplasmic relocation of cytochrome-c and Smac/DIABLO can be seen in HCT116wt cells. In contrast, in HCT116shDR5 cells, the release of cytochrome-c and Smac/ DIABLO was blocked. The results of this analysis are shown in Figure 2.1.5. These results mean that inhibition of cytochrome-c and/or lack of Smac/DIABLO release is one of the molecular causes for HCT116shDR5 cells' resistance to 5-FU.

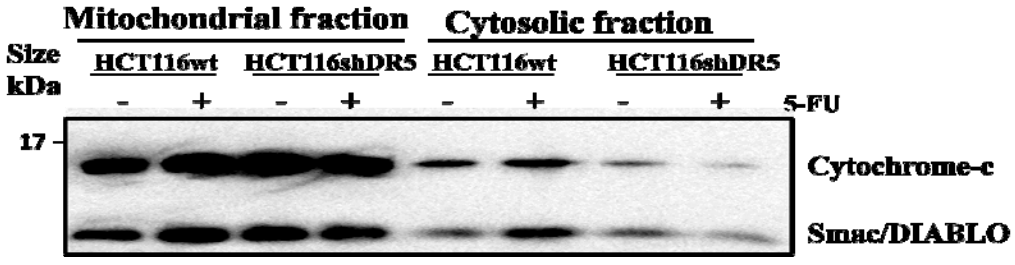


Figure 2.1.5 Release of mitochondrial proteins was blocked in HCT116shDR5 cells in response to 5-FU. HCT116wt and HCT116shDR5 cells were treated with or without 5-FU for 12 h, then cells were fractionated and subjected to Western Blot analysis using indicated antibodies.

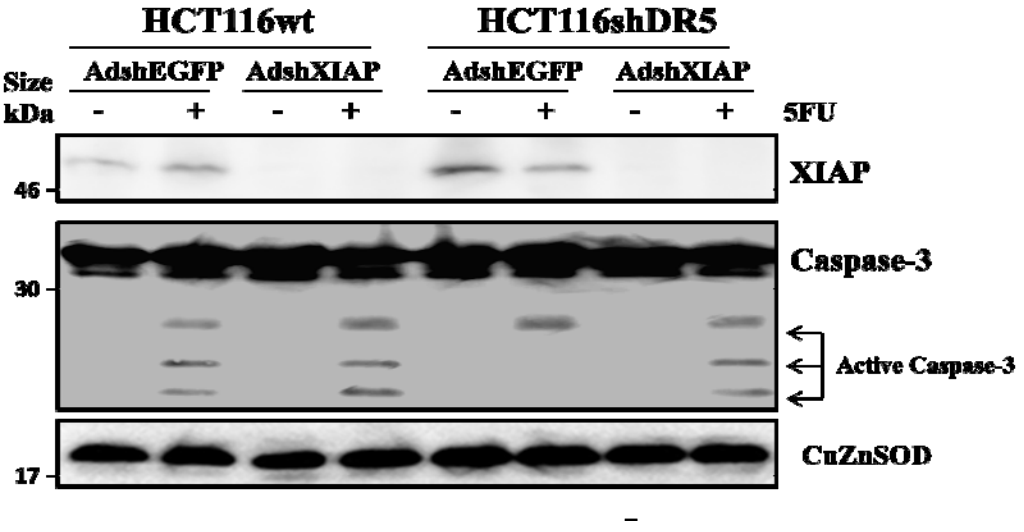
2.1.6 Down-regulation of XIAP can sensitize HCT116shDR5 cells to 5-FU

The mammalian inhibitor of apoptosis protein (IAP) family of proteins has 8 members, including XIAP, c-IAP1, c-IAP2, survivin, NIAP etc (Ashhab et al., 2001; Deveraux and Reed, 1999; Vucic et al., 2000). XIAP can negatively regulate apoptosis by directly inhibiting caspases activity. It can bind to caspase-9, caspase-7, caspase-3 and prevent the activation of the caspase cascade (Roy et al., 1997; Tamm et al., 1998). The activation of caspase-3 by caspase-8 can be blocked by XIAP through interaction between XIAP and partially processed caspase-3 (reviewed by Goyal, 2001). We compared the protein levels of XIAP in HCT116wt and HCT116shDR5 cells. As shown in Figure 2.1.6 A, XIAP was expressed similarly in both cell types. Therefore, the basal level of XIAP is not the reason for caspase-3 inhibition. One of the proteins released from mitochondria during apoptosis is Smac/DIABLO which can bind and neutralize the inhibitory activity of XIAP (Chai et al., 2001).

In our study, the release of cytochrome-c and Smac/DIABLO proteins can only be detected in HCT116wt cells, but not in HCT116shDR5 cells. To mimic the function of Smac/DIABLO, we knocked down XIAP by using DNA-directed RNA interference (RNAi) via adenoviral (AdshXIAP)-mediated expression of small hairpin RNAs (shRNA) directed at XIAP. 48 h after viral transduction, XIAP expression was assessed by Western Blot and found to be down-regulated to almost non-detectable levels (Figure 2.1.6 A). To account for any side effects of adenoviral infection and/or shRNA expression, an adenoviral vector (AdshEGFP) containing a shRNA construct against EGFP was used as a control. Cells transduced with AdshXIAP or AdshEGFP, and were treated with 5-FU 48 h after transduction for 48 h, and apoptosis was measured (Figure 2.1.6 B). The sensitivity of HCT116shDR5 cells to 5-FU was considerably increased when XIAP protein levels were decreased. Furthermore, we tested caspase-3 activation in HCT116wt and HCT116shDR5 cells that were transduced with AdshXIAP and subsequently treated with 5-FU. The Western Blot results show that caspase-3 activity was increased in HCT116shDR5 cells transduced with AdshXIAP as compared to cells transduced with the AdshEGFP control vector

after treated with 5-FU. These results point to a lack of mitochondrial activation and consequent a lack of pro-apoptotic protein Smac/DIABLO in the cytosol as the molecular cause for the 5-FU resistance in HCT116shDR5 cells.

A



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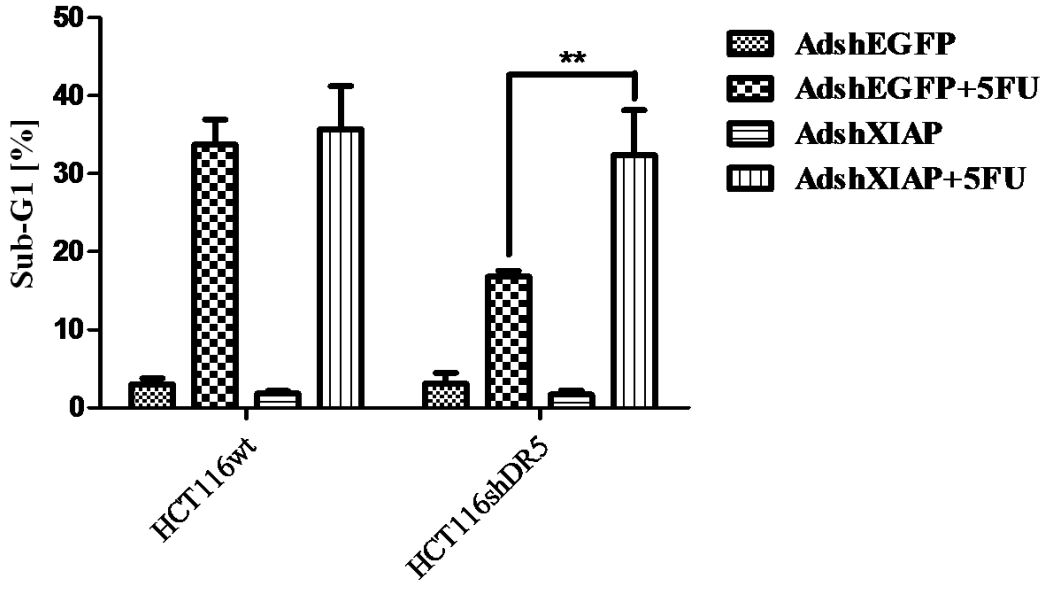


Figure 2.1.6 Knockdown of XIAP can sensitize HCT116shDR5 cells to 5-FU. HCT116wt and HCT116shDR5 cells were transfected with AdshEGFP or AdshXIAP. 48 h after transfection, cells were treated with or without 5-FU for another 48 h. **A.** Then whole-cell extracts of cells were analyzed by Western Blot using indicated antibodies. CuZnSOD was used as a loading control. **B.** Whole cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The mean of three experiments is shown in each column, and bars correspond to S.E. * $P<0.05$, ** $P<0.01$.

2.1.7 Bid cleavage and Bax activation is inhibited in the HCT116shDR5 cells

As 5-FU resistance is localized around the mitochondrial pathway and factors, we analyzed members of the Bcl-2 family. The Bcl-2 family consists of proteins with pro- and anti-apoptotic activity. Bax is a pro-apoptotic member of the Bc1-2 protein family which can accelerate apoptosis induced by a variety of stimuli (Oltvai et al., 1993). During apoptosis, the cytosolic and monomeric Bax translocates to the mitochondrial membranes and causes the loss of mitochondrial membrane potential and subsequent cytochrome-c and Smac/DIABLO release and further caspase activation (Goping et al., 1998; Hsu et al., 1997). Moreover, it has also been shown that apoptotic stimuli induce a conformational change of Bax prior to its translocation to mitochondria where it inserts as a homodimer (Hsu and Youle, 1998; Murphy et al., 2000; Nechushtan et al., 1999). Several studies have implied that Bax protein level and its status appeared to be a determinant marker for the response to 5-FU therapy (Nita et al., 1998; Ogura et al., 1999; Sturm et al., 1999). We therefore asked whether this Bax conformational change was also inhibited in the HCT116shDR5 cells after 5-FU treatment. In order to investigate the Bax conformational change, immunoprecipitation (IP) analysis was carried out with the anti-Bax monoclonal antibody 6A7 that specially recognizes the conformationally changed Bax protein (Hsu et al., 1997; Hsu and Youle, 1998; Murphy et al., 2000). It has been reported that non-ionic detergents can cause Bax conformation change, so we used two previously reported detergents containing lysis buffers, which are sufficient to cause Bax conformational change, as positive controls (Cheng et al., 2001; Hsu and Youle, 1998). To this end, we found that the Bax undergoes conformation change after 5-FU treatment only in HCT116wt but not in HCT116shDR5 cells (Figure 2.1.7 A).

Bid, a “BH3-only” pro-apoptotic member of the Bcl-2 family, plays an important role in connecting death signals initiated from death receptors to the intrinsic pathway (reviewed by Esposti, 2002). In a normal cell, Bid is predominantly located in the cytosol. After receiving the stimuli, Bid can be proteolytically cleaved by caspase-8 in an unstructured loop, exposing a new amino terminal glycine residue, which allows

posttranslational N-myristoylation of Bid, thereby facilitating its translocation to the mitochondrial outer membrane (Zha et al., 2000). After being cleaved, Bid can yield a p15 C-terminal truncated fragment (tBid) that can translocate to the mitochondria, where it induces the conformational change of Bax leading to the release of mitochondrial proteins (Korsmeyer et al., 2000; Martinou and Green, 2001). As we found that the Bax conformational change and the release of cytochrome-c, Smac/DIABLO activity were all inhibited in HCT116shDR5 cells, we investigated Bid cleavage in both cell types. By Western Blot analysis, we found that in HCT116wt cells, 5-FU can induce Bid cleavage after 48 h treatment, while in HCT116shDR5 cells the Bid cleavage was blocked (Figure 2.1.7 B).

A

B

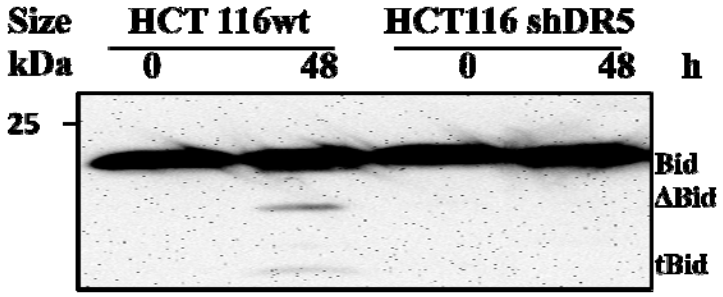


Figure 2.1.7 Bax was not activated due to the inhibition of Bid cleavage A. Cells were treated with 5-FU for the indicated time, conformationally active Bax protein was immunoprecipitated (IP) with anti-Bax 6A7 antibody and the precipitates were immunoblotted with anti-Bax antibody. Cell lysates by two detergent lysis buffers were used as positive controls. **B.** HCT116wt and HCT116shDR5 cells were treated with 5-FU for indicated time. Then whole-cell extracts of cells were analyzed by Western Blot.

2.1.8 Bid is constitutively phosphorylated in HCT116shDR5 cells

The results described above showed that the apoptosis signaling pathway was blocked above and/or at the mitochondrial level in the HCT116shDR5 cell after 5-FU treatment. In addition, due to failure of Bid cleavage despite caspase-8 activation was nearly not affected, so we sought to understand the mechanisms behind the reduced Bid activation.

The activity of BH3-only proteins can be regulated by posttranslational modifications. For example, Bad can be phosphorylated by protein kinase A and Akt. Activation of those protein kinases can promote phosphorylation and inactivation of Bad through allowing its sequestration by the phosphoserine-binding 14-3-3 protein (Zha et al., 1996). Phosphorylation of Bid has been shown to prevent its cleavage by caspase-8 (Degli Esposti et al., 2003; Desagher et al., 2001). During Fas-induced apoptosis, there is a dephosphorylation process of Bid and its subsequent cleavage, while Bid was constitutively phosphorylated and uncleaved in chronic cholestasis and other models of hepatic apoptosis resistance (Vogel et al., 2006). So we tested the Bid phosphorylation status in the HCT116wt and HCT116shDR5 cells after 5-FU treatment. We used a specific phospho-Bid antibody that is able to recognize the phosphorylated Serine 78 site (S78) of Bid. We found that in HCT116wt cells, Bid was either not phosphorylated at this site (S78) or constantly and rapidly dephosphorylated allowing subsequent cleavage after 5-FU treatment, whereas Bid was phosphorylated in HCT116shDR5 cells (Figure 2.1.8).

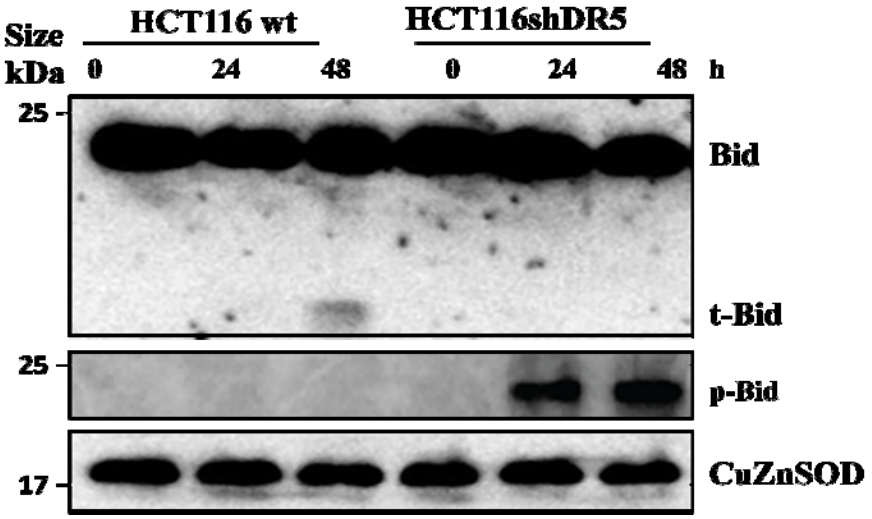
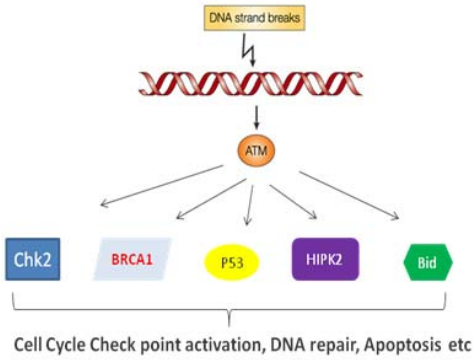


Figure 2.1.8 Bid is not dephosphorylated in HCT116shDR5 cells after 5-FU treatment. HCT116wt and HCT116shDR5 cells were treated with 5-FU for the indicated time. Then whole-cell extracts of cells were analyzed by Western Blot using indicated antibodies. CuZnSOD was used as a loading control

2.1.9 Inhibition of ATM kinase could abrogate Bid phosphorylation and rescue the apoptosis in HCT116shDR5 cells in response to 5-FU

ATM kinase can be activated following DNA damage (reviewed by Abraham, 2001; Molinari, 2000). Activated ATM kinase can phosphorylate a wide spectrum of substrates (Figure 2.1.9 A). The functional consequences of ATM activation have been associated with many cellular events like cell cycle check point activation, DNA repair, apoptosis etc. Bid has also been found as a new substrate of ATM (Kamer et al., 2005). To test if ATM also phosphorylates Bid in our system, we treated HCT116wt and HCT116shDR5 cells with ATM kinase inhibitor KU-55933 prior to the addition of 5-FU. Figure 2.1.9 B shows that ATM inhibitor KU-55933 could antagonize Bid phosphorylation in HCT116shDR5 cells after 5-FU treatment (Figure 2.1.9 B). At the same time, the Bid cleavage was rescued in HCT116shDR5 cells (Figure 2.1.9 C). The apoptosis rate was increased as well (Figure 2.1.9 D). These data suggest that ATM is the major kinase on mediating the phosphorylation of Bid in response to 5-FU and Bid phosphorylation can confer resistance to cleavage and apoptosis.

A



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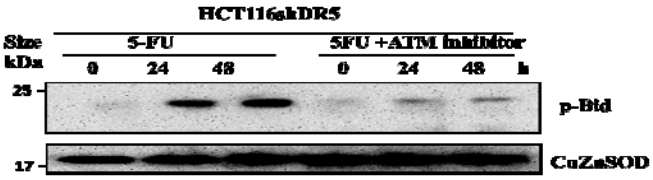

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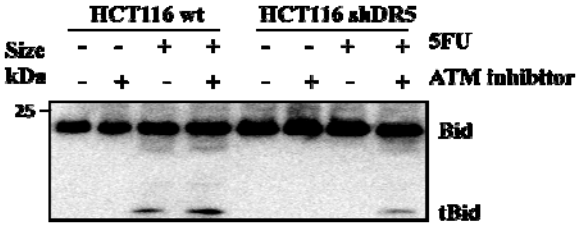

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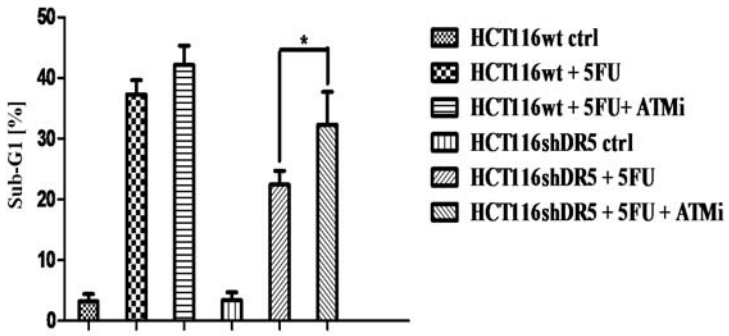

Figure 2.1.9 ATM kinase phosphorylate Bid in response to 5-FU. A. Schematic illustration of ATM kinase and its substrates and the function of ATM. **B** and **C.** HCT116wt and HCT116shDR5 cells were treated with 5-FU alone or 5-FU plus ATM inhibitor KU-55933. Then whole-cell extracts of cells were analyzed by Western Blot. **D.** Cells were prepared as described in B, then the whole cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The mean of three experiments is shown in each column, and bars correspond to S.E. * $P<0.05$, ** $P<0.01$.

2.1.10 NF-κB pathway is not activated in either HCT116wt or HCT116shDR5 cells after 5-FU treatment

NF-κB has been studied intensively for its role in immune and inflammatory process (reviewed by Hayden and Ghosh, 2008). The role of NF-κB in the regulation of apoptosis has also received a great deal of attention (reviewed by Hinz et al., 2001). NF-κB is a homo- or hetero-dimeric transcription factor that controls the expression of various genes involved in inflammatory, anti-apoptotic and immune responses. Numerous data showed that NF-κB controls expression of several inhibitors of apoptosis (IAPs), including c-IAP1, c-IAP2, and X chromosome-linked IAP (XIAP) (Irmler et al., 1997; Liston et al., 1996). Overexpression of NF-κB could suppress apoptosis (Beg and Baltimore, 1996; Liu et al., 1996a). Genetic studies also showed that mice deficient in RelA, a major activating subunit of NF-κB, die from massive apoptosis of hepatocytes in the liver (Beg and Baltimore, 1996). NF-κB is activated in several types of cancer cells and constitutive activation of NF-κB could confer resistance to apoptosis in these cells (Hinz et al., 2001). It has also been reported that NF-κB could be activated by death receptor ligands, chemotherapeutic drugs, or ionizing radiations (Das and White, 1997; Wang et al., 1996a). In the quiescent state, NF-κB remains inactive in the cytoplasm through binding to the inhibitory protein IκB (reviewed by Hayden and Ghosh, 2008). Activation of IKKß can induce phosphorylation of IκB, and subsequent proteosomal degradation. The degradation of IκB releases NF-κB to translocate into the nucleus where it exerts transcriptional functions (Zhang et al., 2009).

Since we found that apoptosis was attenuated in HCT116shDR5 cells, we wonder if NF-κB might play a role in the response of HCT116 cells to 5-FU. In contrast to treatment with TNF-α, we could not detect any phosphorylated IκB after 5-FU treatment (Figure 2.1.10 A). To further confirm this, a luciferase assay was performed with a plasmid containing NF-κB binding sites that control a reporter luciferase gene. As shown in Figure 2.1.10 B, we were unable to find any signs of NF-κB activation. All these data indicate that NF-κB was not activated by 5-FU in both HCT116wt and HCT116shDR5 cells.

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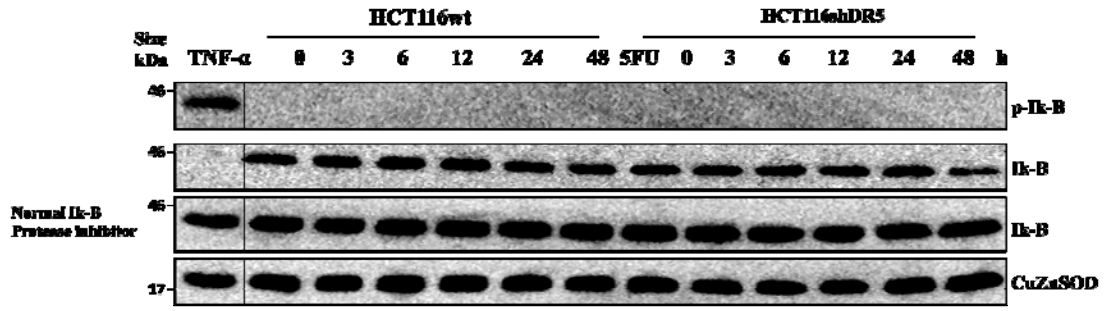

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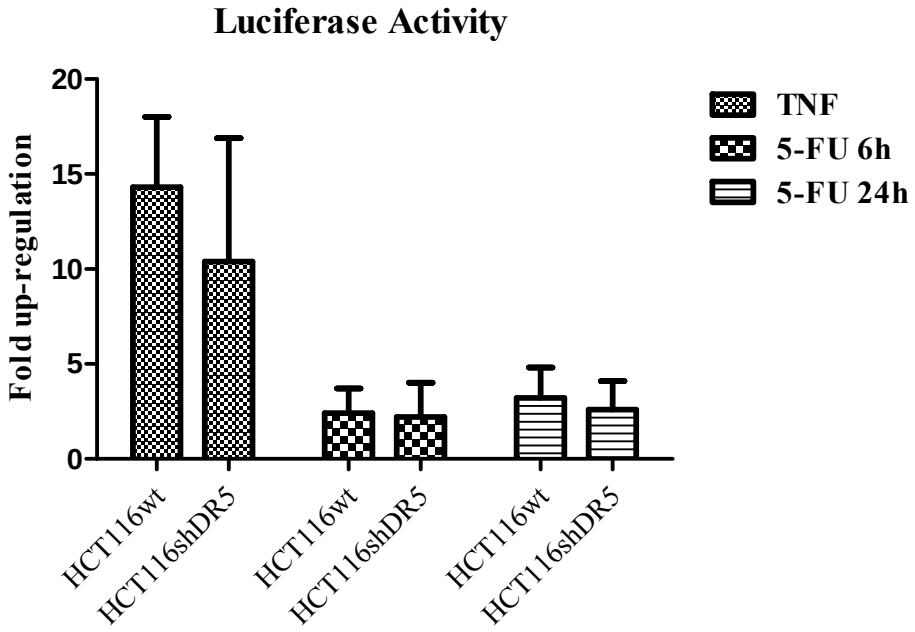

Figure 2.1.10 NF-κB pathway is not activated in HCT116 cells in response to 5-FU A. HCT116wt and HCT116shDR5 cells were treated with 5-FU for indicated time. Then whole cell extracts were analyzed by Western Blot with indicated antibodies. CuZnSOD was used as a loading control. Cells treated with TNF-α for 0.5 h were used as a positive control. **B.** Luciferase activity of an NF-κB–luciferase reporter plasmid after HCT116wt and HCT116shDR5 cells treated with 5-FU for the indicated times, Cells treated with TNF-α for 0.5 h were used as positive controls. The mean of three experiments is shown in each column, and bars correspond to the S.E.

2.1.11 JNK pathway is activated in HCT116wt but not HCT116shDR5 cells after 5-FU treatment

We ruled out the possibility that NF-κB activation caused the block of apoptosis after 5-FU treatment in HCT116shDR5 cells. In addition, the caspase-8 activation was not affected either. Therefore, we try to find out other cellular signaling pathways that might be involved in. Many studies suggested that JNK pathway is an important intracellular signaling that modulates the apoptosis machinery (reviewed by Chang et al., 2009; Davis, 2000). It has also been reported that DNA damage can induce the activation of the MAP kinase signal pathways, like JNK (Hamdi et al., 2005). We sought to determine whether JNK pathway was activated after 5-FU treatment. We assessed whole cell lysates from 5-FU treated HCT116wt and HCT116shDR5 cells by Western Blot using the antibody against the phosphorylated form of JNK. We found that 5-FU treatment increased the expression levels of phosphorylated JNK in the HCT116wt cells after 24 h. In contrast, in the HCT116shDR5 cells there was no up-regulation of phosphorylated JNK (Figure 2.1.11 A). We also measured the phosphorylation status of the JNK substrates, c-Jun and ATF2 (apoptosis transcription factor 2). In line with the JNK activation, phosphorylation of c-Jun and ATF2 can only be seen in the HCT116wt cells 24 h after 5-FU treatment (Figure 2.1.11 A). Two different mitogen-activated protein kinases (SEK-1/MKK4 and MKK7) have been reported as direct, upstream activators of JNK (Derijard et al., 1995; Van Antwerp et al., 1996). Therefore, we tested whether SEK-1/MKK4 was activated in HCT116wt but not HCT116shDR5 cells. As expected, 5-FU treatment led to the phosphorylation of SEK-1/MKK4 in the HCT116wt cells only. Interestingly, another stress-activated kinase p38, exhibited activity in both cell lines 24 h after the 5-FU treatment (Figure 2.1.11 B). These results indicated that activation of the JNK pathway might play an important role in response to 5-FU treatment.

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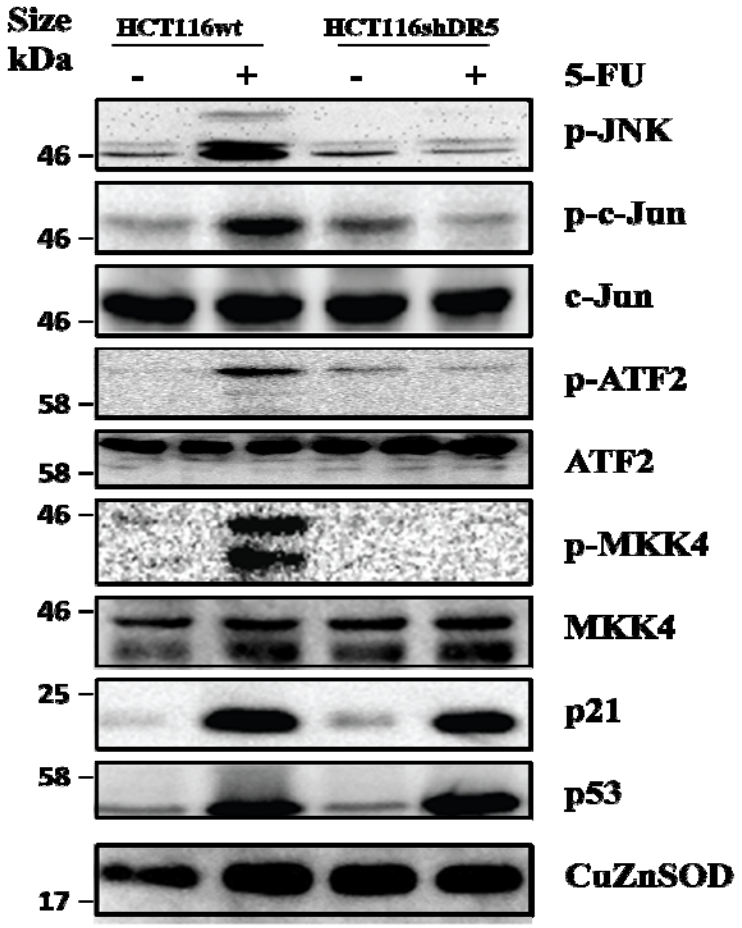

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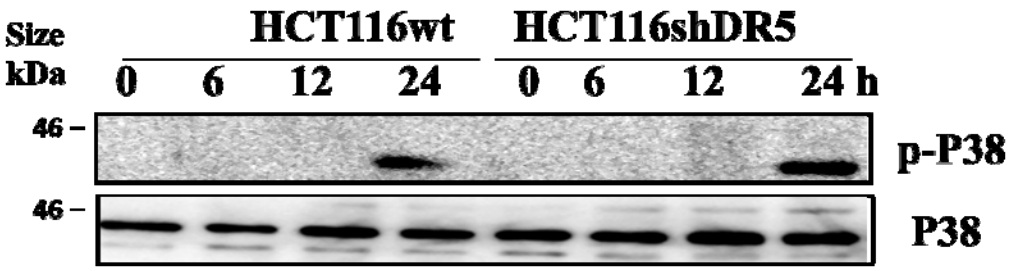
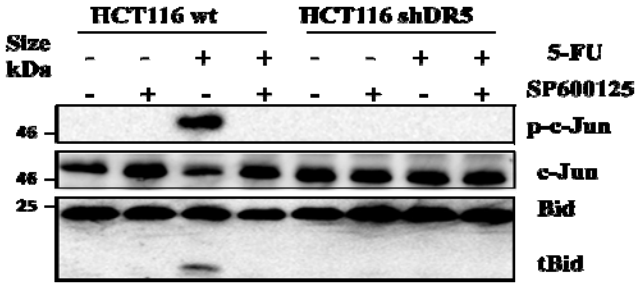


Figure 2.1.11 5-FU activates JNK kinase in HCT116wt but not HCT116shDR5 cells. A and **B**. HCT116wt and HCT116shDR5 cells were incubated with 5-FU for the indicated time, Then whole cell lysates were subjected to Western Blot analysis using the respective antibodies as indicated.

2.1.12 JNK pathway activation is related to the 5-FU-induced apoptosis and Bid cleavage in HCT116wt cells

To determine whether JNK activation is required for 5-FU induced apoptosis in HCT116wt cells, we examined the effect of SP600125 which is an inhibitor of JNK. Treatment of HCT116wt cells with 5-FU resulted in phosphorylation of the JNK substrate c-Jun, and pretreatment with SP600125 can block this effect of 5-FU (Figure 2.1.12 A). Interestingly, the Bid cleavage was also inhibited by SP600125 (Figure 2.1.12 A) and the apoptosis rate dropped from around 40% to 20% after 5-FU treatment for 48 h (Figure 2.1.12 B). All those results indicated that JNK activation is not only required for 5-FU induced apoptosis but might also affect the phosphorylation status of Bid.

A



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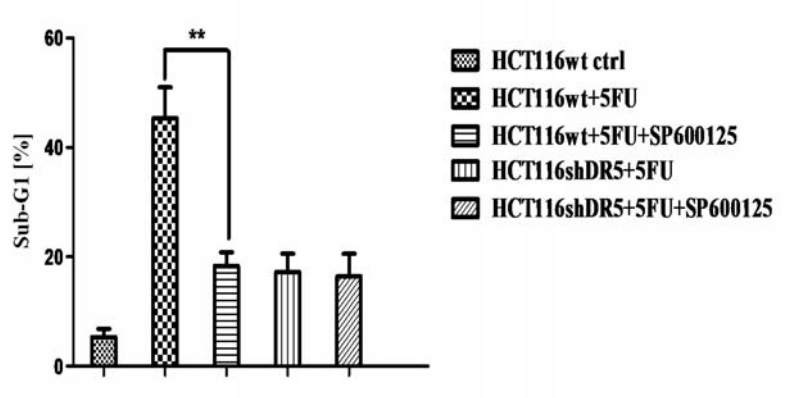


Figure 2.1.12 JNK activation is required for 5-FU induced apoptosis and Bid cleavage. HCT116wt and HCT116shDR5 cells were incubated first for 30 min with or without 20 μM SP600125 and then for 48 h in the presence or absence of 5-FU. **A.** The whole cell lysates were then subjected to Western Blot analysis with indicated antibodies **B.** Whole cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The mean of three experiments is shown in each column, and bars correspond to S.E. * $P<0.05$, ** $P<0.01$.

2.1.13 DR5 is also needed for JNK activation in U2OS cells

In order to test whether DR5 was generally involved in JNK activation induced by 5-FU, we examined JNK activation in two U2OS cell lines (osteosarcoma cells) in which DR4 and DR5 were over-expressed, respectively. Western blot results showed that wild-type U2OS cells express relatively low levels of DR5 when compared to the U2OS DR5-expressing cells. Interestingly, after 5-FU treatment JNK was only activated in the DR5-expressing U2OS cells but not in wild-type and DR4-expressing cells. This result indicates that the requirement of DR5 in 5FU-induced JNK activation may be general and not cell-type specific

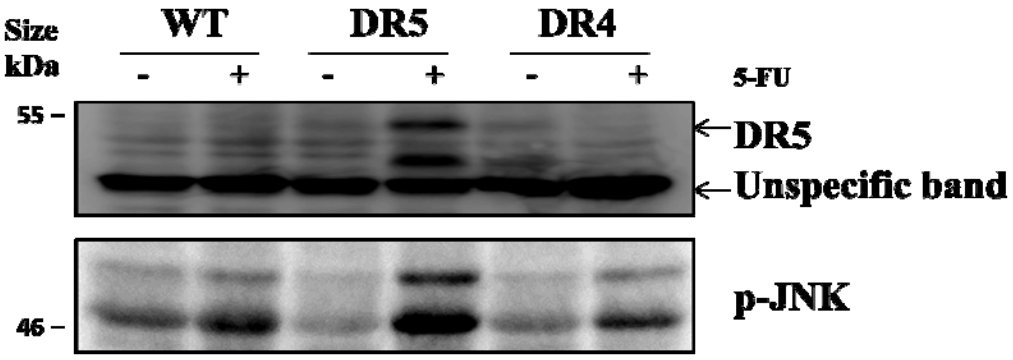


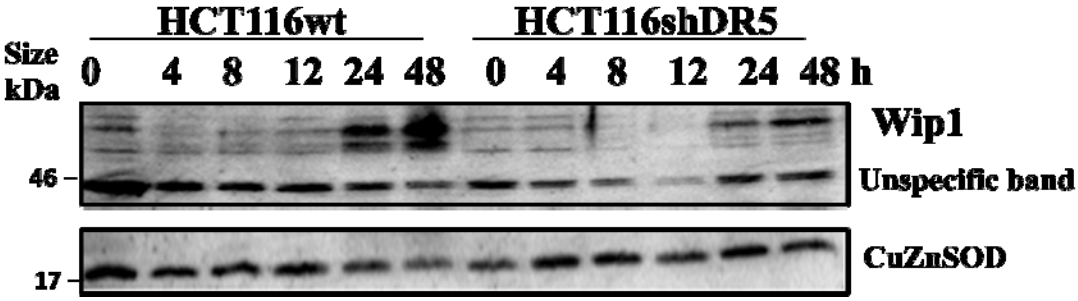
Figure 2.1.13 DR5 is also required for JNK activation induced by 5-FU in U2OS cells. U2OS cells with stable expression of DR4 and DR5 were incubated with 5-FU for 24 h. The whole cell extracts were analyzed by Western Blot with indicated antibodies.

2.1.14 Wip1 was induced in the HCT116 wt cells after 5-FU treatment but delayed in HCT116shDR5 cells

Protein phosphorylation levels are controlled by the opposing actions of kinases and phosphatases. Since we were unable to detect the phosphorylation of Bid in HCT116wt cells after 5-FU treatment, we hypothesised that Bid could be dephosphorylated in the HCT116wt cells after 5-FU treatment. The required dephosphorylation of Bid could be mediated by a putative Bid phosphatase.

To date, no phosphatase that can dephosphorylate Bid has been identified so far. We hypothesised that Wip1 might be the phosphatase that is able to act on Bid based on several reasons. Firstly, Bid phosphorylation site (Ser78) is a SpQ motif which is a preferred target of Wip1 (reviewed by Chew et al., 2009; Lu et al., 2008a). Secondly, since we found that JNK is activated after 5-FU treatment in HCT116wt cells, and recently it has been reported that Wip1 can be up-regulated by JNK activation (Song et al., 2010). Thirdly, Wip1 has been shown to inhibit the kinase activity of ATM which is responsible for the phosphorylation of Bid (Lu et al., 2008b). In order to test our hypothesis, the levels of Wip1 in 5-FU treated HCT116wt and HCT116shDR5 cells were examined. As shown in Figure 2.1.14 A, in HCT116wt cells, the Wip1 induction at protein level was observed as early as 12 h after 5-FU treatment. However, the Wip1 induction was delayed in HCT116shDR5 cells, which could only be detected around 48 h after the treatment. We also used qRT-PCR to examine Wip1 induction in response to 5-FU. Wip1 was initially identified as a member of p53-target genes family (Choi et al., 2000). Recently Wip1 has been found to be up-regulated independent of p53 although in a delayed manner (Song et al., 2010). To test this, we used one line named HCT116p53-/- in which p53 was knocked out, as a control. In line with the Western Blot results, Wip1 transcripts increased in HCT116wt cells 6 h after 5-FU treatment, which were not observed in HCT116shDR5 (Figure 2.1.14 B). We could not see the up-regulation of Wip1 transcripts in HCT116p53-/- either, it might because of the time point is too early. In a previous study, it was found that Wip1 up-regulation can only be detected 24 h later in p53 knock-out cells (Song et al., 2010).

A



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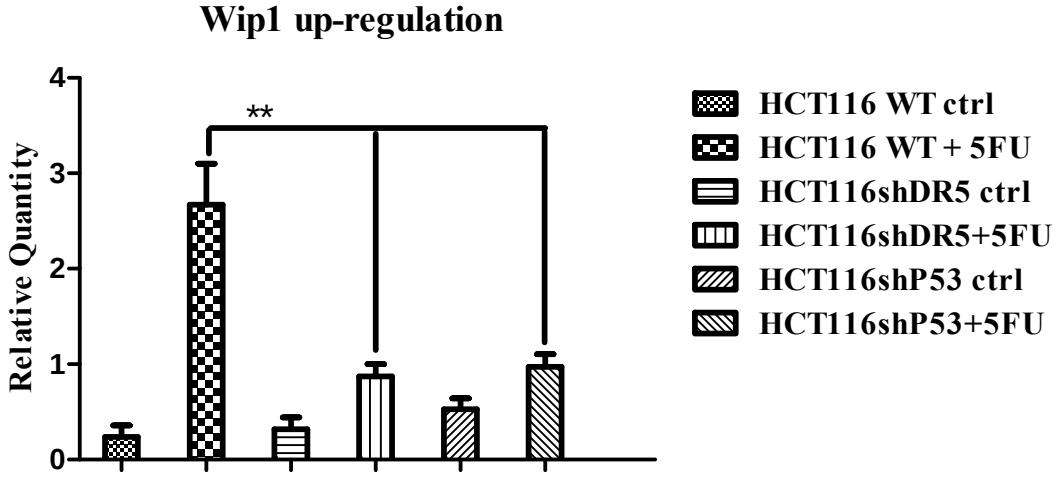

Figure 2.1.14 Induction of Wip1 was delayed in response to 5-FU in HCT116shDR5 cells. A. HCT116wt and HCT116shDR5 cells were treated with 5-FU for the indicated time. Then whole cell extracts were analyzed by Western Blot with indicated antibodies. CuZnSOD was used as a loading control. **B.** Wip1 mRNA transcripts were analyzed via qRT-PCR after 5-FU induction for 6 h. Data indicate the level of Wip1 transcripts relative to a control gene, GAPDH. The mean of three experiments is shown in each column and bars correspond to S.E. ** $P<0.01$.

2.1.15 Over-expression of Wip1 impairs Bid phosphorylation in HCT116shDR5 cells

To examine whether Wip1 can affect Bid phosphorylation, we over-expressed Wip1 in HCT116shDR5 cells. At the same time, cells were incubated with or without 5-FU for 48 h. As shown in Figure 2.1.15 A and B, when Wip1 was over-expressed, the Bid cleavage was rescued and the apoptosis rate was also increased around 10% ($P<0.05$).

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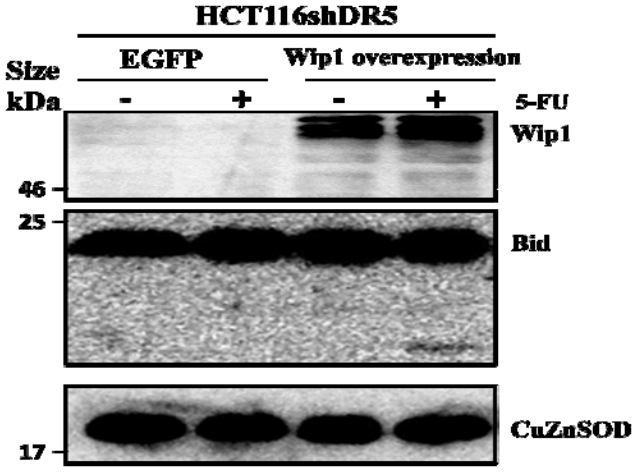

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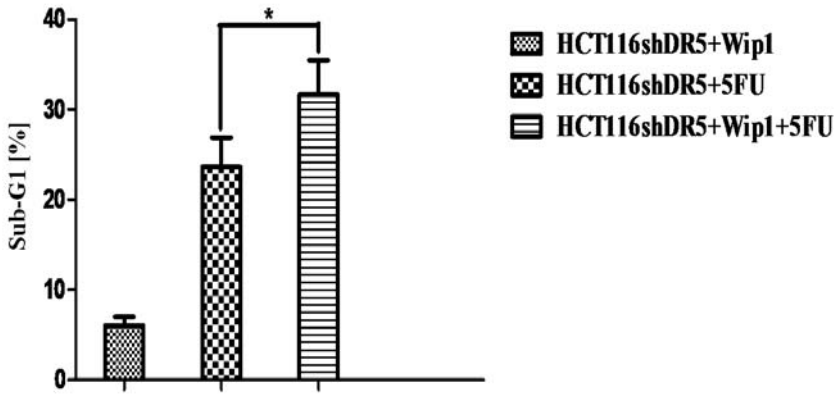

Figure 2.1.15 Overexpression of Wip1 can rescue Bid cleavage in HCT116 shDR5 cells after 5-FU treatment. A. HCT116shDR5 cells were transfected with Wip1 construct. Treated with or without 5-FU for 48 h, then the whole cells extracts were analyzed by Western Blot using indicated antibodies. CuZnSOD was used as a loading control. **B.** Cells were treated as described in A, then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The mean of three experiments is shown in each column, and bars correspond to S.E. * $P<0.05$, ** $P<0.01$.

2.1.16 Depletion of Wip1 leads Bid phosphorylation and confers resistance to 5-FU

To complement the Wip1 over-expression experiments, we investigated the Bid phosphorylation status in Wip1-depleted cells. Using two distinct Wip1 shRNA vectors, we created two stable Wip1 knockdown HCT116 cell lines named HCT116shWip1-1 and HCT116shWip1-2 respectively. Both Wip1 shRNAs were tested and showed greater than 90% knockdown efficiency (Figure 2.1.16 A). In both HCT116shWip1 cells, Bid keeps being phosphorylated after 5-FU treatment. The total levels of Bid protein remained constant, and the cleavage of Bid was inhibited. We also checked the caspase activation pathway. Similar to the HCT116shDR5 cells, caspase-3 activation was also inhibited in HCT116shWip1. It means that the mitochondrial pathway was also inhibited in the HCT116shWip1 cells after 5-FU treatment, and the Bid phosphorylation was the likely cause for this block. Moreover, the apoptosis rate of HCT116shWip1 after 5-FU treatment was only around 12%, which was even lower than HCT116shDR5 cells (Figure 2.1.16 B). It might due to the fact that Wip1 can still be up-regulated in HCT116shDR5 cells by p53 although in a less and delayed manner, but in HCT116shWip1 cells the Wip1 was fully depleted.

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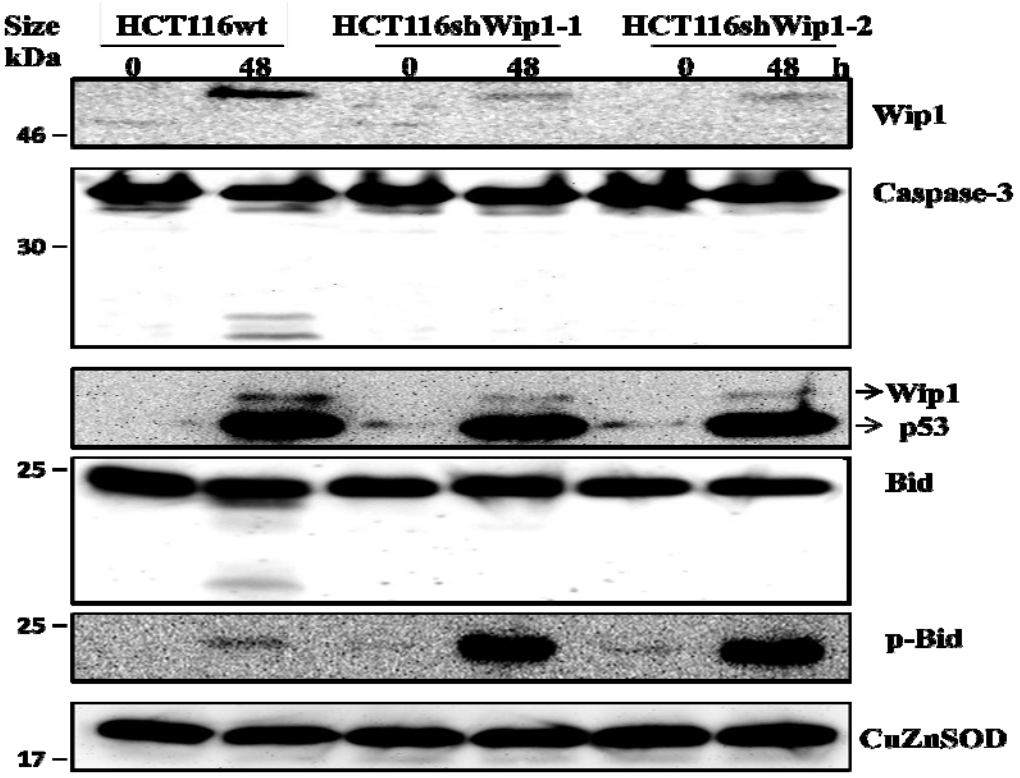

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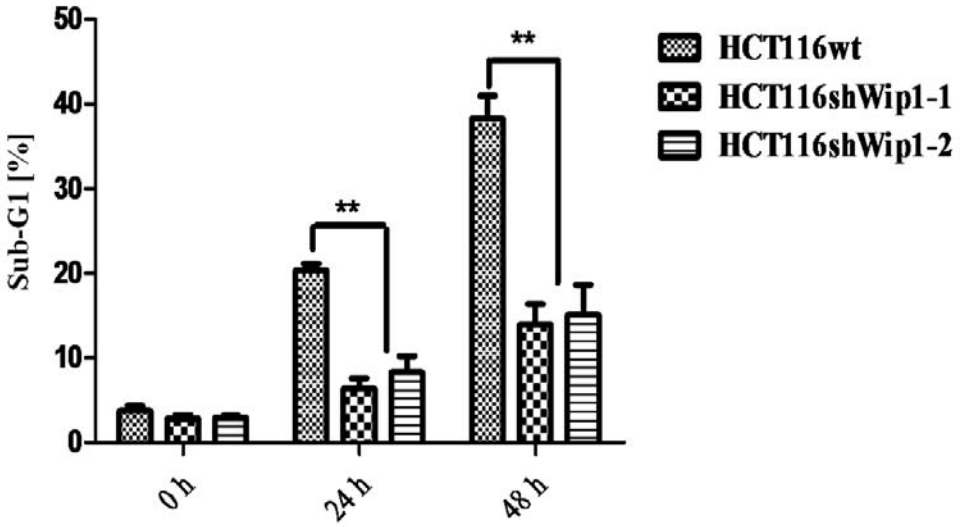

Figure 2.1.16 Depletion of Wip1 can cause resistance to 5-FU A. HCT116wt and HCT116shWip1 cells were treated with or without 5-FU for indicated time, then whole cell extracts were analyzed by Western Blot with indicated antibodies. CuZnSOD was used as a loading control. **B.** Cells were treated with 5-FU for indicated time, then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The mean of three experiments is shown in each column, and bars correspond to S.E. * $P<0.05$, ** $P<0.01$.

2.1.17 5-FU induced up-regulation of Wip1 is partially mediated by JNK activation

Although p38-MAPK-p53 was the first reported pathway of Wip1 induction, recently it has also been found that JNK-c-Jun can also mediate Wip1 induction (Song et al., 2010). We therefore examined the specificity of JNK-mediated Wip1 induction in response to 5-FU treatment. Pretreatment with JNK inhibitor SP600125 decreased Wip1 protein expression, as shown in Figure 2.1.17. Since Wip1 can be induced by p53, we used HCT116p53-/- cells to determine whether p53 or JNK is the major inducer of Wip1. We found that in HCT116p53-/- cells, Wip1 can still be induced, but the level was lower compared to wild type cells and after inhibition of JNK the induction of Wip1 was totally blocked (Figure 2.1.17). At the same time, inhibition of JNK also resulted in less Wip1 induction in HCT116wt cells. These results implied that both JNK activation and p53 can up-regulate Wip1. Inhibition of JNK can attenuate the Wip1 induction by 5-FU.

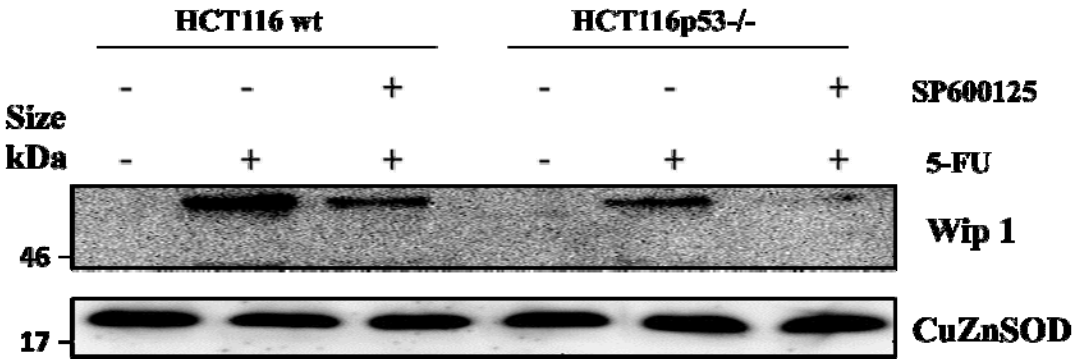

Figure 2.1.17 Wip1 can be induced by p53 and JNK. HCT116wt and HCT116p53-/- cells were pretreated for 6 h with or without the JNK specific inhibitor SP600125 (20 μM). Then treated with 5-FU for 48 h, total cell lysates were prepared and subjected to Western Blot using indicated antibodies. CuZnSOD was used as the loading control.

2.1.18 Wip1 induction in HCT116p53-/- and HCT116p53-/-shDR5 cells

In earlier study, Wip1 up-regulation was found to be regulated solely by p53 (Choi et al., 2000). Recently, it has been found Wip1 can also be regulated by the JNK pathway (Song et al., 2010). In our study, we found that Wip1 up-regulation was attenuated in HCT116shDR5 cells which also have defects in JNK activation in response to 5-FU. In order to further elucidate the manner of Wip1 up-regulation. We took advantage of HCT116p53-/- cells. We found that DR5 can still be up-regulated although delayed in a p53 independent manner after 5-FU treatment (Figure 2.1.18-1 A). In line with the Western Blot results, qRT-PCR results also showed that DR5 mRNA level was also up-regulated in a delayed manner in HCT116p53-/- cells (Figure 2.1.18-1 B). Additionally, at early time points we observed inhibition of Bid cleavage. Later the Bid cleavage was rescued (Figure 2.1.18-1 A). To further explore this issue. We employed on shRNA approach for stable knockdown of DR5 in the HCT116p53-/- cell with the resultant line named HCT116p53-/-shDR5. We found that JNK activation was totally abolished in HCT116p53-/-shDR5 cells (Figure 2.1.18-2 A). In addition, the Wip1 up-regulation and Bid cleavage was also fully inhibited in HCT116p53-/-shDR5 cells. Like the HCT116shWip1 cells, the HCT116p53-/-shDR5 cells showed almost complete resistance to 5-FU (Figure 2.1.18-2 B). In turn, the combined loss of both p53 and DR5 showed synergistic effects of protecting cells from 5FU-induced apoptosis.

Taken together, these results further confirmed that Wip1 induction is dependent on p53 and JNK activity, and up-regulation of Wip1 could affect the cellular response to 5-FU.

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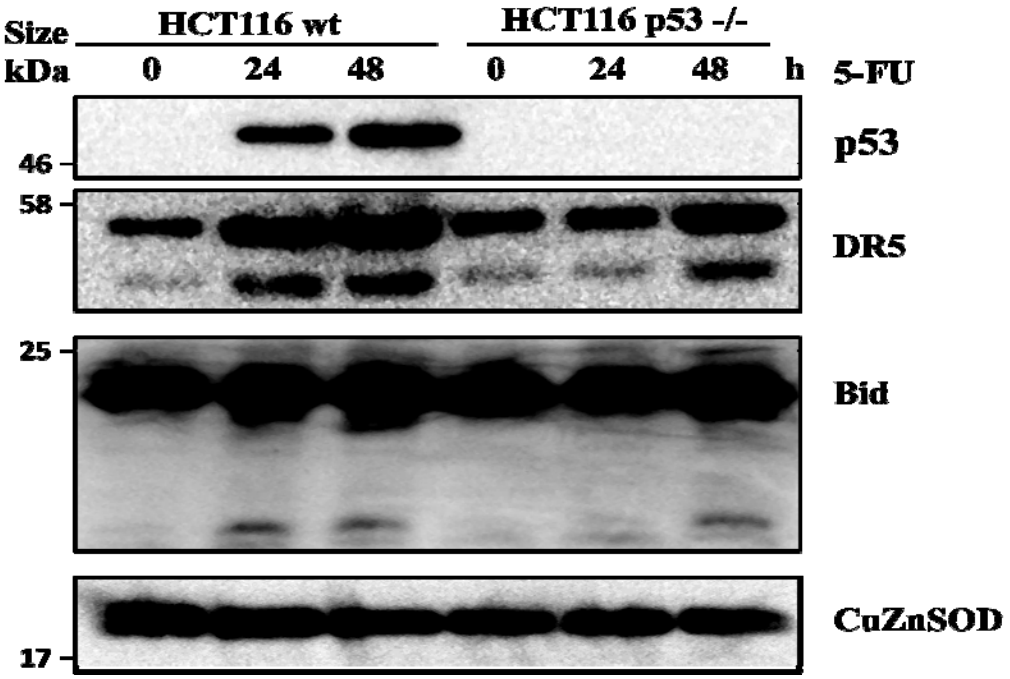

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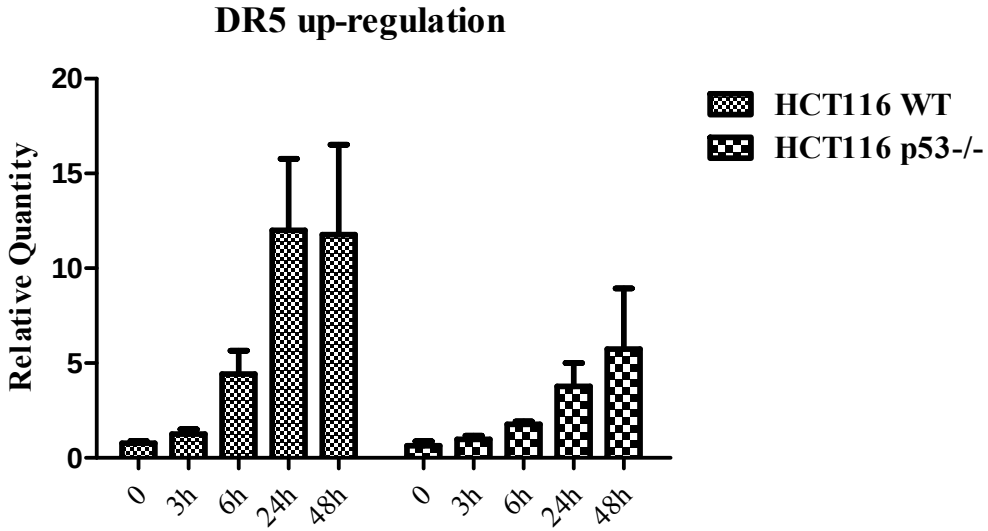

Figure 2.1.18-1 DR5 induction was delayed in HCT116p53-/- cells in response to 5-FU. A. HCT116wt and HCT116p53-/- cells were treated with 5-FU for indicated time. Total cell lysates were then prepared and subjected for Western Blot analysis using indicated antibodies. CuZnSOD was used as a loading control. **B.** DR5 mRNA transcripts were analyzed via qRT-PCR, after HCT116wt and HCT116p53-/- cells treated with 5-FU for the indicated times. The mean of three experiments is shown in each column, and bars correspond to S.E.

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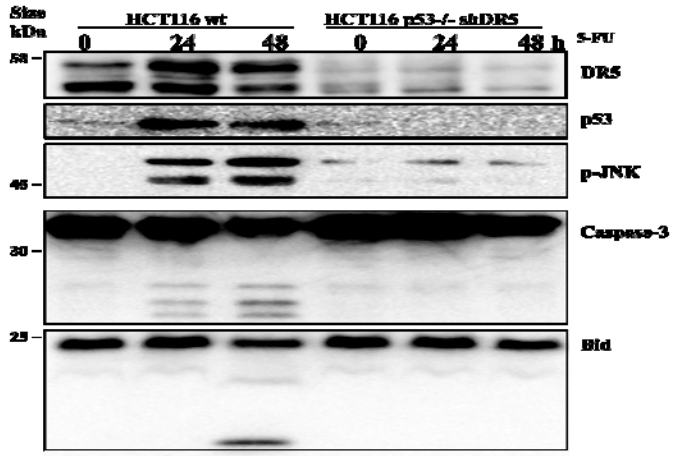

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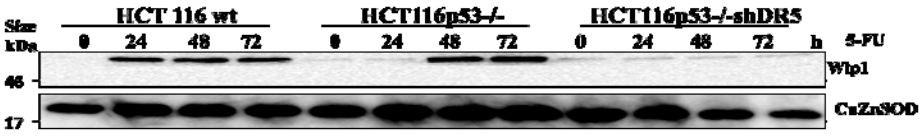

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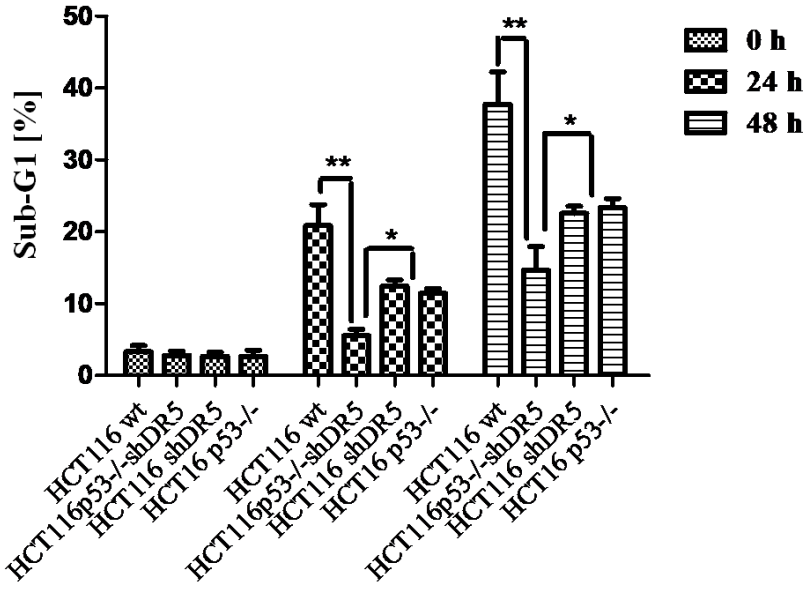

Figure 2.1.18-2 Depletion of both p53 and DR5 synergistically protects cells from 5-FU induced apoptosis. A and **B.** HCT116wt and HCT116p53-/-shDR5 cells were treated with 5-FU for indicated time, Total cell lysates were then prepared and subjected for Western Blot analysis using indicated antibodies. CuZnSOD was used as a loading control. **C.** the whole cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The mean of three experiments is shown in each column, and bars correspond to S.E. * $P<0.05$, ** $P<0.01$.

2.1.19 FADD but not caspase-8 is required for the JNK activation induced by 5-FU

Through binding to its cognate ligand, DR5 can undergo conformational changes that promote interaction between the death domain of the receptor and the adaptor protein FADD. FADD can in turn recruit caspase-8 to form a complex called DISC (death inducing signalling complex) (Kischkel et al., 1995). It has also been found that through binding to TRAIL, DR5 can induce non-apoptotic signalling pathways, such as the various MAP kinase cascades, PKC (Protein kinase C) and the NF-κB (Park et al., 2005; Wajant et al., 2003). Different from the well-established events causing caspase activation, the molecular mechanisms of the kinase-activating function have been controversial. Especially, there are still no reports about the mechanism of how chemoagents activate the kinase pathway. So we tried to elucidate the molecular compenents and events that mediate the 5-FU induced JNK activation.

Firstly, we tried to dissect the role of FADD and caspase-8 in 5-FU induced JNK activation. We made use of stable FADD and caspase-8 knock down clones HCT116shFADD and HCT116shcaspase-8. Although depletion of caspase-8 can protect cells against death induced by 5-FU, this did not influence 5-FU induced JNK activation (Figure 2.1.19 A). In line with previous reports that FADD depletion can attenuate the activation of JNK by TRAIL and TNF (Natoli et al., 1997; Varfolomeev et al., 2005), the JNK activation was also diminished in HCT116shFADD cells in response to 5-FU (Figure 2.1.19 A). All these results verified that like DR5, FADD is needed for 5-FU induced JNK activation. In agreement with previous studies (Natoli et al., 1997; Varfolomeev et al., 2005), we found the activation of JNK by ligands TRAIL and TNF was also diminished in HCT116shFADD cells (Figure 2.1.19 A). Notably, we also found that the up-regulation of Wip1 was also attenuated in HCT116shFADD after 5-FU treatment. This further confirmed that Wip1 up-regulation is partly dependent on JNK activation.

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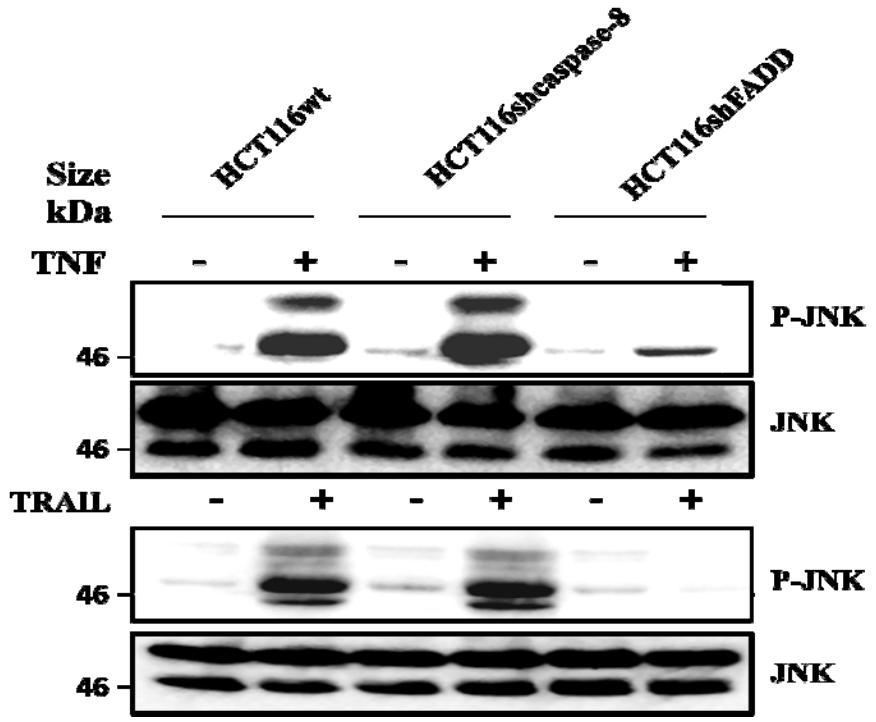

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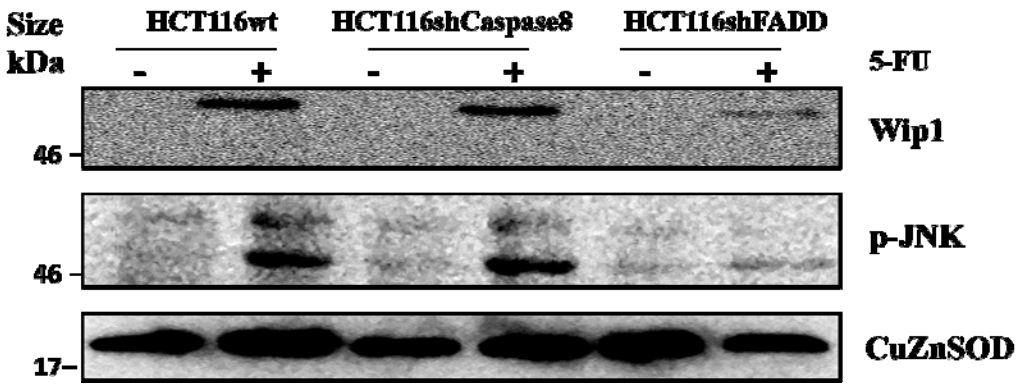

Figure 2.1.19 FADD is required for JNK activation induced by 5-FU. A and **B.** HCT116wt, HCT116shcaspase-8 and HCT116shFADD cells were treated with TNF-α (10 ng/ml), TRAIL (10 ng/ml) for 30 min or with 5-FU for 24 h. Total cell lysates were then prepared and subjected for Western Blot analysis using indicated antibodies. CuZnSOD was used as a loading control.

2.1.20 FADD can physically interact with ASK1, TRADD, RIP1 and TRAF2 but not MAP4K5

Since FADD is indispensable for JNK activation. We hypothesised that FADD may form a complex with DR5 that can stimulate JNK activation. To test this, we determined whether FADD can physically interact with other proteins in a transfection-based co-immunoprecipitation assay. Myc-tagged FADD was co-expressed with different candidate proteins in 293 cells and immunoprecipitated with anti-Myc antibody. The immune complexes were subjected to western blot with corresponding antibodies. First, we tested ASK1 (apoptosis signal-regulating kinase 1). ASK1 (apoptosis signal-regulating kinase 1) is a MAPKKK that activates the SEK1-JNK and MKK3/MKK6-p38 signaling cascades (Ichijo et al., 1997; Wang et al., 1996a). A kinase-inactive mutant of ASK1 can reduce TNF and FasL-induced JNK activation and apoptosis, suggesting that ASK1 might be a pivotal component in JNK activation (Chang et al., 1998; Nishitoh et al., 1998). ASK1 was found to associate with FADD (Figure 2.1.20 A). Next, we tested TRADD (TNFR-associated death domain protein) which is also an adaptor protein with death domain. TRADD has been found in association with FADD and TNFR1 to form a complex to activate JNK pathway by TNF (Holler et al., 2000). Expression of TRADD can also augment JNK activation with certain stimuli (Eliopoulos et al., 1999). As expected, TRADD can also be detected with FADD (Figure 2.1.20 B). TRAF2 (TNF receptor-associated factor 2) is a member of the TRAF protein family. To date, six members of the TRAF family have been found (Nishitoh et al., 1998). Over-expression of TRAF2 has been reported be able to activate JNK (Song et al., 1997). TRAF2 has been reported to associate with ASK1 (Nishitoh et al., 1998). Additionally, TNF signaling to JNK was defective in TRAF2-deficient cells (Yeh et al., 1997), dominant negative TRAF2 can also inhibit the TNF-induced JNK (Liu et al., 1996b; Natoli et al., 1997). As shown in Figure 2.1.20 C, TRAF2 can also interact with FADD. Recent studies suggest that FADD can associate with TRAF2, RIP1 to form a signaling complex, which is indispensable for activation of JNK after TRAIL treatment (Varfolomeev et al., 2005). RIP1 (receptor-interacting protein 1) is a death domain containing kinase that can be

recruited by TRADD then binds to TNFR1 and is essential for full JNK activation by TNF (Devin et al., 2003). There are also studies that indicate that RIP1 is important for JNK activation by TRAIL or FasL (Kreuz et al., 2004; Lin et al., 2000). So we determined whether RIP1 can also bind to FADD. Not surprisingly, RIP1 was also found to associate with FADD (Figure 2.1.20 D).

MAP4K5 also termed GCK-related (GCKR) enzyme, is a MAP kinase kinase kinase kinase that belongs to the GCKs (germinal center kinases) family. MAP4K5 is an upstream activator of JNK pathway. For example, MAP4K5 can link TNF signaling to JNK activation (Chadee et al., 2002) and MAP4K5 can also interact with TRAF2 which is required for TNF signaling to MAP4K5 (Shi and Kehrl, 2003). All those information make MAP4K5 an interesting candidate in our model for JNK activation. Howerver, we were unable to co-immunoprecipitate MAP4K5 with FADD (Figure 2.1.20 E).

In summary, we found that FADD can bind to ASK1, TRADD, TRAF2, RIP1 but not MAP4K5. These findings indicate that there might be a complex, which is responsible for the activation of JNK, composed of those proteins. Knock-down of FADD can cause inhibition of JNK activation, it might be due to FADD acting as a platform to recruit those proteins to form the complex. Given that the depletion of FADD or DR5 both showed the same defect in the activation of JNK, so the mechanism may be similar. Due to technical reasons, we were unable to immunoprecipitate DR5, so whether DR5 can also bind those proteins is still unknown and further work is needed to test this.

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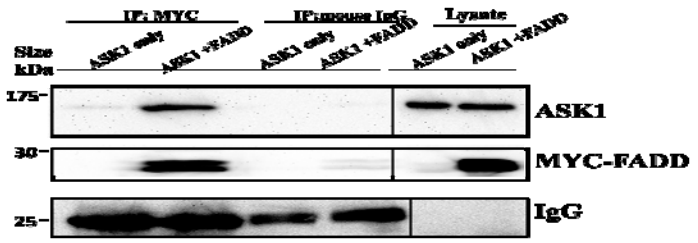

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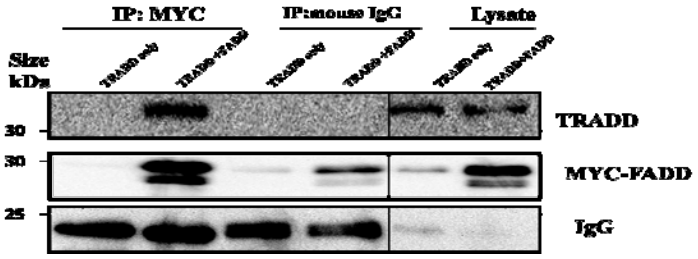

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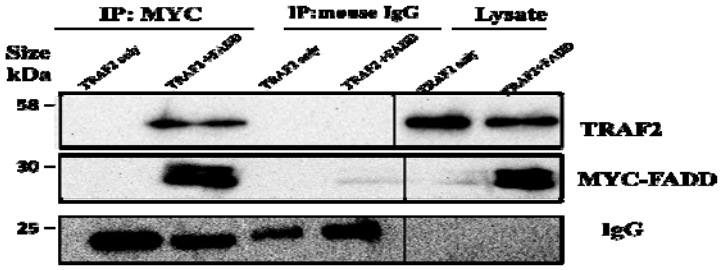

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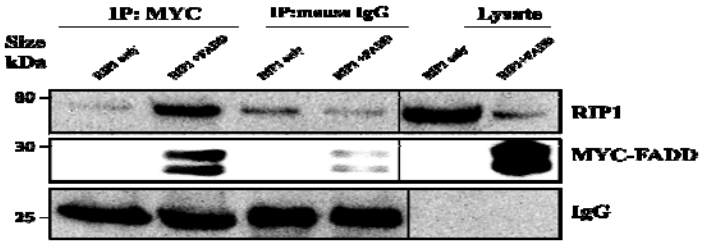

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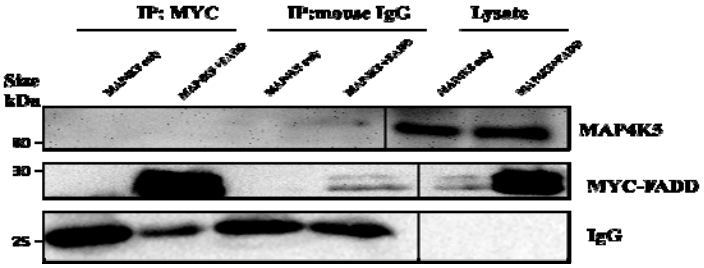

Figure 2.1.20 FADD can physically interact with ASK1, TRAF2, RIP and TRADD but not MAP4K5. **A.** ASK1 **B.** TRADD **C.** TRAF2 **D.** RIP1 but not **E.** MAP4K5 can co-immunoprecipitate FADD from cellular extracts. The indicated constructs were expressed in 293 cells, and Myc-tagged FADD was immunoprecipitated with α-Myc antibodies and analyzed by Western Blot with the indicated antibodies.

Part II

Enhanced anti-cancer efficacy of MSC-mediated delivery of sTRAIL in combination with 5-FU or silencing of XIAP

2.2.1 Soluble TRAIL variants are expressed and secreted

As full length TRAIL does not contain a secretory signal peptide it is a membrane protein. In order to render it into a secreted and soluble transgene product, we made use of the signal peptide from the human Fibrillin-1 (hFib) gene and ligated it to the Furin cleavage site (Furin CS), an Isoleucine Zipper (ILZ) and the apoptosis-inducing moiety of the TRAIL (aa114-aa289) (Figure 2.2.1 A). The Furin CS facilitates removal of the signal peptide, while the ILZ gives rise to trimeric TRAIL representing a more potent form as compared to TRAIL monomers (Kim et al., 2006b). Aside from the wild-type soluble TRAIL construct (sTRAIL-wt) containing the consensus TRAIL cDNA, we also designed two TRAIL-receptor specific variants, one selective for DR4 and one for DR5. One amino acid sequence change was introduced into the TRAIL segment, namely D269H (Aspartic acid at position 269 changed to Histidine) to generate the DR5-specific mutant (sTRAIL-DR5), whereas the DR4-specific mutant (sTRAIL-DR4) was generated by an S159R (Serine at position 159 changed to Arginine) amino acid modification (Figure 2.2.1 A). These expression constructs were transfected into 293 cells, the expression of sTRAILs were assessed by Western Blot (Figure 2.2.1 B) and the levels of sTRAILs in the supernatant were measured by ELISA (Figure 2.2.1 C).

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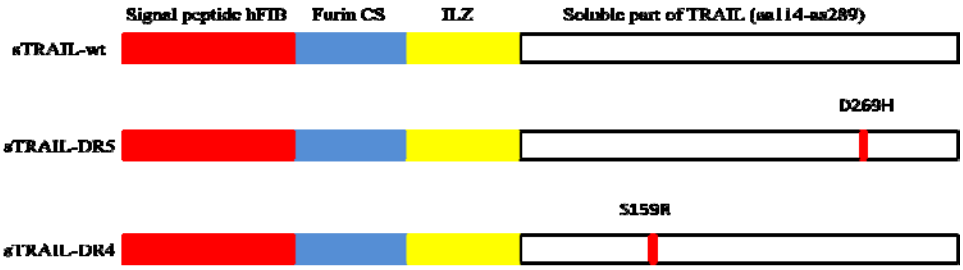

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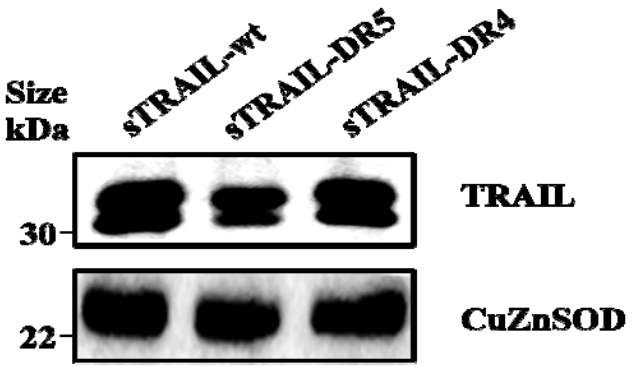

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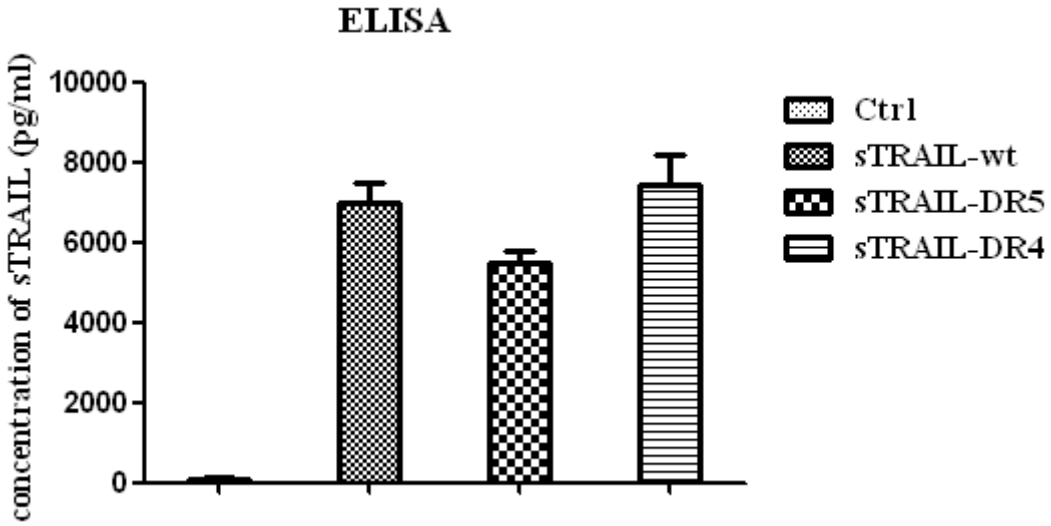

Figure 2.2.1 Soluble TRAILs expressed in 293 cells. A. Schematic drawing of the sTRAIL-wt, sTRAIL-DR5, and sTRAIL-DR4 constructs. The signal peptide sequences hFIB was ligated to the Furin CS and ILZ stretch and ectodomain sequence of TRAIL **B.** Each sTRAIL expression construct described in A was transiently transfected into 293 cells. 24 h after transfection, the whole cell lysates of the transfected cells were prepared and analyzed by Western Blot for TRAIL, CuZnSOD was used as a loading control **C.** Results of TRAIL enzyme-linked immunosorbent assay (ELISA) analyses demonstrating secretion of sTRAIL into the supernatant of transfected 293 cells.

2.2.2 TRAIL variants trigger increased apoptosis

After having transfected 293 cells with the three constructs expressing sTRAIL-wt, sTRAIL-DR4 and sTRAIL-DR5, respectively, the supernatants were collected 24 h later. The concentrations of sTRAIL were measured by ELISA and adjusted to 2 ng/ml. Then the supernatants were transferred onto different tumour cells and we found differences with regard to their propensity to undergo apoptosis via DR5 or DR4.

Human ovarian cancer A2780 cells, human pancreatic BxPc-3 cells, human colorectal cancer cells HCT116 and Colo205 cells showed increased apoptosis when treated with supernatants from sTRAIL-DR5 construct-transfected 293 cells (Figure 2.2.2 A), whereas human pancreatic carcinoma Colo357 cells and human cervical cancer Hela cells exhibited elevated cell death rates with sTRAIL-DR4 (Figure 2.2.2 B). Noteworthy, Colo205 cells showed a great preference to DR5-selective sTRAIL, which can still induce apoptosis rates of 90% even with only 0.5 ng/ml while sTRAIL-wt and sTRAIL-DR4 showed almost no apoptosis-inducing activity at the same concentration (Figure 2.2.2 A).

Next, we tested the specificity of these TRAIL variants on HCT116 parental cells and cells with stable RNAi-silenced DR5 and DR4 named HCT116shDR5 and HCT116shDR4, respectively that were derived from HCT116 cells (Figure 2.2.2 C). When these cells were treated with supernatants from 293 cells secreting sTRAIL-DR5 or sTRAIL-DR4, we found that HCT116shDR5 cells showed markedly reduced apoptosis in response to sTRAIL-DR5 whereas the level of apoptosis was even slightly increased with sTRAIL-DR4 and almost unaffected with sTRAIL-wt (Figure 2.2.2 C). In contrast, HCT116shDR4 cells exhibited significantly decreased apoptosis with sTRAIL-DR4, while the apoptosis levels were elevated with sTRAIL-DR5 and almost no difference could be detected with sTRAIL-wt (Figure 2.2.2 C). Those findings indicate that the sTRAIL variants indeed induce apoptosis via one of the two receptors.

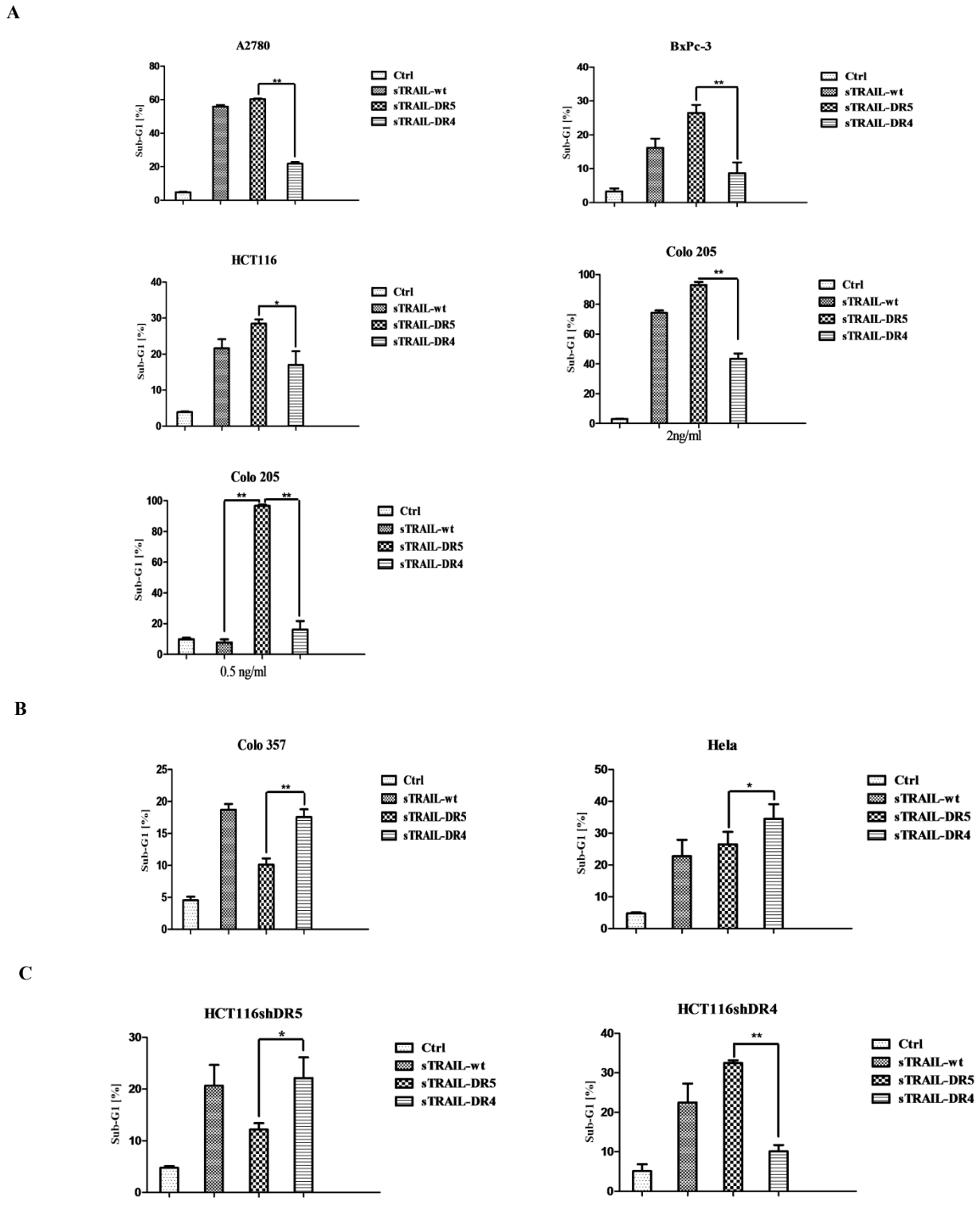

Figure 2.2.2 Biological activity of sTRAIL-wt, sTRAIL-DR5 and sTRAIL-DR4 in different cancer cells. A. A2780, BxPc-3 and HCT116 cells **B.** Colo357 and Hela cells **C.** HCT116shDR5 and HCT116shDR4 were incubated with the EGFP (Ctrl), sTRAIL-wt, sTRAIL-DR5, and sTRAIL-DR4 supernatants (2 ng/ml) for 24 h, then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. * $p<0.05$, ** $p<0.01$. Data are the mean ± S.E from three independent experiments.

2.2.3 MSCs can express and secrete soluble TRAIL and induce apoptosis in Colo205 cells via DR5

Having established functionality and specificity of the sTRAIL variants, we generated adenoviral vectors containing the sTRAIL expression cassettes. These adenoviral vectors, termed Ad.sTRAIL-wt, Ad.sTRAIL-DR5 and Ad.sTRAIL-DR4, respectively, were used to transduce murine MSCs, which had been characterized by their capacity for adipogenic, osteogenic and chondrogenic differentiation as well as FACS analysis with an array of surface marker antibodies. The resulting MSCs expressing the sTRAIL variants were named MSC.sTRAIL-wt, MSC.sTRAIL-DR5 and MSC.sTRAIL-DR4, respectively. 48 h after the adenoviral transduction, the levels of sTRAIL were determined by Western Blot, the adenovirus expressing the DsRed was used as a control (Figure 2.2.3 A). Then we mixed MSC.sTRAILs with Colo205 cells at cellular ratios of 1:5, 1:10 and 1:20 respectively. Among all those MSC.sTRAILs, MSC.sTRAIL-DR5 could induce significantly more apoptosis than other MSC.sTRAILs (Figure 2.2.3 B). These results further demonstrated the higher susceptibility of Colo205 cells to apoptosis through a DR5-mediated pathway. Furthermore, we collected the supernatants of MSC.sTRAIL-wt, MSC.sTRAIL-DR5, MSC.sTRAIL-DR4 and the supernatants were diluted at the dilutions of 1:10; 1:20; 1:50 respectively and then applied to Colo205 cells. We found that the supernatant of MSC.sTRAIL-DR5 triggered significantly more apoptosis than the two others (Figure 2.2.3 B). We also assessed apoptosis at the molecular level. In line with the cell death rates, we found that MSC.sTRAIL-DR5 induced caspase-8 activation more profoundly than MSC.sTRAIL-wt, whereas MSC.sTRAIL-DR4 exhibited almost no activation of caspase-8 (Figure 2.2.3 C). These results were also supported by caspase-3 Western Blots that showed full caspase-3 activation in MSC.sTRAIL-DR5-treated Colo205 cells, wheras significantly less could be measured in MSC.sTRAIL-wt stimulated cells and nearly no activation could be seen in the MSC.sTRAIL-DR4 treated samples (Figure 2.2.3 C).

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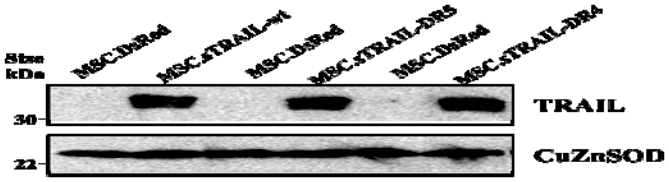

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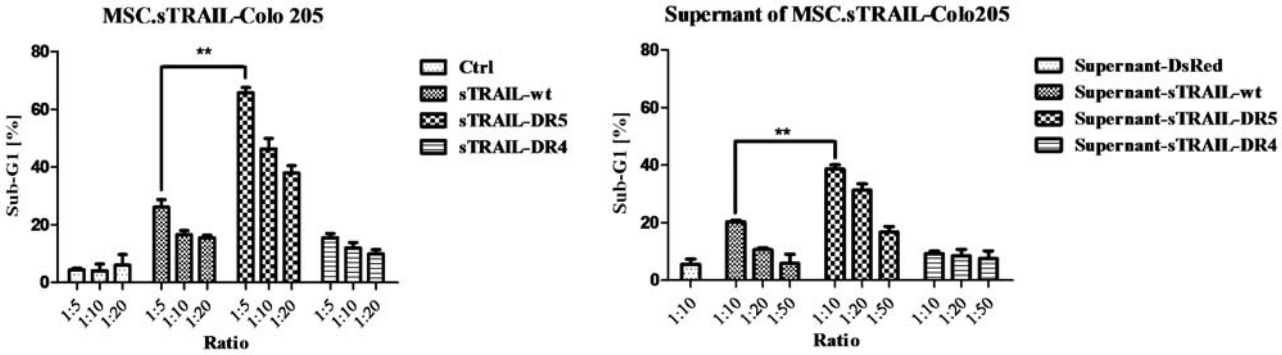

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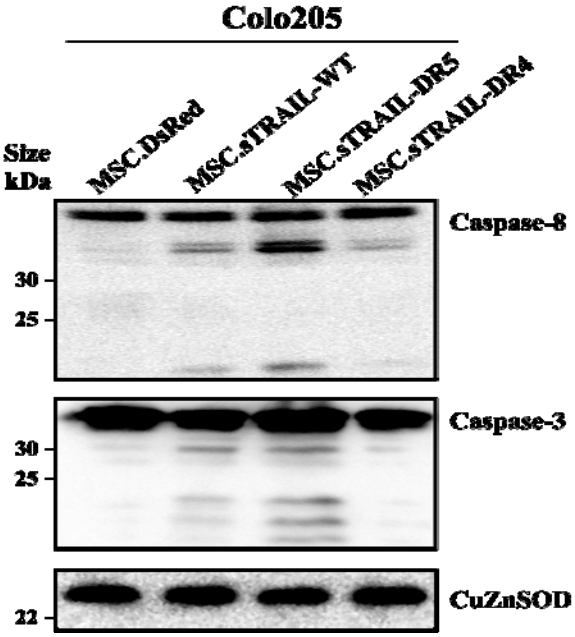
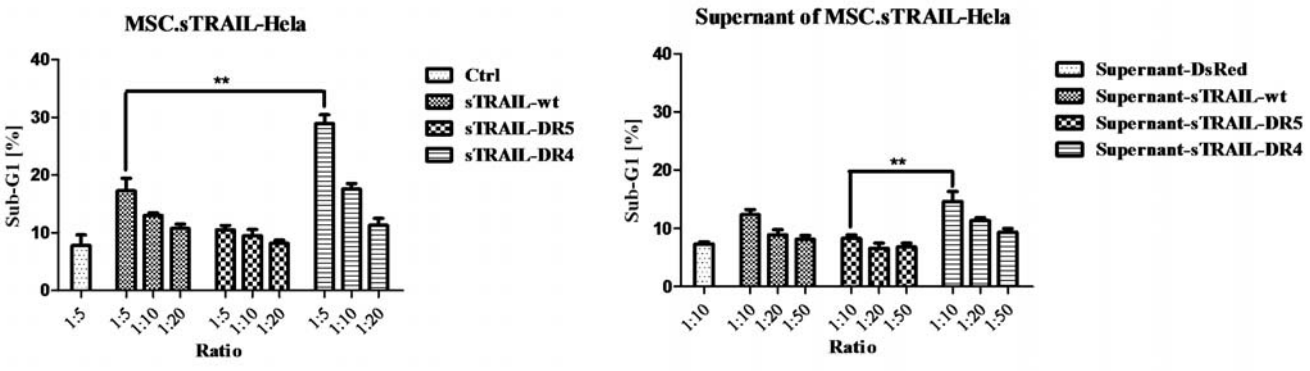


Figure 2.2.3 Effects of MSC.sTRAIL on Colo205 cells A. Expression of sTRAILs in MSC.sTRAIL-wt, MSC.sTRAIL-DR5, MSC.sTRAIL-DR4 were demonstrated by Western Blot **B**. MSC.DsRed, MSC.sTRAIL-wt, MSC.sTRAIL-DR5 and MSC.sTRAIL-DR4 and the supernants were mixed with Colo205 cells at different ratios for 24 h, then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. * $p<0.05$, ** $p<0.01$. Data are the mean ± S.E from three independent experiments. **C**. Caspase-8 and caspase-3 activation of the highest mix ratio (1:5) was determined by Western Blot analysis.

2.2.4 Hela cells undergoes apoptosis via DR4

On the other side, in Hela cells addition of MSC.sTRAIL-DR4 gave rise to markedly higher cell death rates compared to MSC.sTRAIL-wt and MSC.sTRAIL-DR5 (Figure 2.2.4 A). In support of these findings, the supernatants of MSC.sTRAIL-DR4 transferred onto Hela cells resulted in higher apoptosis rates than supernatants from MSC.sTRAIL-wt and MSC.sTRAIL-DR5 (Figure 2.2.4 A). Western Blot results revealed the caspase-8 activation is the strongest in MSC.sTRAIL-DR4 treated cells. These finding are in accordance with the apoptosis results (Figure 2.2.4 B). In addition, caspase-3 Western Blot also showed that MSC.sTRAIL-DR4 caused activation as evident by the appearance of the active fragments (p17/p15) (Figure 2.2.4 B). In summary, expression and secretion of sTRAIL and variants can effectively induce apoptosis in different cancer cells.

A



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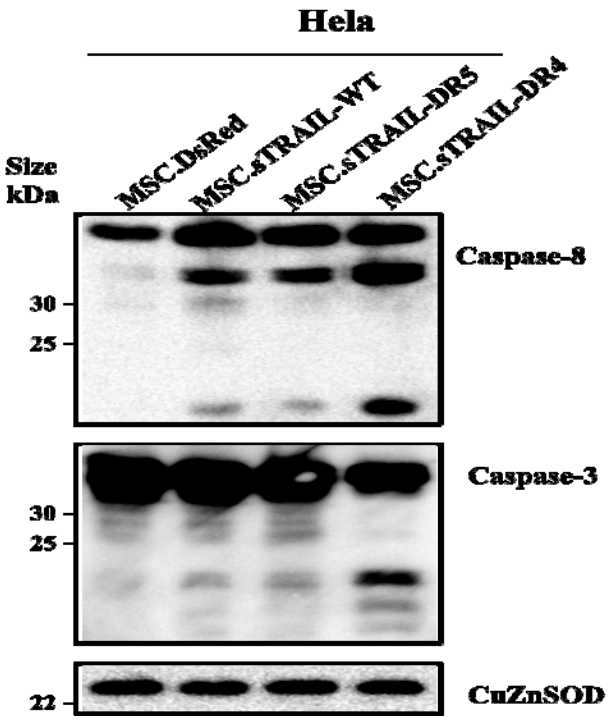


Figure 2.2.4 Effects of MSC.sTRAIL on Hela cells **A**. MSC.DsRed, MSC.sTRAIL-wt, MSC.sTRAIL-DR5, MSC.sTRAIL-DR4 and the corresponding supernatants were mixed with Hela cells at different ratios for 24 h. Then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. * $p<0.05$, ** $p<0.01$. Data are the mean ± S.E from three independent experiments. **B**. Caspase-8 and caspase-3 activation of the highest mix ratio (1:5) was determined by Western Blot.

2.2.5 Combination of 5-FU and MSC.sTRAIL enhances apoptosis in different HCT116 colon cancer cells

In pursuit of enhancing the effects of MSC-mediated delivery of sTRAIL variants further, we tested a combination treatment consisting of MSC.sTRAILs and a conventional cytotoxic anti-cancer drug 5-FU, which is commonly used in the treatment of colorectal cancer. We pre-treated HCT116wt, HCT116shDR5, and HCT116shDR4 cells with 5-FU at 10 μM. This concentration alone did not cause significant apoptosis. Then the cells were treated with MSC.sTRAIL-wt, MSC.sTRAIL-DR5 and MSC.sTRAIL-DR4 for another 24 h. When we measured apoptosis in HCT116wt cells, we found that pre-treatment with 5-FU caused increased apoptosis levels compared to treatment with MSC.sTRAILs alone. Cell death rose from around 10% in MSC.sTRAIL-wt treated cells to around 20% in 5-FU/MSC.sTRAIL-wt samples and from 20% to 30% for 5-FU/MSC.sTRAIL-DR5 stimulated cells (Figure 2.2.5 A). In HCT116shDR4 cells, cell death rose from 15% in MSC.sTRAIL-wt treated cells to 20% in 5-FU/MSC.sTRAIL-wt samples, and 30% to 40% in 5-FU/MSC.sTRAIL-DR5 stimulated cells. (Figure 2.2.5 A, B, C). Interestingly, in MSC.sTRAIL-DR4 treated samples there was only a slight increase in apoptosis rates after 5-FU treatment in all cells. In HCT116shDR5 cells after being treated with all three sTRAILs there was almost no increase in apoptosis. Taken all these findings together, a combined treatment of 5-FU and MSC.sTRAIL and in particular MSC.sTRAIL-DR5, resulted in synergistic apoptotic effects. (Figure 2.2.5 B).

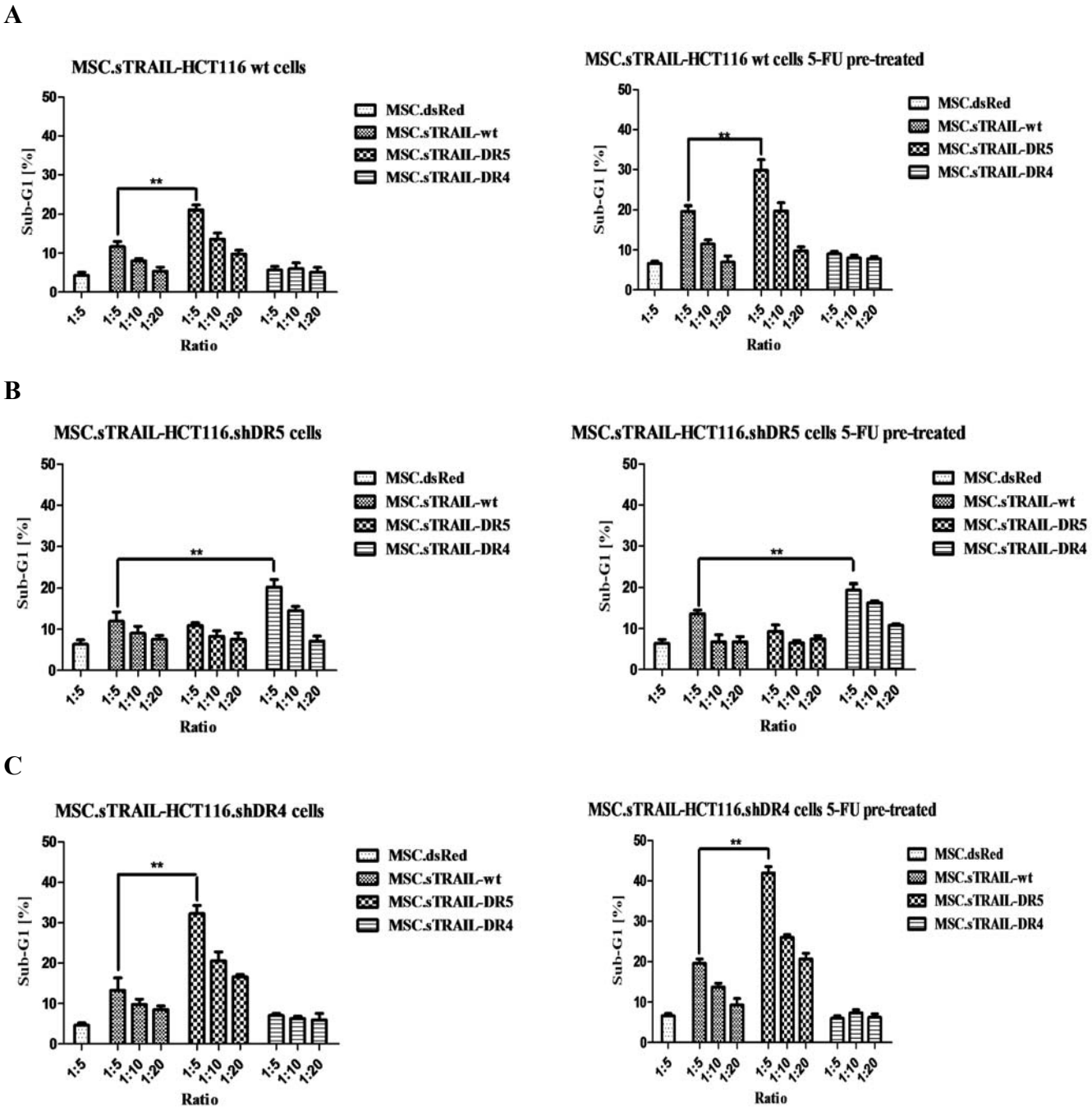
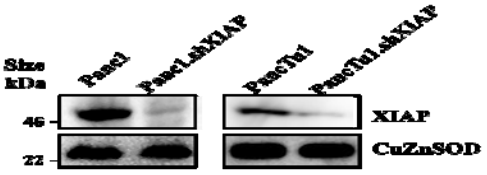


Figure 2.2.5 5-FU can enhance MSC.sTRAIL induced apoptosis in HCT116 cells **A**. HCT116wt cells **B**. HCT116.shDR5 cells **C**. HCT116.shDR4 were pretreated or not with 10 μM 5-FU for 24 h before mixing with MSC.DsRed, MSC.sTRAIL-wt MSC.sTRAIL-DR5, MSC.sTRAIL-DR4 at indicated ratios for another 24 h, then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. The cell apoptosis rate was increased after pretreated with 5-FU in HCT116wt cells and HCT116shDR4 cells but not HCT116shDR5 cells. * $p<0.05$, ** $p<0.01$. Data are the mean ± S.E from three independent experiments.

2.2.6 Pancreatic cancer cells are TRAIL resistant and can be sensitised by XIAP silencing

Many cancer cells are TRAIL resistant and this resistance manifests a significant hurdle to the successful therapeutic use of TRAIL. We analyzed two pancreatic cancer cell lines, Panc1 and PancTu1, and found them to be resistant to TRAIL (Mohr et al., 2010). Overcoming this resistance combined with effective TRAIL delivery offers a potential new treatment regimen. Inhibition of XIAP has been shown to be an effective TRAIL sensitization approach. Therefore, we generated stable XIAP knock-down clones in the two TRAIL resistant pancreatic cancer cells, Panc1 and PancTu1. These clones were named Panc1.shXIAP and PancTu1.shXIAP respectively, and were shown to harbour no detectable XIAP levels by Western Blot (Figure 2.2.6 A). When we treated PancTu1.shXIAP and Panc1.shXIAP cells with MSC.sTRAIL-wt, MSC.sTRAIL-DR5 and MSC.sTRAIL-DR4, we found significantly increased apoptosis levels in the XIAP knock-down clones (Figure 2.2.6 B). Furthermore, after transferring the supernatants, we also found that the supernatant triggered more apoptosis in the XIAP knock-down cells than the parental cells (Figure 2.2.6 B).

A



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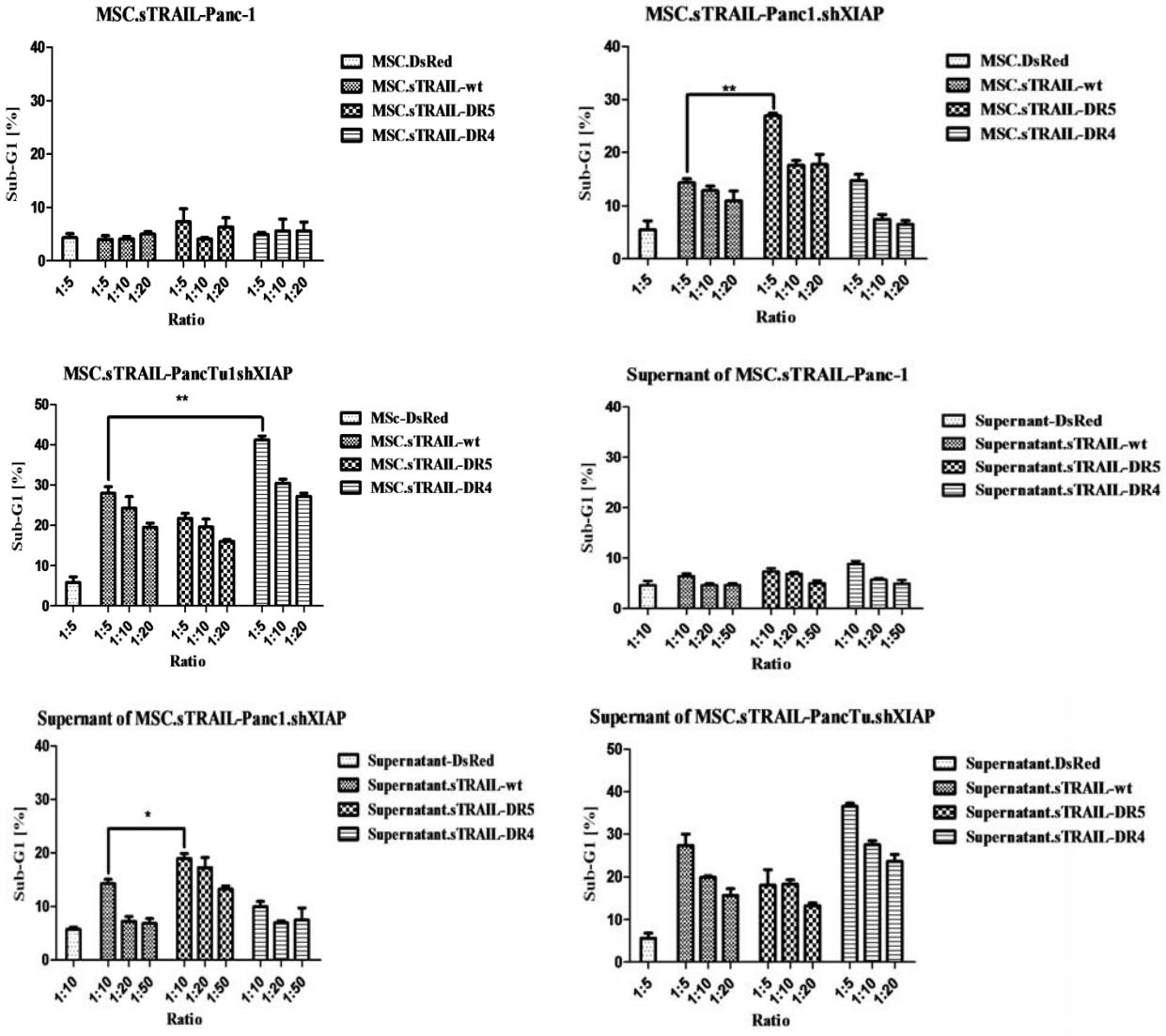


Figure 2.2.6 MSC.sTRAIL can kill XIAP-silenced cancer cells. **A**. Western Blot for XIAP in Panc1.shXIAP and PancTu1.shXIAP clones in which XIAP was stably knocked down. Depicted are parental cells (Panc1 and PacTu1), clones with silenced XIAP (Panc1.shXIAP and PancTu.shXIAP) **B**. Mixing of MSC.DsRed, MSC.sTRAIL-wt, MSC.sTRAIL-DR5, MSC.sTRAIL-DR4 and their supernatants at different ratios with Panc1, Panc1.shXIAP, and PancTu.shXIAP cells for 48 h, then the cells were collected and the apoptotic fraction was recognized as sub-G1 population of cells by flow cytometry. * $p<0.05$, ** $p<0.01$. Data are the mean ± S.E from three independent experiments.

CHAPTER3 DISCUSSION

3.1 Silencing of DR5 can confer resistance to 5-FU

Specific and selective apoptosis induction in cancer but not normal cells is a major goal of cancer therapy (reviewed by Gerl and Vaux, 2005; Schulze-Bergkamen et al., 2009). Most current anti-cancer chemotherapies kill tumour cells through activating the intrinsic and/or extrinsic pathway of apoptosis. Indeed, the ability of cells to escape apoptosis is considered as an essential hallmark of cancer (reviewed by Gerl and Vaux, 2005). Understanding how tumour cells execute apoptosis and how they evade apoptotic death might enable a more efficient approach to anti-cancer therapy.

5-FU is a widely used chemotherapeutic drug that has been used for treatment of many different primary and metastatic malignancies. 5-FU exerts its function through inhibiting thymidilate synthase, thereby causing thymine-deficient stress and subsequent DNA and/or RNA damage which is considered to be a common initial event in apoptosis (reviewed by Debatin and Krammer, 2004). The DNA damage can then be propagated by the cellular stress response (reviewed by Rich et al., 2000). However, one limit of using such chemotherapeutic drugs is drug resistance. Plenty of efforts have been made to unveil the mechanisms behind the resistance. It has been found that thymidylate synthase (TS) protein overexpression can cause resistance to 5-FU (Copur et al., 1995; Longley et al., 2003). 5-FU sensitivity can also be influenced by many other factors such as: expression levels of dihydropyrimidine dehydrogenase, the genetic status of p53, DNA mismatch-repair system and multiple molecular events, and the levels of anti-apoptotic Bcl-2 proteins (reviewed by Longley et al., 2003). Recently, miRNAs have also been implicated in the 5-FU resistance of cancer cells. For instance, miR-21 represses hMSH2 expression and miR-193a-3p represses SRSF2, which could affect the response to 5-FU (Ma et al., 2012; Valeri et al., 2010). However, the molecular mechanisms of 5-FU resistance in tumour cells are still largely unknown. Therefore, identification and evaluation of new molecular targets and/or signaling pathways involved in 5-FU resistance would allow designing of novel agents and improved scheduling regimes that can improve the tumour response to cancer chemotherapy.

There are two pathways can lead to apoptosis named extrinsic and intrinsic pathway, respectively. The relative contributions of the extrinsic and the intrinsic apoptotic pathway to drug-induced apoptosis are still controversial (reviewed by Debatin, 1999; Kaufmann and Earnshaw, 2000; Kaufmann and Vaux, 2003). Cytotoxic drugs like cisplatin, etoposide, induced apoptosis through Fas/FADD pathway but independent of FasL (Micheau et al., 1999). The extrinsic pathway has also been implicated in 5-FU treatment since FasL/Fas system can be activated in colon carcinoma cells (Houghton et al., 1997). The expression level of Fas has also been found related to the sensitivity of cancer cells to chemotherapy agents (Iijima et al., 1997). Moreover, even ultraviolet light also induce apoptosis through the activation of Fas pathway (Rehemtulla et al., 1997). However, at the same time it has also been found that drug-induced apoptosis is not related to the Fas/FasL signaling pathway in lung cancer cells (Ferreira et al., 2000).

As a membrane bound protein, DR5 was firstly found as a DNA damage-inducible p53-regulated death receptor gene (Wu et al., 1997). DR5 can also be induced by many different stimulus like γ-irradiation, UV and 5-FU (Burns et al., 2001; Fei et al., 2002; Wang and El-Deiry, 2004). DR5 was identified as a TRAIL receptor that was able to bind TRAIL and trigger apoptosis through the extrinsic apoptotic pathway. DR5 mutations have been found in neck and head, breast and lung cancer. In head and neck cancer, a truncating mutation in the death domain was common, while loss of function mutations in the death domain have been observed in lung cancer (Lee et al., 1999; Pai et al., 1998; Shin et al., 2001).

Since it has been discovered, the function of DR5 in the process of apoptosis has been intensively investigated. Through up-regulation of DR5, one could reverse the Bax-deficient tumour cells resistance to TRAIL (Wang and El-Deiry, 2003a). When DR5 was over-expressed, it could induce apoptosis in a ligand-independent manner (Screaton et al., 1997). However, the role of DR5 in apoptosis might not only be in extrinsic pathway. It has been found DR5 null mice are more resistant to radiation induced apoptosis (Wang and El-Deiry, 2003a). Additionally, using RNA interference to knockdown DR5, it was found that silencing of DR5 can promote colon tumour

xenograft growth in nude mice and confer resistance to 5-FU (Wang and El-Deiry, 2004). These observations suggest that DR5 may be a critical determining factor for tumourigenesis and promoting sensitivity to chemotherapy. Therefore, DR5 might be used as a biomarker for clinical chemotherapy.

In this study, we generated stable DR5-silenced HCT116 cells using RNAi. In accordance with previous reports, we found that inactivation of DR5 functions decreased apoptotic responses to 5-FU. The apoptosis rates of HCT116shDR5 cells were only around half of HCT116wt cells in response to 5-FU. This feature seems to be specific for DR5, because depletion of DR4 cannot protect cells from 5-FU induced apoptosis and even increased the apoptosis rate a little. We confirmed that the function of DR5 was diminished in HCT116shDR5 cells by using soluble wild-type TRAIL and a DR5-specific TRAIL variant. Next, we studied the molecular events within the 5-FU induced apoptosis signaling cascade in more details. We found that the activation of the caspase-8 was almost the same in both HCT116wt and HCT116shDR5 cells. In the death receptor induced apoptosis pathway, the caspase-8 is recruited to form the DISC (death-inducing signaling complex). During cytotoxic drug-induced apoptosis it has been suggested that caspase-8 can be activated independently of death receptors (Engels et al., 2000; Ferreira et al., 2000; Wesselborg et al., 1999). Similarly, we found that caspase-8 activation in response to 5-FU is independent of death receptor and is located upstream of caspase-3 activation, because the time point of caspase-8 activation is earlier than caspase-3 activation. Therefore there might be another mechanism and/or complex similar to the DISC that can activate caspase-8 in response to 5-FU. Very recently, a novel cell death-inducing complex named “Ripoptosome” has been identified, which can be formed in response to cytotoxic stimuli and independent of ligand/receptor systems (Tenev et al., 2011).

In our study, although the caspase-8 activation was not affected, we observed that caspase-3 activation was attenuated in HCT116shDR5 cells. Caspase-3 plays a central role in the execution of cell death. Loss of caspase-3 inhibits drug-induced apoptosis and reconstitution of capase-3 can reverse drug resistance in breast cancer cells with acquired drug resistance (Friedrich et al., 2001). The activation of

executioner caspases like caspase-3 is a two-step mechanism. First, an initial proteolytic cleavage is needed, which can separate the large subunit from the small subunit. Usually, the activity of the caspase is still inhibited by its own prodomain and removal of the prodomain is a prerequisite for full enzymatic activity and occurs in a autocatalytic step (Roy et al., 2001). In addition to being activated by caspase-8, caspase-3 can also be activated by caspase-9 which binds to cytochrome-c released from mitochondria and Apaf-1 to form a complex called the "apoptosome".

We concluded that the resistance of HCT116shDR5 to 5-FU was due to caspase-3 activation being inhibited. To elucidate the underlying mechanisms, we analysed the upstream factors involved in caspase-3 activation.

3.2 Mitochondrial pathway is inhibited in HCT116shDR5 cells after 5-FU treatment

Cells can be classified into type I or type II (Scaffidi et al., 1998). In type I cells, the activation of caspase-8 can fully activate executioner caspase-3 and induce apoptosis. In type II cells, activation of caspase-8 only is not sufficient, therefore the mitochondrial pathway is required to enhance caspase-8 cleavage and apoptosis. A pivotal event in the mitochondrial pathway is the release of mitochondrial proteins such as cytochrome-c and Smac/DIABLO. The released cytochrome-c with dATP and Apaf-1 can form an oligomeric complex named "Apoptosome". This complex activates caspase-9, followed by activation of the effector caspases like caspase-3, caspase-6 and caspase-7 (reviewed by Loeffler and Kroemer, 2000; Susin et al., 2000b).

In our study, we detected the release of cytochrome-c from mitochondria into the cytosol in HCT116wt cells in response to 5-FU, however this release cannot be detected in HCT116shDR5 cells. At the same time, we also observed a lack of release of Smac/DIABLO into the cytosol in HCT116shDR5 cells which mean that the activity of XIAP is not neutralized leading to inhibition of caspase-3 and blocking of apoptosis. Smac/ DIABLO is a structural homologue of the *Drosophila* proteins, Reaper, Hid and Grim. Normally, Smac/DIABLO is localized in mitochondria and is

released into the cytosol during the early stages of apoptosis. Smac/DIABLO can antagonize XIAP, thereby rescuing the caspase activity (Ekert et al., 2001). The physiological function of Smac/DIABLO is still unknown and *DIABLO*-/- mice appeared normal (Okada et al., 2002).

XIAP belongs to the Inhibitor-of-Apoptosis Proteins (IAP) family, whose members contain baculoviral repeat (BIR) domains and, in some cases, a zinc RING-finger domain (reviewed by Deveraux and Reed, 1999). IAPs are thought to be able to inhibit apoptosis through direct inhibition of caspases. To date, XIAP is the only member of the IAP family to directly inhibit the cascade (Wright and Duckett, 2005). It is still controversial as to whether other members of the IAP family can also inhibit capases, although cIAP1 and cIAP2 have been shown to bind to but not necessarily inhibit caspases (reviewed by Eckelman et al., 2006).

XIAP can inhibit the active form of caspase-9 (p35/p12 heterotetramer) through an interaction between its BIR3 domain and the small subunit (p12) of caspase-9 (reviewed by Deveraux and Reed, 1999; Srinivasula et al., 2001). XIAP can also inhibit active caspase-3 and -7 through the BIR2 domain along with a few critical residues (Huang et al., 2001; Riedl et al., 2001). XIAP maintains caspase-3 primarily in its partially processed p20/p12 form, whereas in the presence of Smac/DIABLO, p20/p12 caspase-3 disassociates from XIAP and as a result, can undergo autocatalytic processing to remove its prodomain and generate the fully mature p17/p12 form (Sun et al., 2002). So XIAP can inhibit both extrinsic and intrinsic pathways by inhibiting effector caspases (Bratton et al., 2001).

It has been found that XIAP down-regulation in colon carcinoma cells allowed a conversion from a Type II to a Type I cell in response to TRAIL in short-term apoptosis assays (Ndzengue and Bann, 2008). Additionally, depletion of XIAP can render hepatocytes to sensitise apoptosis independent of Bid, and afforded a switch from Type II to Type I signaling (Jost et al., 2009). Another study in Jurkat cells generated similar results (Shawgo et al., 2009). Hence, this suggests that in Type II cells, the mitochondrial pathway for XIAP neutralization rather than caspase-9 activation is more important in the process of apoptosis induction.

We used an adenoviral vector expressing a small hairpin directed against XIAP (AdshXIAP) to knock-down XIAP to mimic the release of Smac/DIABLO. Knock-down of XIAP in HCT116shDR5 cells resulted in increased apoptosis reaching almost the levels of HCT116wt cells. At the same time, the caspase-3 activity was also increased in HCT116shDR5 cells transduced with AdshXIAP as compared with cells treated with the AdshEGFP control vector. In our experiments, down-regulation of XIAP alone is enough to rescue apoptosis. Hence, although it was generally assumed that type II cells require caspase-9 activity to increment effector caspase activation, there is a growing body of evidence that Smac/DIABLO release and XIAP inhibition are more important. Our data confirmed that the inhibition of the mitochondrial pathway is the cause for the resistance of HCT116shDR5 to 5-FU.

There are several models that can explain the release of mitochondrial proteins during apoptosis. One model is that permeability transition pore, which is composed of ANT, cyclophilin D and VDAC (voltage-dependent anion channel), is needed for the protein release. Another model is the swell of inner membrane caused by ions and water entry into matrix. So the outer matrix can physically rupture (Feldmann et al., 2000; Vanderheiden, 1999). Both of these models conflict with the results of studies of Bax:Bak double deleted mice (Dewson and Kluck, 2009). Currently, it is widely accepted that pro-apoptotic Bcl-2 family members like Bak and Bax can multimerize to form a pore in the outer mitochondrial membrane (Dewson and Kluck, 2009). Normally, Bax is mainly located in the cytosol and recruited to the OMM (outer mitochondrial membrane) when it receives the signals of apoptosis (Gilmore et al., 2000; Goping et al., 1998). Bax deficiency can cause colorectal cancer cells to become resistant to 5-FU (Zhang et al., 2000). However, even in healthy cells there are still detectable amounts of Bax on the mitochondria. Furthermore, Bax movement to mitochondria does not guarantee a cell to undergo apoptosis (Nechushtan et al., 1999; Valentijn et al., 2003). So Bax must undergo conformational change to activate apoptosis. By using a specific Bax antibody that is able to recognize the conformational change of Bax, we observed that the Bax conformation changed in HCT116wt cells, which was blocked in HCT116shDR5 cells after 5-FU treatment.

The activation of Bax is tightly regulated by the Bcl-2 family members. For example; pro-apoptotic Bid, Bim and Bad can positively activate the Bax and Bak while the anti-apoptotic Bcl-2 and Bcl-xl can inhibit it (Letai et al., 2002; Willis et al., 2005). As for the mechanism of Bax activation is still controversial. Currently, two models have been proposed, one is that Bax is constantly being checked by the anti-apoptotic activity of Bcl-2 like proteins. This inhibition can be relieved by pro-apoptotic BH3-proteins. The other model is that BH3-proteins like Bid, Bim and Puma directly activate Bax, but anti-apoptotic Bcl-2 proteins sequester this process in healthy cells. Not just the BH-3 proteins, two novel activators have been identified recently. One is named IRF3 (interferon regulatory factor 3), which is a transcription factor that can induce interferon expression and activate Bax and/or Bak (Chattopadhyay et al., 2010). Another one was called endophilin B, which can activate Bax/Bak independently of its role in mitochondrial fission (Etxebarria et al., 2009). In addition, non-protein factors such as mild heat, detergents and high pH could also activate Bax and Bak (Khaled et al., 2001; Pagliari et al., 2005). Among all those factors that can activate Bax, the tBid which is generated by the cleavage of Bid is the most potent one.

Bid is a pro-apoptotic Bcl-2 family member. There is still little information about the physiological role of Bid. *Bid*-/- mice develop normally, but display symptoms similar to human CMML (chronic myelomonocytic leukemia) with deregulated myeloid homeostasis (Zinkel et al., 2003). *Bid*-/- deficient MPCs (myeloid progenitor cells) show increased mitomycin c-induced chromosomal abnormalities (Zinkel et al., 2005). Mounting evidence indicates that Bid has a unique role in the connection of the extrinsic pathway to the intrinsic pathway. Initially, Bid was found to be the substrate of caspase-8 in the Fas/FasL signaling pathway (Li et al., 1998; Luo et al., 1998). Later, Bid was also found to be cleaved in response to other death stimuli like staurosporine, UV radiation, etoposide etc (reviewed by Slee et al., 2004). During this process, Bid can be cleaved within an unstructured loop to expose a new amino-terminal glycine that undergoes post-translational myristoylation (Zha et al., 2000). The myristoylated Bid can then translocate to the mitochondria where

truncated tBid can insert into membrane and activate Bax (Zha et al., 2000). Additionally, Bid without cleavage has also been shown can be targeted to mitochondria through its high affinity binding to the mitochondria-specific lipid cardiolipin, although whether this binding can activate Bax is still unknown (Lutter et al., 2000). tBid can activate Bax through two patterns. On the one hand, tBid can open the N-terminal moiety of Bax directly (Cartron et al., 2004). On the other hand, tBid translocation to mitochondria can promote Bax recruitment and activation (Chipuk and Green, 2003; Giam et al., 2008; Walensky et al., 2006). It has also been proposed that Bid might have the capacity to form membrane pores directly or through the binding to other channel proteins (Chou et al., 1999; McDonnell et al., 1999; Schendel et al., 1999). The analysis of the solution structure of Bid revealed that Bid also contains regions that structurally resemble bacterial toxins which can form pores (Schendel et al., 1999).

Furthermore, Bid can change the lipid composition of the outer mitochondrial membranes through exertion of its lipid transfer activity (Esposti, 2002). Fukazawa et al, showed that overexpression of Bid can induce chemosensitization even in the absence of functional p53 (Fukazawa et al., 2003). In addition, Bid was found to be a target of p53 and Bid-null mice embryonic fibroblasts are more resistant to 5-FU (Sax et al., 2002). Recently, Bid has been found in a DNA damage sensor complex following replicative stress thus may have a role in the stabilization of replication fork (Liu et al., 2011). Bid can also induce several other prominent mitochondrial dysfunctions, like mitochondrial depolarization (Zamzami et al., 2000), mitochondrial cristae reorganization (Ding et al., 2004; Kim et al., 2004b; Scorrano et al., 2002) and mitochondrial permeability transition (Zhao et al., 2003). All these findings suggest that Bid plays an important role in the intrinsic pathway.

In line with previous reports, we found that the Bid can be cleaved into tBid in the process of apoptosis induced by 5-FU. Till now, numerous studies have been carried out to investigate the role of Bid during DNA damage-induced apoptosis. Most of those studies showed that Bid is indispensable for DNA damage-induced apoptosis, whereas only one study reported that Bid plays no role in DNA damage-induced

apoptosis (Kaufmann et al., 2007). The difference of genetic background may explain the discrepancy, because this study used cells that were cultivated from *Bid*-/- mice with a C57BL/6 background.

We found that Bid cleavage was blocked in HCT116shDR5 cells. It was demonstrated that murine Bid could be phosphorylated by casein kinase I and II leading to inhibition of cleavage by caspase-8 (Desagher et al., 2001). In human cells, Bid could also be phosphorylated in response to DNA damage and diminishing of this phosphorylation increased susceptibility of cells to DNA damage (Kamer et al., 2005). Sustained phosphorylation of Bid caused by ATM kinase can also confer resistance to FasL/Fas-induced apoptosis *in vivo* (Vogel et al., 2006). Since we found that Bid cleavage was inhibited in HCT116shDR5 cells, we checked the Bid phosphorylation status and found that Bid Ser78 site kept phosphorylation in HCT116shDR5 but not HCT116wt cells after 5-FU treatment. In order to test if this phosphorylation was caused by ATM kinase, we used a specific ATM inhibitor. We found that the Bid cleavage and apoptosis rate could be rescued to some extent although it could not be fully rescued after inhibition of the ATM kinase. It might be due to some other kinases being able to phosphorylate Bid at other sites. For example, phosphorylated murine Bid at Thr59 site, which is regulated by CK2, can cause resistance to caspase cleavage in *in vitro* assays (Desagher et al., 2001). Using a CK2 inhibitor DRB or overexpression of a dominant-negative CK2a catalytic subunit can overcome this resistance (Hellwig et al., 2010). Suppression of ATM kinase activity can sensitise cancer cells to death, as has been previously described (Rainey et al., 2008; Truman et al., 2005). Currently, targeted cancer therapy based on the inhibition of ATM activity is currently being carried out in clinical trials (O'Connor et al., 2007). Thus our findings provide more insight into this potential therapeutic approach.

Phosphorylation is an important and reversible mechanism that can regulate protein function. Nearly one third of mammalian cellular proteins can be modified by protein phosphorylation (reviewed by Blom et al., 1999). Most proteins are found to be phosphorylated at serine and/or threonine residues, and some proteins involved in signal transduction can also be phosphorylated at tyrosine sites (reviewed by Blom et

al., 1999). Phosphorylation can confer resistance to proteolytic cleavage in many proteins. For example, signal-induced phosphorylation of two serine residues just beyond the aspartic acid residue which is recognized by caspase-3 can inhibit IκB cleavage by caspase-3 (Barkett et al., 1997).

It is quite common that Bcl-2 family members are subjected to phosphorylation modification. For example, dependent on the target sites, phosphorylation can stabilize or enhance the degradation of Mcl-1 (Kodama et al., 2009; Maurer et al., 2006). Phosphorylation of Bcl-xl can inhibit its anti-apoptotic ability (Upreti et al., 2008). Bax can be inactivated by phosphorylation (Gardai et al., 2004). Bcl-2 has two sites Thr74 and Ser87 that can be phosphorylated by ERK. Phosphorylation on these two sites can also prevent the proteolytic degradation thereby increasing the anti-apoptotic ability of Bcl-2 (Breitschopf et al., 2000; Dimmeler et al., 1999). The exact mechanism of how phosphorylation can cause resistance to caspases is still unknown. It is possible that phosphorylation decreases accessibility of protease to its recognition site. The phosphorylation can also change the conformation of proteins thereby affecting the protein-protein interaction. For example, phosphorylation of the p53 amino-terminus can disrupt the MDM2-p53 interaction (Shieh et al., 1997; Unger et al., 1999).

In Bid, there are several potential phosphorylation sites around the caspase and granzyme cleavage sites. Although both caspase-8 and caspase-3 have the ability to cleave Bid (Engels et al., 2000), biochemical studies shown that Bid is a better substrate for caspase-8 than for caspase-3 (Esposti, 2002; Li et al., 1998). Therefore, although we observed less activation of caspase-3 in HCT116shDR5 cells, it is unlikely to be the reason for inhibition of Bid cleavage but rather the consequence of it. Besides caspases, Bid is also susceptible to cleavage by a variety of other proteases like other granzyme-B, lysosomal proteases and calpain (Bossy-Wetzel and Green, 1999; Chen et al., 2001). Whether phosphorylation affects their cleavage or not is still unknown. In our system, other proteases were not likely involved in Bid cleavage, because the Bid cleavage was completely abolished in HCT116shcaspase-8 cells.

In summary, here we found that Bid's Ser78 site phosphorylation plays an important role in cellular responses to 5-FU. This phosphorylation might be a protective response and can raise the threshold at which mitochondria releases proteins to induce cell death.

3.3 JNK pathway is activated in HCT116wt cells but not HCT116shDR5 cells after 5-FU treatment

After 5-FU treatment, we also observed JNK activation in HCT116wt cells but not in HCT116shDR5 cells. JNK activation is involved in many biological responses such as cytokine production, the inflammatory response, actin reorganization, cell transformation and metabolism (reviewed by Krishna and Narang, 2008). JNK exerts its function through phosphorylation of substrates in a stimuli-specific and cell type-specific pattern. A spectrum of stimuli including UV and γ-irradiation, inflammatory cytokines such as TNF-α, DNA damage agents (etoposide, 5-FU, cisplatinum) and heat shock can trigger JNK activation. The activation of JNK may further induce more extensive DNA damage thus amplifying the death response (Zou et al., 2007). The role of JNK in apoptosis can be both positive and negative, depending on the cell type and stimuli context (reviewed by Chang and Karin, 2001; Davis, 2000). Strong and sustained JNK activation is generally associated with enhancement of apoptosis, whereas transient JNK activation can result in cell survival (reviewed by Chang and Karin, 2001).

To establish the role of JNK in 5-FU caused apoptosis in HCT116wt cells, we made use of a specific JNK inhibitor named SP600125. The presence of SP600125 attenuated 5-FU induced apoptosis of HCT116wt cells, indicating that 5-FU-induced apoptosis is JNK-dependent in HCT116wt cells. JNK activation may contribute to apoptosis through both the extrinsic and intrinsic pathway. For example, JNK signaling can increase apoptosis through the up-regulation of FasL (Chen and Wilson, 1998). JNK can also regulate the mitochondrial pathway. It has been reported that activation of JNK can induce cytochrome-c release and caspase activation in pramanicin-treated Jurkat cells (Kutuk et al., 2005). Other reports suggested that JNK

can directly regulate the translocation of Bax from the cytoplasm to the mitochondria in response to various stimuli (Stadheim and Kucera, 2002; Yuan et al., 2003). Blocking JNK by either dominant-negative mutant (DN-JNK) or treated with the JNK inhibitor SP600125 can abrogate the release of Smac/DIABLO, caspases activation and apoptosis (Chauhan et al., 2003). Inhibition of JNK can also abolish the activation of caspase-3 and PARP cleavage induced by GSE (grape seed extract) (Gao et al., 2009). In addition, JNK inhibitor or JNK siRNA can abrogate 2-methoxyestradiol-mediated caspase activation and apoptosis (Gao et al., 2005a). JNK might also delay caspase-9 activation through inhibiting the apoptosome formation, thus attenuating caspase-3 activation (Tran et al., 2007). All these data indicate that JNK plays a critical functional role in the mitochondrial pathway, although the exact mechanism is still unclear.

The mitochondrial pathway is tightly regulated by proteins of the Bcl-2 family. Numerous lines of evidence indicate that JNK can also regulate the status of Bcl-2 proteins. For example, the conformational change and the mitochondrial redistribution of Bax were diminished in JNK deficient cells (Lei et al., 2002). Bax might be a substrate of JNK although the site of phosphorylation has not been found yet (Kim et al., 2006a). The potential Bax phosphorylation mediated by JNK could cause Bax activation (Kim et al., 2006a). The BH3-only protein Bad can also be phosphorylated by JNK *in vitro*. Distinct from Akt and PKA that phosphorylate Bad at Serine sites, a novel Thr201 residue can be phosphorylated by JNK (Yu et al., 2004). The function of Bad phosphorylation is still controversial, because in transfection assays Bad-mediated apoptotic activity can be either increased or decreased by this type of phosphorylation (Donovan et al., 2002; Yu et al., 2004). The pro-apoptotic phosphorylation of Bad has also been questioned (Zhang et al., 2005). JNK can phosphorylate structurally related BH3-only proteins like Bmf and Bim too (Lei and Davis, 2003; Putcha et al., 2003). The phosphorylation of Bmf and Bim has been reported to lead to increased apoptosis. Interestingly, the transcription of Bim can also be regulated by JNK, through JNK-dependent AP-1 activity, leading to JNK-dependent apoptosis (Harris and Johnson, 2001; Whitfield et al., 2001).

Anti-apoptotic members of the Bcl-2 family like Bcl-2, Bcl-xl, and Mcl-1 can also be phosphorylated by JNK *in vitro*. Transfection studies indicate that phosphorylation of these proteins can suppress the anti-apoptotic functions (reviewed by Liu and Lin, 2005). At the same time, overexpressed Bcl-2 and Bcl-xl could suppress the activation of JNK as well as the apoptosis (Chen and Tan, 1998). So the cross-talk between the JNK and Bcl-2 family members may exist widely. As for the JNK regulation of the status of Bid, it has been reported that after being exposed to UV radition, Bid can be proteolytically processed in a caspase independent but JNK dependent manner (Tournier et al., 2000). Inhibition of JNK by NAC (N-acetyl-L-cysteine) can decrease the cleavage of Bid without any effect on the caspase-8 processing (Ohtsuka et al., 2003). Additionally, a new study indicate that with inhibited NF-κB signaling, TNF can cause JNK-dependent processing of Bid to a novel form (jBid) that can specifically induce the release of Smac/DIABLO (Deng et al., 2003). However, the mechanism employed by JNK to regulate Bid cleavage is still unknown. Here we found that blockage of JNK activation can inhibit Bid cleavage, this added to our knowledge about the relationship between Bid and JNK.

3.4 Wip1 can be up-regulated by JNK activation and then in turn dephosphorylate Bid

We revealed that JNK activation could up-regulate the phosphatase Wip1 which in turn could negatively regulate the Bid phosphorylation status. After DNA damage, Wip1 can be transcriptionally induced by p53 (Fiscella et al., 1997). A cyclic AMP response element (CRE) and a p53 response element were found in the 5'-UTR of the *WIP1* gene (Rossi et al., 2008; Song et al., 2010). Binding of CREB to CRE can cause basal expression of Wip1 and directs transcription initiation at upstream sites, while the p53 response element is required for the p53-dependent transcription. Many pathways like JNK and NF-κB are also able to induce the up-regulation of Wip1 (Lowe et al., 2010; Song et al., 2010). It has also been found that Wip1 could be negatively regulated by a micro-RNA, miR-16 (Zhang et al., 2010). In line with a recent report indicating that JNK activation can activate the Wip1 (Song et al., 2010),

we found that JNK pathway and p53 cooperate to activate Wip1. When compared to the HCT116wt cells, the level of Wip1 transcripts and proteins increased at a much lower pace in HCT116shDR5 cells although the amount of the p53 made no difference. In stressed cells, JNK phosphorylates and activates c-Jun, ATF-2 and enhances their transcriptional regulation of target genes (Buschmann et al., 2001). From our results, we concluded that JNK activation is required for the early induction of Wip1. Noteworthy is that the lack of JNK activation or p53 alone only delays Wip1 expression. Only depletion of both p53 and JNK activation can result in total inhibition of Wip1 induction. The JNK pathway may not only up-regulate Wip1.

Wip1 belongs to the Type 2C protein phosphatases family (PP2C). Wip1-deficient mice have some postnatal abnormalities, including male reproductive organ atrophy and a reduction in male fertility and longevity (Choi et al., 2000). Several other PP2C family members have been reported to dephosphorylate Bcl-2 family members. For example, PP2Cα and PP2Cß were able to dephosphorylate and thus activate Bad (Klumpp et al., 2002; Krieglstein et al., 2003). In mammalian cells, the phosphatase Wip1 has been shown to act on multiple ATM substrates such as p53, Chk1, Chk2 (reviewed by Le Guezennec and Bulavin, 2010; Lu et al., 2008a). In addition, it has also been demonstrated that Wip1 can directly dephosphorylate ATM at Ser1981 and this is critical for resetting ATM phosphorylation as cells face DNA damage (Shreeram et al., 2006).

Here, we found that Wip1 can dephosphrylate Bid. The activity of Wip1 on this site is consistent with a previous report that showed Wip1 targets other ATM substrates containing the canonical pS/TQ site (Kim et al., 1999). We showed that deficiency of Wip1 phosphatase results in accumulation of phosphorylated Bid. There are three possible mechanisms regarding how Wip1 dephophorylates Bid: (1) Bid is directly dephosphorylated by Wip1. (2) Wip1 dephosphorylates Bid indirectly through dephosphorylation and inactivation of ATM. (3) a combination of both. Further research is needed to unearth the details. Since Bid phosphorylation might also regulate the cell cycle and DNA damage checkpoints, and Wip1 has also been shown to target multiple DNA damage checkpoint proteins, it would also be interesting to

investigate the effect of Wip1 on the cell-cycle regulating activity of Bid.

In contrast to a study indicating that depletion of Wip1 could increase apoptosis (Xia et al., 2009), we found the absence of Wip1 confers resistance to 5-FU. This could be due to cell type and stimulus-specific response. Xia et al. used different cell lines and stimuli, therefore the experiments are not comparable. In previous reports, Wip1 suppressed apoptosis mainly through down-regulation of p53 (Bulavin et al., 2004; Takekawa et al., 2000). However, in our study we failed to find any decrease of p53 activity after induction of Wip1. Very recently, a report was published, which is very similar to our findings, suggesting that Wip1 can promote apoptosis in absence of p53 (Goloudina et al., 2012). Furthermore, it has also been shown that Wip1 could inhibit the NF-κB pathway by directly dephosphorylating the p65 subunit on Ser536 (Chew et al., 2009). NF-κB can increase anti-apoptotic gene expression and abolish the pro-apoptotic signaling ability of p53, which leads to cell survival (reviewed by Hayden and Ghosh, 2008; Perkins, 2007). Taken together, the role of Wip1 may be diverse and dependent on cell type and stimulus. Our study uncovers a pro-apoptotic function of Wip1 that acts through Bid.

p53 is able to activate both cell-cycle arrest and apoptosis in most cell types in response to DNA damage. Over 50% of human tumours have a mutation or depletion in p53 and because inactive p53 cannot mediate a damage-induced apoptotic response these tumours are chemo- and radio-resistant (Baker et al., 1989; Nigro et al., 1989). *In vivo* studies using the p53-null mice and cells confirmed that p53 can mediate apoptosis. The p53-null mice embryonic fibroblast cells (MEFs) are also resistant to oncogene overexpression and chemotherapeutic agents induced apoptosis (Clarke et al., 1993; Lowe et al., 1993a). The depletion of p53 in thymocytes and intestinal stem cells can confer resistance to radiation-induced apoptosis (Lowe et al., 1993a). As for the mechanism, several targets have been proposed to mediate p53-dependent apoptosis through transcriptional activation, including DR5, Bax, PIDD, Noxa, Puma, Bak, Bid, Fas, DR4, and caspase-6 (Burns et al., 2001; MacLachlan and El-Deiry, 2002; Muller et al., 1998; Wu et al., 1997). In addition to transcriptional activation, p53 may also contribute to apoptosis through transcriptional repression of

anti-apoptotic genes such as *Survivin* and *Bcl-2* (Hoffman et al., 2002; Wu et al., 2001). p53 can also regulate apoptosis independently of its transcriptional activity. Similar to pro-apoptotic Bcl-2 family members, p53 can translocate to mitochondria and directly activate Bax (Chipuk and Green, 2003). After DNA damage, p53 can also activate Bak through neutralisation of Mcl-1, which is an anti-apoptotic Bcl-2 family member (Erster and Moll, 2004).

Here we found that in p53-deficient cells, DR5 can still be up-regulated but in a delayed manner. It might be because DR5 can also be induced by transcription factors other than p53. For example, DR5 up-regulation can be mediated by the transcription factor STAT1 (signal transducer and activator of transcription 1) (Meng and El-Deiry, 2001). When cells were treated with glucocorticoids and interferon (IFN)-γ, which can induce STAT1, DR5 expression was increased in cells with mutated p53. As an oncogenic transcription factor, Myc could also increase DR5 expression and antagonise increased apoptosis (Wang and El-Deiry, 2004). The transcription factor CCAAT/enhancer binding protein (CHOP) also plays a role in DR5 expression and is induced by the farnesyltransferase inhibitor, lonafarnib (Sun et al., 2007). Inhibition of CHOP expression can prevent the induction of DR5 expression by lonafarnib. In HCT116p53-/- cells, the Wip1 up-regulation can still be observed although in a delayed manner. However, in HCT116p53-/-shDR5 cells the Wip1 up-regulation was completely blocked. In line with previous reports, we also found that depletion of p53 or DR5 alone can confer relative resistance to 5FU in HCT116 cells (Bunz et al., 1999). Interestingly, concomitant loss of DR5 and p53 offers additional protection from apoptosis induced by 5-FU. The apoptosis rate of HCT116p53-/-shDR5 cells was similar to HCT116shwip1 cells, since the induction of Wip1 was completely inhibited in HCT116p53-/-shDR5 cells. In conclusion, our findings further confirmed the function of Wip1 in the promotion of apoptosis in response to 5-FU.

3.5 Molecular mechanism of JNK activation after 5-FU treatment

Earlier studies have unveiled the mechanisms of death-inducing ligands FasL and TRAIL to activate the JNK pathway (Sheridan et al., 1997; Wallach et al., 1999). However, there is little known about the mechanisms of how chemo-agents activate the JNK pathway.

Depletion of DR5 can cause block of JNK activation and this block can confer resistant to 5-FU. Therefore, further research about components of JNK pathway may be helpful to better understand the mechanism of JNK activation and provide hints for chemo-resistant mechanism. We used 293 cells as a model system to investigate this. Varfolomeev et al found that TRAIL activates JNK pathway through promoting the formation of a secondary signaling complex composed of FADD, RIP1 and TRAF2 (Varfolomeev et al., 2005). In addition, Micheau et al found that FADD is also indispensable for cytotoxic drugs induced apoptosis (Micheau et al., 1999). In their study FADD is indispensable for cell death induction and this secondary complex formation. Interestingly, our functional investigation also revealed that 5-FU requires FADD for cell death induction and JNK activation. So there might be a similar complex with FADD that is able to activate the JNK pathway.

We found that FADD was able to bind TRAF2, ASK1, RIP1, TRADD but not MAP4K5. ASK1 is a member of the MAP3K family that can specifically activate a cascade ending with JNK activation (Zhang et al., 2004). It can associate with TRAF2 leading to the activation of ASK1 (Nishitoh et al., 1998). However, to our knowledge, it has not been reported before that ASK1 can bind to FADD, it has been shown that ASK1 can form a high molecular mass complex (>1500kDa) through its C-terminal coiled-coil (CCC) domain (Noguchi et al., 2005). But the details of this complex are still unknown. TRAF2 is an adaptor protein that is able to bind to TNFR1. TRAF2 plays important role in JNK activation. For example, TNF-α induced JNK activation was significantly impaired in the absence of TRAF2 (Yeh et al., 1997). CD40-induced JNK activation was also dependent on TRAF2 in splenic B-cells (Ahonen et al., 2002). Different groups using *TRAF2* knockout mice or transgenic mice expressing a

dominant negative TRAF2 mutant protein have also demonstrated that TRAF2 is absolutely required for activation of JNK by TNF (Lee et al., 1997; Yeh et al., 1997).

As kinase pathway adaptors, TRADD and RIP1 have been reported to bind to DR4 and DR5 in overexpression model systems (Ashkenazi and Dixit, 1998). We confirmed that TRADD and RIP1 can also physically interact with FADD. Recently, it was found that DNA damaging agents such as etoposide, ionizing radiation, neocarcinostain, doxorubicin, or camptothecin can induce functional interaction of RIP1, FADD and Caspase-8 (Biton and Ashkenazi, 2011). So there might be a similar complex composed of FADD, ASK1, TRAF2, TRADD and RIP1, which may account for the activation of JNK by 5-FU. However, caspase-8 maybe not required for the formation of this complex, since we found that in caspase-8 deficient cells the JNK activation by 5-FU was not affected.

Until recently, the role of caspase-8 in kinase activation was still controversial, because published studies with the caspase-8 null mice mainly focused on its role in apoptosis signaling pathway, and little is known about the role of capase-8 in kinase activation *in vivo*. Studies in mutant Jurkat T cell lines with deficiency in caspase-8 revealed that caspase-8 is required for kinase activation by death receptor ligands (Wajant et al., 2000). At the same time, other studies utilizing siRNA techniques have also confirmed the requirement for caspase-8 in JNK activation by FasL, although TRAIL was not investigated (Imamura et al., 2004). In contrast, ectopic expression of a dominant-negative caspase-8 mutant did not interfere with activation of JNK by TNF, FasL, or TRAIL (Wajant et al., 1998). Therefore, the role of capase-8 in JNK activation is likely to be dependent on the type of cell and stimulus. MAP4K5 can form a complex when co-expressed with TRIP (TRAF interacting protein) and TRAF2 leading to activation of JNK (Chin et al., 1999). These findings make MAP4K5 a putative candidate to participate in the activation of JNK in our system. However, we failed to find any FADD bindings to MAP4K5.

In summary, we found FADD can interact with ASK1, TRAF2, TRADD and RIP1 but not MAP4K5, potentially comprising a complex leading to JNK activation. It would be interesting to further investigate the role of this potential complex.

3.6 Conclusions of Part I

Colorectal cancer is treated with various anti-cancer drug cocktails of which almost all include 5-Fluorouracil (5-FU). 5-FU exerts its effect by causing apoptosis in tumour cells. However, a problem in its clinical use is the development of chemo-resistance, which at the molecular level can be caused by apoptosis resistance. In the present study, we found that the characteristics of 5-FU-induced apoptosis in HCT116 cells are reminiscent of the death receptor-mediated apoptosis in so called type II cells where death receptor signaling to effector caspases is amplified by recruiting mitochondria into an amplification loop that involves Bid cleavage by caspase-8, Bax activation and the release of Smac/DIABLO. The intrinsic pathway is indispensible for 5-FU-induced apoptosis in HCT116 cells and silencing of DR5 can surprisingly cause the block of the intrinsic pathway. Further analysis revealed that the block of the intrinsic pathway was caused by inhibition of Bid cleavage and activation in the HCT116shDR5 cells. Thus, the molecular point of resistance is the Bid protein. To this end we demonstrated that cleavage of Bid and its activation may be negatively regulated by phosphorylation which is at least partially mediated by the ATM kinase. Furthermore, we also found that JNK is activated in the HCT116wt cells, but not in HCT116shDR5 cells after 5-FU treatment. JNK is involved in the process of regulating Bid phosphorylation. JNK activation can lead to the up-regulation of Wip1 which is a serine/threonine phosphatase of the type 2C protein phosphatatse family (PP2C). Wip1 can in turn antagonize the phosphorylation of Bid. Based on our findings, we now propose a model in which DR5 is not the initial inducer of cytotoxic drug-induced apoptosis, as activation of the initiator caspase-8 is not affected by silencing of DR5, but rather functions as a pace-maker through activation of JNK that in turn affords effective Bid processing and apoptosis (Figure 3.1).

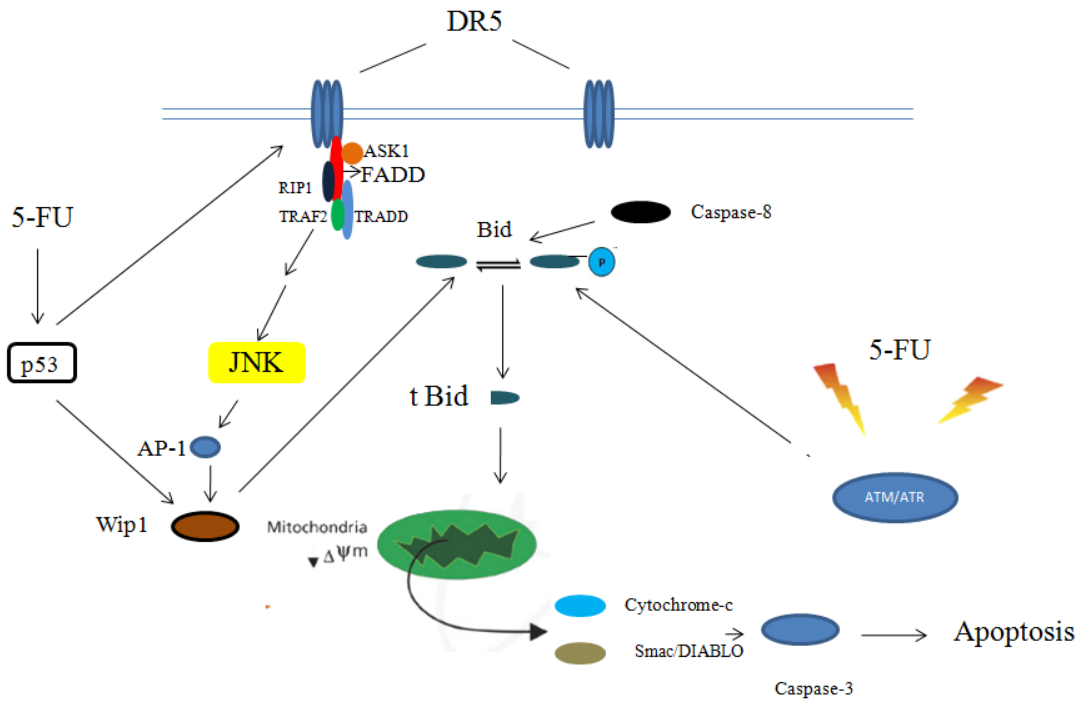


Figure 3.1 Proposed model of the DR5-regulated 5-FU induced apoptosis in HCT116 cells. After sensing cellular stress the ATM/ATR kinase is activated and Bid is phosphorylated. Phosphorylation of Bid causes resistance to the cleavage by capase-8. 5-FU-induced up-regulation of DR5 leads to the activation of JNK. Through binding to DR5, FADD and other components can form a complex, which is needed for the JNK activation. Activated JNK cooperates with p53 leading to the expression of Wip1 which can antagonize Bid phosphorylation. After being dephosphorylated, Bid can be cleaved into tBid, then move to mitochondria and trigger the intrinsic pathway.

3.7 Variants soluble TRAIL induce apoptosis in different cancer cells

Specifically inducing apoptosis in tumour cells but not normal cells is a promising therapeutic approach for cancer. TRAIL has been shown to induce apoptosis in a wide spectrum of cancer cells *in vitro* and to specifically limit tumour cell growth without killing normal cells and tissues *in vivo*. These results point to a strong potential of TRAIL as an anti-cancer weapon.

Full-length TRAIL is expressed as a trans-membrane protein and functions by binding to cell surface receptors of targeted cells (Ashkenazi and Dixit, 1999). Usually, secreted proteins have an advantage over non-secreted proteins as it generates access to more target cells. Therefore, a TRAIL protein secreted into circulation or culture supernatant may have better effects. In order to develop TRAIL as a secreted protein, we fused a secretion signal sequence derived from the human Fibrillin-1 (hFIB) protein to the TRAIL ectodomain sequence. Due to the requirement of trimerization of TRAIL for apoptotic activity, we added an isoleucine zipper (ILZ), a derivate of the leucine zipper, as a trimerization domain (Kim et al., 2004a). In addition, in order to remove the signal peptide, a Furin cleavage site (FCS) was added. Based on this secreted soluble TRAIL version we developed two variants that can selectively bind to DR4 and DR5, respectively.

Our DR5-selective variant was developed by introducing a mutation D269H. By crystal structure analysis, this variant was shown to have higher affinity to the DR5 receptor when compared to wild-type TRAIL (van der Sloot et al., 2006). This highly selective variant has been shown previously to be very efficient to induce cell death in an ovarian carcinoma xenograft mice model (Duiker et al., 2009). As for the DR4-selective variant, a mutation S159R was introduced. It has been shown that this mutation allows Arg-159 to create a hydrogen bond with the backbone oxygen atom of Cys-113 of DR4 (Reis et al., 2010). Therefore, this mutation can cause a loss in interaction energy between TRAIL and DR5 and a gain in interaction energy between TRAIL and DR4, offering an explanation for the increase in DR4 binding specificity (Reis et al., 2010).

In the present study, by using different cancer cell lines, receptor-selective behavior of these variants was demonstrated. Mounting evidence indicates that different cancer cells can display a preference for either DR4 or DR5 in apoptosis signaling. For example, colon and breast cancer cell lines mainly signal through DR5, whereas primary lymphoid malignancies mainly through DR4 (Kelley et al., 2005; van der Sloot et al., 2006). In our study, we found that A2780, HCT116, Colo205 and BxPC cells preferentially underwent apoptosis through DR5 while in Colo357 and Hela cells, apoptosis was signaled mainly through DR4. These findings indicate that targeting of DR4 or DR5 in different cancer cell lines can result in enhanced apoptosis. Therefore, these sTRAIL variants are also expected to have increased *in vivo* activity. However, the mechanism that causes different tumour cell lines to show different susceptibility toward DR4 or DR5 stimulation is still unknown. The relative contribution of each death receptor to TRAIL-induced apoptosis is not fully understood either. Usually, the expression levels of DR4 and DR5 receptors do not generally show a direct correlation with sensitivity to TRAIL (reviewed by Ashkenazi, 2002). For example, although CLL cells express DR4 and DR5 at similar levels, only DR4 was found to be mainly responsible for TRAIL signalling.

Taken together, it highlights the importance to indentify the TRAIL receptor subtype that may preferentially or exquisitely transmit the apoptotic signal in a given cell.

3.8 MSCs expressed sTRAIL variants induce apoptosis in cancer cells

TRAIL-based cancer therapies using rhTRAIL and delivery of TRAIL using a herpes simplex virus or and adenovirus have been described before (Lee et al., 2002; Shah et al., 2003). However, many problems still hamper the clinical application; such as systemic toxicity, the short biological half-life, insufficient delivery into outgrowing glioma cells infiltrating the brain parenchyma, and virus-mediated immune reactions (Ashkenazi and Herbst, 2008; Griffith et al., 2000). In recent years, many studies have shown that MSCs can be used in experimental tumour therapies, owing to their intrinsic anti-tumour properties or in combination with the expression of therapeutic transgenes. For example, injection of MSCs expressing full-length TRAIL can lead to growth retardation of human lung and breast carcinoma xenografts (Loebinger et al., 2009; Mohr et al., 2008). Various groups also have reported the use of MSCs producing sTRAIL in models of glioblastoma (Menon et al., 2009; Sasportas et al., 2009). In the present study, we designed adenoviral vectors that expressed the secretable form of trimeric TRAIL, which were then used to transduce MSCs to generate sTRAIL secreting stem cells named MSC.sTRAIL-wt, MSC.sTRAIL-DR4 and MSC.sTRAIL-DR5 respectively. After the mixture of such MSCs with tumour cells we found that the TRAIL variants had divergent effects on the different cancer cells. In line with the results we described above, in Colo205 and HCT116 cells MSC.sTRAIL-DR5 showed the best effects, whereas MSC.sTRAIL-DR4 induced more apoptosis in Hela cells.

3.9 Silencing of XIAP enhance the effects of MSC.sTRAIL in pancreatic cancer cells

Despite the potent tumour-specific apoptosis-inducing abilities of TRAIL, many malignant tumour cells are resistant to TRAIL. Therefore, identification and understanding of the factors that mediate TRAIL resistance in tumour cells are important and great efforts have been taken in the past years in this dimension. In our study, we found the pancreatic cancer cell lines Panc1 and PancTu1 are resistant to TRAIL. Pancreatic cancer is one of the leading causes of cancer deaths in the Western world with steadily increasing numbers (reviewed by Parker et al., 1997).

Numerous studies indicated that XIAP, which is known to be frequently up-regulated in cancers, can contribute to TRAIL-resistance in various cancer cell lines (Braeuer et al., 2006; Fulda and Debatin, 2004). In particular, pancreatic cancers are known to be aggressive and resistant to apoptosis at least in part owing to high XIAP levels (Vogler et al., 2008). XIAP blocks apoptosis by binding to and inhibiting caspase-3 and caspase-9. Moreover, XIAP has also been implicated in the metastasis process (reviewed by Mehrotra et al., 2010). It has been reported that silencing of XIAP by RNA interference (RNAi) can significantly enhance TRAIL-induced apoptosis in pancreatic cancer cells both *in vitro* and *in vivo* (Vogler et al., 2009; Vogler et al., 2008). XIAP knock-down can also sensitise to taxane therapy in prostate, lung and breast cancer models (LaCasse et al., 2006; McManus et al., 2004). Furthermore, silencing of XIAP can overcome the mitochondrial block caused by high anti-apoptotic Bcl-2 levels in metastatic SW620 cells (Ndozangue-Touriguine et al., 2008).

Our group also found that knock-down of XIAP can significantly reduce the metastatic properties (Mohr et al., 2010). Even in the rather TRAIL-sensitive cell line Colo357, knock-down of XIAP could further increase the TRAIL-induced cell death (Vogler et al., 2007). All these findings make XIAP an attractive target for cancer therapy. To this end, we generated stable XIAP knock-down clones by RNAi in the two different pancreatic cancer cells line (Panc1.shXIAP and PancTu1.shXIAP) and treated them with our array of MSC.sTRAIL variants. We found that in contrast to the

wild-type parental cells, XIAP knock-down pancreatic tumour cells were sensitive to the effects of MSC-produced sTRAIL. We found that PancTu1.shXIAP responded better to DR4 specific sTRAIL, which is in line with a previous report (Stadel et al., 2010). In contrast, MSC.sTRAIL-DR5 induced more apoptosis in Panc1.shXIAP cells. Thus, we concluded that pancreatic tumour cells do not exclusively undergo apoptosis via DR4, although it may be the case for the majority of pancreatic cancers. Further studies are needed to clarify the TRAIL-receptor preference in pancreatic cancer cells. Currently, several XIAP inhibitors are in clinical tests. The therapeutic potential of MSCs is also being tested in a number of phase I/II/III clinical trials (reviewed by Bernardo and Fibbe, 2012). The outcome of these trials was generally positive and the application of MSCs was safe and without adverse effects (Salem and Thiemermann, 2010). Taken together, we have illuminated the therapeutic potential of MSCs expressing and secreting TRAIL-receptor-specific variants of sTRAIL in cancer.

3.10 Combination treatment with 5-FU can enhance the effects of MSC.sTRAIL

In order to increase the effects of MSC-mediated sTRAIL further we pretreated tumour cells with 5-FU. 5-FU is principally used in the treatment of colorectal cancer, in which it has been the established form of chemotherapy for decades. The synergism of TRAIL and 5-FU has been described in numerous reports (Mizutani et al., 2002; von Haefen et al., 2004). In the present study, we tested 5-FU pre-treatment followed by MSC.sTRAILs in HCT116 colorectal cancer cells. We found that following the treatment with low doses of 5-FU, we detected greatly enhanced apoptosis when the HCT116 cells were subsequently subjected to MSC.sTRAIL. This finding may be of clinical relevance since high concentrations of 5-FU are toxic *in vivo*, and this synergy was achieved with sub-toxic concentration of 5-FU. As for the molecular mechanisms behind this synergic effect, it could be due to the up-regulation of TRAIL receptor expression and/or down-regulation of the anti-apoptotic protein c-FLIP (Ganten et al., 2004; Ganten et al., 2005). 5-FU could also enhance the expression of Bax. The up-regulation of Bax can in turn enhance the apoptotic ability of TRAIL (Nita et al., 1998). It has also been proposed that 5-FU treatment could enhance the recruitment of FADD and pro-caspase-8 to the DISC, while FLIP recruitment was decreased (Lacour et al., 2003).

In our scenario, it is more likely due to the up-regulation of DR5 caused by 5-FU. Although 5-FU is known to activate both DR4 and DR5 (Ganten et al., 2004), we found DR5 to be induced significantly more than DR4. We also found that synergistic effects of TRAIL and 5-FU can be seen in both HCT116wt and HCT116shDR4 cells, while they were the most potent in the HCT116shDR4 cells treated with MSC.sTRAIL-DR5. At the same time, the synergistic effect can barely be observed in the HCT116shDR5 cells due to the lack of DR5. It indicates that a combination of 5-FU with a specific TRAIL variant can give rise to optimised tumour cell killing activity.

3.11 Conclusions of Part II

TRAIL is a promising cancer therapy owing to its tumour specificity. However, many cell lines derived from a variety of tumour types show resistance to TRAIL-mediated apoptosis, thus limiting the efficacy of TRAIL therapy. Here we showed that different tumour cell lines preferentially transduce apoptosis through one of the death-inducing TRAIL receptors but not the other. Thus using death receptor-selective TRAIL can offer a better apoptosis-inducing effect in specific cells. In addition, we also showed that through the combination of TRAIL with chemotherapeutics like 5-FU or down-regulation of XIAP, the apoptosis-inducing ability of TRAIL could be enhanced. Furthermore, the capacity of MSCs transduced with adenoviral vector expressing TRAIL for inducing apoptosis in different tumour cells supports the potential of these cells to become a powerful delivery vector for the treatment of cancer. These findings are of great interest for the clinical application of stem cells and/or TRAIL-based cancer therapy.

CHAPTER4 FUTURE PERSPECTIVE

4.1 Future perspective

Collectively, the results from part I suggest that loss of DR5 can lead to inhibition of JNK activation and Bid phosporylation resulting in resistance to cytotoxic drug induced apoptosis. Therefore, DR5, JNK, Wip1 and Bid phosphorylation status would be valuable diagnostic tools to predict responsiveness of cancer cells to cytotoxic drugs. Moreover, it would be interesting to examine whether ATM/ATR is the sole Bid-kinase that is implicated in drug-resistance. Other putative kinases could be interesting new targets. We also found that JNK-dependent up-regulation of Wip1 could dephosphorylate Bid and restore the process of apoptosis. How Wip1 acts on Bid and the mechanism of how phosphorylation can inhibit the activation of Bid are still elusive and more investigations into this would help us to better understand the role of Wip1 and the regulation of Bid activation. Furthermore, the role of DR5 in the activation of JNK after 5-FU treatment is also unclear. Mechanistically, further analysis of the DR5-associated components that assemble upon 5-FU treatment and lead to JNK activation is one of the next challenges. To this end, JNK can also be activated by TRAIL or TNF. However, it appears that different JNK isoforms are activated (data not shown). Whether these different JNK isoforms can also increase the expression of Wip1 is unknown. Identification of these differences will increase our understanding of the apoptosis-promoting function of JNK.

In part II, we demonstrated that sub-toxic concentrations of a cytotoxic drug could enhance the apoptosis-inducing activity of MSC-delivered soluble TRAIL, especially when normal TRAIL was substituted with a DR5-selective variant. As shown in Figure 4.1, this synergistic effect of the combined treatment was caused by enhanced DR5 expression. Simultaneously, we found that DR5 is able to activate the JNK pathway, so the application of MSC.sTRAIL and in particular MSC.sTRAIL-DR5 may augment the activation of JNK, which might also contribute to the synergistic effect. In this context, JNK activation would accelerate the cross-talk between the extrinsic and intrinsic pathway via Bid. It would be interesting to test whether a combination treatment of 5-FU with MSC.sTRAIL-DR5 can give

rise to sensitisation via JNK and not only via increased caspase-8 activation. However, there are several other questions that remain unresolved. It is still unknown how certain tumour cells preferentially transduce apoptosis via one TRAIL receptor but not the other. So far, we have been unable to predict which receptor-selective treatment would be the most appropriate. A variant TRAIL, which can activate both death receptors but not bind to decoy receptors, might be the optimal option. TRAIL resistance mechanisms are also not fully understood. Besides XIAP, there are other factors that might affect the responsiveness, such as NF-κB, ERK, and p38 pathways. These alternative signaling events may impact on the cellular response to TRAIL and could be further explored as potential resistance mechanisms. As for the delivery method, a remaining question is whether an alternative system could be more efficient than the adenoviruses transduced MSCs to deliver and express TRAIL? Research into these questions might be helpful to reveal the full potential of TRAIL.

In the last decades, significant progress has been made in the understanding of the cellular pathways regulating apoptosis. Although a foundation to support induction of apoptosis as a therapeutic tool is in place, much remains to be learned about the underlying molecular mechanism. To this end, our findings have several implications. First, our studies are relevant for the design of molecular targeted therapies to increase the efficacy of 5-FU and TRAIL. Second, from a mechanistic point of view, our findings provide insights into the cross-talk between the extrinsic and intrinsic pathways. So our results are expected to be helpful for the development of combination regimens for human cancers and to potentially predict therapeutic responses.

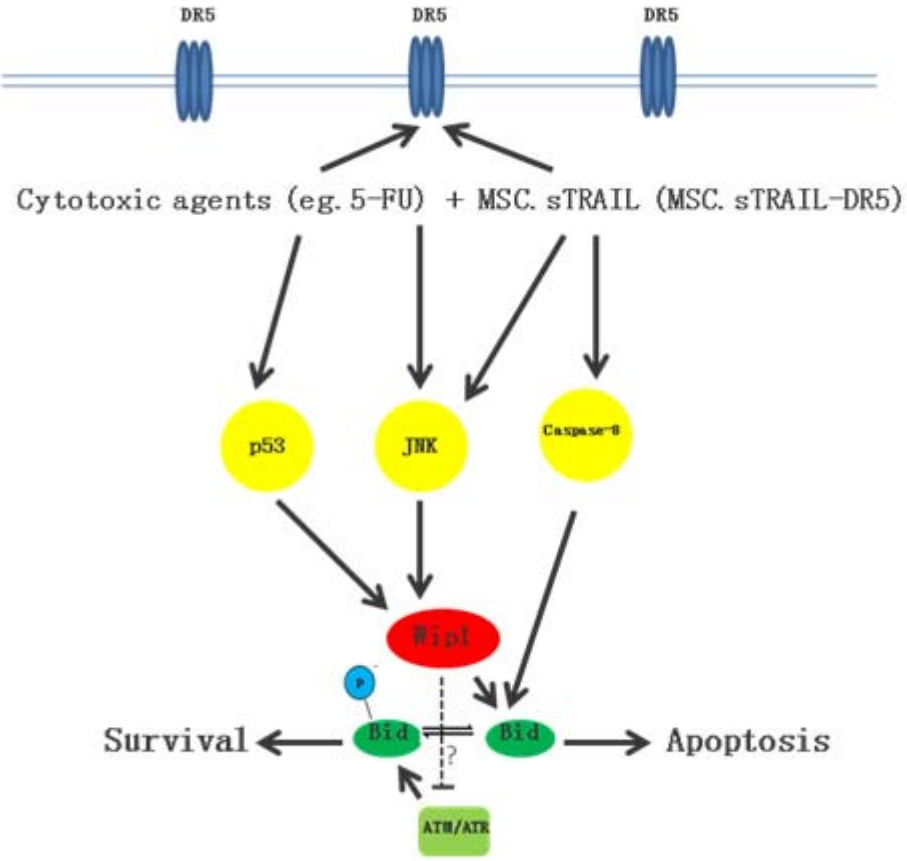


Figure 4.1 Schematic model of how targeting of DR5 leads to synergistic induction of apoptosis. Cytotoxic drugs like 5-FU lead to increase in DR5 expression thereby increasing the apoptosis-inducing activity of TRAIL. TRAIL can be delivered by MSCs (MSC.sTRAIL), TRAIL variants that are specific for DR5 have even higher activity (MSC.sTRAIL-DR5). Furthermore, cytotoxic compounds activate the JNK pathway and up-regulate the level of p53, which synergistically regulate the expression of Wip1. ATM/ATR kinase mediates the phosphorylation of Bid. Wip1 can antagonize the phosphorylation of Bid and allow apoptosis to ensue. MSC.sTRAIL/MSC.sTRAIL-DR5 activate the extrinsic apoptotic pathway acting through caspase-8 as well as the JNK pathway via DR5, which both signal through Bid and result in enhanced apoptosis.

CHAPTER5 MATERIALS AND METHODS

Chemical Reagents	Manufacturer
5-FU (5- Fluorouracil)	Sigma, St.Louis,MO,USA
6-aminodexanoic acid	Sigma, St.Louis,MO,USA
β- mercaptoethanol	Sigma, St.Louis,MO,USA
Acrylamide:N,N′-Methylenebisacrylamide 29:1	Sigma, St.Louis,MO,USA
Ammonium persulfate	Sigma, St.Louis,MO,USA
Ampicillin sodium salt	Sigma, St.Louis,MO,USA
Agarose	Sigma, St.Louis,MO,USA
Blasticidine S hydrochloride	Sigma, St.Louis,MO,USA
Bradford reagent	Sigma, St.Louis,MO,USA
Bromophenol Blue	Sigma, St.Louis,MO,USA
BSA (Bovine Serum Albumin)	Roth, Karlsruhe,Germay
Calcium chloride	Sigma, St.Louis,MO,USA
Caspase Inhibitor (z-VAD fmk)	Santa Cruz Biotechnology, CA, USA
DMSO	Sigma, St.Louis,MO,USA
DNA ladders (100bp; 1kbp)	New England Biolabs, UK
DNA loading buffer 6×	New England Biolabs, UK
Enhanced Chemiluminescent Detection Reagent	Thermo Scientific, Waltham, MA, USA
Ethanol	Sigma, St.Louis,MO,USA
Fetal Calf Serum (FCS)	Invitrogen, Carlsbad, CA, USA
Glycerol	Sigma, St.Louis,MO,USA
Glycine	Sigma, St.Louis,MO,USA
SP600125 (JNK inhibitor)	Sigma, St.Louis,MO,USA
HEPES (4-(2-hydroxyethyl)-1-piperazineethan	Sigma, St.Louis,MO,USA
KU-55933(ATM inhibitor)	Sigma, St.Louis,MO,USA
Kanamycin sulfate	Sigma, St.Louis,MO,USA
LB Broth	Sigma, St.Louis,MO,USA
LB Agar	Sigma, St.Louis,MO,USA
Magnesium chloride	Sigma, St.Louis,MO,USA
Methanol	Sigma, St.Louis,MO,USA
NP40	Sigma, St.Louis,MO,USA
Penicillin/Streptomycin	Sigma, St.Louis,MO,USA
Phosphate Buffered Saline (PBS)	Sigma, St.Louis,MO,USA
Ponceau S solution	Sigma, St.Louis,MO,USA
Potassium acetate	Sigma, St.Louis,MO,USA
Potassium chloride	Sigma, St.Louis,MO,USA
Propidium Iodide solution	Sigma, St.Louis,MO,USA
Protease Inhibitor Cocktail	Roche, Basel, Switzerland
PVDF membrane	GE healthcare, UK
Recombinant Human TRAIL	R&D system, Minneapolis, MN, USA
Skimmed Milk	Marvel, Chivers, Dublin, Ireland
Sodium chloride	Sigma, St.Louis,MO,USA

Sodium citrate	Sigma, St.Louis,MO,USA
Sodium dodecyl sulfate (SDS)	Sigma, St.Louis,MO,USA
Sucrose	Sigma, St.Louis,MO,USA
SYBR-Master Mix	Applied Biosystems, La Jolla, CA, USA
TNF-α	Peprotech, Rocky Hill, NJ, USA
Trypsin/EDTA solution	Sigma, St.Louis,MO,USA
Tris	Sigma, St.Louis,MO,USA
Triton X-100	Sigma, St.Louis,MO,USA
Tween	Sigma, St.Louis,MO,USA

Enzymes	**Manufacturer**
T4 ligase enzyme	New England Biolabs, UK
Gateway LR Clonase enzyme	Invitrogen, Carlsbad, CA, USA
Restriction enzymes	New England Biolabs, UK

Antibodies

Primary Antibodies	**Serial Number**	**Manufacturer**
ASK1	D11C9	Cell Signaling Technology, Beverly, MA, USA
ATF2	20F1	Cell Signaling Technology, Beverly, MA, USA
Bax	D2E11	Cell Signaling Technology, Beverly, MA, USA
Bax	6A7	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Bid	91508	R&D Systems, Minneapolis, MN, USA
Caspase-3	31A893	Imgenex, San Diego, CA, USA
Caspase-8	1C12	Cell Signaling Technology, Beverly, MA, USA
c-Jun	60A8	Cell Signaling Technology, Beverly, MA, USA
CuZnSOD	N/A	The Binding Site, Brimingham, UK
Cytochrome-c	6H2.B4	BD Clontech, Franklin Lakes, NJ, USA
FADD	N/A	Cell Signaling Technology, Beverly, MA, USA
IκB	M-364	Santa Cruz Biotechnology, Santa Cruz, CA, USA
MAP4K5	D66E2	Cell Signaling Technology, Beverly, MA, USA
Mice Ig G	N/A	Sigma, St.Louis,MO,USA
MKK4	5C10	Cell Signaling Technology, Beverly, MA, USA
Myc	N-262	Santa Cruz Biotechnology, Santa Cruz, CA, USA
p21	12D1	Cell Signaling Technology, Beverly, MA, USA
p38	7D6	Cell Signaling Technology, Beverly, MA, USA
p53	DO-1	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Phospho-ATF2	11G2	Cell Signaling Technology, Beverly, MA, USA
Phospho-Bid	S78	Bethyl Laboratories, Brimingham, UK
Phospho-c-Jun	54B3	Cell Signaling Technology, Beverly, MA, USA
Phospho-IκB	14D4	Cell Signaling Technology, Beverly, MA, USA
Phospho-JNK	98F2	Cell Signaling Technology, Beverly, MA, USA
Phospho-MKK4	C36C11	Cell Signaling Technology, Beverly, MA, USA
Phospho-p38	3D7	Cell Signaling Technology, Beverly, MA, USA

Smac/Diablo	56/Smac/DIABLO	BD Pharmingen, Franklin Lakes, NJ, USA
TRADD	7G8	Cell Signaling Technology, Beverly, MA, USA
TRAF2	C192	Cell Signaling Technology, Beverly, MA, USA
TRAIL	N/A	Peprotech, Rocky Hill, NJ, USA
TRAIL-R1/DR4	B-N28	Santa Cruz Biotechnology, Santa Cruz, CA, USA
TRAIL-R2/DR5	D4E9	Cell Signaling Technology, Beverly, MA, USA
Wip1	F-10	Santa Cruz Biotechnology, Santa Cruz, CA, USA
XIAP	28/hILP/XIAP	BD Pharmingen, Franklin Lakes, NJ, USA

Secondary Antibodies	**Manufacturer**
Mouse anti-rabbit IgG-HRP (horseradish peroxidase)	Santa Cruz Biotechnology, Santa Cruz, CA.
Rabbit anti-sheep IgG-HRP (horseradish peroxidase)	Santa Cruz Biotechnology, Santa Cruz, CA
Goat anti-mouse IgG-HRP (horseradish peroxidase)	Santa Cruz Biotechnology, Santa Cruz, CA

Cell Culture Media	**Manufacturer**
Dulbeccos Modified Eagle Medium (DMEM)	Invitrogen, Carlsbad, CA, USA
McCoy's 5A	Lonza, Allendale, NJ, USA
RPMI 1640	Invitrogen, Carlsbad, CA, USA

Plasmids	**Manufacturer**
pcDNA3	Invitrogen, Carlsbad, CA, USA
pENTR™/U6	Invitrogen, Carlsbad, CA, USA
pENTR™-1A	Invitrogen, Carlsbad, CA, USA
pAd/BLOCK-iT-DEST™	Invitrogen, Carlsbad, CA, USA
pAd/CMV/V5- DEST™	Invitrogen, Carlsbad, CA, USA
pEGFP-N1	Clontech Laboratories, Anaheim, CA
pCMV6-XL5 Wip1	Origene, Rockville, MD, USA
pCMV6-Entry MYC-tagged FADD	Origene, Rockville, MD, USA
pCMV6-XL4 Ask1	Origene, Rockville, MD, USA
pCMV6-XL5 TRADD	Origene, Rockville, MD, USA
pCMV6-XL4 TRAF2	Origene, Rockville, MD, USA
pCMV6-XL4 RIP1	Origene, Rockville, MD, USA
pCMV6-XL4 MAP4K5	Origene, Rockville, MD, USA
pGL4 NF-κB Firefly luciferase reporter	Promega, Madison, WN, USA
pRL Renilla luciferase control reporter	Promega, Madison, WN, USA

Cell lines	Source	Description
HCT116	Cell line stock of the lab (ATCC)	human colorectal tumour cells
HCT116shDR5	Cell line stock of the lab	human colorectal tumour cells
HCT116shFADD	Cell line stock of the lab	human colorectal tumour cells
HCT116shcaspase-8	Cell line stock of the lab	human colorectal tumour cells
HCT116p53-/-	Cell line stock of the lab (gift from Bert Vogelstein)	human colorectal tumour cells
HCT116p53-/-shFADD	Cell line stock of the lab	human colorectal tumour cells
HCT116shWip1	Cell line stock of the lab	human colorectal tumour cells
Colo205	Cell line stock of the lab (ATCC)	human colorectal tumour cells
Hela	Cell line stock of the lab (ATCC)	human cervix tumour cells
Panc1	Cell line stock of the lab (ATCC)	human pancreatic tumour cells
PancTu1	Cell line stock of the lab (ATCC)	human pancreatic tumour cells
293	Cell line stock of the lab (ATCC)	human embryonic kidney cells
A2780	Cell line stock of the lab (ATCC)	human ovarian tumour cells
BxPc	Cell line stock of the lab (ATCC)	human pancreatic tumour cells
Colo357	Cell line stock of the lab (ATCC)	human pancreatic tumour cells
U2OS	Cell line stock of the lab (gift from Oliver Micheau)	human osteosarcoma cells
Primary Cells		
MSCs	Cell stock of the lab	mice mesenchymal stem cells

Commercial Kits	Manufacturer
GenElute™ Plasmid Miniprep Kit	Sigma, St.Louis,MO,USA
GenElute™ Gel Extraction Kit	Sigma, St.Louis,MO,USA
GenElute™ PCR Clean-Up Kit	Sigma, St.Louis,MO,USA
NucleoBond^{TM} Xtra maxi-prep Kit	Macherey-Nagel, Düren, Germany
RNeasy Kit	Qiagen, Darmstadt, Germany
Human TRAIL/TNFSF10 Quantikine ELISA Kit	R&D system, Minneapolis, MN, USA
Dual-luciferase Reporter Assay Kit	Promega, Madison, WN, USA
Quick-Change mutagenesis Kit	Stratagene, La Jolla, CA, USA
Superscript First-Strand Synthesis for Reverse Transcriptase (RT)-PCR kit	Invitrogen, Carlsbad, CA, USA

Oligonucleotides

The following small hairpin (sh) RNA motif was used to knock-down target genes

Caspase-8	5′- GGGTCATGCTCTATCAGAT-3’
DR5	5′-GCTAGAAGGTAATGCAGACTCTGCCATGTC -3’
DR4	5′-GCTGTTCTTTGACAAGTTGC-3’
EGFP	5’-GTACAACTACAACAGCCAC-3’
FADD	5’- GTGCAGCATTTAACGTCATAT-3’
Wip1	5′-GACCATAAGCCAGAACTTCC-3’
XIAP	5′-GTGGTAGTCCTGTTTCAGC-3’

Primers of RT-PCR

Wip1

Forward	5’-GAGGCGATCGCCATGGCGGGGCTGTACTCG-3’
Reverse	5’- GCGACGCGTGCAAACACAAACAGTTTTCCT-3’

Primers of cloning hFIB signal peptide part

Forward 5’-GATCCATGCCGTCTTCTGTCTCGTGGGCATCCTCCTGC
TGGCAGGCCTGTGCTGCCTGGTCCCTGTCTCCCTGGCTG-3’

Reverse 5’-AATTCAGCCAGGGAGACAGGGACCAGGCAGC ACAGGCCTT
GCCAGCAGGAGGATGCCCCACGA CGGCATG-3’

Primers of cloning Furin CS (furin cleavage site) part

Forward 5’-AATTCAGCGCCAGGAACAGGCAGAAGAGGG-3’

Reverse 5’-TCGACCCTCTTCTGCCTGTTCCTGGCGCTG-3’

Primers of cloning ILZ (isoleucine-zipper sequence) part

Forward 5’-TCGACAGGATGAAGCAGATCGAGGACAAGATCGAGGAG
ATCCTGAGCAAGATCTACCACATCGAGAACGAGATCGATC
GCCAGGATCAAGATCAAGAAGCTGATCGGCGAGAGGC-3’

Reverse 5’-TCGAGCCTCTCGCCGATCAGCTTCTTGATCCTGGCGATCTC
GTTCTCGATGTGGTAGATCTTGCTCAGGATCTCCTCGATCTA
TGCTCCCGTATCGGTGTATGTGTCCTCGATCTGCTTCATCC-3’

Primers of cloning full-length TRAIL

Forward 5’-GCACGTCGACCAGGATCATGGCTATGATGG -3’

Reverse 5’-CGTGAGCGGCCGCCAGGTCAGTTAGCCAACT-3’

Primers of cloning soluble TRAIL

Forward 5’-CTCGAGGTGAGAGAAAGAGGTCCTCAGAGAG-3’

Reverse 5’-GCGGCCGCTTAGCCAACTAAAAAGGCCC-3’

Primers of create variant TRAILs

DR5-selective TRAIL (D269H)

Forward 5'-AATGAGCACTTGARAGACATGCACCATGAAGCCAGTTT-3'

Reverse 5'-AAACTGGCTTCATGGTGCATGTCTATCAAGTGCTCATT-3'

DR4-selective TRAIL (S159R)

Forward 5'-TGGGAATCATCAAGGAGGGGGCATTCATTCCTGAG-3'

Reverse 5'-CTCAGGAATGAATGCCCCCTCCTTGATGATTCCCA-3'

Solutions:

Tris Buffer Saline (TBS): Tris-HCl 25 mM pH 8.0, NaCl 137 mM.

TBS-Tween: TBS with 1% Tween 20. PBS-Tween: PBS with 1% Tween 20

Tris-EDTA (TE): Tris-HCl 10 mM, EDTA 1 mM pH 8.0.

TAE buffer (50×): EDTA 50 mM pH 8.0, Tris-Acetate 2 M, 57.1 ml Glacial Acetic Acid.

Cellular lysis buffer: Tris-HCl 50 mM pH 7.4, 10% Glycerol, 0.5% NP40, NaCl 150 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, KCl 1 mM and Complete Protease Inhibitor Cocktail.

Anode I buffer: Tris-HCl 300 mM, 20% Methanol.

Anode II buffer: Tris-HCl 25 mM, 20% Methanol.

Cathode buffer: Tris-HCl 25 mM, 6-Aminodexanoic Acid 40 mM, 20% Methanol.

SDS-PAGE running buffer (10×): Tris-HCl 250 mM, Glycine 1.92 M, SDS 0.1%

Protein loading buffer: Tris-HCl 65 mM pH 6.8, 10 % Glycerol, 4 % SDS, 4 % β-Mercaptoethanol, 0.2 % Bromophenol Blue.

Antibody dilution buffer: 3% Bovine Serum Albumin (BSA) in TBS-Tween.

Western blot blocking buffer: 3-5% Skimmed Milk or 3% BSA in TBS-Tween.

Annealing buffer: Potassium Acetate 500 mM pH7.4, Magnesium Acetate 10 mM, HEPES-KOH 150 mM.

Nicoletti buffer: 0.1% Sodium Citrate, 0.1 % Triton X-100, 50 µg/ml Propidium Iodide.

HBS: NaCl 140 mM, Na_2HPO_4 1.5 mM, HEPES 50 mM pH 7.2, sterilised and stored at 4ºC.

$CaCl_2$ solution (calcium phosphate transfection): 250 mM, sterilised.

$CaCl_2$ solution (preparation of competent *E.coli* cells): 15 mM pH 6.7, sterilised.

CLAMI (Cell Lysis And Mitochondria Intact) buffer: Sucrose 250 mM, KCl 80 mM, 50 µg/ ml Digitonin in PBS.

RIPA buffer: Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 2 mM, 1% NP40, 0.1% SDS.

Methods

Cell Culture

HCT116 cells and their isogenic daughter cell line HCT116p53-/-, and their derived clones HCT116p53-/-shDR5 (generated by Andrea Mohr), HCT116shDR5 (generated by Andrea Mohr), HCT116shCaspase-8 (generated by Andrea Mohr), HCT116shFADD (generated by Andrea Mohr), HCT116shWip1 were grown in McCoy's medium supplemented with 10% FBS and 100 U/ml penicillin and 100 g/ml streptomycin. Colo205 cells, Hela cells, PancTu1 cells, Panc1 cells, U2OS cells as well as 293 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and 100 U/ml penicillin and 100 g/ml streptomycin. A2780 cells, BxPc cells and Colo357 cells were grown in RPMI-1640 medium supplemented with 10% FBS and 100 U/ml penicillin and 100 g/ml streptomycin. MSCs cells (generated by Stella Maris Albarenque) were grown in low-glucose DMEM supplemented with 20% FBS and 100 U/ml penicillin and 100 g/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37^0 C. When cells reached 80-90% confluence, they were washed with PBS, trypsinised and resuspended in fresh medium. An appropriate fraction of cells solution were transferred into a new culture flask with fresh medium for further cultivation or seeded for further experiments. Cells were frozen down at -80^0 C in 1 ml FBS containing 10% DMSO, aliquot into 1.5 ml screw-cap tubes. When recovering cells, the frozen cells were gently and entirely pipetted into 10 ml media and plated. After cells had attached to the culture flask, usually after 24 h, the medium was changed.

Cell Counting

Cell numbers were counted by using a microscope counting chamber (hemocytometer). The number of cells in one set of 16 corner squares is equivalent to the number of cells $\times 10^4$/ ml

Drug Treatment

For apoptosis induction, 5-FU was used at concentration of 200 μM. ATM inhibitor KU-55933 treatment was carried out at concentration of 10 μM. JNK inhibitor SP600125 treatment was carried out at concentration 20 μM.

Transfection of Cells

For all kinds of tranfections, cells were prepared one day earlier. To check the efficiency of the tranfection, pEGFP plasmid was also transfected and percentage of green fluoresence cells was checked 24 h or 48 h after the fluoresence transfection by microscopy. For the transfection of HCT116 cells, we used the FuGENE reagent. Firstly, the cells were seeded in a 6-well plate and then transfected at a density of around 70-80%. Secondly, fresh medium was added before the transfection and serum-free medium was used to dilute the plasmid DNA to achieve a concentration of 20 μg/ml. Then 100 μl of the diluted DNA was mixed with 8 μl FuGENE. The mixture was placed at room temperature for more than 30 min, then the mixture was added into medium. For the transfection of 293 cells, we used the calcium phosphate transfection method. Before the transfection, fresh 2% FCS containing medium was added. For each well of 6-well plate, 0.5 ml HBS were aliquoted into a sterile 1.5 ml Eppendorf tube. In a separate tube 5 μg of plasmid DNA were mixed with 250 μl $CaCl_2$ (2.5 mM) and enoμgh distilled water to bring the total volume to 0.5 ml. The $CaCl_2$/DNA mix was then added to the HBS drop-wise under vortexing. After 45 min of incubation at room temperature, the mixture was slowly added to the media. 4 h later, the medium was removed and the cells were washed with PBS and fresh medium added.

Luciferase Assay

Cells were seeded at 1×10^5 cells/well in a 6-well plate and left to adhere overnight. Next day, cells were transiently transfected with around 1 μg of the NF-κB firefly luciferase reporter plasmid and 0.1 μg of Renilla luciferase control plasmid by using the FuGENE reagent. 24 h after transfection, cells were treated with TNF-α for 30 min as a positive control or 5-FU for 6 h and 24 h, respectively, and harvested. Then luciferase activity was measured by using the dual-luciferase reporter assay kit according to the manufacturer's instructions.

Generation of stable cell clones

HCT116 cells transfected using the FuGENE reagent. 24 h after the transfection, cells were transferred to 15 cm tissue culture dishes and kept under selection with corresponding antibiotics. After about 3 weeks, isolated clones were visible and picked with cloning discs and transferred to 24-well plates until confluence was arrived. The clones were then scaled-up and tested by Western Blots. Once stable clones were established, they were grown in media without antibiotics.

Transduction of MSCs

Murine MSCs were seeded at 1×10^5 cells/well in a 6-well plate and left to adhere overnight. Cells were transduced with viral vector at 100 pfu/cell by spinning plates at 1500 rpm for 1.5 h at 37^0 C. The cells with virus were cultured overnight, then washed off and new medium added.

Mixing of MSCs with tumour cells

Tumour cells were plated in 6-well plates at 10^5 cells per well and left overnight to adhere. Then cells were pre-treated with 10 μM 5-FU or left untreated. Adenovirus-transduced murine MSCs were then added 24 h later at different ratios. Another 24 h or 48 h later, cells were collected for apoptosis assays.

Western Blot

After being harvested, the cells were washed once in PBS and lysed in cellular lysis buffer on ice for 45 min. Then the whole lysates were centrifuged at 12,000 rpm for 15 min. The supernatant was used for Western Blot. Protein concentrations in the supernatants were determined by Bradford assay, using BSA as the standard. Equal amounts of protein (50 μg) were subjected to SDS-PAGE on a 12% gel before being transferred onto PVDF membrane. Transfers were carried out at 50 mA constant current for 1 h. Transfer and loading of protein was routinely checked by Ponceau S staining. After transfer of proteins, the PVDF membrane was blocked (shaking) in blocking buffer (5% skimmed milk or 3% BSA in PBS, dependent on the protein to be detected) for 1 h at room temperature. The membrane was then incubated with the primary antibody diluted in PBST at 4^0 C overnight. Then the membrane was washed with milk-PBST and PBST three times for 10 min each. Next, the membrane was incubated for 1 h at room temperature with the secondary antibody. Blots were then washed again with milk-PBST and PBST three times for 10 min each. Finally, the enhanced chemiluminescence (ECL) solution was added for 1 min. The signal was recorded and analyzed by using the FluorChem Chemiluminescent Imaging System (Alpha Innotech).

Immunoprecipitation

For Bax immunoprecipitation, equal amounts of protein from whole cell lysates (800 µg) were used for immunoprecipitation. All samples were brought to a final volume of 450 µl with cellular lysis buffer. Samples were then rotated for 5 h at 4^0 C with 5 µl of monoclonal antibodies (Bax 6A7) and 150 µl anti-rabbit IgG magnetic beads. Then beads supernatant were precipitated by a magnetic field. The beads were then washed five times with the cellular lysis buffer. Finally, the last supernatant was removed and 25 µl of 5×loading buffer added. The beads were incubated in the loading buffer at 95-100° C for 5 min, and then centrifuged at 12000 rpm for 5 min. The supernatants were subjected to Western Blot and Bax was detected with a normal Bax primary antibody. For FADD co-immunoprecipitation, the protocol is basically the same as for Bax immunoprecipitation, however the cells were lysed in RIPA buffer, before the lysates were incubated with anti-Myc antibody. The respective proteins were detected with primary antibodies against ASK1, TRADD, TRAF2, RIP1, and MAP4K5 respectively.

Preparation of subcellular fractions

In order to separate the cytosolic and mitochondria fractions, cells were washed in ice-cold PBS. The cells were then lysed using CLAMI (Cell Lysis And Mitochondria Intact) buffer on ice for 5 min, cell suspension was centrifuged at 1500 rpm for 5 min at 4° C. The supernatant was removed and stored at -20° C as the cytosolic fraction. The pellet was re-suspended in cellular lysis buffer and placed on ice for another 15 min. The suspension was centrifuged at 12,000 rpm for 15 min at 4° C and the supernatant was stored as the mitochondrial fraction at -20° C.

Flow cytometry analysis

For the apoptosis assays using flow cytometry, cells were harvested and centrifuged at 1500 rpm for 5 min. Then the cells were washed once with 1×PBS and lysed in Nicolleti buffer containing PI. The fluorescence signal was measured using a laser that supplies light at γ=488 nm in the FL3 (propidium iodide detection) channel. Cells with normal DNA content show typical G1-M-G2 cell-cycle specific DNA content histograms. Cells with decreased (hypodiploid) DNA content (sub-G1) are considered apoptotic cells. 2000 events were measured for each sample.

Plasmid DNA preparation

Minipreps were performed to obtain up to 10 µg plasmid DNA from overnight cultures inoculated from a single colony into 5 ml LB-Broth with antibiotics and grown at 37^0 C. The bacteria were then harvested and using GenElute™ Plasmid Miniprep kit according to the manufacturer´s protocols. Maxipreps were performed in order to get more plasmid DNA. Bacteria from a small overnight culture are grown in 200 ml LB-Broth overnight at 37^0 C. Then the bacteria were harvested lysed and DNA purified using the NucleoBond™ Xtra maxiprep kit according to the manufacturer´s instructions

DNA/RNA quantification

DNA/RNA quantification was measured by using a Nanodrop™ spectrophotometer (Thermo Scientific). The DNA/RNA concentrations can be read at λnm A260. The quality can be assessed by the ratio of λnm A260:A280.

Gel Electrophoresis

Unless otherwise stated, 1% agarose gels were prepared using electrophoresis grade agarose in 1×TAE buffer. These were electrophoresed using tanks in 1×TAE solution using 60-120 voltage. Prior to loading, samples were mixed with 6×DNA-loading buffer. To estimate the size of a DNA fragment a DNA marker (100 bp or 1 kb ladders) was analysed in parallel. The DNA bands were visualized with ultraviolet light.

DNA recovery from agarose gel

DNA bands were recovered from agarose gel by using the GenElute™ Gel Extraction kit according to the manufacturer´s instructions.

Enzymatic reactions of DNA

Restriction digestion, ligations were performed with buffers and protocols supplied with the enzymes. When necessary, the purification was carried out after the digestion by using GenElute™ PCR Clean-Up kit according to the manufacturer's instructions.

Preparation of chemically competent *E.coli* cells

E.coli strain Top10 (genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-) was used for cloning procedures. Heat shock competent cells were prepared from 200 ml cultures grown at 370 C, when the λnm A600 spectrophotometer reading was 0.6. The culture was chilled on ice for 30 min, harvested and washed in ice-cold $CaCl_2$ solution (15 mM). After a second centrifugation, cells were washed again with $CaCl_2$ solution (15 mM), then resuspended in $CaCl_2$ (15 mM) with 10% DMSO, aliquoted and snap frozen at -800 C. Alternatively, subcloning efficiency competent cells DH5α (genotype: F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96relA1 λ-) were purchased from Invitrogen.

Transformation of *E.coli*

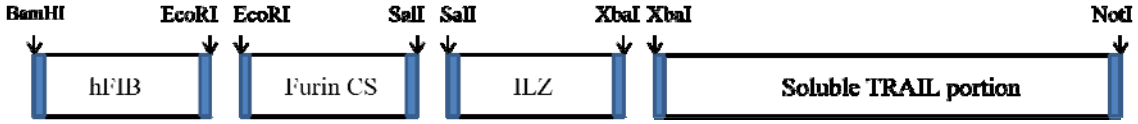
An aliquot of competent *E.coli* cells (50 µl or 100 µl) was thawed on ice. 0.5-20 µl ligation reaction or mini-prep plasmid DNA was added to the cells. After mixing the DNA and competent cells, the mixture was incubated on ice for 30 min, then the mixture was incubated in a water bath at 42^0 C for 90 s, before mixture was placed back on ice for 2 min. Then 400 µl LB-Broth without antibiotics were added and the mixture was incubated and shaken for 45 min at 37^0 C. Then the mixture was plated onto LB plates containing the required antibiotics for selection and incubated overnight at 37^0 C.

siRNA design and construct short hairpin expression vector

The oligos of the target gene were designed by the Invitrogen "BLOCK-iT™ RNAi Designer" (http://rnaidesigner.invitrogen.com/rnaiexpress/). The primers (sense and antisense for each motif) were dissolved at a concentration of 2 µg/ul. 13 µl of each were then hybridized with 5×annealing buffer and cloned into the pENTR™/U6 vector linearized with BbsI and EcoRI. The reaction system is composed of 13 µl primers (sense and anti-sense each), 10 µl annealing buffer and 14 µl dH_2O. The mixture was placed on a hot water bath above 90^0 C and then left to cool down to room temperature. Then 1 µl of the mixture was ligated to 100 ng pENTR™/U6 vector. The plasmid resulting from the ligation was then checked for the short hairpin insert by cleavage with NdeI and SalI enzymes. These small-hairpin RNA expression vectors were then tested for their functionality by transfecting 293 cells. 48 h after the transfection, 293 cells were harvested and protein levels of the respective target gene were tested by Western Blot. The shRNA motifs in pENTR/U6 vector can be subcloned into the pBLOCK-iT™-DEST vector or pAd/BLOCK-iT™-DEST vector. They were used to generate stable knockdown clones or produce adenoviral vectors.

Cloning of sTRAIL constructs

The full-length human TRAIL was cloned into BamHI/EcoRI sites of pcDNA3 by RT-PCR from cell RNA, using primers (the primers carried additional restriction enzyme sites 5'-BamHI and 3'-EcoRI) as described earlier. Then the soluble portion of TRAIL (amino acids 114-281) was amplified by PCR and subcloned into the XbaI/NotI sites of the pcDNA3 vector resulting in pcDNA3.sTRAIL. Then the signal peptide sequence (hFIB) was fused with the furin cleavage site (Furin CS), isoleucine-zipper sequence (ILZ) and cloned into the BamHI/XbaI sites of the pcDNA3.sTRAIL vector. The resulting plasmid was termed pcDNA3.hFIBsTRAIL-wt, simply referred to sTRAIL-wt. The sTRAIL-DR5 (D269H) and sTRAIL-DR4 (S159R) were generated by using the Quick-Change site-directed mutagenesis kit according to the manufacturer's instructions and confirmed by DNA sequencing.



Restriction enzyme cleavage sites of hFIB.sTRAIL

pBLOCK-iT cloning

For the production of stable clones, the shRNA motifs were subcloned from the pENTR^{TM}/U6 vector into pBLOCK-iT^{TM}-DEST vector by using the Gateway LR Clonase enzyme according to the manufacturer's instructions.

Generation of Adenovirus vectors expressing a short hairpin

For the production of the adenoviral vectors carrying the short hairpin expression cassettes, Small hairpin RNAs were subcloned from the pENTR^{TM}/U6 vectors into the pAd/BLOCK-iT^{TM}-DEST plasmid by using the Gateway LR Clonase enzyme according to the manufacturer's instruction.

Generation of Adenoviral vectors expression protein

Recombinant E1/E3-deleted adenoviral vectors with the cytomegalie virus (CMV) promoter/enhancer element expressing soluble TRAILs and EGFP were constructed. Firstly, pcDNA3.hFIBsTRAIL and pEGFP.N1 plasmids were digested with BamHI and NotI. Then the fragments are ligated into pENTR^{TM} 1A and then into pAd/CMV/V5-DEST^{TM} plasmid using Gateway LR Clonase enzyme according to the manufacturer's instructions.

RNA preparation

Total RNA was isolated from cells with the RNeasy Kit according to the manufacturer's instructions. Then RNA was aliquoted and stored at -80^0 C.

cDNA synthesis

cDNA was synthesised from 1 µg of total RNA using the Superscript First-Strand Synthesis for Reverse Transcriptase (RT)-PCR kit in a 20 µl volume. The reaction was carried out according to the manufacturer's instructions. cDNA was stored at -20^0 C

Real-Time PCR

Real-Time PCR was performed by using Applied Biosystems StepOne Plus Real-Time PCR System. The 25 µl PCR system composed of 10.5 µl primers (5 pM), 10 ng cDNA, and 12.5 µl SYBR master mix. The PCR reaction was incubated in a 96-well plate at 95^0 C 20 s, followed by 40 cycles of 95^0 C 3 s, 60^0 C 30 s then followed by 95^0 C 15 s, 60^0 C 60 s and 95^0 C 15 s. The results were analyzed by using Excel.

TRAIL Enzyme-Linked Immunosorbent Assay (ELISA)

To examine secreted sTRAIL, the culture supernatants of transfected 293 cells were collected by centrifugation at 15,000 rpm for 20 min at room temperature. The supernatants were then diluted 20-fold and the commercial Human TRAIL/TNFSF10 Quantikine ELISA Kit was used according to the manufacturer's instructions.

Statistical Analyses

If not otherwise stated, experiments were performed in triplicate or duplicate. Experimental values are expressed as mean value ± standard error (S.E.). For significance analyses Student's t-tests, were used and $p < 0.05$ was considered significant and $p < 0.001$ as highly significant

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