Mammalian Reovirus Infection Changes the Expression of Bcl-xL Protein in H1299 Cell Line Independent of p53

by

Lou Wang

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Department of Medical Microbiology and Infectious Diseases

University of Manitoba

Winnipeg, Manitoba

Canada

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aa	amino acid
Bcl-2	B cell lymphoma/leukemia 2
Bcl-xL	B-cell lymphoma/leukemia-extra large
Bcl-xS	B-cell lymphoma/leukemia-extra Short
BRV	fusogenic baboon reovirus
BSA	bovine serum albumin
cDNA	copy deoxyribonucleic acid
CPE	cytopathic effect
D-buffer	dialysis buffer
ddH_20	double-distilled water
DISC	Death Inducing Signaling Complex
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DOC	deoxycholate
DR	death receptor
dsRNA	double-stranded RNA
DTT	dithiothreitol
EGFR	epithelial growth factor receptor
FCS	fetal calf serum
GADD45α	growth arrest and DNA damage 45α
HPI	hours post infection
ISVP	intermediate subviral particle
MDM-2 gene	murine double minute 2 oncogene

MEM	minimum essential medium
MOI	multiplicity of infection
mRNA	messenger RNA
MRV	mammalian reovirus
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFU	plaque forming units
PI	post infecting
Rb	retinoblastoma
RPA	RNA protection assay
SDS	sodium dodecyl sulphate
STAT	Signal transducer and activator of transcription
T1L	type 1 Lang
T2J	type 2 Jones
T3A	type 3 Abony
T3D	type 3 Dearing
TNFR	tumor necrosis factor receptor
TRAIL	(TNF)-related apoptosis-inducing ligand
UV	ultraviolet
VDAC	voltage dependent anion-selective channel
Vertrel®XF	1,1,1,2,3,4,4,5,5,5-decafluoropentane

ABSTRACT

Mammalian reovirus (MRV) is a prototype virus of Reoviridae family. MRV virions are composed of two concentric protein capsids that surround a genome of 10 segments of dsRNA. It has been shown that MRV can manipulate host gene expression and further induce apoptosis and cell cycle arrest in various cell lines. However, the detailed molecular mechanisms by which MRV regulates the expression of host cells are largely unknown. P53 is an important transcriptional factor which modulates the expression of more than 130 genes controlled in cell stress-response. We aimed to examine the molecular mechanisms underlying the effect of MRV infection on the expression of host genes and the possible role of p53 in the interaction of MRV and host cells. Prototype serotype 3 reovirus strain Dearing (T3D) and serotype 1 strain Lang (T1L) were used to infect different cell lines, respectively, H1299 (p53-null and p53 positive), HT1080 (p53 mutant and p53 positive) and HCT116 (p21 deficient and 14-3-3σ deficient).

By comparing the virus replication curve of T1L and T3D in these cell lines, we found that MRV can replicate with a similar pattern in both p53-defective and p53-positive cell lines which indicated that p53 does not have significant impact on MRV replication in these cell lines.

We further found that the level of Bcl-xL protein, which has been shown to be able to inhibit apoptosis, was increased in H1299 cell lines (both p53-null and p53 positive) infected by T3D, but decreased in the same cell lines infected by T1L. A

similar change of Bcl-xL protein was not observed In HCT116 and HT1080 cell lines with MRV infection. Fifty four T1L×T3D reassortants were used to map which gene or gene combination was responsible for the changes of Bcl-xL protein. We found that the expression of Bcl-xL protein in H1299 cell line infected by MRV was majorly controlled by the S1 gene segment which encodes the σ1 cell attachment protein and the σ1s non structural protein, while minorly controlled by L3 gene segment of MRV.

CHAPTER I: INTRODUCTION

1.1 Virus replication and cellular response

1.1.1 Virus infection

Viruses are the simplest and smallest known living organisms. Viruses are ultramicroscopic infectious agents. They cannot replicate themselves outside of host cells. Generally, most viruses are made up of protein and nucleic acid, and some viruses also have envelops around them. The origins of virus is not very clear. The size of virus particle ranges from about 20 to 400 nm (nanometers) in diameter (Marx and Jean L, 1991).

While viruses can not replicate by themselves, host cells are big virus-assembly factories that can provide workshop and resources for virus replication. There are two strategies taken by viruses to infect host cells. One is “Hit and run” infection; the other is “Hit and stay” infection (Hilleman, 2004). “Hit and run” viruses can induce lysis of host cells and apoptosis to rapidly release many offspring viruses. These viruses then infect other cells, tissues, individuals and even species. They have high levels of infectivity, such as influenza, rhinoviruses (the common cold), and measles. “Hit and stay” viruses, such as hepatitis C virus (HCV), human papillomavirus (HPV), Epstein-Barr virus (EBV), take a milder method; they even sometime sacrifice their virulence to maintain a persistent infection. “Hit and stay” viruses can inhibit apoptosis and induce cell cycle arrest. To get maximum replication, viruses

must manipulate the function and gene expression of host cells. By manipulating host cells, viruses can get 3 benefits：(1) replicating themselves by using the energy, materials and subcellular structures; (2) inducing lysis or apoptosis of host cells to release progeny, then infecting other hosts; or inhibiting apoptosis or inducing cell cycle arrest to increase infection course in order to facilitate maximum replication; (3) Changing host surface antigens to avoid immune responses. Growing evidence shows that there are changes in gene expression of host cells infected by viruses in the cytoplasm, such as in reoviruses. It appears that viruses take advantage of some mechanisms to change host gene expression. For instance, the reovirus u2 protein can induce the accumulation of interferon regulatory factor-9 in nucleus and disrupt its function, as a result, interferon-stimulated gene expression in host cells was repressed (Sherry B., 2009). Or this changing gene expression can be due to host cellular responses (Mossman et al. 2001; Hiscox JA, 2007)

1.1.2. Cell responses

The above may be just one side of the whole story. Host cells also develop mechanisms to resist viral invasion. For example, host cells can secrete cytokines like interferon to inhibit viral infection. Apoptosis induction also can be regarded as a host defense method to limit viral infection (Schwarz *et al,* 1998). Viruses, as intracellular parasites, depend upon the host cell for different steps in their life cycles. The cellular genes are probably involved in the whole course of virus cycles from viral binding to cell receptors to release of progeny virus (Op De Beeck and

Caillet-Fauquet, 1997). By insertional mutagenesis, one group selected 111 reovirus-resistant clones from rat intestinal epithelial cells. These clones contained mutant genes involved in particular metabolic or signaling pathways (Organ *et al,* 2004). Thus, the alteration of host gene expression can change the viral life cycle. The transcription and expression of host gene are very complicated. p53 as a transcriptional factor may play a very important role in the course of viral infection.

1.2 P53

P53 is an important transcriptional factor. It functions as a tumor suppressor to maintain cell stability. It plays an important role in host cells during many kinds of stress. It has multiple functional roles. P53 was firstly identified by several different groups respectively in 1979. p53 was found to bind to large T antigen which was an oncogenic protein encoded by SV40 virus, hence, p53 was believed to be an oncogene at that time. Ten years later, It was finally identified as a tumor suppressor gene by Baker in 1989 (Baker et al, 1989). It was further shown that SV40 virus used its large T antigen to bind to p53 and block p53-indcued cell cycle arrest and apoptosis, so to induce the transformation in host cells (Pipas and Levine, 2001)..

1.2.1 Structure of p53

When resolved in SDS-PAGE, p53 is located around 53 kilodaltans (kD).The mass of the protein is about 43.7kD because the proline residues slow the migration of the protein in gel (Ziemer *et al,* 1982). Human p53 protein has 383 amino acids,

including 7 main domains: (1) N-terminal transcription-activation domain (Gaulton *et al*, 1989B), functions to activate transcription factors; (2) activation domain 2 (AD2), functions related to apoptotic activity; (3) Proline rich domain, important for the apoptotic activity of p53; (4) central DNA-binding core domain (DBD)，contains one zinc atom and several arginine amino acids; (5) nuclear localization signaling domain; (6) homo-oligomerisation domain (OD) Tetramerization is essential for the activity of p53 in vivo; (7) C-terminal domain involved in down regulation of DNA binding of the central domain.

Normally, p53 is present at low levels in unstressed cells, as an inactive protein with a very short half-life (20-30minutes) (Proctor *et al*, 2008). Multiple conditions can trigger increased expression of p53, including DNA damage, UV radiation, oncogenic signaling, hypoxia and virus infection. Binding of activated p53 to particular DNA sequences can up or down regulate the transcription of hundreds of genes involved in various cellular processes that lead to cell-cycle arrest, senescence, differentiation, DNA repair and apoptosis (Beattie *et al,* 2005; Giono and Manfredi*,* 2006). The type and amount of stress determines the cellular processes by various mechanisms.

1.2.2 Regulation of p53

The murine double minute oncogene (MDM-2 gene) is the major negative regulator of p53. P53 can regulate the transcription of MDM-2, which is a slow process (lasting several hours). But then MDM-2 protein can bind to p53, which can inactivate the transcriptional function of p53 and ubiquitinate p53. The second

process is rapid. The auto-regulatory loop keeps the level of MDM-2 and p53 protein oscillating, which can be observed in vivo in various cells (Lev Bar-Or *et al,* 2000). In addition to MDM-2, MDM-X (MDM-4) is a regulator of p53, which can directly negatively manipulate p53 and positively regulate MDM-2 (Parant *et al,* 2001). There are 10 positive or negative feedback loops in the regulation of p53 (Harris *et al,* 2005). In these loops, activity or/and amount of related proteins can be regulated by p53; these proteins in turn can manipulate the activity of p53 in cells. Amongst them, seven are negative feedback loops that down regulate p53 activity, including MDM-2, Cop-1, Pirh-2, p73 delta N, cyclin G, Wip-1 and Siah-1. The other three are positive feedback loops, including PTEN-AKT, p14/19 ARF and Rb (Harris *et al*, 2005). Posttranslational modification is another important way to regulate p53 activity and function, including phosphorylation and dephosphrylation, acetylation and deactivation, methylation and demethylation, ubiquitination and de-ubiquination, sumoylation, neddylation, glycosylation or ADP-ribosylation at different sites. Multiple modifications may happen in p53 (Bode *et al*, 2004).

1.2.3 The function of p53

Activated p53 manipulates the two major cell cycle checkpoints, in G1/S or G2/M by inducing the expression of p21, 14-3-3 sigma, Cdc-25C and GADD45 (el-Deiry *et al*, 1992). The G1/S arrest consists of two phases, the first is not related to p53, but the second is p53-dependent. P21 gene product is the major effecter in the p53-mediated G1arrest by inhibiting cyclin E-cdk2. This cyclin-dependent kinase

works on the Rb protein to inhibit its binding to E2F1, which promotes the transcription of genes involved in preparing a cell from G1 to S phase. 14-3-3σ is partly responsible for the p53-mediated G2 arrest. The protein can bind to CDC25C and keep it in the cytoplasm, which can prevent it from activating cyclin B-CDC2 in nucleus. Hence, the cell is blocked in the G2 phase. The p53 gene can regulate apoptosis both the extrinsic and intrinsic pathways. In the extrinsic pathway, p53 can increase the expression of Fas (secreted protein) (Michalak *et al*, 2005) and DR5 (the TRAIL receptor, a membrane protein). These proteins then can activate caspase 8 and Bid. In the intrinsic pathway, p53 can up-regulate the expression of pro-apoptotic Bax and down-regulate that of anti-apoptotic BcL-2 (Miyashita and Reed*,* 1995; Selvakumaran *et al,* 1994). P53 can move out of the nucleus, translocate to the mitochondria and interact with Bcl-2 and Bcl-xL to promote the release of cytochome c (Mihara *et al*, 2003)

In addition to cell cycle arrest, senescence and apoptosis, some genes regulated by p53 produce secreted proteins including PAI, maspin and thrombospoindin. These proteins communicate signals to adjacent cells informing them of a stress response (Komarova *et al,* 1998) and changing the extra cellular matrix (Nishizaki *et al,* 1999) .The function of other genes regulated by p53 are related to DNA repair and inactivation of reactive oxidative intermediates and peroxides (Sandra *et al*, 2005).

1.2.4. P53 mutation

P53 mutation occurs in wide variety of human tumors. About 80% of p53 gene

mutations are missense mutation resulting in the synthesis of a stable protein without specific DNA-binding activity and accumulating in the cytoplasm of tumor cells (Soussi, 2007). The DNA binding domain is located in the central region of the p53 protein which is easy to be mutated (Kato *et al,* 2003). There are two classes of mutations: one affects amino acids involved in the protein-DNA interaction; the other affects the conformation allowing binding to hsp70 (Cho *et al,* 1994). Most mutant p53s are defective in trans-activation and may exert a dominant negative effect by preventing wild type p53 from binding to the promoter of its target genes (Willis *et al,* 2004). Some mutant p53s can partially bind DNA and still bind to a subset of p53 response elements (Rowan *et al,* 1996).

1.3. Reovirus

1.3.1 History and family

Mammalian reoviruses (MRVs) were firstly isolated in the respiratory and enteric tracts of a healthy human in the early 1950s. Mammalian reoviruses are the prototypic members of the Orthoreovirus genus, one of the 12 genera of Reoviridae family. The members of Reoviridae have similar structures, genome segment and protein composition. The Ortheoreoviruses contain 4 species: nonfusogenic mammalian reovirus (MRV); fusogenic avian reovirus (Carvalho *et al,* 1982); fusogenic Nelson Bay reovirus and fusogenic baboon reovirus (BRV). Mammalian reoviruses consist of 3 serotypes. Each serotype has a prototypic strain: strain Lang

(T1L) for serotype 1; strain Jones for serotype 2 and strain Dearing for serotype 3 (W K Joklik, 1980).

1.3.2 Morphology and Structure

The Reovirus genome is composed of 10 double stranded RNA (dsRNA) segments, which are enclosed in two concentric capsids consisting of 8 different proteins. The 10 RNA segments show different mobility by polyacrylamide gel electrophoresis due to different numbers of base pairs. They are classified into 3 large segments (L1, L2 and L3), 3 medium segments (M1, M2 and M3) and 4 small segments (S1, S2, S3 and S4) (W K Joklik, 1980).

Reoviruses exist in three forms: the mature virion, intermediate (or infectious) subviral particle (ISVP) and Core. All of them contain the whole copy of segmented genome, but are different in morphology, protein composition, physiochemical and biological properties. The mature virion has 8 structural proteins organized into core (inner capsid) and outer capsid. Outer capsid consists of 600 copies of μ1 or μ1C, 36 copy of σ1 and 600 copies of σ3 proteins. The outer particle works as a gene delivery system and determines the susceptibility of viruses to various inactivating agents. The core is made up of 120 copies of λ1, 60 copies of λ2, 12 copies of λ3, 24 copies of μ2, and 150 copies of σ2 proteins. The virion is the major form released from infected host cells. After intact reovirus virion enters the respiratory and enteric tracts or enters the cytoplasm of infected cells, proteolysis then changes the virion into ISVPs and then the core. ISVPs consist of 7 kinds of structural proteins.

Compared to virion, ISVPs lack the σ3, and the μ1C protein is changed into δ and φ. In addition, the structure of σ1 also is changed. All of these changes can increase the infectivity of ISVPs. The core does not have infectivity outside of host cells due to losing the outer capsid with its binding domain. Because the core has the genome of reovirus and enzymes for virus replication, it can initiate virus replication in cytoplasm (W K Joklik, 1980; Tyler *et al*, 2001).

1.3.3 Life cycle of MRV

1.3.3.1 Entry into cell

The entry of MRV into host cells is mediated by the binding of virus protein to the surface protein of host cells. The outer capsid protein, σ1 is identified as the cell-binding protein. There are several cellular surface molecules serving as receptors for σ1, including sialic acid, multiple sialylated glycoproteins, epidermal growth factor receptors, junction adhesion molecule (JAM). Whether there are other receptors remains unknown. During the attachment of reovirus to host cells, σ1 undergoes a conformational change, which is necessary for viral entry and subsequent disassembly. After attachment, the intact virion may be taken up by receptor-mediated endocytosis. Once virions enter the endosomes, the endosomes will fuse with lysosomes, where virions will be converted into ISVPs and further Core by acid pH and proteolysis. ISVPs are also extracellular created by proteolysis in intestinal tract. The ISVPs are capable of directly penetrating the plasma membrane,

independent of the receptor mediated endocytosis. Then core particles will enter cytosol from endosome, in which the cleavage of the outer capsid protein σ3 is a prerequisite (Tyler *et al*, 2001).

1.3.3.2 Transcription and replication

In the cytosol, the viral particles lose all remaining outer capsid proteins，and then become the viral core. The core particles contain all viral genomic segments and multiple enzymes for viral replication. The λ2 protein undergoes a conformational change that open the λ2 channels, which are important for further transcriptase activation. Reovirus replication has two stages, which are primary and secondary transcription (Tyler *et al*, 2001).

The primary transcription happens in the two hours after infection (Zweerink and Joklik, 1970). In the beginning, only 4 (L1, M3, S3 and S4) of 10 dsRNA gene segments are transcribed to produce mRNA. Then corresponding proteins (λ3, μNS, σNS and σ3) are translated by host ribosomes. These proteins then act on the core by unknown mechanisms to promote transcription of all 10 genes. The initial positive-stand transcripts contain 5'-methylated caps and lack polyadenylated tails. The cap structures are added to nascent mRNAs by the λ2 proteins as the mRNA is extruded through the λ2 channel (Luongo*,* 2002). Then full complement of viral proteins will be translated based on these nascent mRNAs, which also serve as the templates for minus-strand synthesis. These proteins then associate with the ten different mRNAs to form replicase complexes. Every complex packages only one

copy of each gene segment into a particle without an outer capsid and the inner core protein by an unknown mechanism.

The newly produced core-like particles then start the secondary transcription. Ninety five percent of all transcription is produced by these nascent particles (Ito *et al,* 1972). The mRNAs made during secondary transcription do not have cap structure. These uncapped mRNA may be translated in the late stage of infection. The synthesis of viral proteins is also supposed to be controlled by unknown mechanism (Skup and Millward, 1980)

1.3.3.3 Assembly and release

MRV assembly probably involves several stages. The mRNA assortment complex consisting of σ3 protein and non-structural proteins μNS and σNS may form the earliest reovirus particle (Antczak *et al*, 1992). The next particle is RNase sensitive replicase particles, containing both inner and outer capsid proteins, but lack non-structural proteins. This particle has an ssRNA-dependent dsRNA polymerase which is involved in minus-strand synthesis (Morgan *et al*, 1975). Once the minus-strand is synthesized, the replicase particle becomes RNase resistant (Zweerink and Joklik, 1976). When additional copies of the outer caspid proteins are added to virion particles, the assembly is completed. Some studies showed that many reovirus structural proteins exhibit some degree of self-assembly in the absence of viral RNA or other viral proteins (Xu *et al*, 1993). The exact process is not clear. When host cell is dead, the progeny virions then will be released.

1.3.4 Effect of MRV on host cell

The infection of reovirus can inhibit the synthesis of cellular DNA (Gaulton *et al,* 1989A; Roner *et al*.1985), cytoskeleton structure (Sharpe *et al*, 1982) and change the number of growth factor receptors (Strong *et al*, 1993) and their signal transduction capability.

1.3.4.1. Reovirus can induce apoptosis in host cell

Reovirus can induce apoptosis in a wide variety of cultured cells in vitro and in target tissues in vivo, including the heart and CNS (Clarke *et al,* 2003).

Reovirus strains have different capacity to induce apoptosis in various cultured cell lines. It has been investigated in murine L929 fibroblasts and Madin-Darby canine kidney (MDCK) cells. The prototype type 3 (T3D) reovirus strain, Dearing (T3D) has higher capacity to induce apoptosis than the prototype type 1(T1L) reovirus strain, Lang (T1L). Using T1L×T3D reassortant viruses, some studies showed that the S1 and M2 gene segments were the determinants of reovirus-induced apoptosis in L929 and MDCK cells, S1 segment alone as a determinant in Hela cells (Tyler *et al*, 1996; Rodgers *et al*, 1997; Connolly *et al,* 2001). The S1 gene segment encodes the viral attachment protein σ1 and the nonstructural protein σ1s. UV-inactivated reovirus virons, containing σ1 but lacking σ1s, can still induce apoptosis in host cells. This proved that σ1 is required for reovirus to induce apoptosis instead of σ1s (Tyler *et al*, 1995). The major outer capsid protein μ1/μ1c, encoded by M2 gene segment, also

contributes to apoptosis. Treating cells with monoclonal antibodies directed against σ1, μ1, or σ3 before infection can inhibit apoptosis (Virgin *et al,* 1994; Rodgers *et al,* 1997; Connolly *et al,* 2001). It not only delineates that virus proteins like σ1, μ1 contribute to apoptosis by reovirus infection, but also indicates that some steps like viral cell attachment or virus uncoating in reovirus infection have relation to apoptosis.

There are two processes induced by reovirus infection that control apoptosis induction: viral receptor engagement and onset of transcriptional activation. Blocking the viral binding to host cells can inhibit apoptosis. Replication-incompetent UV-treated T3D virions can induce apoptosis, but the efficiency is lower compared to T3D. These results tell us that viral-cell attachment plays a partial role in apoptosis induced by reovirus and that apoptosis is not related to viral replication. Using protease inhibitor E64, an endosomal acidification inhibitor which can block the transformation of virion to ISVPs, can inhibit apoptosis induced by reovirus virions. However E64 can not inhibit the apoptosis induced by ISVPs. The result suggests that apoptosis is induced by intracellular processes in viral infection except for viral-cell attachment (Connolly *et al*, 2002). Ribavirin, a guanosine nucleoside, can inhibit the reovirus dsRNA-dependent RNA polymerase, blocking the synthesis of viral single-stranded (ss) and dsRNA (ds). Treating cells with ribavirin cannot block apoptosis induced by T3D. Apoptosis induced by different reovirus temperature sensitive (ts) mutants in Hela and L929 cells, respectively, ts mustants with replication block in entry of progeny (tsA201), assembly of core particles (tsC447), assembly of outer capsid (tsB352, tsG453), dsRNA synthesis (tsD357, tsE320), and empty virus

component (devoid of dsRNA genome) at non-permissive temperature indicates that the reovirus-induced apoptosis is related to processes of replication cycle before viral transcription (Ban *et al*, 1998).

Reovirus-induced apoptosis in different cell types shares similar basic characteristics by external receptor and internal mitochondrial pathways (Clarke *et al*, 2005). Activation of cell surface death receptors, members of the tumor necrosis factor receptor (TNFR) superfamily, is the first step of apoptosis triggered by reovirus in all cell lines. The binding of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) to the cell surface death receptor (DR) 4 (also called TRAIL-R1) and DR5 (also called TRAIL-R2) can initiate apoptosis in a wide variety of cells including L929 cells, human embryonic kidney (HEK293) cells, and several human cancer cell lines (Clarke *et al,* 2000; Clarke *et al,* 2001). Soluble DR4 or DR5, anti-TRAIL antibodies, and the overexpression of DcR-1 (decoy receptor) can completely block apoptosis induced by reovirus in various cells. In contrast, anti-TNF and Fas Ligand antibodies and soluble forms of non-TRAIL related death receptors all fail to inhibit reovirus-induced apoptosis (Kominsky *et al*, 2002). These results indicate that the reovirus induced apoptosis is initiated by the TRAIL signaling pathway, by which a wide variety of viruses including measles, hepatitis, influenza, respiratory syncytial virus, cytomegalovirus mediate apoptosis in host cells (Clarke *et al,* 2005). Apoptosis of TRAIL-sensitive cells (Hela cell) can be induced by the supernatant from T3D reovirus-infected cells, which can be inhibited by treating the supernatant with soluble DR5, but not by anti-reovirus antibody (Clarke *et al,* 2000). The activity of the

supernatant to induce apoptosis appears 24 hours post-infection and increases through 48 hours post-infection. This suggests that TRAIL released from infected cells can presumably stimulate infected cells (autocrine pathway) or uninfected cells (paracrine pathway) to secret TRAIL. Further, reovirus infection can activate the target molecules downstream of TRAIL receptor attachment, such as caspase 8 and caspase 3 (Richardson-Burns *et al*, 2002; Kominsky *et al*, 2002a).

Reovirus failed to induce apoptosis in Bcl-2-overexpressing MDCK cells indicating the involvement of mitochondrial signaling pathway in reovirus-induced apoptosis (Rodgers *et al*, 1997; Kominsky *et al*, 2002) . The release of mitochondrial pro-apoptotic factors including cytochrome c and Smac/DIABLO from mitochondria to the cytoplasm of infected cells is the direct evidence (Kominsky *et al*, 2002; Clarke *et al*, 2004). Apoptosis inducing factor (AIF) is not detected in the cytoplasm or nucleus of infected cells, which indicates that the release of this pro-apoptotic factor is selective (Kominsky *et al*, 2002). On the other hand, apoptosis induction in the mitochondrial pathway involves changes in mitochondrial transmembrane potential, which reflects the loss of integrity of the outer mitochondrial membrane. Alteration of the mitochondrial transmembrane potential is not found in HEK293 cells (Kominsky *et al,* 2002) or monkey kidney CV-1 cells (Sharpe, *et al*, 1982) infected by MRV.

In cells infected by reovirus T3D, activated caspase 8, one of downstream events of TRAIL-receptor pathway in apoptosis, promotes the cleavage of the Bid (one of members of Bcl-2 family) into truncated from of protein (tBid). Activated tBid can then translocate to the mitochondria and facilitate the release of pro-apoptotic

mitochondrial factors. This is evidence linking death-receptor and mitochondrial apoptotic pathways (Clarke and Tyler, 2003).

1.3.4.2. Reovirus can induce cell cycle arrest in host cells

Mammalian reovirus infection can induce cell cycle arrest in both the G1 (Saragovi *et al,* 1999) and G2/M phase (Poggioli *et al*, 2000). Reovirus strains differ in their capacity to induce cell cycle arrest. T3D and type 3 Abney (T3A) have greater extent of cell cycle arrest than theT1L strain in the inhibition of DNA synthesis, G1 and G2/M arrest. The S1 and M2 genes are the determinants needed to inhibit DNA synthesis and cause G1 and G2/M arrest and were mapped by using T1L×T3D and T1L×T3A reassortant viruses (Gaulton *et al*, 1989A; Tyler *et al*, 1996; Poggioli *et al*, 2000). The G1 arrest is not a common feature of reovirus infection, which is not seen in HEK-293 cells (Poggioli *et al*, 2000). UV-inactivated reovirus virons, containing σ1 but lacking σ1s, can induce G1 arrests in R1.1 thymoma cells. Furthermore，G1 arrest can be induced in thymoma cells by purified T3D σ1 protein alone. This finding shows that σ1, instead of σ1s, is the determinant for reovirus-induced G1 arrest (Saragovi *et al*, 1999). The mechanism of G1 arrest induced by T3D σ1 protein is not clear, which may involve inactivation of p21ras. The reovirus-induced G2/M phase cell cycle arrest has been shown in various cell lines, including HEK293 ,MDCK, C127,and Hela cells (Poggioli *et al*, 2001). T3C84-MA which has normal function of the T3D σ1 protein but lacks the T3D σ1s protein can induce apoptosis, but not G2/M arrest (Tyler *et al,* 1995), which indicates that σ1s protein is necessary for

reovirus-induced G2/M arrest.

Poggioli reported the altered expression of genes involved in the cell cycle in HEK293 cells by using DNA microarray technology after reovirus infection. They found that Type 3 MRV can change expression of 10 genes (such as N-Ras, cyclin E) involved in G1/S transition, 11 (such as wee-1, chk-1) genes participating in G2/M transition and 4 genes (such as BUB-3, MPP11) in mitotic spindle checkpoint. Type 1 MRV influences a smaller number of genes where expression is involved in cell cycle arrest (Poggioli *et al*, 2002).

1.3.5 Progress in clinical research and application

Reovirus naturally prefers to infect intestinal epithelial cells in vivo. These cells have a high capability for proliferation and division. In comparison, normal cells with slow proliferation remain resistant to reovirus infection. Furthermore, studies found that epithelial growth factor receptor (EGFR) minus cells were relatively resistant to reovirus replication, while transfection of the cells with EGFR can dramatically increase the reovirus infection in these cells (Strong *et al*, 1993). The Ras singling pathway, a major pathway downstream of EGFR, plays an integral role in reovirus infectivity (Strong *et al*, 1998). Persistent Ras activation is commonly found in tumor cells while normal cells do not have the pathway activated (Strong *et al*, 1998). So reovirus is a good candidate for cancer therapy due to the preference for growing in tumor cells. Initial experiments found that more than 80% of cell lines from various tumor types were sensitive to reovirus-mediated killing (Coffey *et al,* 1998). So far,

phase Ⅰ and Ⅱ trials of reovirus therapy have been completed in various tumors and histopathological analysis showed that reovirus specifically induced tumor cell death while adjacent normal tissue remained unaffected (Thirukkumaran *et al,* 2003).

1.3.6 MRV reassortant and genetic mapping

Due to the segmented nature of reovirus genome, if different parent reoviruses co-infect the same host cell, this can result in the progeny virions containing a mixed set of gene segments from the two parents. If two strains infect the same cell processed a completely random assortment, the resulting progeny would have 2^n (where n is the number of gene segments) possible gene combinations of genome segments from two parents. In theory, two parent reoviruses will result in 2^{10} (1,024) different gene combinations. However, only 3-25% progeny reassortants are produced from co-infection (Fields, 1971).

There are 83 T1L×T3D reassortants in our lab. The reassortants have been generated and characterized by Dennis Drayna, Earl Brown and Kevin Coombs. Many of these reassortants have been used in many different labs to genetically map a variety of different phenotypes to different reovirus gene segments (Nibert, *et al*, 1996).

1.4 Bcl-x

The Bcl-x gene is a member of the Bcl-2 family.

1.4.1 Bcl-2 family

The Bcl-2 family can be divided into 3 groups (Brunelle and Letai, 2009): (1) anti-apoptotic multi-domain proteins (Bcl-2,Bcl-xL,Mcl-1,Bcl-w,Bfl-1,Bcl-B and so on), which contain four BH domains (BH1, BH2, BH3 and BH4); (2) pro-apoptotic multi-domain proteins (Bax, Bak and Bok), which contain three BH domains (BH1, BH2, BH3), and (3) the pro-apoptotic BH3-only protein family, which includes BAD, BID, BIM, NOXA, BIK, HRK, PUMA and so on. The members of Bcl-2 family share sequence homology in conserved regions, called Bcl-2 homology (BH) domains. In addition to BH domains, several members of Bcl-2 family have a transmembrane domain which can help the protein to localize to the subcellular membranes such as the mitochondrial outer membrane, endoplasmic reticulum (ER) and nuclear membranes. The proteins of Bcl-2 family participate in multiple protein-protein interactions among each other and with other cellular proteins, which results in biological function, such as apoptosis, cell cycle arrest and so on. The level and function of the Bcl-2 family proteins involves regulation of transcription, translation, post-translational control, alternative splicing and intracellular redistribution (Cory *et al*, 2002). Alternative splicing can provide complexity of protein and functional diversification of the Bcl-2 family proteins. For example, Bcl-2 has two isoforms that is Bcl-2α and Bcl-2β. It can happen in other members, including Bcl-2, Bcl-x, Mcl-1, Bfl-1, Bax, Bim, PUMA, Bcl-G, Bcl-rambo, and Bid. The alteration can influence the function and subcellular distribution of the protein. There are several mechanisms involved in post-translational regulation. Posttranslational regulation occurs in the

unstructured loop between the BH3 and BH4 domains in BCL-2 and Bcl-xL. The domain can be phosphorylated, which inactivates the survival function of the proteins (Chang *et al,* 1997). The domain can be subjected to caspase-mediated photolytic cleavage, which results in the removal of BH4, promoting apoptosis (Cheng *et al,* 1997; Clem *et al,* 1998).

1.4.2 Protein product of Bcl-x gene.

In the mouse it has been shown that there are at least 4 different exons located upstream of the unique open reading frame in Bcl-x gene. It suggests that the choice of promoter and alternative splicing may provide an important mechanism for regulation of the Bcl-x gene. So far, five alternative splicing variants of the Bcl-x gene are known: Bcl-xL, Bcl-xS, Bcl-xβ, Bcl-xγ and Bcl-xAK. BcL-xL, consisting of BH1, BH2, BH3 and BH4, a 233-amino acid protein, has anti-apoptotic function. Bcl-xS has 177 amino acids, lacking 63 amino acids sequence from positions 126 to 188 of the Bcl-xL, containing BH3 and BH4, plays a pro-apoptotic function. The Bcl-xβ, 227 amino acid protein having 4 BH domains, was identified as pro-apoptotic function in rat promyeloid cells (Shiraiwa *et al*, 1996) and as anti-apoptotic function in mouse neuronal cells (Ban *et al*, 1998). The Bcl-xγ is a 235 amino-acid protein, containing 4 BH domains, predominantly in T cells, can inhibit apoptosis (Yang *et al*, 1997). Bcl-xAK, a 138 amino acid, containing BH2 and BH4, is a special member in Bcl-2 family because it can induce apoptosis without a functional BH3 domain (Hossini *et al*, 2006). Bcl-xL, Bcl-xS, and Bcl-xAK have the transmembrane domain (Acs *et al*,

1971), so they can locate in cytosol and subcellular membrane. Bcl-xβ and Bcl-xγ lack the structures, so they are located in cytosol.

1.4.3 Bcl-xL

Experiments show that the expression of Bcl-xL is the major form of Bcl-x gene in several kinds of tumor cells (Hossini *et al*, 2006). Post translational modification can occur on Bcl-xL, such as phosphorylation or deamination. The site of phosphorylation is Ser63 within the unstructured loop between the BH3 and BH4 domains. The phosphorylation can be the result of the disruption of microtubules caused by photodynamic therapy (Xue *et al*, 2003) or chemotherapeutics, including paclitaxel, vincristine, vinblastien (Poruchynsky *et al,*1998). In addition to phosphrylation, Bcl- xL is also subject to deamidation at asparagines 52 and 66 (Takehara and Takahashi, 2003). These two kinds of modification can inactivate the survival function of Bcl-xL.

The mechanism of accurate regulation of expression of Bcl-xL protein is not very clear. But some studies show that STAT (especially STAT3 or 5), NF-κB and dysfunction of p53 play an important role in the regulation of Bcl-xL protein (Grad *et al,* 2000; Lee *et al,* 2008).

The main function of Bcl-xL is to maintain the stability of cells by anti-apoptosis and stabilize the cell cycle checkpoints. Bcl-xL is present in the cytoplasm, and then it translocates to the outer mitochondrial membrane. It can inhibit the extrinsic and intrinsic apoptotic pathways. The BH1, BH2 and BH3 domain of Bcl-xL are capable of binding the BH3 domains of pro-aptototic proteins. In this way, Bcl-xL inhibits the

formation of chain structure between Bax and Bak, further preventing the disruption of the mitochondrial membrane and release of apoptotic mediators. Bcl-xL can also inactivate mitochondrial voltage dependent anion-selective channel (VDAC) and prevent the mitochondrial membrane permeability (Shimizu *et al,* 2000). In the extrinsic pathway, Bcl-xL can inhibit caspase-8 cleavage and disrupt DISC formation (Wang *et al,* 2004).

In addition to anti-apoptotic function, Bcl-xL plays a role in stabilizing cell cycle checkpoint. The overexpression of Bcl-xL can stabilize the G2/M cell cycle arrest. The progression from G2 into mitosis is regulated by the serine-threonine kinase cdk1 (cdc2). Experiments show that Bcl-xL colocalizes and binds to cdk1 in the nucleus during the G2/M cell cycle checkpoint. The flexible loop domain of Bcl-xL can inhibit cdk1 kinase activity (Schmitt *et al*, 2007).

1.5 Objective of this present study

Reovirus infection results in apoptosis, cell arrest, alteration of cytoskeletal structure and inhibition of protein synthesis in a wide variety of cells as discussed above. It is unavoidable that reovirus infection changes the expression of host genome. In the course of infection, reovirus replicates just in the cytoplasm. Thus, it is interesting to study how the reovirus infection changes the expression of host genome. Previously, the mRNA levels of different cellular genes GADD45α, p21, mcl 1, bax, p53, TRAIL, DR3, caspase-8, RIP, FAS, FASL in H1299 (p53-null) cells after infection with reovirus were tested in our lab. We found that reovirus can change the

expression of GADD45-α and Bcl-x mRNA by a p53-independent pathway. p53, as mentioned above, plays a critical role in the apoptosis and cell growth arrest induced by various virus infections through regulating the expression of host genes. However, the role of p53 in reovirus infection is largely unknown. Therefore, it is interesting to study the mechanisms by which reovirus infection changes the expression of Bcl-x protein with a specific interest in the role of p53 during this process.

To better understand the direct relationship between virus infection and expression of host genes, we need to carry out experiments in both p53-null and p53-positive background. H1299 (p53 positive and p53-) (Chen *et al*, 1996), HT1080 (p53 positive and p53 mutation) and HCT116 (p21 deficient and 14-3-3σ deficient)) (Waldma *et al*, 1995) provide us with valuable tools to study the relationship of p53 and changes of Bcl-x protein expression in different cell lines after reovirus infection. It is also possible that we can further find some p53-independent cellular response pathways. Mammalian reovirus can replicate in various mammalian cell lines. It can result in changed condition of host cell. The symptoms of MRV infection are usually subclinical in humans or limited to young animals, so it is accordingly safe to work with. There are 83 T1L×T3D reassortant reovirus with identified gene maps available in our lab. All of the above provide us with good material to investigate the reovirus infection and host cell responses.

My hypothesis is that: MRV infection can change the expression of Bcl-xL protein expression by p53 independent pathway.

The summary of research direction in this study

To examine changes of the expression of gene

and protein of Bcl-x in host cells induced by MRV

(T1L and T3D) infection in p53 wild type and null background

To evaluate the impact of p53 on this change

To investigate which viral gene or protein leads

to this change by reassortant of T1L x T3D mapping

Chapter II MATERIALS AND METHODS

2.1 Stock cell lines and viruses

2.1.1 Stock cell line.

(1) mouse fibroblast: L929: cells were grown in suspension cultures at a density of 4.5～5.0×10^5 cells/ml in Jokliks modified minimal essential medium（J-MEM） (Gibco,Grand Island,NY) supplemented with 6% fetal calf serum (FCS) (Intergen,Purchase,NY),and 2mM L-glutamine. The L929 cells generally grow to～ 1 ×10^6 each day and are passaged every day. (2) Human lung carcinoma cell lines: H1299 (p53-null) and H1299 (p53 positive) (transfected with p53 plasmid to express wild-type p53 protein transiently) cell lines (kindly gift provided by Dr. X. Chen) were cultured with D-MEM (Gibco, Grand Island, NY) with 10% FCS and 2mM L-glutamine and H1299 (p53 positive) cells were also cultured with 1ug/ml of Tetracycline. (3) human colon carcinoma cell lines: HCT116 (p21 deficient) cells and HCT116(14-3-3σ deficient) cells (kindly gift provided by Dr. Michael R. Mowat) (4) human fibroblastoma cell lines: HT1080 (p53 positive) and HT1080 (p53 mutant) cells (kindly gift provided by Dr. Michael R. Mowat) (5) TLA-HEK293T^{TM} Cell lines: 293-T cells (6) carcinomic human alveolar basal epithelial cell lines: A594 cells were cultured with D-MEM with 10%FCS and 2mM L-glutamine. (7) myelogenous leukaemia cell line: K562 cells were cultured with 1640 media with10% FCS.

2.2.2. Virus passaging and plaque assay

Prototype mammalian reovirus type 1 Lang (T1L), type 3 Dearing (T3D) and T1L ×T3D reassortants are laboratory stocks. Virus infections were carried out either in monolayer (grown in the presence of 5%CO_2 at 37℃) or suspension (grown at 33.5 ℃), supplemented with the media described above plus 100U/ml penicillin,100ug/ml amphotericin-B as previously described (Coomb *et al*, 1994).

2.2.2.1 Virus passaging

5ml L929 cell suspension were added into T25 Corning flasks (Fisher, Nepean.ON) at 0.5×10^6 cells/ml and incubated overnight or 4×10^6 cells were incubated for 1 to 2 hours to allow attachment, the cells were infected with reovirus strains with 0.5ml of plaque-purified P_0 stock. The virus was allowed to adsorb to the cell monolayer for 1hour at room temperature with regular rocking every 10 minutes. After adsorption, the infected cells were supplemented with 4.5 ml of MEM supplemented with antibiotics and were incubated at 37℃. The cells were examined daily for CPE. When 80-90% CPE was observed, flasks were frozen at -80℃. They were frozen and thawed three times, and then the P_1 cell lysates were collected. The viruses for P_2 and P_3 stocks were cultured as above for P_0-P_1 passage by using different sizes of flasks.

2.2.2.2 Virus amplification.

To purify large amounts of virus particles, batch infections of more than 500 ml

were set up. For 1L batch infection, 6.5×10^8 cells were centrifuged at $100\times g$ for 10 minutes. The cell pellet was re-suspended to a final concentration of $1\text{-}2\times10^7$ cells /ml with culture media and infected with virus from a P_2 or P_3 stock at a multiplicity of infection (MOI) of 3-5 plaque forming unit (PFU)/cell. After adsorption for 1 hour at room temperature with periodic swirling every 10 minutes, the infected cells were diluted to 6.5×10^5 cells /ml with 75% fresh prepared MEM containing antibiotics and 25% pre-adapted media. The suspension of infected cells was incubated at 33.5℃ for 65 hours for most strains. Some slower growing viruses required longer incubation times. In these cases, the infected cells were fed fresh prepared media at 48 hours. Cells were harvested when there was 60% cytopathic effect (CPE) determined by Trypan Blue exclusion using a hemocytometer. After incubation, cells were centrifuged at $2200\times g$ for 20 minutes in a fixed-angle rotor (JA-10) in a Beckman RC centrifuge (Beckman, Mississauga, ON). Cells were resuspended in 12ml homogenization buffer (HO buffer) (10Mm Tris, 259mM NaCl, 10mM β-mercap-toethanol and pH adjusted to 7.4,) and transferred to a 30ml COREX tube and total volume was recorded. This mixture could by used immediately for viral purification or frozen at -80℃ for later treatment.

2.2.2.3 Virus purification

Cells were sonicated in COREX tube for 10 seconds. The cell membranes were disrupted with the addition of 1/50 sample volume of 10% DOC (desoxycholate) followed by vortexing and incubation on ice for 30min. Vertrel® XF

(1,1,1,2,3,4,4,5,5,5-decafluoropentane) was added into the sample to extract virus. Virus was further purified in 1.20-1.45g/cc cesium chloride gradients. Virus bands were harvested and dialyzed extensively against D-buffer (Dialysis buffer, 150mM NaCl, 15mM $MgCl_2$, 10mM Tris and pH adjusted to7.4) to remove cesium chloride. Virus was collected into appropriate vessels and stored at 4℃ or frozen with the addition of 25% glycerol at -80℃.

2.2.2.4 Plaque Assay.

On day0, 1.2×10^6 L929 Cells were plated onto Corning Costar 6-Well or 0.5×10^6 L929 cells for 12-welll dishes (Fisher ,Nepean .ON) and incubated overnight at 37℃ with 5% CO_2 to form monolayers (If the cell monolayers were prepared the same day as the virus infection, the numbers of each well were doubled to add into the cells and cultured for 3-4hours).

On day 1, serial 1:10 dilutions of virus stocks were made in gel saline (127mM NaCl, 0.2mM $CaCl_2$, 0.8mM $MgCl_2$, 19mM HBO_3, and 0.1mM $Na_2B_4O_7$, 0.3% wt/vol gelatin). The cell monolayers were infected in duplicate with 100ul/6-well (50ul/12-well of each cell dilution) and viruses allowed to attach to cells for 1hour with periodic rocking every 10 minutes, each well was overlaid with 3ml/6-well (1ml/12-well) of a 50:50 ratio of 2% agar and 2×Medium199 (M199) supplemented with 6% FCS and a final concentration of 2mM L-glutamin,100U.ml of penicillin,100ug/ml streptomycin sulfate and 100ug/ml amphotericin-B. The infections were incubated at 37 and 5% CO_2. On day3, cells were fed with 2ml/6-well

(0.8ml/12-well) fresh agar/M199 (prepared as above). Cells were strained with a 0.04% neutral red solution (neutral red added to a 1:1 ratio of 2×PBS to 2% agar) on day6 or day7 (depending on the plaque sizes). Viral plaques were counted 15-18 hours later and titers calculated.

2.2.2.5. Virus purification:

To replenish viral stocks, viral plaques were isolated and picked with a sterile Pasteur pipette. The viral/agar plugs were re-suspended in 0.5ml MEM supplemented with antibiotics and placed at 4℃ for 24 hours to allow viral diffusion into the media for P_{0-1} stock. The stock was used for an additional round of plaque purification to yield P_{0-2} stock. The double plaque purified stock（P_{0-2}）was amplified twice (P_1,P_2) and harvested by freeze/thaw three times.

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2.3 Infection of host cell

H1299 (p53 positive and p53-), HT1080 (p53 positive and p53 mutation) and HCT116 (p21 and 14-3-3σ deficient)) cells were individually set up in 6-well plate. When the confluence arrived to 70%, the cells were infected with T1L, T3D at MOI=0, 0.5, 2.5,10,25,100 PFU/Cell according to standard methods previously mentioned. At different time points, the cell sample was harvested for virus titer and Western blotting.

2.4 Virus titer test

The samples were stored at -80℃ followed by freeze and thaw for at least 3 times and then tested by standard plaque assay.

2.5 Immunoprecipitation and in-gel or in-solution digestions

2.5.1 Preparation of cell lysate

The cell monolayers were washed with cold PBS twice, and scraped from the plates. The suspension cells were centrifuged, and then washed by cold PBS twice. After washing, pelleted cells were harvested.

2.5.1.1 Subcellular fractionation

The cells were dissolved in NP-40 lysis buffer (150mM NaCl,1%Nonidet P-40, 50mM Tris and pH adjusted to 8.0) protease inhibitors (Pepstatin A 1μg/ml, Leupeptin 10 μg/ml, Aprotinin 2μg/ml, PMSF 1mM, EDTA 5mM, EGTA 1mM, Na Fluoride 10 mM, Na Orthovanadate 1mM) at 4℃ for 20min, followed by centrifugation at 9300×*g* and the supernatants harvested. The supernatants were from cytosol and the pellets were from the other parts except cytosol (mostly from nuclear and mitochondrial membranes). Then the pellets were washed with PBS followed by centrifugation at 9300×*g* for 20min twice. The pellet were resuspended by RIPA buffer (150mM NaCl,1.0% NP-40 0.5% sodium deoxycholate, 0.1%SDS, 50mM Tris and pH adjusted to 8.0) with protease inhibitors. The samples were sonicated for 10 seconds on ice followed by centrifugation 9300×*g* for 30min and the supernatants harvested.

2.5.1.2. Whole cell extract

To get the whole cell lysate, the fresh cells pellet was directly resuspended with RIPA buffer. Then the samples were sonicated for 10 seconds on ice followed by centrifugation at 9300×*g* for 20min and the supernatants harvested.

2.5.2 Immunoprecipitation

The protein A/G-agarose (Cat.#20421, Pierce) was washed with buffer (150mM Nacl, 50mM Tris and pH adjusted to 8.0) twice to remove ethanol followed by Blocking buffer (1%Serum Albumin, 150mM NaCl, 50mM Tris and pH adjusted to 8.0) incubation at 4℃ for 1 hour. Then 100ul anti-Bcl-xl primary antibody (Sc634, Sant Cruz) was added and incubated with the protein A/G-agarose at 4℃ for 2 hours. Then cell lysates were incubated with the antibody-A/G-agarose complex at 4℃ overnight. On second day, the samples were centrifuged and washed by washing buffer 5 times. Finally the pellet containing precipitated protein was harvested for the next step.

2.5.3 In-solution digestion

The pellets were diluted with water up to 100ul, and then 10ul 100mM dithiothreitol (DTT) were added. Then the sample was incubated at 56℃ for 1 hour. After the samples were cooled down to room temperature, 17ul of 550mM iodoacetamide was added into each sample. Each sample was incubated in room

temperature for 40min. The sample was then treated with17ul 100mM DTT and 2.0ug Trypsin, followed incubated at 37℃ overnight. On the second day, 10ul of 1%TFA (Trifluoroacetic acid) was added per 100ul sample. Then the sample was centrifuged at 9300×*g* for 1min.The supernatant was vacuum-dried. Then the sample was checked by Mass spectrometry.

2.5.4 In-gel digestion

For the each sample, an equal volume of 2X Laemmli buffer (4% SDS, 10% 2-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris and pH adjusted to 6.8) was added followed by heating at 95℃ for 5min to elute precipitated protein and antibody. Then the samples were centrifuged and the supernatants were harvested. The samples were loaded onto 12% polyacrylamide gels, subjected to electrophoresis (detail preparation see **2.7.1**). The protein gels were washed by water for 30min, and then stained by gel staining solution (Cat.#24590 Pierce) overnight. Further the gels were washed by water until the bands became clear. The gels bands were cut into small pieces. 100mM ammonium bicarbonate and acetonitrile (ACN) were used to wash the gel pieces until the staining color disappeared. Gel pieces were further vaccum-dried. 100ul 10mM DTT was added onto the dry gel piece and incubated at 56℃ for 45min. Then the volume was recorded of DTT absorbed by each gel piece. 50ul 55mM iodoacetamide was added into each gel piece, and then keep at room temperature for 30min. According to the volume of DTT absorbed by the gel, same volume of 50ng/ul Trypsin was added onto each gel piece. The whole

tubes were incubated at 37℃ overnight. On second day, 1%TFA and ACN were used to wash the digested peptides out of the gel piece. Then the supernatants containing peptides were further vacuum-dried. Then the samples were checked by Mass spectrometry.

2.6 Transfection of Bcl-xL shRNA into target cells

The Bcl-xL shRNA vectors RHS3979-9600907 (CCGGGCTCACTCTTCAGTCG GAAATCTCGAGATTTCCGACTGAAGAGTGAGCTTTTTG), RHS397-9600908 (CC GGGTGGAACTCTATGGGAACAATCTCGAGATTGTTCCCATAGAGTTCCACTTTTT G), RHS397-9600909 (CCGGGTTTAGTGATGTGGAAGAGAACTCGAGTTCTCTTC CACATCACTAAACTTTTTG), RHS3979-9600910 (CCGGAGAGCTTTG AACAGGAT ACTTCTCGAGAAGTATCCTGTTCAAAGCTCTTTTTTG), RHS3979-9600911(CCGG CGACGAGTTTGAACTGCGGTACTCGAGTACCGCAGTTCAAACTCGTCGTTTTT) and pLKO.1 empty vector RHS4080 (as negative control) were purchased from Thermo Scientific. The lentiviral packaging plasmids psPAX2 (Plasmid 12260) and pMD2.G (Plasmid 12259) were purchased from Add Gene.

2.6.1. Replication and purification of plasmid

The *E.coli* containing plasmid was cultured in 2XLB Broth media (LB-Broth-Lenno x 20g/l, Peptone 10g/l, Yeast Extract 5g/l) with 100ug/ul Ampicillin at 30°C over night. Plasmid Maxi kit (No.12163, Qiagen) was used for extraction of plasmid DNA. Finally the plasmid DNA was stored in 10 mM Tris (pH adjusted to 8.5) at 4°C or

-80℃.

2.6.2. Packaging lentiviral particles

TLA-HEK293T cells were plated at 5.5x10^6cells per 100mm plate before transfection. Each Bcl-xL vector or pLKO.1 vector (9ug) was mixed with psPAX2 (4.5ug) and pMD2.G (9ug) with 112.5ul Arrest-In™ transfection reagent (ATR1740, Thermo scientific). Then the mixture was diluted by DMEM media (without serum and antibiotic) to 2ml, and was incubated at room temperature for 20min. The growth media was aspirated from TLA-HEK293T cells. The 2ml mixture and 1ml media without serum were added to TLA-HEK293T cells. The cells were returned to the CO_2 incubator at 37℃ for 6 hours. Then additional 7 ml of complete DMEM with 10% serum and 200U/ml of penicillin, 200ug/ml streptomycin sulfate and 200ug/ml amphotericin-B was added to cells. At 48 and 72 hours, the old media was harvested which contains lentiviral particles. The media was centrifuged at 1900×*g* for 20 minutes at 4°C to pellet cell debris. The supernatants were centrifuged at 50000×*g* for 1.5 hours at 4°C. The pellet was diluted by 1ml DMEM media without serum.

2.6.3 Transfecting cell lines

The concentration of puromycin needed to select for resistance has been tested in A539 and H1299 cells before transfection. In this study, the resistant concentrations were 1ng/ml for A549 cells, and 3ng/ml for H1299 cells. A549 and H1299 cells were set up at 2.5x10^5 cells per well in 6-well plates before transfection.

The cells were washed with PBS twice. 100 ul media with Lentiviral particles was add to each well, and then 1ml fresh media without serum was added to each well. After 8 hours, 2 ml complete media was added into each well. After 48 hours post transfection, puromycin was added into each well to select transfected cells.

2.7 Western blotting

The expression of Bcl-x protein was analyzed by Western blotting (immunobloting)

2.7.1 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to separate proteins according to their electrophoretic mobility. The SDS gel was prepared in the Mini-PROTEAN Tetra Cell system (-8000 Bio-Rad). 12% separating SDS gels were poured and allowed to polymerize for 40min. Then stacking SDS gels were added on the top of separating gel. The preparation of protein samples was described in section **2.5.4.** The glass clamped gels were set up and filled with gel running buffer (25mM Tris-HCl, 200mM glycine and 0.1%SDS). Each well was loaded with 200ug of protein samples. The gels were run was at 120V for 2 hours for protein separation in MINI-PROTEAN Tetra cell system. The samples were run until the bromophenol blue marker approached the bottom of separating gel; the gels were taken out of the glass plates for transfer of proteins for membranes.

2.7.2 Transfer of protein from SDS page to PVDF membrane

The gels were soaked in transblot buffer (48mM Tris, 39mM glycine, 0.0375% SDS and 20%MeOH) for several minutes after SDS-PAGE. At same time, the transfer membranes (ImmoblinTM,Millipore, Bedford, MA) were first wetted with 100% MeOH for 1minute, then ddH_2O for 1minute, and finally soaked in Transblot buffer for 5min. LKB 2117 Multiphor II Electrophoresis Unit was used for transferring. The transferring sandwich was arranged according to the following order: 4 pieces of filter paper, PVDF membrane, gel, and another 4 pieces of filter paper placed from anode to cathode direction.

2.7.3 Incubation of PVDF membrane with antibodies and developing after transfer,

Membranes were washed once with ddH_2O, then stained with 2% Ponceau-S(3-hydroxy-4[2-sulfo-4-(-4-suflophenylazo0-phenylazo)-2,7-naphthalenedi sulfonic acid (Sigma, Oakvile, Ontario), Membranes were rinsed by ddH2O until protein bands became visible, and soaked in blocking buffer (5% Skim milk in TBST buffer (20mM Tris-base,100 Mm NaCl and 0.05%Tween20) at 4℃ over night. The PVDF membranes were washed in TBST buffer for 1 min. Then rabbit anti-Bcl-xL/S polycolonal antibody (S493, Sant Cruz) at 1:1000 dilution in blocking buffer was used to incubated with the membrane for 4 hours at 4℃. The membrane was washed three times with TBST (10min/time) to remove weakly bound primary antibodies. Then goat-anti-rabbit-IgG coupled to horseradish perioxidase at 1:10000 dilution in

blocking buffer was incubated with the membrane at room temperature for 50min. The membrane was washed three times with TBST (15min/time) to remove non-specifically bound antibodies. ECL solution was used to develop the PVDF membrane in Image machine (AlphaInnotech, San Leandro, USA). The antibodies then were stripped using a stripping buffer (1.5% glycine, 0.1%SDS, 0.5% Tween 20, pH adjusted to 2.2). The membrane was wash with PBS twice, and then TBST twice. The expression of GAPDH protein was detected using a similar protocol as mentioned. The antibody used was mouse anti-GAPDH antibody (NB300-328, NOVUS Biologicals) at 1:10000 dilution, and Goat-anti-mouse IgG antibody (ab6728, Abcam)

2.8. In Vitro Deamidation Reaction

The treatment was modified from Takehara and Takahashi, 2003. The H1299 cells were treated by alkaline buffer (150 mM NaCl,1.0% NP-40 0.5% sodium deoxycholate, 0.1% SDS, 25mM glycine-NaOH, pH10), with or without added proteinase inhibitors) , or with neutral Buffer (150 mM NaCl,1.0% NP-40 0.5% sodium deoxycholate, 0.1% SDS ,50 mM Tris and pH adjusted to 8.0 with or without proteinase inhibitor). The samples were sonicated for 10 seconds on ice followed by centrifuge 9300×*g* for 20min and the supernatants harvested. Each sample was then incubated at 4℃ or 37℃ for 4 or 16 hours.

CHAPTER III: RESULT

3.1 Morphology of Cells infected by MRV

To study whether p53 has any effect in the responses to MRV infection, we recorded the morphological changes of various cell lines (H1299, HT116 and HT1080) at different time points (6, 24, 48 and 72 HPI, data not shown in other time points except 48 HPI). Figure 1 showed the morphological changes at 48 hours post infection at MOI=10 PFU/cell. We found that p53 positive and defective cell lines exhibited distinctive morphologies after MRV infection.

p53 positive HCT 1080 and H1299 cell lines showed characteristic features of cytopathic changes after infection with MRV. Compared to their controls without infection, MRV-infected, p53-positive cells had a round shape, shrunken cell membrane, debris from dead cells floating in the media or loosely attached and the nucleus became swollen or shrunken. I further found that T1L or T3D MRV caused slightly different types of CPE, especially in p53 positive HT1080 cell lines. Greater cell debris, more blank areas without attachment of cells and lower density of attached cells were observed in cells infected by T1L, while after infection with T3D, the predominant cells founded was small rounded, shrinking or swelling and very loose attached to the wall of plates. The number of attached cells in the T3D infection was relatively higher than that in T1L infection.

Figure 1

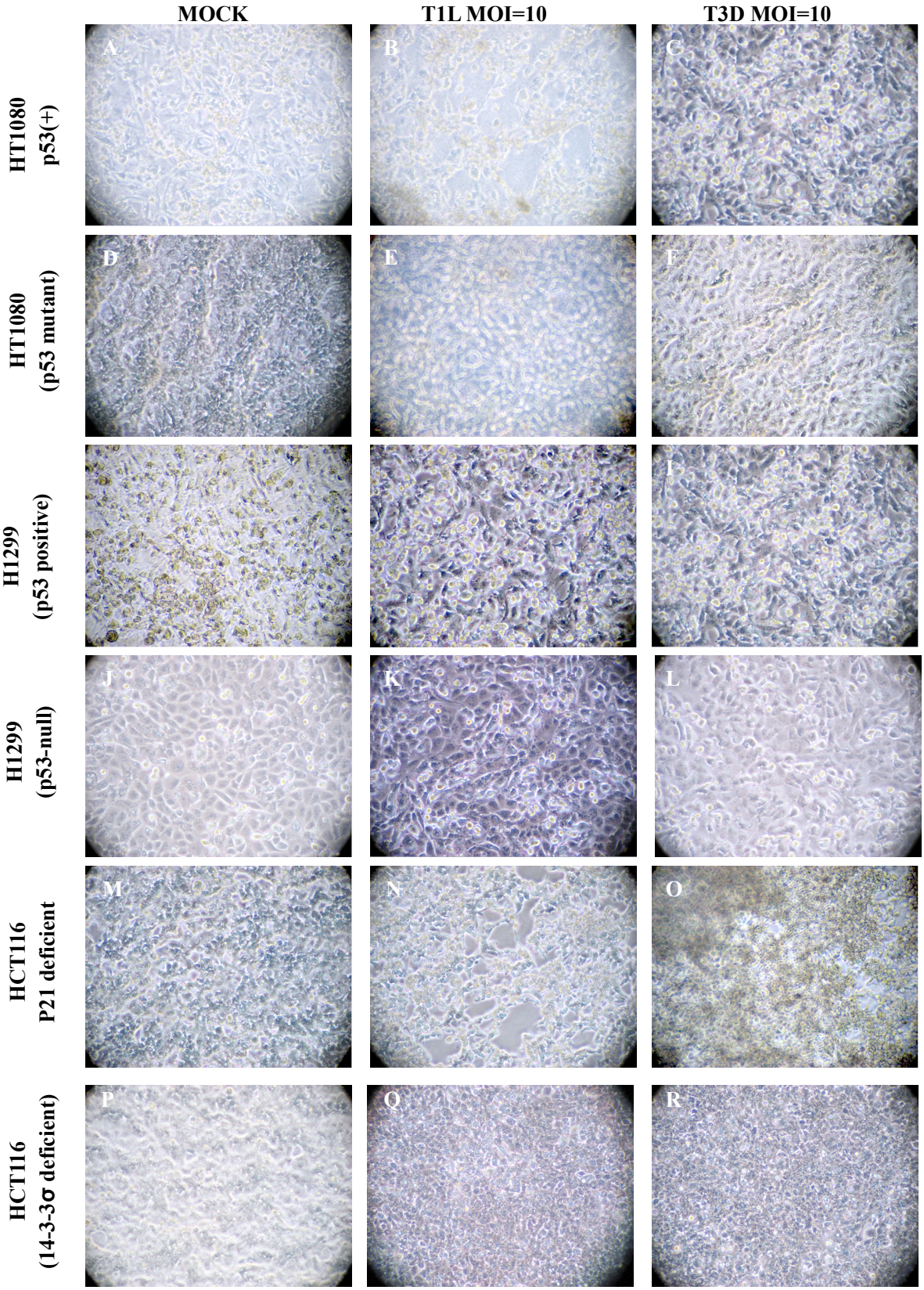

MOCK
T1L MOI=10
T3D MOI=10
HT1080 p53(+)
HT1080 (p53 mutant)
H1299 (p53 positive)
H1299 (p53-null)
HCT116 P21 deficient
HCT116 (14-3-3σ deficient)
A
B
C
D
E
F
I
J
K
L
M
N
O
P
Q
R

Figure1. Six cell lines infected with reoviruses T1L or T3D at MOI=10 PFU/cell at 48 hours post infection. A. HT1080 (p53 positive) without infection; **B.** HT1080 (p53 positive) infected by T1L; **C.**HT1080 (p53 positive) infected by T3D; **D.** HT1080 (p53 mutant) without infection; **E.** HT1080 (p53 mutant) infected by T1L; **F.** HT1080 (p53 mutant) infected by T3D; **G.** H1299 (p53 positive) without infection; **H.** H1299 (p53 positive) infected by T1L; **I.** H1299 (p53 positive) infected by T3D; **J.** H1299 (p53-null) without infection; **K.** H1299 (p53-null) infected by T1L; **L.** H1299 (p53-null) infected by T3D. **M.** HCT116 (p21 deficient) without infection; **N.** HCT116 (p21 deficient) infected by T1L; **O.** HCT116 (p21 deficient) infected by T3D; **P.** HCT116 (14-3-3σ deficient) without infection; **Q.** HCT116 (14-3-3σ deficient) infected by T1L; **R.** HCT116 (14-3-3σ deficient) infected by T3D;

After infection with MRV, p53 defective cell lines exhibit different morphology from p53 positive cell lines by becoming brighter, larger and rounded, but not swollen or shrunk, nor dislodged from the wall of culture plate. Moreover, p53 defective cell lines caused less CPE irrespective of doses of MRV than p53 positive cell lines. This implied that different pathways could be involved in the responses to MRV infection in p53 positive and defective cell lines.

Furthermore, we found that, compared to lower MOI, higher doses of MRV infection at different time points resulted in more cell death and CPE in both p53 positive and defective cells lines. The appearance of CPE was also earlier in cells with higher dose of MRV infection.

At 72 hours, the media color in HCT116 (p21 and 14-3-3σ deficient) and HT1080 (p53 mutant and p53 positive) became yellow and cells were dislocated from the wall in clusters, even with Hepes buffer used to adjust the media pH. It is probable that cells replicated at a very fast speed that metabolized product influenced the morphology and growth of cells beyond the effect of virus infection. It was observed that, with a slower growth rate compared with HT1080, H1299 (p53-null and p53 positive) cell lines cultured for 72 hours with or without MRV infection have similar morphologies as those cultured for 48 hours. Thus, it is possible that the metabolism of cell line H1299 plays a less role compared with the virus effect.

Having observed p53 positive and defective cells respond distinctively after MRV infection, we furthered looked at the morphological changes of HCT116 (p21 and 14-3-3σ - deficient) cell lines with MRV infection. It was reported that HCT116 cell

lines deficient in p21 failed to maintain stable G1/S and G2/M arrest, and eventually underwent apoptosis, while HCT116 (14-3-3σ deficient) cell line could maintain G1/S arrest, but not G2/M arrest (Chan *et al*, 2000). As shown in Figure 1, we found that these two cells lines showed different morphologies after MRV infection. HCT116 (p21-deficient) had a round shape with shrunken cell membrane and swollen or shrunken nucleus. HCT116 (14-3-3σ deficient) cell lines were bright, bigger and round after infection. Moreover, there was more cell debris from dead cells floating in the media in HCT116 (p21-deficient) cells lines.

3.2 MRV growth curve in HT1080 and HCT116

The replication of MRV in HT1080 or HCT116 cell lines was tested at MOI=10. The virus titers were tested by standard plaque assays after cells were harvested at different time points.

We found (Figure 2A) that the amplification of MRV in HT1080 (p53 mutant) and HT1080 (p53 positive) cells reached quickly to the plateau within 24-36 hours and the titers were maintained until 48 hours, and then the titers of virus dropped slowly between 48-72hours. In HCT (p21 or 14-3-3σ deficient) cell lines, the titers of virus increased slowly to the plateau within 36-48 hours. Then the titers of virus slightly went down between 48-72 hours (Figure 2B).

Paired t test was used to compare the capacity of each MRV strain in different cell lines. We found that the titters of MRV, whether T1L or T3D, had no statistically significant differences ($p>0.05$) in HT1080 and HCT116 cell lines at different time

Figure 2.

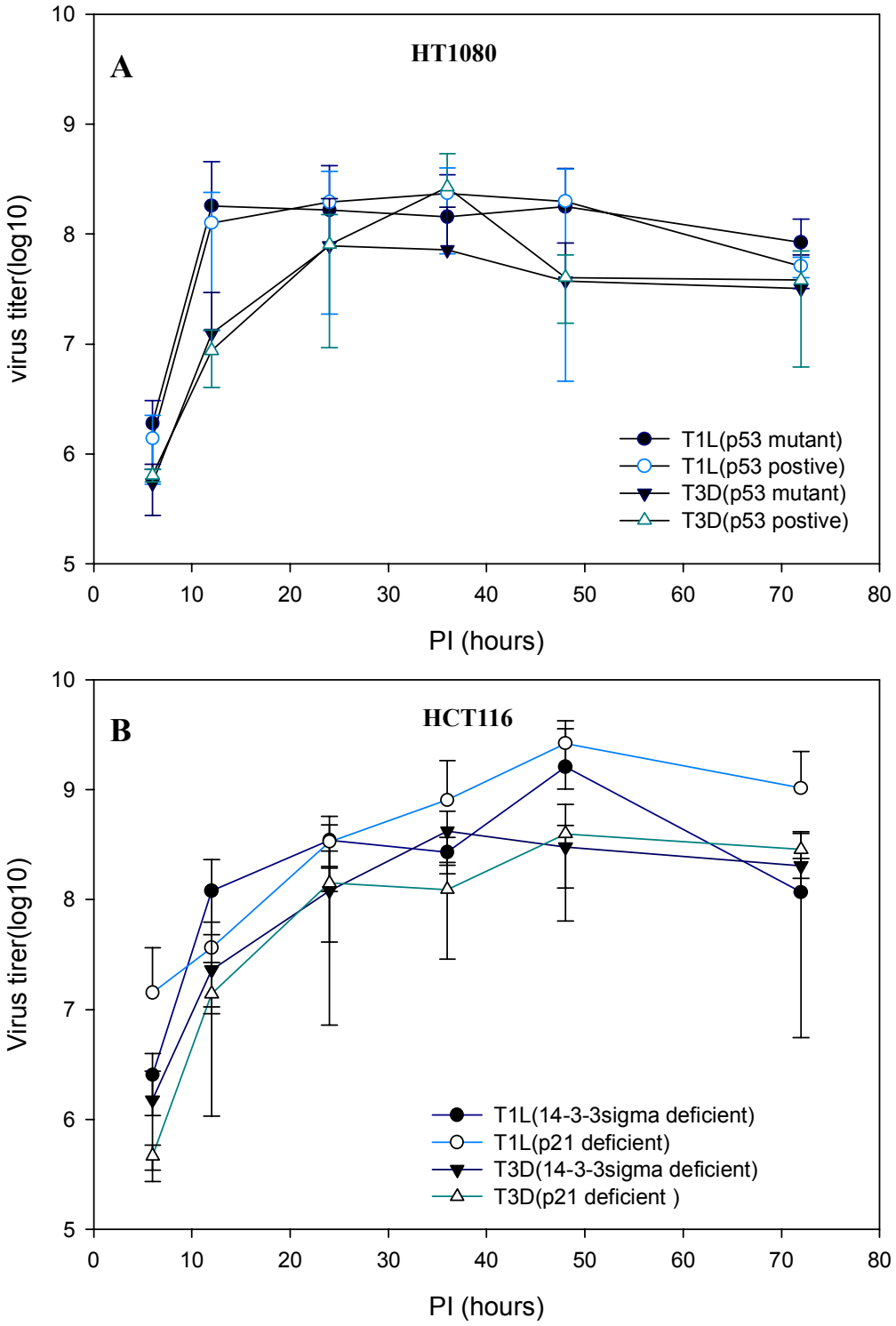
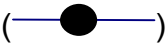
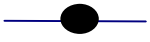
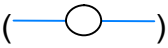

A
HT1080
virus titer(log10)
PI (hours)
T1L(p53 mutant)
T1L(p53 postive)
T3D(p53 mutant)
T3D(p53 postive)
B
HCT116
Virus tirer(log10)
PI (hours)
T1L(14-3-3sigma deficient)
T1L(p21 deficient)
T3D(14-3-3sigma deficient)
T3D(p21 deficient)

Figure 2. Reovirus replication in different cell lines. HCT116 (p21 or 14-3-3σ deficient) and HT1080 (p53 mutant and p53 positive) were infected by T1L or T3D at MOI=10. The virus cultures were harvested at different time points and the titers were measured by a plaque assay. The data represents the average of three experiments (n=3) and the error bars represent standard deviation of the mean. **A**.virus replication in HT1080 cell lines. T1L (p53 defective) (); T1L (p53 positive) (); T3D (p53 defective) (); T3D (p53 positive) () **B.** virus replication in HCT116 cell lines; T1L (14-3-3σ deficient) (); T1L (p21 deficient) (); T3D (14-3-3σ deficient) (); T3D (p21 deficient) ()

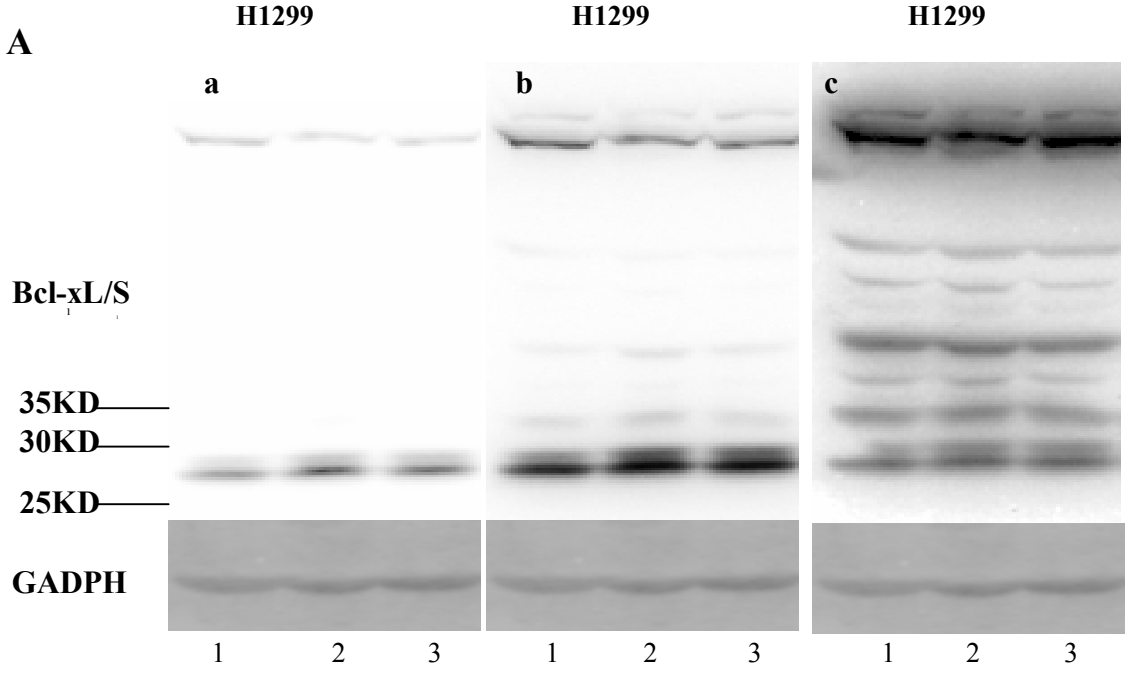
points.

In our lab, previous data showed that MRV (T1L or T3D) has similar capability to replicate in p53-null and p53 positive H1299 cell lines. Thus, MRV can replicate in both p53, 14-3-3σ, p21-defective and p53 positive cell lines, and the presence or absence of p53 in cell lines had no significant effect on MRV replication.

3.3. Identification of Bcl-xL protein by western blot

The polyclonal antibody (Sc-634, Santa Cruz) can detect both Bcl-xL and Bcl-xS (the most common splice variants of Bcl-x gene). The molecular weights of the two proteins are around 28kD and 18kD. First, we needed to confirm the position of the two proteins by western blot. I tested the samples from normal cultured p53- H1299 at 24, 48 and 72 hours by western blot. I obtained just one band around 26-35kD (Figue3Aa) if the membrane was developed for 1min. If the membrane was developed for 5min, I got several bands (Figure3Ab), but the most obvious band was still the band of 28kD. According to the molecular weight, I deduced the band of 28kD is Bcl-xL protein; I could not detect the expression of Bcl-xS (around 18kD). It was reported that Bcl-xS protein expression was very low compared to the expression of Bcl-xL from previous studies (Mozzetti *et al*, 2000; Hossini *et al*, 2006). The detection of Bcl-xL/S protein by western blot was repeated on the same PVDF membrane. Compared with the first time detection (Figure 3Aa+b), PVDF membrane was incubated with primary antibody for longer time and developed by ECL for 7min. No band around 18kD could be found, but more bands higher than 28kD were seen

Figure 3


A
H1299
H1299
H1299
a
b
c
Bcl-xL/S
35KD
30KD
25KD
GADPH
1 2 3 1 2 3 1 2 3

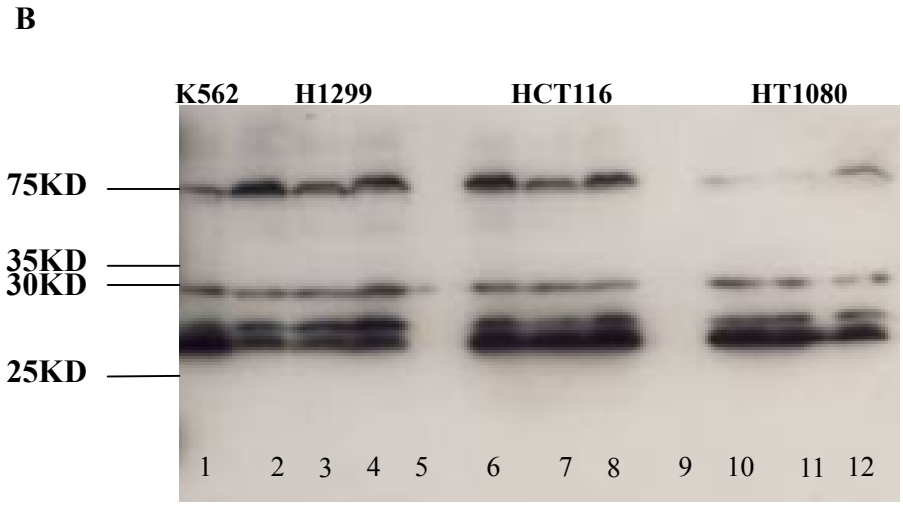

B
K562
H1299
HCT116
HT1080
75KD
35KD
30KD
25KD
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3. Bcl-x protein detected by western blotting. **A** shows the expression of Bcl-x protein in H1299. **a, b** and **c** are result of same PVDF membrane after different development conditions. After the transfer of protein from 10%SDS gel to PVDF membrane, the membrane was incubated with blocking buffer for more than 6 hours at 4℃, and then incubated with a 1:1000 dilution of primary antibody for 4 hours at 4 ℃. Then the PVDF was incubated with a 1:10000 dilution of secondary antibody for 1 hour at room temperature. Finally, the membrane was developed by ECL. **a** corresponded to 1 min exposure to film; **b** was corresponded to 5 min exposure to film; **c**: the membrane was incubated with primary antibody again for 2 hours at room temperature. Then it was incubated with secondary antibody for 1 hour. Finally the membrane was developed again by ECL for 7min. **1** is H1299 (24 hours incubation), **2** is H1299 (48 hours incubation) and **3** is H1299 (72 hours incubation). **B** shows the expression of Bcl-x protein in K532, H1299, HCT116 and HT1080 cells. Lane **1** is K562, **2** is H1299 (24 hours incubation), **3** is H1299 (48 hours incubation),**4** is H1299 (72 hours incubation), **6** is HCT116 (24 hours incubation), **7** is HCT116 (48 hours incubation), **8** is HCT116 (72 hours incubation), **10** is HT1080 (24 hours incubation), **11** is HT1080 (48 hours incubation),**12** is HT1080 (72 hours incubation).

(shown in Figure 3Ac).

A positive control (sc2203, K562 whole cell lysate, Sant Cruz) was further used to ascertain the location of Bcl-xL/S in H1299, HCT116 and HT1080 by western blot. The first band in the developed membrane was found to be the band around 28kD. So it is probable that the band at 28kD is the major Bcl-xL protein, and other Bcl-x proteins have no or little expression in these cell lines. The other bands are non-specific cross-reacting proteins.

As an alternate way to confirm the location of Bcl-x proteins by western blot, we decided to do immunoprecipitation and Mass spectrometry test. The first time, the samples from cytosol or non-cytosol parts of p53-null H1299 were used for immunoprecipitaion. Figure 4A show the migration of immunoprecipitate and antibody in the 12%SDS page. I found the high-signal bands around 50kD and 28KD which correspond to the antibody control. These two bands are heavy and light antibody chains. The band above the heavy chain band was BSA (used in blocking buffer in immunoprecipitation). Each band was cut and separated for in-gel digestion and mass spectrometry check. After mass spectrometry check, I did not found any peptide from Bcl-x proteins, but found some protein that can interact with Bcl-x proteins, such as 14-3-3 protein, voltage-dependent anion channel protein (VDAC). Finally, we used K562 whole cell lysate for immunoprecipitation because the cell line was used as positive control the company recommended. The pellet of cells harvested in eppendorf centrifuge tube was nearly 1ml, so the amount of protein of K562 cells lysis used was very high. After immunoprecipitation, half the sample was

Figure 4.

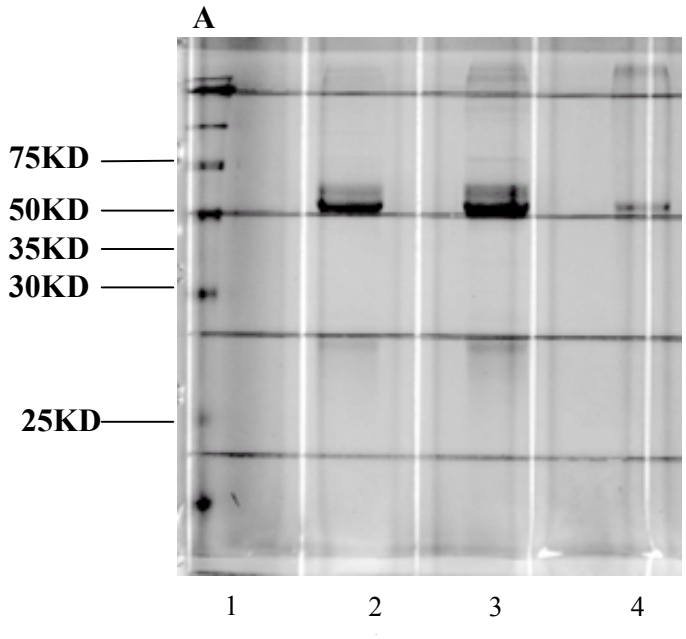

A
75KD
50KD
35KD
30KD
25KD
1
2
3
4
B
75KD
50KD
35KD
30KD
25KD
1
2
3
4

Figure 4. Immunoprecipitation of Bcl-x. **A** is the result from H1299 cell line. Lane **1** is rainbow marker, lane **2** is immunoprecipitate from cytosol lysates(100ul antibody was used), lane **3** is immunoprecipitate from non-cytosolic lysates (100ul antibody was used), lane **4** is antibody as control (10ul antibody). The 4 lines across the image (from left side to right side) come from the labeled scale in container for gel staining. **B** is the result from K562 cell line. Lane **1** is rainbow marker; Lane **2** is immunoprecipitae from whole cell lysate (200ul antibody was used); lane **3** is antibody as control (20ul antibody); lane **4** is rainbow marker. In **B**, other kind of container without scale was used to stain the gel, so B did not have the scale bands.

tested by in-solution digestion and mass spectrometry examination, another half sample was tested by in-gel digestion and mass spectrometry examination (Figure 4B).

The result of in-gel digestion was very similar to the first time, and we did not find any peptide from Bcl-x proteins. In the in-solution digestion, I found the two Bcl-x peptides ^{7}ELVVDFLSYK^{15} and ^{79}EVIPMAAVK^{87} (shown in table1). ^{7}ELVVDFLSYK^{15} is located in BH4, while ^{79}EVIPMAAVK^{87} is located in the joint region between BH4 and the Hix-2 (in the BH3 domain) by comparing the protein sequence (shown in table 1). From the results of immunoprecipitation and mass spectrometry, we knew that the primary antibody can directly bind to protein of Bcl-x or other proteins which can interact with the protein of Bcl-x. However, these two peptides were also presented in Bcl-xL, Bcl-xS, Bcl-xβ, Bcl-xγ and Bcl-xAK. Therefore, we are not sure about the location of Bcl-xL protein by Western blot.

The molecular weight of Bcl-xS and Bcl-xAK is around 18-20Kd (Hossini, *et al*, 2006). So these two proteins are not found at 28kD position. We need to make sure which isoform of Bcl-xL, Bcl-xβ, Bcl-xγ that the band at 28kD represent to. Then Bcl-x shRNA was used to reduce the expression of Bcl-x proteins on Western blot. We used five different Bcl-xL shRNAs to transfect A549 cells and H1299 cells. As shown in Figure 5, shRNAs (No. 9600911 and No. 9600907) completely blocked expression of the 28kD protein; shRNAs (No. 9600908 and No. 9600910) partially blocked expression of the 28kD protein; shRNA (No. 9600909) did not affect the 28kD band. By comparing the structure of Bcl-x mRNA (as shown in Table 1), we found that

Figure 5.

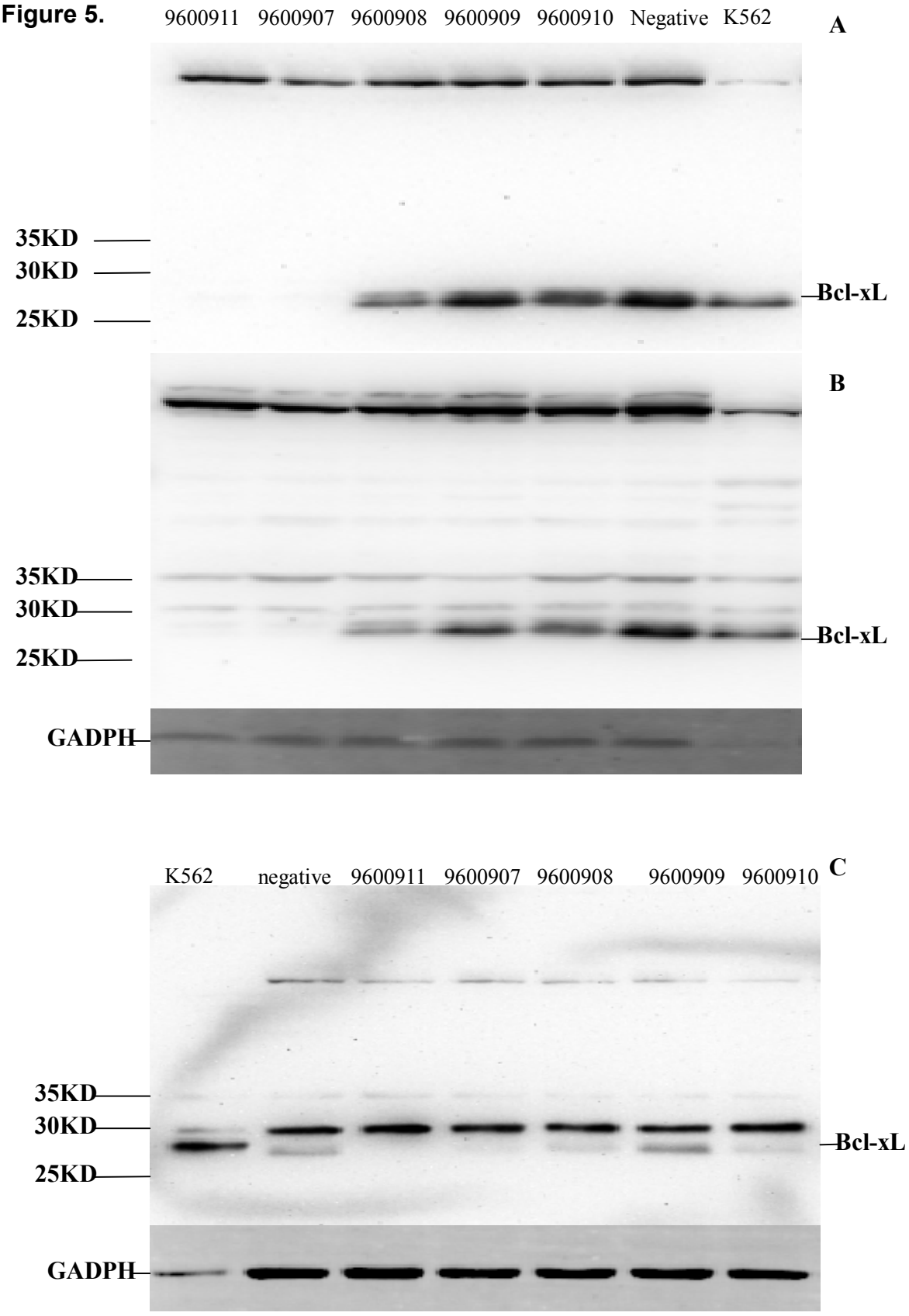

9600911
9600907
9600908
9600909
9600910
Negative
K562
A
35KD
30KD
25KD
Bcl-xL
B
35KD
30KD
25KD
Bcl-xL
GADPH
K562
negative
9600911
9600907
9600908
9600909
9600910
C
35KD
30KD
25KD
Bcl-xL
GADPH

Figure 5. Bcl-x protein expression in cells transfected by Bcl-xL shRNA. The cells were transfected by indicated Lenti-viral particles expressing Bcl-xL shRNA or without shRNA at MOI=1 PFU/cell. Then the transfected cells were selected by puromycin for one week. The cell lysates were analyzed with Western blotting to detect the Bcl-xL proteins expression. **A**: A594 Cell lines developed 1 min in ECL; **B**; same PVDF membrane, developed 5 min in ECL. **C**. H1299 cell lines.

Table 1. The sequences of Bcl-x mRNA and proteins

																										RHS3979-9600909**															
Bcl-xL/S/AK RNA	5'....	atg	tct	cag	agc	aac	cgg	gag	ctg	gtg	gtt	gac	ttt	ctc	tcc	tac	aag	ctt	tcc	cag	aaa	gga	tac	agc	tgg	agt	cag	ttt	agt	gat	gtg	gaa	gag	aac	agg	act	gag	gcc	cca	gaa	ggg
Bcl-xL protein	N...	M	S	Q	S	N	R	(E	L	V	V	D	F	L	S	Y	K)*	L	S	Q	K	G	Y	S	W	S	Q	F	S	D	V	E	E	N	R	T	E	A	P	E	G
Bcl-xS protein	N...	M	S	Q	S	N	R	(E	L	V	V	D	F	L	S	Y	K)*	L	S	Q	K	G	Y	S	W	S	Q	F	S	D	V	E	E	N	R	T	E	A	P	E	G
Bcl-xAK protein	N...	M	S	Q	S	N	R	(E	L	V	V	D	F	L	S	Y	K)*	L	S	Q	K	G	Y	S	W	S	Q	F	S	D	V	E	E	N	R	T	E	A	P	E	G
Bcl-xβ RNA	5'....	atg	tct	cag	agc	aac	cgg	gag	ctg	gtg	gtt	gac	ttt	ctc	tcc	tac	aag	ctt	tcc	cag	aaa	gga	tac	agc	tgg	agt	cag	ttt	agt	gat	gtg	gaa	gag	aac	agg	act	gag	gcc	cca	gaa	ggg
Bcl-xβ protein	N...	M	S	Q	S	N	R	(E	L	V	V	D	F	L	S	Y	K)*	L	S	Q	K	G	Y	S	W	S	Q	F	S	D	V	E	E	N	R	T	E	A	P	E	G
Bcl-xγ RNA	5'....	atg	tct	cag	agc	aac	cgg	gag	ctg	gtg	gtt	gac	ttt	ctc	tcc	tac	aag	ctt	tcc	cag	aaa	gga	tac	agc	tgg	agt	cag	ttt	agt	gat	gtg	gaa	gag	aac	agg	act	gag	gcc	cca	gaa	ggg
Bcl-xγ protein	N...	M	S	Q	S	N	R	(E	L	V	V	D	F	L	S	Y	K)*	L	S	Q	K	G	Y	S	W	S	Q	F	S	D	V	E	E	N	R	T	E	A	P	E	G
						5					10					15					20					25					30					35					40

Bcl-xL/S/AK RNA		act	gaa	tcg	gag	atg	gag	acc	ccc	agt	gcc	atc	aat	ggc	aac	cca	tcc	tgg	cac	ctg	gca	gac	agc	ccc	gcg	gtg	aat	gga	gcc	act	ggc	cac	agc	agc	agt	ttg	gat	gcc	cgg	gag	gtg
Bcl-xL protein		T	E	S	E	M	E	T	P	S	A	I	N	G	N	P	S	W	H	L	A	D	S	P	A	V	N	G	A	T	G	H	S	S	S	L	D	A	R	(E	V
Bcl-xS protein		T	E	S	E	M	E	T	P	S	A	I	N	G	N	P	S	W	H	L	A	D	S	P	A	V	N	G	A	T	G	H	S	S	S	L	D	A	R	(E	V
Bcl-xAK protein		T	E	S	E	M	E	T	P	S	A	I	N	G	N	P	S	W	H	L	A	D	S	P	A	V	N	G	A	T	G	H	S	S	S	L	D	A	R	(E	V
Bcl-xβ RNA		act	gaa	tcg	gag	atg	gag	acc	ccc	agt	gcc	atc	aat	ggc	aac	cca	tcc	tgg	cac	ctg	gca	gac	agc	ccc	gcg	gtg	aat	gga	gcc	act	ggc	cac	agc	agc	agt	ttg	gat	gcc	cgg	gag	gtg
Bcl-xβ protein		T	E	S	E	M	E	T	P	S	A	I	N	G	N	P	S	W	H	L	A	D	S	P	A	V	N	G	A	T	G	H	S	S	S	L	D	A	R	(E	V
Bcl-xγ RNA		act	gaa	tcg	gag	atg	gag	acc	ccc	agt	gcc	atc	aat	ggc	aac	cca	tcc	tgg	cac	ctg	gca	gac	agc	ccc	gcg	gtg	aat	gga	gcc	act	ggc	cac	agc	agc	agt	ttg	gat	gcc	cgg	gag	gtg
Bcl-xγ protein		T	E	S	E	M	E	T	P	S	A	I	N	G	N	P	S	W	H	L	A	D	S	P	A	V	N	G	A	T	G	H	S	S	S	L	D	A	R	(E	V
						45					50					55					60					65					70					75					80

														RHS3979-9600911**																									
Bcl-xL/S/AK RNA		atc	ccc	atg	gca	gca	gta	aag	caa	gcg	ctg	agg	gag	gca	ggc	gac	gag	ttt	gaa	ctg	cgg	tac	cgg	cgg	gca	ttc	agt	gac	ctg	aca	tcc	cag	ctc	cac	atc	acc	cca	ggg	aca	gca	tat
Bcl-xL protein		I	P	M	A	A	V	K)*	Q	A	L	R	E	A	G	D	E	F	E	L	R	Y	R	R	A	F	S	D	L	T	S	Q	L	H	I	T	P	G	T	A	Y
Bcl-xS protein		I	P	M	A	A	V	K)*	Q	A	L	R	E	A	G	D	E	F	E	L	R	Y	R	R	A	F	S	D	L	T	S	Q	L	H	I	T	P	G	T	A	Y
Bcl-xAK protein		–	P	M	A	A	V	K)*	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Bcl-xβ RNA		atc	ccc	atg	gca	gca	gta	aag	caa	gcg	ctg	agg	gag	gca	ggc	gac	gag	ttt	gaa	ctg	cgg	tac	cgg	cgg	gca	ttc	agt	gac	ctg	aca	tcc	cag	ctc	cac	atc	acc	cca	ggg	aca	gca	tat
Bcl-xβ protein		I	P	M	A	A	V	K)*	Q	A	L	R	E	A	G	D	E	F	E	L	R	Y	R	R	A	F	S	D	L	T	S	Q	L	H	I	T	P	G	T	A	Y
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Bcl-xγ protein		I	P	M	A	A	V	K)*	Q	A	L	R	E	A	G	D	E	F	E	L	R	Y	R	R	A	F	S	D	L	T	S	Q	L	H	I	T	P	G	T	A	Y
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			RHS3979-9600910**																																						
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Bcl-xL protein		Q	S	F	E	Q	V	V	N	E	L	F	R	D	G	V	N	W	G	R	I	V	A	F	F	S	F	G	G	A	L	C	V	E	S	V	D	K	E	M	Q
Bcl-xS protein		Q	S	F	E	Q	V	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Bcl-xAK protein		…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Bcl-xβ RNA		cag	agc	ttt	gaa	cag	gta	gtg	aat	gaa	ctc	ttc	cgg	gat	ggg	gta	aac	tgg	ggt	cgc	att	gtg	gcc	ttt	ttc	tcc	ttc	ggc	ggg	gca	ctg	tgc	gtg	gaa	agc	gta	gac	aag	gag	atg	cag
Bcl-xβ protein		Q	S	F	E	Q	V	V	N	E	L	F	R	D	G	V	N	W	G	R	I	V	A	F	F	S	F	G	G	A	L	C	V	E	S	V	D	K	E	M	Q
Bcl-xγ RNA		cag	agc	ttt	gaa	cag	gta	gtg	aat	gaa	ctc	ttc	cgg	gat	ggg	gta	aac	tgg	ggt	cgc	att	gtg	gcc	ttt	ttc	tcc	ttc	ggc	ggg	gca	ctg	tgc	gtg	gaa	agc	gta	gac	aag	gag	atg	cag
Bcl-xγ protein		Q	S	F	E	Q	V	V	N	E	L	F	R	D	G	V	N	W	G	R	I	V	A	F	F	S	F	G	G	A	L	C	V	E	S	V	D	K	E	M	Q
						125					130					135					140					145					150					155					160

											RHS3979-9600908**																														
Bcl-xL/S/AK RNA		gta	ttg	gtg	agt	cgg	atc	gca	gct	tgg	atg	gcc	act	tac	ctg	aat	gac	cac	cta	gag	cct	tgg	atc	cag	gag	aac	ggc	ggc	tgg	gat	act	ttt	gtg	gaa	ctc	tat	ggg	aac	aat	gca	gca
Bcl-xL protein		V	L	V	S	R	I	A	A	W	M	A	T	Y	L	N	D	H	L	E	P	W	I	Q	E	N	G	G	W	D	T	F	V	E	L	Y	G	N	N	A	A
Bcl-xS protein		…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	D	T	F	V	E	L	Y	G	N	N	A	A
Bcl-xAK protein		…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	E	P	W	I	Q	E	N	G	G	W	D	T	F	V	E	L	Y	G	N	N	A	A
Bcl-xβ RNA		gta	ttg	gtg	agt	cgg	atc	gca	aag	tgg	atg	gcc	act	tac	ctg	aat	gac	cac	cta	gag	cct	tgg	atc	cag	gag	aac	ggc	ggc	tgg	gta	aga	acc	aag	ccc	ctt	gtg	tgt	ccc	ttt	tct	ttg
Bcl-xβ protein		V	L	V	S	R	I	A	S	W	M	A	T	Y	L	N	D	H	L	E	P	W	I	Q	E	N	G	G	W	V	R	T	K	P	L	V	C	p	F	S	L
Bcl-xγ RNA		gta	ttg	gtg	agt	cgg	atc	gca	gct	tgg	atg	gcc	act	tac	ctg	aat	gac	cac	cta	gag	cct	tgg	atc	cag	gag	aac	ggc	ggc	tgg	ggg	tgt	gag	tgg	agg	tac	acc	cct	cag	atc	tgt	ctt
Bcl-xγ protein		V	L	V	S	R	I	A	A	W	M	A	T	Y	L	N	D	H	L	E	P	W	I	Q	E	N	G	G	W	G	V	S	G	G	T	P	L	R	S	V	F
						165					170					175					180					185					190					195					200

																												RHS3979-9600907**										
Bcl-xL/S/AK RNA		gcc	gag	agc	cga	aag	ggc	cag	gaa	cgc	ttc	aac	cgc	tgg	ttc	ctg	acg	ggc	atg	act	gtg	gcc	ggc	gtg	gtt	ctg	ctg	ggc	tca	ctc	ttc	agt	cgg	aaa	tga	...	...	........3'
Bcl-xL protein		A	E	S	R	K	G	Q	E	R	F	N	R	W	F	L	T	G	M	T	V	A	G	V	V	L	L	G	S	L	F	S	R	K	...	...	...C'	
Bcl-xS protein		A	E	S	R	K	G	Q	E	R	F	N	R	W	F	L	T	G	M	T	V	A	G	V	V	L	L	G	S	L	F	S	R	K	...	...	...C'	
Bcl-xAK protein		A	E	S	R	K	G	Q	E	R	F	N	R	W	F	L	T	G	M	T	V	A	G	V	V	L	L	G	S	L	F	S	R	K	...	...	...	...C'
Bcl-xβ RNA		gcc	tct	ggt	cag	aga	tcc	cca	aca	gcc	ctt	ctt	ctg	tat	ctc	ttt	ctg	ttg	tgt	tgg	gtg	att	gtt	gga	gac	ggt	gat	agt	tga	...	...	...	...	...	...	...	...	3
Bcl-xβ protein		A	S	G	Q	R	S	P	T	A	L	L	L	Y	L	F	L	L	C	W	V	I	V	G	D	V	D	S	...	...	...	...	...	...	...	...	...	...C'
Bcl-xγ RNA		cag	aag	gct	tgt	tca	agt	gcc	agg	agt	ggc	gga	gca	cgt	ttg	tga	tcc	cag	cct	ttg	gga	ggt	gga	aac	aga	agg	atc	gga	agt	tca	agg	ccc	tcc	tca	gct	att	ata	3
Bcl-xγ protein		R	R	L	V	Q	V	P	G	V	A	E	H	V	C	D	P	S	L	W	E	V	E	T	E	G	S	E	V	Q	G	P	P	Q	L	L	...	...C'
						205					210					215					220					225					230							

The sequence data referred from Bcl-xL (genebank CAA80661.1), Bcl-xS (genebank CAA80662.1), Bcl-xAK (AM Hossini, *et al* 2006), Bcl-xβ (Swiss-Prot: P53563.2) and Bcl-xγ (not reported in human, the sequence from mouse, GenBank: U51277.1)

* The peptides were detected by massspectrometry from in-solution digestion after immuniprecipitation by anti-Bcl-xL/S antibody (sc493). The peptieds are [7]ELVVDFLSYK[16] and [78]EVIPMAVK[86]

** The targeted sites (shown in red color) of Bcl-x mRNA by 5 kinds of shRNA.

shRNA (No.9600907) blocked the expression of Bcl-xL, Bcl-xS, Bcl-xβ and Bcl-xγ but not Bcl-xAK. (Thus, Bcl-xAK can be excluded, besides, the molecular weight of this protein does not match); shRNA (No. 9600911) blocked the expression of Bcl-xL, Bcl-xS and Bcl-xAK, but not Bcl-xβ or Bcl-xγ (so Bcl-xβ and Bcl-xγ can be excluded). Bcl-xS can also be excluded due to mismatched molecular weight. Finally, we believe that the 28kD bands correspond to Bcl-xL.

Careful inspection of the band around 28kD showed it actually had two bands (the gap is around 1-2kD). Takehara showed that human Bcl-xL was deamidated at asparagines 52 and 66, and alkaline treatment of Bcl-xL induced deamidation *in vitro* (Takehara and Takahashi, 2003). Whole cell lysates (with or without proteinase inhibitor) from H1299 cell line were treated by different pHs. I found that Bcl-xL protein at pH=10 lagged around 1kD compared to the Bcl-xL protein treated at pH =7 both with and without proteinase inhibitor added (Figure 6). This result suggested that the two bands around 28KD represented Bcl-xL either deamidated or undeamidated.

3.4 The expression of Bcl-xL protein in p53 defective and positive cell lines infected by MRV

Three sets of cell lines H1299 (p53-null and p53 positive), HT1080 (p53 mutant and p53 positive) and HCT116 (p21 or 14-3-3σ deficient) were used to test the impact of MRV infection on the expression of Bcl-xL protein. P53 defective and positive H1299 cells were infected by T1L or T3D at different doses of MOI=0, 0.5, 2.5,10, 25 and 100 PFU/cell, and harvested at different time points (24, 48, 72, 96 hours post

Figure 6.

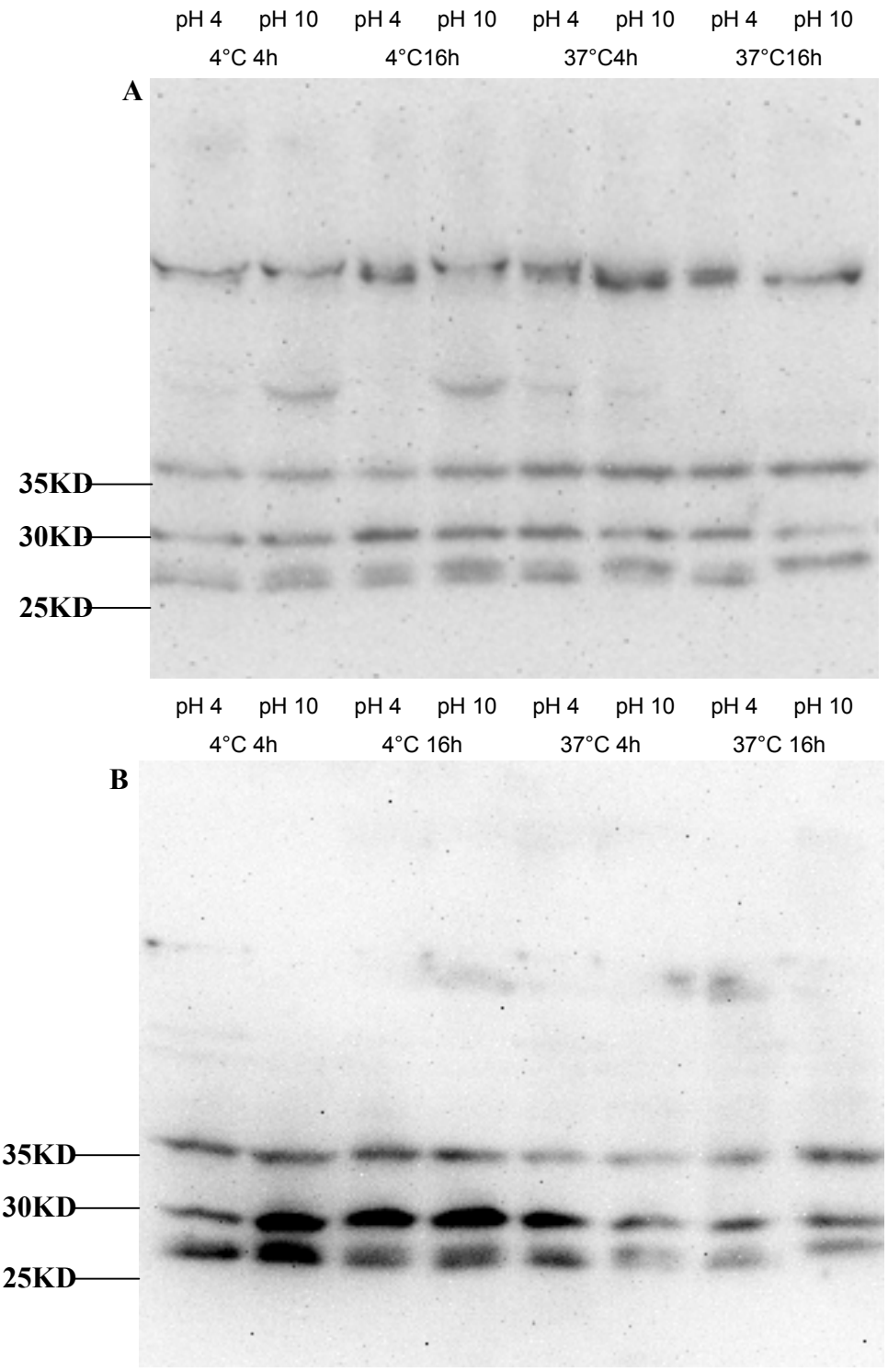

pH 4
pH 10
pH 4
pH 10
pH 4
pH 10
pH 4
pH 10
4°C 4h
4°C16h
37°C4h
37°C16h
A
35KD
30KD
25KD
pH 4
pH 10
pH 4
pH 10
pH 4
pH 10
pH 4
pH 10
4°C 4h
4°C 16h
37°C 4h
37°C 16h
B
35KD
30KD
25KD

Figure 6. Deamindation of Bcl-xl treatment. The treatment was modified from Takehara (Takehara and Takahashi, 2003), The H1299 cells were treated by alkaline buffer (pH adjusted to10), or neutral Buffer (pH adjusted to 7.0).The samples were sonicated for 10 seconds on ice followed by centrifugation at 9300×*g* and the supernatants harvested. Each sample was incubated at 4℃ or 37℃ for 4 or 16 hours. Proteinase inhibitor was added to half of the samples (Figure 6B) or not (Figure 6A). Then the expression of Bcl-xL expression was analyzed by Western blot. The two picture show that Bcl-xL protein in pH=10 lagged around 1kD compared the Bcl-xL protein at pH=7 both with or without proteinase inhibitor at 4 or 37℃ for 16 hours.

Infection) for Bcl-xL protein analysis by Western blot. Figure 7A shows that the expression of Bcl-xL protein in H1299 (p53-and p53 positive) cell lines infected by MRV. Scion image® was used to analyze the density of Bcl-xL protein and GADPH protein in Western blot. According to the densities of Bcl-xL protein and GADPH protein of each sample, we calculated the fold changes of Bcl-xL protein caused by MRV infection compared to control (Table 2). The formula used follows:

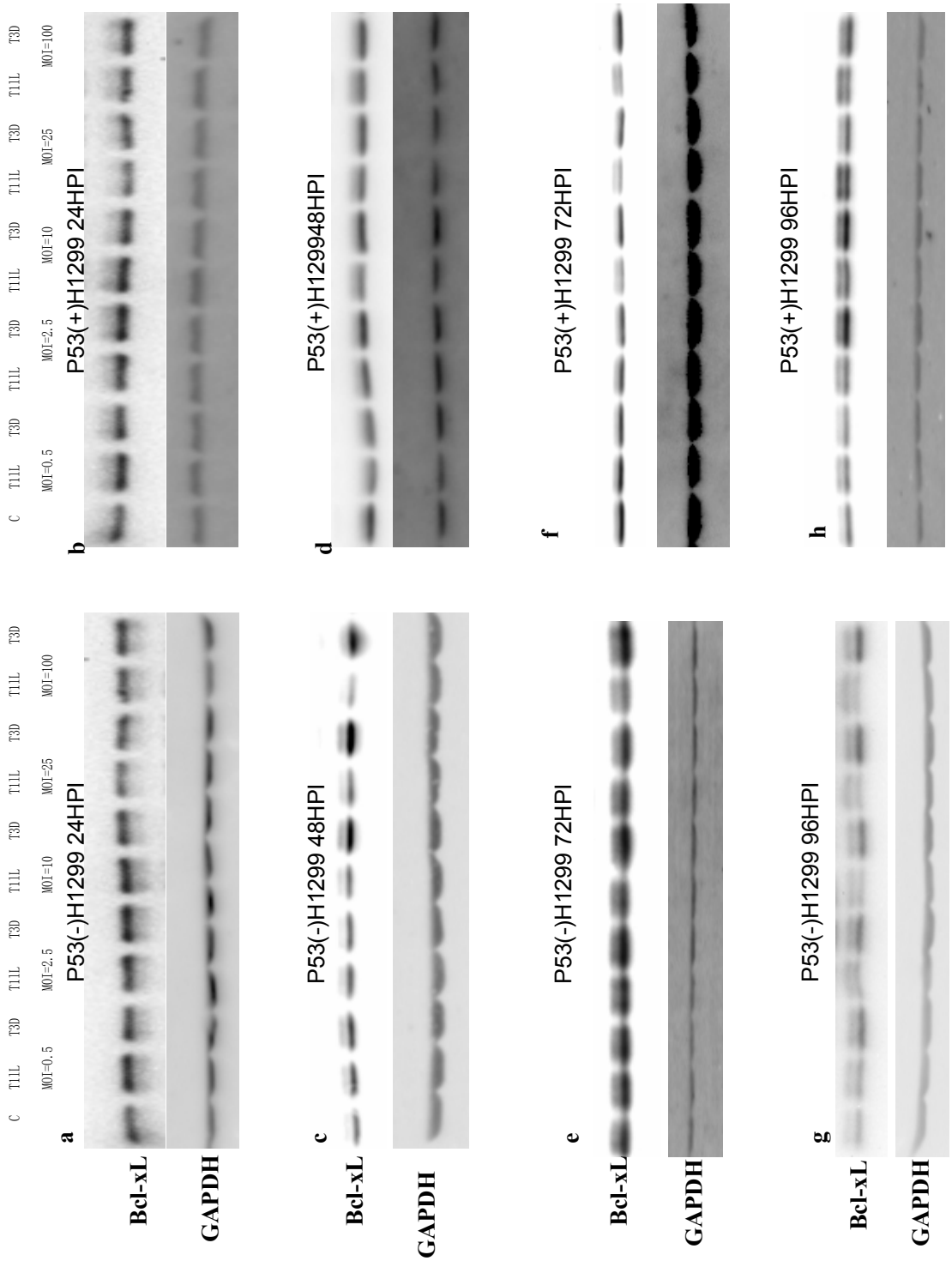
$$\text{Fold} = \frac{\text{Density of Bcl-xL protein in each MRV treatment / density of Bcl-xL in control}}{\text{Density of GAPDH protein in each MRV treatment / density of GAPDH in control}}$$

As shown in Figure 7, the MRV infection changed the expression of Bcl-xL protein in both p53-null and p53 positive H1299 cell lines. In order to compare the capacity of T1L and T3D to induce changes in the level of Bcl-xL protein compared to controls at same time point and same infection doses (same MOI), one way ANOVA F test and q test were used, as shown in table 3. At 24 hours post infection, there was no significant difference of Bcl-xL expression between each T1L and T3D at any of the MOIs in H1299 cells. However, at 48 hours post infection, MRVs were able to exert distinctive effect on the expression of Bcl-xL. At 48 hours with high MOI doses (10, 25,100 PFU/cell), the expression of Bcl-xL protein was increased by T3D infection, while decreased by T1L infection in both p53-null and p53 positive H1299 cell lines. Similar trend was found at 72 and 96 hours post infection at low MOI doses.

To understand the role of p53 on the expression change of Bcl-xL caused by

Figure 7

A


C T11L T3D T11L T3D T11L T3D T11L T3D
MOI=0.5 MOI=2.5 MOI=10 MOI=25 MOI=100
a
P53(-)H1299 24HPI
Bcl-xL
GAPDH
c
P53(-)H1299 48HPI
Bcl-xL
GAPDH
e
P53(-)H1299 72HPI
Bcl-xL
GAPDH
g
P53(-)H1299 96HPI
Bcl-xL
GAPDH
b
P53(+)H1299 24HPI
d
P53(+)H129948HPI
f
P53(+)H1299 72HPI
h
P53(+)H1299 96HPI

B.

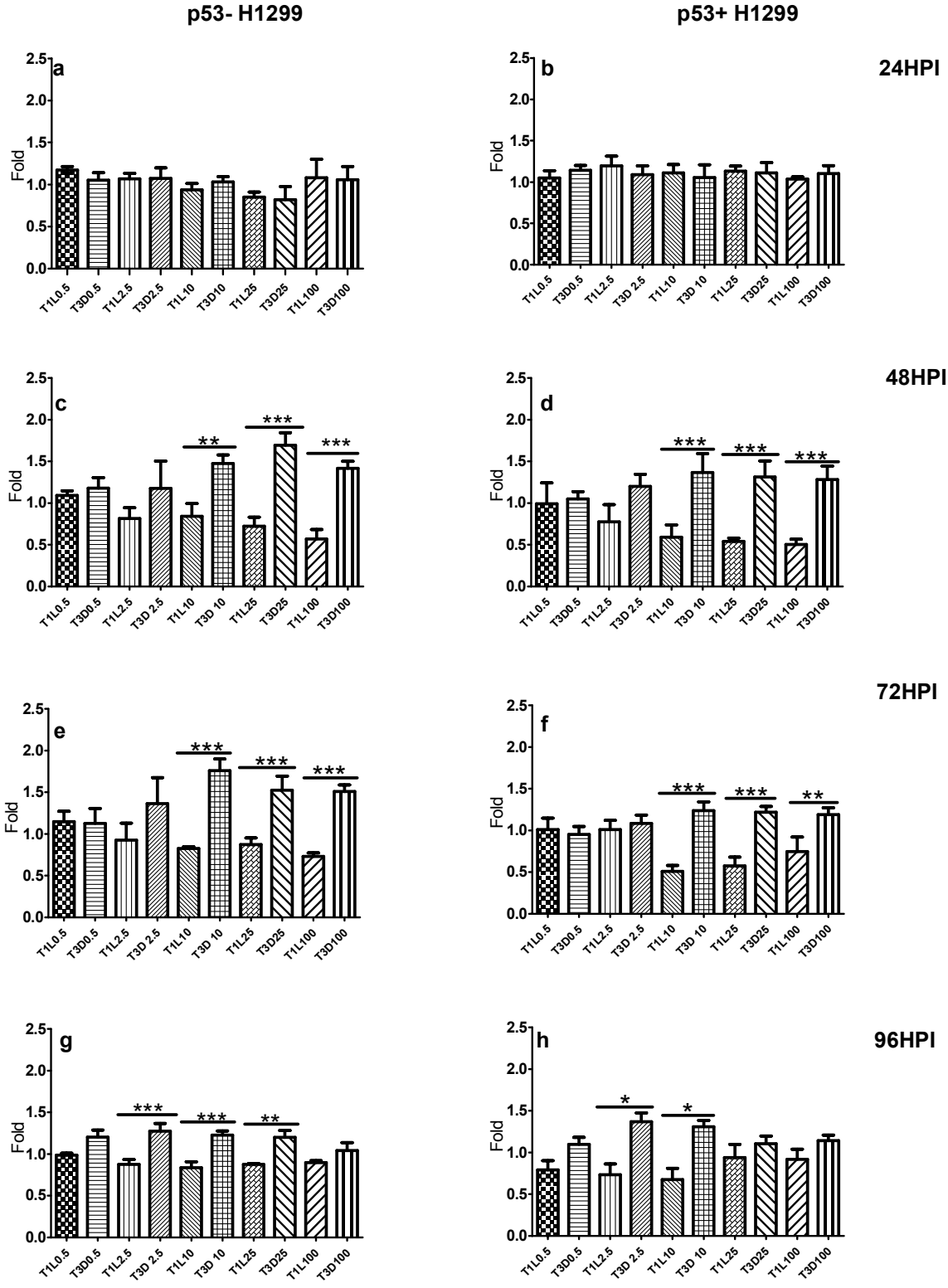

p53- H1299
p53+ H1299
24HPI
48HPI
72HPI
96HPI
a
b
c
d
e
f
g
h
Fold
T1L0.5
T3D0.5
T1L2.5
T3D2.5
T1L10
T3D10
T1L25
T3D25
T1L100
T3D100

Figure 7. Western blot detection of Bcl-xL protein in p53-null and p53 positive H1299 after MRV infection. The cells were infected by reoviruses T1L or T3D at MOI=0.5, 2.5,10,25 and 100 PFU/cell. Cells were harvested at 24, 48, 72 and 96 hours post infection. The cell lysates were analyzed by Western blotting to detect the levels of Bcl-xL protein, with GADPH as control. The protein densities were analyzed by scion image® program and the results were expressed as the mean fold changes of Bcl-xL protein of the infected cells divided by the control of GADPH level from three independent experiments (n=3). Error bars indicate standard deviation of the mean. **A** shows representative original western blotting results. **B** is made by graphpad prism 5 according to the relative density fold changes of Bcl-xL: **a** H1299 p53 (-) at 24HPI, **b** H1299 p53 (+) at 24HPI, **c** H1299 p53 (-) at 48HPI, **d** H1299 p53 (+) at 48HPI, **e** H1299 p53 (-) at 72HPI, **f** H1299 p53 (+) at 72HPI, **g** H1299 p53 (-) at 96HPI, **h** H1299 p53 (+) at 96HPI. The data were analyzed by ANOVA F-test and q test (by Graphpad Prism 5). The asterisks indicate statistically significant difference of Bcl-xL protein level between cells infected with T1L and T3D at the same MOI dose. Single asterisks mean $p<0.05$; double asterisks mean $p<0.01$; triple asterisks mean $p<0.001$.

Table 2. P values of comparison of the relative fold change of Bcl-xL induced by T1L and T3D at different MOIs*

H1299 p53(-) 24HPI

	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
T1L0.5	*0.959*	*0.988*	*0.899*	*0.029*	*0.966*
T1L2.5	*1.000*	*1.000*	*1.000*	*0.273*	*1.000*
T1L10	*0.966*	*0.911*	*0.991*	*0.966*	*0.959*
T1L25	*0.525*	*0.398*	*0.659*	*1.000*	*0.503*
T1L100	*1.000*	*1.000*	*1.000*	*0.215*	*1.000*

H1299 p53(+)24HPI

	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
T1L0.5	*0.974*	*1.000*	*1.000*	*0.999*	*1.000*
T1L2.5	*1.000*	*0.940*	*0.760*	*0.985*	*0.974*
T1L10	*0.077*	*0.077*	*0.077*	*0.077*	*0.077*
T1L25	*1.000*	*1.000*	*0.994*	*1.000*	*1.000*
T1L100	*0.940*	*1.000*	*1.000*	*0.996*	*0.998*

H1299 p53(-) 48HPI

	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
T1L0.5	*1.000*	*0.883*	*0.250*	***0.003***	*0.749*
T1L2.5	*0.549*	*0.098*	***0.005***	***<0.001***	*0.052*
T1L10	*0.611*	*0.121*	***0.007***	***<0.001***	*0.066*
T1L25	*0.372*	***0.050***	***0.002***	***<0.001***	***0.025***
T1L100	***0.030***	***0.002***	***<0.001***	***<0.001***	***0.001***

H1299 p53(+)48HPI

	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
T1L0.5	*1.000*	*0.927*	*0.325*	*0.661*	*0.647*
T1L2.5	*0.716*	*0.176*	***0.017***	***0.060***	*0.058*
T1L10	*0.252*	***0.034***	***0.003***	***0.010***	***0.009***
T1L25	*0.233*	***0.031***	***0.002***	***0.009***	***0.008***
T1L100	*0.162*	***0.019***	***0.001***	***0.006***	***0.005***

H1299 p53(-) 72HPI

	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
T1L0.5	*1.000*	*0.879*	***0.020***	*0.184*	*0.242*
T1L2.5	*0.986*	*0.224*	***0.001***	*0.150*	*0.969*
T1L10	*0.768*	*0.060*	***<0.001***	***0.003***	***0.004***
T1L25	*0.940*	*0.137*	***0.001***	***0.008***	***0.012***
T1L100	*0.435*	***0.018***	***<0.001***	***0.001***	***0.001***

H1299 p53(+)72HPI

	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
T1L0.5	*1.000*	*0.998*	*0.301*	*0.399*	*0.606*
T1L2.5	*1.000*	*0.998*	*0.301*	*0.399*	*0.606*
T1L10	***0.002***	***<0.001***	***<0.001***	***<0.001***	***<0.001***
T1L25	***0.009***	***<0.001***	***<0.001***	***<0.001***	***<0.001***
T1L100	*0.487*	*0.053*	***<0.001***	***0.001***	***0.001***

H1299 p53(-) 96HPI

	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
T1L0.5	*0.395*	*0.105*	*0.267*	*0.416*	*1.000*
T1L2.5	***0.042***	***0.007***	***0.024***	***0.046***	*0.731*
T1L10	***0.016***	***0.003***	***0.009***	***0.017***	*0.459*
T1L25	***0.042***	***0.007***	***0.024***	***0.046***	*0.731*
T1L100	*0.067*	***0.012***	***0.039***	*0.072*	*0.848*

H1299 p53(+)96HPI

	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
T1L0.5	*0.620*	***0.018***	***0.049***	*0.588*	*0.429*
T1L2.5	*0.389*	***0.007***	***0.020***	*0.361*	*0.237*
T1L10	*0.197*	***0.002***	***0.007***	*0.179*	*0.108*
T1L25	*0.991*	*0.174*	*0.352*	*0.987*	*0.949*
T1L100	*0.978*	*0.133*	*0.283*	*0.971*	*0.910*

*The comparison of the relative fold change of Bcl-xL induced by T1L at different MOI with the ones induced by T3D at different MOI was analyzed by One way ANOVA and q test.

Table 3. The comparison of the fold changes of Bcl-xL induced by same MRV with same MOI between p53-null and p53 positive H1299 cell lines*

time point	p value for same MRV with same MOI									
	T1L0.5	T1L2.5	T1L10	T1L25	T1L100	T3D0.5	T3D2.5	T3D10	T3D25	T3D100
48HPI	*0.310*	*0.237*	*0.135*	***0.042***	*0.094*	*0.156*	*0.478*	*0.175*	***0.017***	*0.216*
72HPI	*0.226*	*0.309*	***0.006***	***0.003***	*0.423*	*0.189*	*0.164*	***0.015***	***0.039***	***0.020***
96HPI	*0.092*	*0.184*	*0.174*	*0.364*	*0.439*	*0.212*	*0.266*	*0.210*	*0.245*	*0.223*

*. The relative fold change of Bcl-xL in p53-null and p53 positive were analyzed by paired T test.

MRV infection, the fold expression change of Bcl-xL protein caused by MRV at same MOI and time points in p53- and p53 positive H1299 were analyzed by t test (Table 4).

The presence of p53 does not affect the trend of Bcl-xL expression caused by different MRV strains, but its presence can decrease the expression of Bcl-xL protein in both T3D (48HPI, MOI=25; 72HPI, MOI=10, 25 and 100) and T1L (48HPI, MOI=25, 72HPI, MOI=10 or 25) infected cells. HT1080 (p53 mutant and p53 positive) and HCT116 (p21 or 14-3-3σ deficient) cell lines were also used to test the expression of Bcl-xL after MRV infection (Figure 8 and 9). We could not find a similar trend of the protein as found in H1299 cells. One way ANOVA was also used to analyze the data, and we could not find any statistic significance. Therefore, change did not give the p values from the One way ANOVA test.

3.5 Mapping MRV the gene or gene combination that causes the trend of Bcl-xL expression in p53- H1299 cell line

When cells are infected by two or more reovirus strains, the virus gene segments can mix, and progeny viruses will have different combination of gene segments from parental reoviruses. If different reovirus strains have different capability of virus replication or induce different host cell response, the progeny viruses have biologic polymorphisms, which can be used to map a specific gene segment that is the determinant of the biological phenotype (Hermann *et al*, 2004; Tyler *et al*, 1996). There are 83 MRV T1L×T3D reassortants, whose gene types have been identified

Figure 8

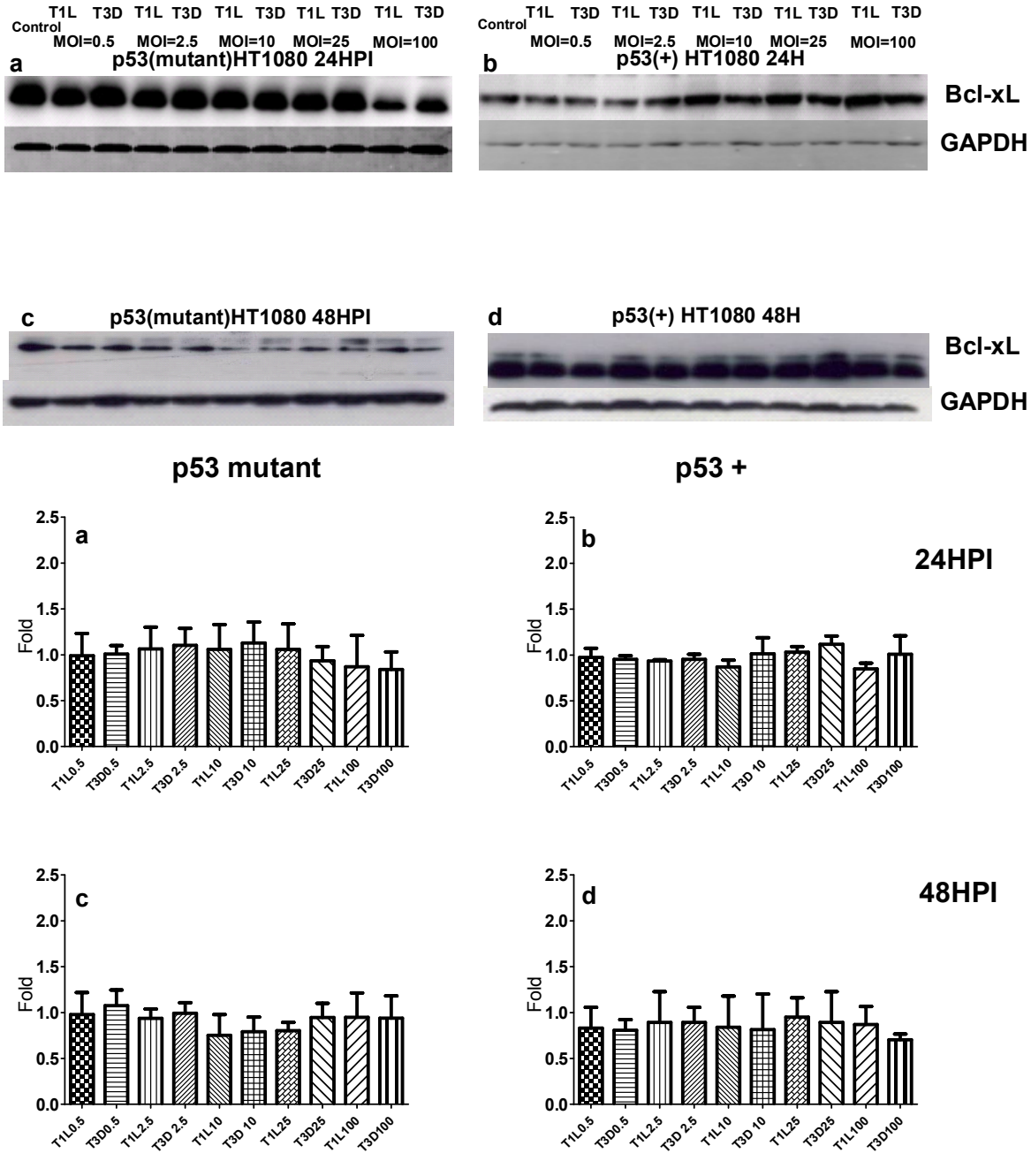
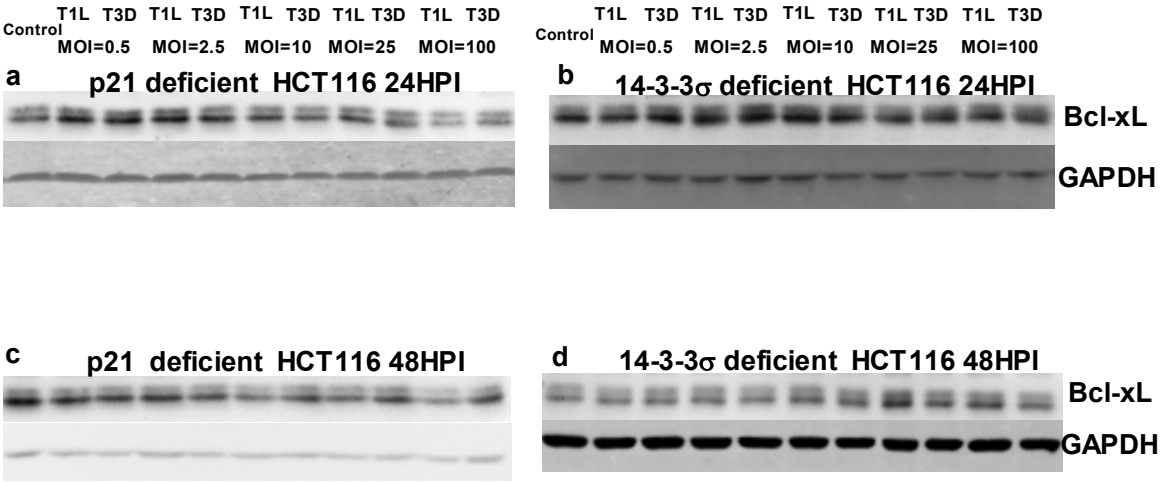

Control
T1L T3D T1L T3D T1L T3D T1L T3D T1L T3D
MOI=0.5 MOI=2.5 MOI=10 MOI=25 MOI=100
a
p53(mutant)HT1080 24HPI
b
p53(+) HT1080 24H
Bcl-xL
GAPDH
c
p53(mutant)HT1080 48HPI
d
p53(+) HT1080 48H
Bcl-xL
GAPDH
p53 mutant
p53 +
a
b
24HPI
c
d
48HPI
Fold
2.5
2.0
1.5
1.0
0.5
0.0
T1L0.5
T3D0.5
T1L2.5
T3D 2.5
T1L10
T3D 10
T1L25
T3D25
T1L100
T3D100

Figure 8. Western bloting detection of Bcl-xL protein in p53 (mutant) and p53 (postive) HT1080 after MRV infection. The cells were infected by reoviruses T1L or T3D at MOI=0.5, 2.5,10,25 and 100 PFU/cells. Cells were harvested at 24 and 48 hours post infection. Then the cell lysates was analyzed by Western blotting to detect the levels of Bcl-xL protein, with GADPH as control. The protein densities were analyzed by the scion image® program and the results were expressed as the mean fold changes of Bcl-xL protein of the infected cells divided by the control of GADPH level from three independent experiments(n=3). Error bars indicate standard deviation of the mean. **A** is representative original western blot result. **B** is made by graphpad prism 5 according to the relative density fold of Bcl-xL **a** HT1080 (p53 mutant) at 24HPI, **b** HT1080 (p53 +) at 24HPI, **c** HT1080 (p53 mutant) at 48HPI, **d** HT1080 (p53 +) at 48HPI.

Figure 9


Control
T1L MOI=0.5
T3D MOI=0.5
T1L MOI=2.5
T3D MOI=2.5
T1L MOI=10
T3D MOI=10
T1L MOI=25
T3D MOI=25
T1L MOI=100
T3D MOI=100
a
p21 deficient HCT116 24HPI
b
14-3-3σ deficient HCT116 24HPI
Bcl-xL
GAPDH
c
p21 deficient HCT116 48HPI
d
14-3-3σ deficient HCT116 48HPI
Bcl-xL
GAPDH

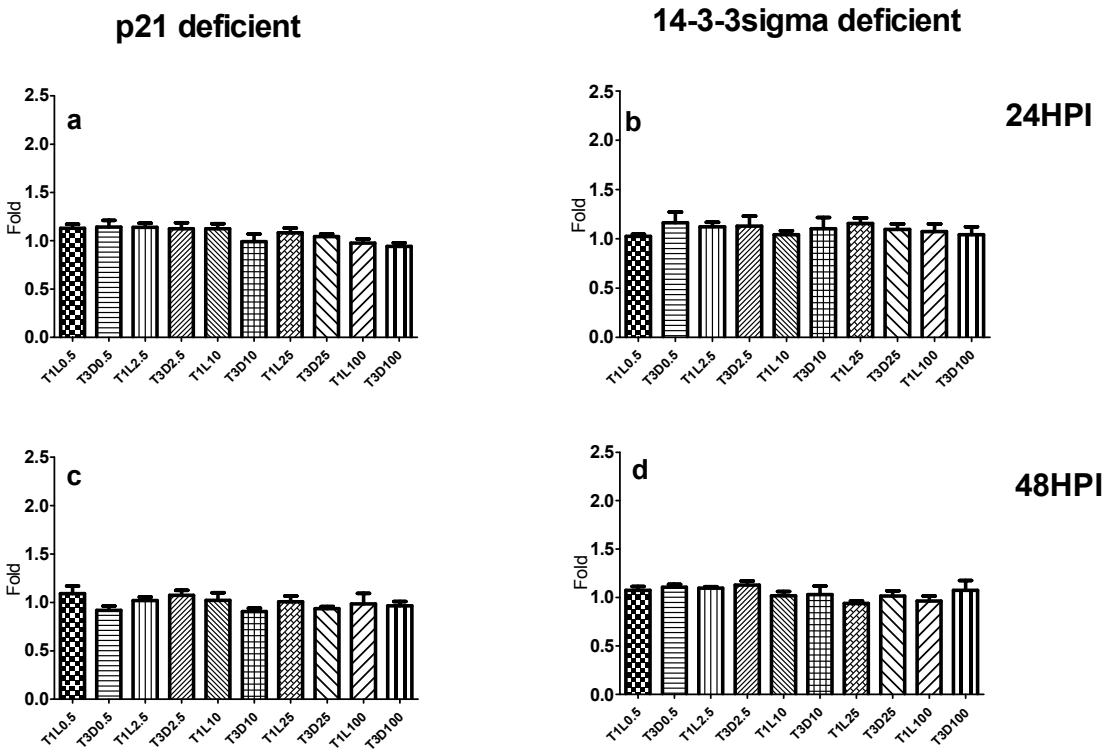

p21 deficient
14-3-3sigma deficient
a
b
24HPI
c
d
48HPI
Fold
2.5
2.0
1.5
1.0
0.5
0.0
T1L0.5
T3D0.5
T1L2.5
T3D2.5
T1L10
T3D10
T1L25
T3D25
T1L100
T3D100

Figure 9. Western bloting detection of Bcl-xL protein in p21 or 14-3-3σ deficient HCT116 after MRV infection. The cells were infected by reoviruses T1L or T3D at MOI=0.5, 2.5,10,25 and 100 PFU/cells. Cells were harvested at 24 and 48 hours post infection. Then the cell lysates were analyzed by Western blotting to detect the levels of Bcl-xL protein, with GADPH as control. The protein densities was analyzed by the scion image® program and the results were expressed as the mean fold changes of Bcl-xL protein of the infected cells divided by the control of GADPH level from three independent experiments(n=3). Error bars indicate standard deviation of the mean. **A** is representative original western blotting result. **B** is made by graphpad prism 5 according to the relative density fold of Bcl-xL. **a** HCT116 (14-3-3σ deficient) at 24HPI, **b** HCT116 (p21 deficient) at 24HPI, **c** HCT116 (14-3-3σ deficient) at 48HPI, **d** HCT116 (p21 deficient) at 48HPI.

(Nibert *et al*, 1996). In this study, 54 MRV reassortants were used to map which gene or gene combination determines the trend of Bcl-xL expression. The p53- H1299 cells were infected by these MRV reassortant viruses at MOI=10 PFU/cell and the cells were harvested at 72 hours post infection for analysis of the expression of Bcl-xL and GADPH (as control) by Western blotting (Figure 10). The experiment was repeated by 3 times. The density of the two proteins was analyzed by Scion image®, and then the fold change of Bcl-xL caused by MRV compared to control cells and GADPH were calculated (the formula shown in **result 4.3**). The relative capacity of each MRV reassortant clone to change the expression of Bcl-xL ranged from 0.28 to 1.65. All clones were inversely ranked from 1 to 56 (for statistical purposes). Then the data were analyzed by different statistic methods (non-parametric Mann-Whitney test and linear regression analysis). The result (Table 4 and 5) show a significant association between the capacity of MRV reassortant to change the expression of Bcl-xL protein and 3 MRV genes: S1 gene (MW test, $p<0.001$and t test, $p<0.001$); L3 gene (MW test$p<0.001$; t test $p<0.001$) and L1 gene (MW test $p=0.001$; t test $p<0.001$). No other gene segments were significantly associated with the capacity of MRV to induce the change of Bcl-xL protein. Further, Linear regression (stepwise) analysis was used to figure out the relationship of expression of Bcl-xL and the 3 genes. We obtained two models: one is that R^2 values of 0.751 ($p<0.001$) for the S1 ($p<0.001$) genes; another is that R^2 values of 0.772 ($p<0.001$) for S1 ($p<0.001$) and L3 genes ($p=0.0.032$).

These results indicate that the S1 gene is the principal determinant of the difference in capacity of T1L and T3D to change the expression of Bcl-xL protein. The

Figure10

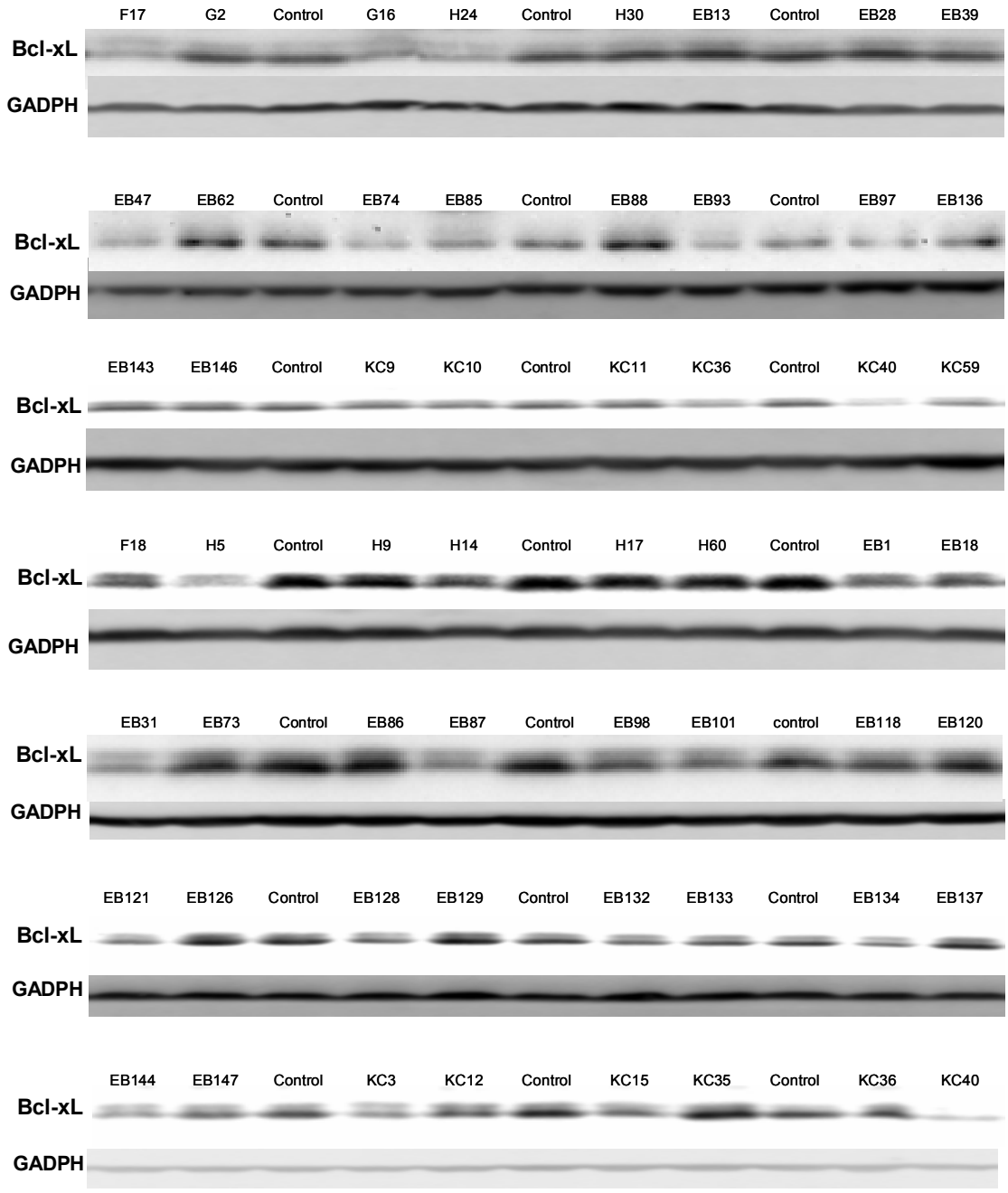

F17 G2 Control G16 H24 Control H30 EB13 Control EB28 EB39
Bcl-xL
GADPH
EB47 EB62 Control EB74 EB85 Control EB88 EB93 Control EB97 EB136
Bcl-xL
GADPH
EB143 EB146 Control KC9 KC10 Control KC11 KC36 Control KC40 KC59
Bcl-xL
GADPH
F18 H5 Control H9 H14 Control H17 H60 Control EB1 EB18
Bcl-xL
GADPH
EB31 EB73 Control EB86 EB87 Control EB98 EB101 control EB118 EB120
Bcl-xL
GADPH
EB121 EB126 Control EB128 EB129 Control EB132 EB133 Control EB134 EB137
Bcl-xL
GADPH
EB144 EB147 Control KC3 KC12 Control KC15 KC35 Control KC36 KC40
Bcl-xL
GADPH

Figure 10. Map which virus gene or gene combination of MRV determines the change expression of the Bcl-xL by detection of Bcl-xL protein by Western blotting in H1299 p53-null infected by MRV reassortants. The H1299 cells were infected by reoviruses reassortants (T1L×T3D) at MOI=10 PFU/cell. Cells were harvested at 72hours post infection. Then the cell lysates were analyzed by Western blotting to detect the levels of Bcl-xL protein, with GADPH as control.

Table 4. Relative fold changes in expression of Bcl-xL protein induced by MRV reassortant viruses

clone	parental source for the following gene segments[a]										Mean of Fold	SD of	Rank[c]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	Change[b]	Fold	
EB128	3	3	1	3	3	3	1	3	3	1	*0.32*	*0.03*	**56**
K40	1	3	1	1	3	1	1	3	3	1	*0.36*	*0.05*	**55**
EB87	1	3	1	1	3	1	1	3	1	1	*0.37*	*0.06*	**53**
F17	1	3	1	1	1	1	1	3	1	1	*0.37*	*0.03*	**53**
EB47	1	3	1	1	1	1	1	1	1	1	*0.37*	*0.01*	**53**
H5	3	3	1	1	1	3	1	3	1	3	*0.38*	*0.06*	**51**
G16	1	1	1	3	1	1	1	3	1	1	*0.39*	*0.01*	**49.5**
EB121	3	3	1	3	1	3	1	3	3	3	*0.39*	*0.07*	**49.5**
EB74	1	3	1	1	1	1	1	1	3	1	*0.41*	*0.05*	**48**
EB93	1	1	1	1	1	1	1	1	3	1	*0.43*	*0.05*	**47**
EB134	1	3	1	3	1	1	1	1	3	1	*0.45*	*0.03*	**46**
EB85	1	1	1	1	1	3	1	3	1	1	*0.46*	*0.01*	**45**
EB18	3	3	1	3	3	3	1	1	3	1	*0.47*	*0.13*	**44**
H24	1	1	1	1	1	1	1	1	1	3	*0.49*	*0.04*	**43**
KC59	1	1	1	3	3	3	1	3	3	3	*0.50*	*0.22*	**42**
KC36	3	1	1	1	1	1	1	3	1	3	*0.54*	*0.11*	**40.5**
EB31	1	1	1	3	1	1	1	3	3	1	*0.54*	*0.08*	**40.5**
F18	1	3	1	1	3	1	1	1	3	3	*0.56*	*0.23*	**39**
KC3	1	3	1	3	1	3	1	1	1	1	*0.57*	*0.08*	**37.5**
EB1	1	3	1	1	3	1	1	1	3	1	*0.57*	*0.10*	**37.5**
EB101	3	3	1	3	1	1	1	1	3	1	*0.60*	*0.08*	**36**
EB98	1	3	1	1	1	1	1	3	1	3	*0.63*	*0.15*	**35**
EB144	1	1	1	1	3	3	1	1	3	1	*0.67*	*0.11*	**34**
EB147	3	3	3	3	3	1	1	3	1	1	*0.68*	*0.12*	**33**
EB126	3	3	1	3	1	1	1	3	3	1	*0.70*	*0.30*	**32**
KC15	1	1	1	1	1	3	1	3	3	1	*0.73*	*0.09*	**31**
H14	1	1	3	1	1	1	1	3	3	1	*0.74*	*0.13*	**30**
KC12	3	3	1	3	3	1	1	3	3	3	*0.77*	*0.03*	**29**
KC11	1	1	1	1	1	1	1	3	3	1	*0.79*	*0.24*	**28**
H17	3	3	1	1	3	3	1	3	3	1	*0.80*	*0.18*	**27**
K9	3	3	1	3	3	3	1	3	3	3	*0.82*	*0.09*	**26**
T1L	1	1	1	1	1	1	1	1	1	1	*0.83*	*0.02*	**25**

K10	1	1	1	1	1	1	1	3	1	1	*0.85*	*0.19*	**24**
EB146	1	1	1	3	1	1	1	1	1	3	*0.93*	*0.08*	**23**
EB132	3	3	1	3	3	1	3	3	3	1	*0.98*	*0.04*	**22**
G2	1	3	1	1	1	1	3	1	1	1	*1.00*	*0.02*	**21**
EB86	1	3	3	3	3	1	3	3	3	1	*1.01*	*0.03*	**20**
EB143	3	1	1	1	1	1	3	1	1	1	*1.02*	*0.09*	**19**
EB62	3	3	3	3	3	3	3	1	3	1	*1.05*	*0.04*	**18**
EB118	3	3	1	1	3	3	3	3	1	1	*1.08*	*0.03*	**17**
EB133	3	3	3	3	1	1	3	3	1	1	*1.12*	*0.08*	**16**
EB136	3	3	3	1	3	1	3	3	3	3	*1.13*	*0.08*	**15**
EB28	3	3	1	3	3	3	3	1	3	3	*1.15*	*0.04*	**14**
EB13	3	3	3	3	3	3	3	3	3	1	*1.16*	*0.03*	**13**
H30	3	3	1	3	3	3	3	3	1	3	*1.17*	*0.03*	**12**
EB97	3	3	1	3	3	3	3	3	3	1	*1.18*	*0.09*	**11**
EB39	1	3	3	1	3	3	3	3	3	3	*1.19*	*0.02*	**9**
EB73	3	3	3	3	3	1	3	3	1	1	*1.19*	*0.11*	**9**
H60	3	3	1	1	3	3	3	3	3	1	*1.19*	*0.18*	**9**
H9	3	3	1	3	1	1	3	3	3	3	*1.20*	*0.11*	**7**
EB120	3	3	3	1	1	3	3	3	1	1	*1.30*	*0.22*	**6**
KC35	1	3	1	1	3	1	3	1	3	1	*1.48*	*0.21*	**5**
EB129	3	3	3	3	3	1	3	1	1	3	*1.51*	*0.03*	**4**
EB88	3	3	3	3	1	3	3	3	3	3	*1.62*	*0.10*	**3**
EB137	3	3	3	3	1	1	3	1	1	1	*1.63*	*0.34*	**2**
T3D	3	3	3	3	3	3	3	3	3	3	*1.76*	*0.14*	**1**
p value	0.001	0.082	<0.001	0.95	0.059	0.234	<0.001	0.657	0.790	0.219			

a Numbers indicate parental sources of gene: 1, T1L; 3, T3D.

b Fold reductions were calculated by comparing the expression of Bcl-xL induced by each clone to control and GADPH

c Ranking was used for statistical purposes.

Table 5. linear regression

		mean data			
Model	1	R^2=0.751		S1	
			p value	***<0.001***	
	2	R^2=0.772		S1	L3
			p value	***<0.001***	***0.032***

L3 gene also plays a smaller independent role in changing the expression of the Bcl-xL protein.

PART IV: DISCUSSION

4.1 MRV replication in p53 defective and p53 positive cell lines

The CPE in host cells infected by viruses involves many cellular processes, such as the synthesis of DNA, RNA and proteins, changes of cytoskeletal structure and membrane integrity (Roizman *et al*, 1996), although the mechanism of CPE has not been very clear. Apoptosis and lysis of host cells infected by MRV are definitely related to CPE caused by virus due to the loss of membrane integrity. In this study, MRV infection can induce more CPE in p53 positive cell lines compared to p53-defective cell lines. Why is so little CPE in p53-defective cell lines infected by MRV? I think that MRV replication alone can not result in CPE or enough CPE. In order to induce CPE, MRV must stimulate or activate some kinds of mechanisms. In this study, the biggest difference of various cell lines is the presence or absence of p53. So p53 was probably playing an important role in CPE of host cells infected by MRV. The characteristic of CPE in this study actually resembles the combined feature of apoptosis, cell lyses, and non-apoptotic cell death. So p53 promotes the course of death of host cells infected by MRV. Normally, the expression of p53 is very low, but it can be activated and transcribed under some stresses such as DNA damage, UV radiation, oncogenic signaling, hypoxia and virus infection. The activated p53 protein can regulate the expression of many genes to induce cell cycle arrest, apoptosis and senescence (Beattie *et al,* 2005; Giono *et al*, 2006). In this study, the cell stress caused by MRV replication activated the expression of p53 which resulted in the

observation in Figure 1.

Why does T1L and T3D infection induce different characteristics of CPE? The feature of CPE induced by T1L is consistent with cell lyses and non-apoptotic death, while the characteristic of CPE induced by T3D can be explained by apoptosis. MRV can induce apoptosis in a wide range of cells both in vivo and in vitro. Different MRV strains have distinctive capability to induce apoptosis (Tyler *et al*, 1996; Rodgers *et al*, 1997; Connolly *et al*, 2001). Thus, the distinctive features of CPE that we have observed in cells infected by T1L and T3D is possibly due to the different capability of each strain to induce apoptosis.

In the course of MRV replication, inclusions are found in the cytoplasm around the nucleus of infected cells. they consist of filaments (Sharpe *et al*, 1982), MRV dsRNA (Silverstein *et al*, 1970), viral structural and non-structural proteins (Becker *et a,* 2003; Yin *et al*, 2004). Viral assembly happens here by recruiting various components to facilitate the process. As a result, complete and incomplete viral particles also exist in the inclusion which can be released after the death or lysis of host cells. In this study, the apoptosis or non-apoptosis (such as lysis) related to CPE can damage the integrity of host plasma membrane resulting in the release of MRV progenies. In the present study, p53 positive cell lines changed to be small round cells (due to apoptosis) or cell debris (due to cell lysis) after infection with MRV. Thus, the virus titers in p53 positive cell lines are from both the cytosol of live cells and contents released from dead cells. In p53 defective cell lines infected by MRV, with a low ratio of CPE, they grow bigger because of the enlargement of the inclusion.

Thus, the virus titers in p53 defective cell lines were mostly from cytosol of live cells. Before testing MRV titer, we would freeze and thaw the sample three times to completely destroy the plasma membrane of host cells. The virus titers tested here include virus both inside and outside of host cells after MRV infection. Therefore, in this study, p53 defective and positive cell lines share similar viral yield, though different severity of CPE was observed in these cell lines. This result suggests that the MRV replication is not directly related to the its features of CPE, which is consistent with a study that reovirus-induced apoptosis is not linked to viral yield (Rodgers *et al*, 1997).

From the morphology of infected cells and the virus growth curve, we can conclude: P53 gene plays an important role in the formation of CPE in cells infected by MRV, but it does not have impact on the replication of MRV in cells. Thus, MRV can replicate in both p53-defective and p53 positive cell lines.

4.2 Locating the position of Bcl-xL in western blotting

MRV can infect a wide range of cell lines and induce biological responses in host cells such as inhibition of DNA, RNA and protein synthesis, apoptosis and cell cycle arrest. Some studies show that different strains may have distinctive capability to do which should be related to their ability to change host genomic expression. Since MRV replicates in the cytoplasm of host cells, one interesting question is how MRV infection manipulates host genomic expression. In the previous study in our lab, we tested mRNA expression of various genes related to the stress responses in p53-null

H1299 cells infected by T1L or T3D. We found that the Bcl-x mRNA of the cells was suppressed by T1L infection, but increased by T3D infection. Hence, I wanted to figure out in this study whether the expression of proteins transcribed by Bcl-x in various cells was changed by T1L and T3D infection, and whether presence or absence of p53 had any effect on this change. Therefore, in this research, we chose to studied the mechanism by which MRV infection manipulates host genomic expression in both p53 positive and defective cell lines.

Due to alternative splicing, 5 proteins transcribed by the Bcl-x gene have been identified, which are Bcl-xL, Bcl-xS (Boise *et al*, 1993), Bcl-xβ (Shiraiwa *et al*, 1996), Bcl-xγ (Ye *et al*, 2002) and Bcl-xAK (Hossini *et al*, 2006). These proteins share very similar structures and they all have the BH4 domain. The antibody (sc 634) we used recognizes S18 in the N-terminus of Bcl-x portions, which is located in BH4. In theory, the antibody can recognize all five types of Bcl-x proteins. Some studies also reported that there was post-translational modification of Bcl-xL protein such as phosphrylation (Basu *et al*, 2003) and deamindation (Deverman *et al*, 2002). As shown in Figure 3, we got one clear band around 28kD if we develop PVDF membrane for 1min, and more bands in the same PVDF membrane if developed longer. Thus, the first thing we needed to do is to figure out which protein each band represented and exclude the non-specific bands. Immunoprecipitation of H1299 cell lysis by antibody sc 463, SDS PAGE, in-gel digestion and Mass spectrometry were used to identify each band. We could not find any peptide from Bcl-x related protein even when we repeated same experiments 3 times. Finally, large amount of lysate of

K562 cell lines (which is the positive control for anti-Bcl-xL antibody in Western blot recommended by Santa Cruz) was used for immunoprecipation. We did not find any protein related to Bcl-x proteins by in-gel digestion, but we found two kinds of peptides related to Bcl-x proteins by in-solution digestion. There are two possible reasons for these results: one reason is related to the very small amount of Bcl-x protein in cells; the other reason is that the peptides related to Bcl-x can not be recovered from SDS gel by trypsin digestion, thus, we could not identify each band in Western blotting by this way.

Then we focused on the bands around 28kD which presented earliest and strongest in Western blotting. Compared to the bands around 28kD, the other bands (whether they were proteins related to Bcl-x or non-specific proteins) were expressed at very low levels. So the bands around 28kD probably represent the proteins transcribed from Bcl-x gene. Some studies showed Bcl-xL is the major form of Bcl-x mRNA in mice (Gonzalez-Garcia *et al*, 1994). So shRNA technology was used. 5 different Bcl-x shRNAs were transected into H1299 and A549 cell lines. As shown in Figure 4, the bands around 28kD can be completely and partly blocked by shRNA in both cell lines. We finally confirmed that the bands around 28kD represent Bcl-xL by comparing the structure of mRNA (Table 1) and molecular weight.

4.3 The expression of Bcl-xL protein in p53-defective and p53 positive cell lines infected by MRV

In p53-null H1299 cell lines, the impact of MRV on the expression of Bcl-xL

protein was most apparent at 48 hours post infection, which is the late phase of MRV infection. T3D infection increased the expression of Bcl-xL protein. The optimum MOI that could induce maximum expression of Bcl-xL was dependent upon time: increased 1.69 fold when MOI=25 at 48 hours, 1.76 fold (the highest) when MOI=10 at 72 hours and 1.27 fold when MOI=2.5 at 96 hours. As for T1L, the optimum MOI that inhibited the expression of Bcl-xL to the lowest level corresponded to: 0.53 fold (the lowest) when MOI=100 at 48 hours, 0.73 fold when MOI=100 at 72 hours and 0.83 fold when MOI=10 at 96 hours. Thus, expression of Bcl-xL is MRV dose dependent and time dependent. Some studies showed that MRV induced apoptosis in many kinds of cell lines, and T3D had a higher capability to induce apoptosis than T1L (Tyler *et al*, 1995; Tyler *et al*, 1996). One important function of Bcl-xL is inhibiting apoptosis (Cheng *et al*, 1996). So in my study, it is possible that the increased expression of Bcl-xL induced by T3D inhibits the apoptosis triggered by T3D. In addition, there was no activation of p53. So I did not find much CPE in H1299 infected by T3D. Due to the lower capability of T1L to induce apoptosis, it can not cause much CPE by T1L itself in spite of the decreased expression of Bcl-xL in p53-null cell infected by T1L. There is not much CPE in p53-null H1299 cells infected by T1L or T3D in my study. In p53 positive H1299 cells, we have similar findings that T3D increased the expression of Bcl-xL and T1L decreased the expression of Bcl-xL protein. Therefore, the trend of Bcl-xL protein expression induced by MRV infection is not affected by the presence of p53, that is to say, MRV infection changes the expression of Bcl-xL protein by p53-independent pathway in H1299 cell lines.

Though the effect of MRV infection on Bcl-xL expression is independent of p53, we can not completely deny the direct effect of p53 on Bcl-xL expression. By comparing the fold change of Bcl-xL expression between p53-null and p53 positive H1299 cell lines with the same MRV strain, same MOI and same time point (Table 5), after the induction of p53 into the p53-null wild-type H1299 cell lines, T3D increased the expression of Bcl-xL to a less extent, while T1L decreased the expression to a greater extent. The inhibitory effect of p53 on Bcl-xL expression has been reported by other groups (Lee *et al*, 2008), which coincides with our finding here. Same experiments were repeated in HT1080 cells, but failed to yield similar results. This disparity may stem from cell line-specific differences.

4.4 Map the gene or gene combination of MRV that caused the trend of Bcl-xL expression in p53-null H1299 cell line.

Different MRV strains can induce different biological effects in many ways. MRV reassortant mapping can help to determine which gene plays the major role in these effects and understand the function of MRV gene segments/proteins.

In this study, 54 types of MRV reassortant and parental viruses (T1L and T3D) were used, and the major genes that control the change of Bcl-xL protein expression in H1299 cells were then mapped by several statistical analyses. Our data showed that S1 was the major determinant and L3 was the minor determinant. The S1 gene was reported to be the major or sole determinant for inducing apoptosis (Connolly *et al*, 2001; Rodgers *et al*, 1997; Tyler *et al*, 1996) and cell cycle arrest (Gaulton *et al*,

1989A; Tyler *et al*, 1996). The S1 gene segment encodes the viral attachment protein σ1 and the nonstructural protein σ1s. The σ1 protein has an important effect on determining phenotype of MRV strains: It decides the serotype of MRV (Weiner *et al*, 1978); it can determine the strain's spread of infection (Kaye *et al*, 1986); it can trigger apoptosis in host cells (Clarke *et al*, 2005) and it determines the resultant diseases caused by MRV (Kaye *et al*, 1986). The nonstructural protein σ1s has also been reported to play a role in the pathogenesis and virulence of MRV. The nonstructural protein σ1s is a determinant of severity of apoptosis in the heart and central nervous system infected by MRV (Hoyt *et al*, 2005), which is probably because nonstructural protein σ1s can elicit a significant cytotoxic T lymphocyte response (Hoffman *et al*, 1996). σ1s also plays a role in inducing G_2/M cell cycle arrest in infected cells (Poggioli *et al*, 2001).

The L3 gene segment encodes the λ1 protein. The protein λ1 is a major structural protein in reovirus core particle. It has ATPase activity (Noble *et al*, 1997), so its function is probably relevant to transcription of reovirus.

4.5. The clinical relationship

The formation and development of tumors are strongly associated with p53 mutation. So far, most clinical treatment of tumors is dependent on p53 pathway. It is a promising to explore new treatments independent of p53 to treat tumors. The reovirus as a treatment for tumors has been used for many kinds of tumors and different clinical trails, but the mechanism of killing is not very clear to date. In this

study, we try to find a p53-indpendent cell response pathway by MRV in p53-defective and normal cell lines as an experimental model.

The interesting finding is that different MRV strains can have completely different influence on the expression of Bcl-xL. Further, we found that the S1 and L3 gene segments were the determinants of these results. It is warranted to further investigate which site(s) of S1 and/or L3 gene segments are critical in this process. High level Bcl-xL expression was found in a wide variety of human tumors, such as multiple myeloma (Tu *et al*, 1998), lung (Reeve *et al*, 1996) and breast cancer (Olopade *et al*, 1997), gastric carcinoma (Kondo *et al*, 1996). Overexpression of Bcl-xL can cause resistance to chemotherapy in the tumor (Lee *et al*, 2009; Liu *et al*, 1999; Minn *et al*, 1995; Reed *et al*, 1996). It will be very helpful in medical application if we can interfere with the tumor growth by inhibiting the expression of Bcl-xL protein in tumor cells by some measures. Since we have found in this study that T1L can decrease the expression of Bcl-xL in H1299, it is possible that combining T1L with chemotherapy may increase the killing of small cell lung cancer. Other applicable methods such as DNA gene transfer viral infection which delivers enhanced expression of S1 and/or L3 gene segments in T1L may suppress the expression of Bcl-xL protein. Further study on other human cancer cell lines will provide us with valuable information. On the other hand, we need to induce the high level expression of Bcl-xL protein under some conditions such as organ transplantation and degenerative diseases because Bcl-xL protein can sustain survival of cells (Li *et al*, 2004; Lee *et al*, 2009). Measures such as DNA transfer as aforementioned will surely

have clinical significance.

4.6. Conclusion

From this study, we conclude the following:

1. MRV can replicate in both p53-defective and p53-positive cell lines. The appearance or absence or defective of p53 does not have any observed impact on MRV replication.

2. p53 can promote the severity of CPE in cells infected by MRV

3. MRV strains have different capacity to change the expression of Bcl-xL protein in p53-null and p53-positive H1299 cells. T3D infection can increase the expression of the protein, but T1L can decrease the expression of the protein.

4. The S1 gene segment which encodes the σ1 cell attachment protein and the σ1s non structural proteins is the primary determinant of the Bcl-xL expression, and L3 gene segment is the minor determinant.

4.7. Future work

We have shown that MRV infection changed the expression of Bcl-xL protein in H1299 cells. Is there any impact of the changed level of Bcl-xL protein on MRV replication or cell responses? To answer this question, we can transfect the plasmid containing Bcl-xL mRNA or Bcl-xL shRNA into H1299 cells to establish new cell lines with either enhanced or blocked expression of Bcl-xL protein. Then we can examine the apoptosis or cell cycle arrest and MRV replication in the new established cell lines

infected by T3D or T1L.

In this study, we showed that that S1 and L3 gene segments are determinants for the expression of Bcl-xL protein. To further confirm the result, we firstly transfect shRNA into H1299 cells, followed by infection with T1L or T3D. The efficacy of the shRNA on blocking the expression of S1 and/or L3 mRNA in T1L and T3D could be confirmed by SDS-PAGE. The difference of the expression of Bcl-xL protein in H1299 cells with or without transfection will provide us strong evidence on the role of S1 and L3 gene segments.

Provided that we have confirmed the role of S1 gene segment, it will be worthwhile to further investigate how proteins encoded by this gene segments, viral attachment protein σ1 and non structural protein σ1s, regulate Bcl-xL protein expression. MRV virions can be treated by ultraviolet to destroy the virus dsRNA genes, plaque assay will then be used to confirm inactivated viruses. UV-treated MRV can enter host cells but lose the capacity of replication. So after infecting H1299 cells, viral structural protein (σ1) can enter cells, but non-structural viral protein (σ1s) will not be produced (Tyler *et al*, 1995). Finally, Western blotting is used to evaluate the level of Bcl-xL expression in H1299 cells infected by untreated or UV-treated MRV virions.

Which site(s) of S1 and L3 gene segments result in the change of expression of Bcl-xL protein is also an interesting question. We could construct 20 kinds of plasmids containing the sequences of one kind of each MRV (T1L and T3D) gene segments. The plasmid containing the sequence of S1 or L3 gene segment could be

modified by site-directed mutagenesis with the Quick-Change Site-Directed Mutagenesis System (Stratagene, La Jolla, CA) to induce point mutation in various locations and verified by sequencing. Then we transfect constructed plasmids containing all gene segments of T1L or T3D with S1 or L3 gene mutated at different sites into host cells. Then viruses could be harvested and used to infect H1299 cell line and the protein level of Bcl-xL could be examined by Western Blot.

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