The Prosurvival Function of Interferon-gamma-inducible GTPase (IRGM3) in Coxsackievirus B3 Infection

by

Zhen Liu

M.Sc., Chinese Center for Disease Control and Prevention (CCDC), 2003

B.M., Beijing University of Chinese Medicine, 2000

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2010

© Zhen Liu, 2010
Abstract

Interferon-γ-inducible GTPase (IGTP, or IRGM3) is a p47 GTPase upregulated in coxsackievirus B3 (CVB3)-infected murine heart and inhibits CVB3-induced apoptosis through activation of the PI3 kinase/Akt pathway. However, the mechanism of this PI3K/Akt activation and other prosurvival functions of IGTP are unknown. In this study, using a doxycycline-inducible Tet-On HeLa cell line that overexpresses IGTP, I have demonstrated that focal adhesion kinase (FAK) is phosphorylated in response to IGTP expression and that transfection of a dominant negative FAK (FRNK) blocks Akt activation. Furthermore, induction of IGTP promoted the NF-κB activation as evidenced by its enhanced nuclear translocation and increased transcriptional activity. However, FRNK transfection and PI3K inhibitor both blocked the IGTP-induced translocation and NF-κB activation. Moreover, silencing NF-κB with siRNAs significantly inhibited the phosphorylation of FAK and Akt. Finally, blocking this survival pathway by FRNK transfection or NF-κB siRNA reduced CVB3 replication and enhanced cell death during CVB3 infection. Taken together, these results suggest that FAK is a mediator upstream of PI3K/Akt and that NF-κB functions as a downstream effector.

As viral infections including CVB3 can cause endoplasmic reticulum (ER) stress and activate a group of coordinated signal pathways termed ER stress response, additional prosurvival mechanisms of IGTP related to ER stress response was explored. It was demonstrated that IGTP expression suppressed either chemical- or CVB3-induced upregulation of GRP78, the ER stress marker. IGTP expression strongly inhibited the activation of both the PERK and ATF6 pathways of ER stress responses. The
suppression of ER stress responses by IGTP also led to attenuated induction of proapoptotic genes CHOP and GADD34. These data were further supported by experiments using IGTP knockout mouse embryonic fibroblast cells, in which the ER stress response induced by CVB3 infection was not relieved after interferon-γ treatment due to the absence of IGTP. Moreover, blocking PI3K/Akt pathway with either the PI3K inhibitor or dominant negative Akt construct significantly diminished the inhibitory effect of IGTP on ER stress response as well as its prosurvival effect. Therefore, IGTP expression relieves the ER stress response via a PI3K/Akt dependent mechanism, which contributes to cell survival and host defense during CVB3 infection.
Table of Contents

Abstract ......................................................................................................................... ii
Table of Contents ........................................................................................................ iv
List of Tables ................................................................................................................ vii
List of Figures .............................................................................................................. viii
List of Abbreviations ................................................................................................. x
Acknowledgements ................................................................................................... xiii
Dedication .................................................................................................................... xiv
Co-authorship statement ............................................................................................ xv

CHAPTER 1: Background. ............................................................................................. 1

1.1 Overview of myocarditis ....................................................................................... 1

1.2 Overview of CVB3 infection ................................................................................ 8
  1.21 General characteristics of coxsackievirus B3 ................................................ 8
  1.22 The pathogenesis of CVB3-induced myocarditis ........................................... 14
  1.23 Treatment of CVB3 infection ....................................................................... 18
  1.24 CVB3-induced apoptosis and cell signaling ................................................ 21

1.3 Interferon-γ inducible GTPase ......................................................................... 27
  1.31 Innate immune response and IFN-γ ............................................................. 27
  1.32 p47 GTPases and IGTP ................................................................................. 29
  1.33 IGTP in CVB3 infection ............................................................................... 34

1.4 Endoplasmic reticulum stress response ............................................................. 37
  1.41 Introduction of ER stress response ............................................................... 37
  1.42 ER stress response and apoptosis ................................................................ 40
CHAPTER 2: Rationale, Hypothesis, Specific Aims and Experimental Design

2.1 Rationale

2.2 Hypothesis

2.3 Specific aims

2.4 Experimental design

CHAPTER 3: Materials and Methods

3.1 Cells, animals and virus

3.2 Antibodies, plasmids and reagents

3.3 Western blot analysis

3.4 Luciferase assay

3.5 Electrophoretic mobility Shift Assay

3.6 Cell fractionation

3.7 Cell viability assay

3.8 Viral plaque assay

3.9 Statistical analysis

CHAPTER 4: The Mechanism of IGTP-induced PI3K/AKT Pathway

4.1 Results

4.2 Discussion

CHAPTER 5: The Effect of IGTP on Endoplasmic Reticulum Stress Response

5.1 Results

5.2 Discussion

CHAPTER 6: Conclusions and Future Directions
6.1 Conclusions.................................................................106

6.2 Future directions.......................................................107

Bibliography.................................................................110

Appendix: List of publications, abstracts and presentations......................122
List of Tables

Table 1. Etiology of myocarditis.................................................................4
Table 2. The family of Picornaviruses: genera, representative species and the diseases
that they cause.................................................................9
Table 3. Nomenclatures and cellular localization of the p47 GTPases........32
Table 4. Decreased host resistance to different intracellular pathogens in mice lacking
p47 GTPases.................................................................33
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Images of coxsackievirus</td>
<td>9</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Genome organization and proteolytic cleavage of viral polyprotein</td>
<td>12</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Overview of CVB3 life cycle</td>
<td>13</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Viral myocarditis: a triphasic disease</td>
<td>15</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Therapeutic strategies for CVB3 infection</td>
<td>19</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Host protein cleavage and apoptotic effect of viral proteases 2A and 3C</td>
<td>24</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Dendrogram of the p47 GTPase family</td>
<td>30</td>
</tr>
<tr>
<td>Figure 8</td>
<td>The PI3K/Akt survival pathway</td>
<td>36</td>
</tr>
<tr>
<td>Figure 9</td>
<td>A schematic of mammalian ER stress response signaling</td>
<td>39</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Virus induced ER stress response</td>
<td>42</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Tet-On IGTP inducible HeLa cell line</td>
<td>48</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Schematic of the experimental design</td>
<td>49</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Overexpression of IGTP induces up-regulation of p-FAK (Tyr397, Tyr925) and p-Paxillin (Tyr-118)</td>
<td>59</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Schematic of FAK-related non-kinase (FRNK)</td>
<td>60</td>
</tr>
<tr>
<td>Figure 15</td>
<td>FRNK overexpression inhibits IGTP-induced upregulation of p-Akt</td>
<td>61</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Expression of IGTP promotes translocation and activity of nuclear transcription factor NF-κB (p65)</td>
<td>64</td>
</tr>
<tr>
<td>Figure 17</td>
<td>FRNK or PI3K inhibitor LY294002 blocks the IGTP-induced NF-κB translocation and activity</td>
<td>66</td>
</tr>
<tr>
<td>Figure 18</td>
<td>NF-κB p50 siRNA inhibits the IGTP-induced upregulation of p-FAK and p-Akt</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 19. FRNK blocks the IGTP-induced cell survival, and decreases viral protein synthesis and viral release ................................................................. 72
Figure 20. NF-kB siRNA blocks the IGTP-induced cell survival, and decreases viral protein synthesis and viral release ........................................... 74
Figure 21. Expression of endogenous IGTP is required for activation of FAK and Akt in murine cardiomyocytes ............................................. 75
Figure 22. IGTP relieves ER stress response triggered by tunicamycin or BFA ...... 82
Figure 23. IGTP inhibits the activation of PERK pathway of ER stress response ...... 84
Figure 24. IGTP inhibits the activation of ATF6 pathway of ER stress response ...... 86
Figure 25. IGTP expression inhibits the ER stress-mediated apoptotic response ...... 88
Figure 26. IGTP inhibits the upregulation of GRP78 that is induced by CVB3 infection in Tet-On/IGTP HeLa cells ......................................................... 90
Figure 27. IGTP inhibits the activation of PERK and ATF6 pathway that is triggered by CVB3 infection in Tet-On/IGTP HeLa cells ................. 91
Figure 28. Preemptive ER stress response induced by tunicamycin facilitates viral protein synthesis and viral release of CVB3 .................. 93
Figure 29. IGTP relieves CVB3-induced ER stress response in mouse embryonic fibroblasts (MEF) ................................................................. 96
Figure 30. The relief of ER stress response by IGTP depends on the activation of PI3K/Akt pathway ................................................................. 98
List of Abbreviations

The following is a list of abbreviations in alphabetical order:

- AKT: also called protein kinase B
- ANF: Atrial natriuretic factor
- AODN: Antisense oligodeoxynucleotide
- Apaf-1: Apoptosis activating factor-1
- Arf1: ADP-ribosylation factor 1
- ATF4: Activating transcription factor 4
- ATF6: Activating transcription factor 6
- β-MHC: Beta-myosin heavy chain
- BFA: Brefeldin A
- CAR: Coxsackievirus and adenovirus receptor
- CHOP: C/EBP homologous protein
- CTL: Cytotoxic lymphocytes
- CVB3: coxsackievirus B3
- DAF: Decay accelerating factor
- DCM: Dilated cardiomyopathy
- DMEM: Dulbecco's modified Eagle's medium
- DMSO: Dimethyl sulfoxide
- DN: Dominant negative
- Dox: Doxycycline
- eIF2α: Eukaryotic translation initiation factor 2-alpha
- eIF4GI: Eukaryotic translation initiation factor 4GI
- EMSA: Electrophoretic mobility Shift Assay
- ER: Endoplasmic reticulum
- ERK: Extracellular signal-regulated kinase
- ESR: Endoplasmic reticulum stress response
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FRNK</td>
<td>FAK-related non-kinase</td>
</tr>
<tr>
<td>GADD34</td>
<td>Growth arrest and DNA damage-inducible protein 34</td>
</tr>
<tr>
<td>GBP</td>
<td>p65 guanylate-binding protein</td>
</tr>
<tr>
<td>GRP78</td>
<td>Glucose-regulated protein 78</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>IRG</td>
<td>Immunity-related GTPases</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGTP</td>
<td>Interferon-γ-inducible GTPase</td>
</tr>
<tr>
<td>IG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry sequence</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol-requiring enzyme 1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor Kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>p70S6K</td>
<td>p70-ribosomal S6 kinase</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase-like ER resident kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque-forming units</td>
</tr>
<tr>
<td>Pi</td>
<td>post-infection</td>
</tr>
<tr>
<td>PKR</td>
<td>RNA-dependent protein kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase PCR</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>Sterol regulatory element-binding protein 1</td>
</tr>
<tr>
<td>Stat1</td>
<td>Signal transducer and activator of transcription 1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRB3</td>
<td>Tribbles-related protein 3</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response, i.e. ESR</td>
</tr>
<tr>
<td>XBP1</td>
<td>X box-binding protein 1</td>
</tr>
</tbody>
</table>
Acknowledgments

I would like to thank the numerous people around me for their great support, their advice, technical expertise, and most importantly their friendship and moral support, without which this work could not have been completed. It has been a great privilege to work under the supervision of Dr. Decheng Yang, and I really appreciate his mentorship, guidance and kindly help. As well, I am sincerely indebted to Dr. Bruce McManus, Dr. Honglin Luo and Dr. Mary Zhang, who have provided constant encouragement and support that spurs me all the time. I would like to thank all members of our group and the cardiovascular research laboratory at the James Hogg iCAPTURE Centre, for their assistance in various aspects of my research, particularly, Elizabeth Walker, Travis Lim, Dr. Jane Yuan, Zongshu Luo, Jingchun Zhang, Dr. Caroline Cheung, Dr. Alhousseynou Sall, Seti Boroomand, Brian Wang, and Dr. David Walker.

I am very grateful to my supervisory committee members, Dr. Haydn Pritchard, Dr. Neil Reiner, Dr. Honglin Luo, and Dr. Bruce McManus for their precious advice, guidance and time throughout the work.

I also appreciate Dr. Taylor at Duke University who generously provided us the IGTP knockout cell lines. I gratefully acknowledge the financial support by the Canadian Institutes of Health Research, the Heart and Stroke Foundation of Canada, and the five year education from Department of Pathology and Laboratory Medicine at the University of British Columbia.
Dedication

To my parents, Wanfu Liu, Cuiping Wang,

my wife, Janny Liang and whole family.
Co-authorship Statement

Chapters 4 and 5 were co-authored as manuscripts for publication. The entries below represent the complete citations for each of these works.


Contribution: I performed most of the experiments and data analysis. I interpreted the results, conceived and wrote the manuscript. Co-authors assisted with research suggestions, additional experiments, and preparation of two figures.

Chapter 5: Liu Z, Zhang HM, Yuan J, Ye X, Stein DA, Taylor GA, Yang D. Interferon-gamma-inducible GTPase relieves endoplasmic reticulum stress response depending on the activation of PI3K/Akt but not the MAPK pathway. 2010

Contribution: I developed the hypothesis, and performed all of the experiments and data analysis. I interpreted the results and wrote the manuscript. Co-authors assisted with research and technical suggestions, material acquisition and manuscript revision.
Chapter 1: Background

The overall aim of this dissertation is to better understand the function of the interferon-γ GTPase (IGTP) in the host responses to coxsackievirus B3 (CVB3) infection. CVB3-induced myocarditis has important clinical relevance in the human population, and further appreciation of the prosurvival function of IGTP can be a part of better understanding of the pathogenesis of CVB3-induced myocarditis and provide new opportunities for therapeutic interventions.

1.1 Overview of myocarditis

Myocarditis is a major cause of sudden unexpected death in young adults less than 40 years old, and contributes significantly to the incidence of heart failure [1]. It is a non-ischemic inflammatory disease of the myocardium [2]. A large number of infectious pathogens, including viruses and bacteria, can cause myocarditis. Among them, CVB3 is the primary causative agent of viral myocarditis [3, 4].

Epidemiology

As early as the 19th century, myocarditis was recognized as a myocardial disorder characterized by immune infiltration and myocyte death [5]. Myocarditis generally causes a decrease in left ventricular myocardial function due to loss of viable myocardium, and evidence of myocarditis has been found in more than 1% of autopsies [6]. It can occur in all age groups, albeit the peak age group of patients with viral myocarditis is young adults, primarily between 20 and 39 years of age [1]. Epidemiological studies have suggested that up to 60% of the general public is exposed to cardiotropic viruses primarily in their early life, and half of them may have an episode of acute viral heart infection [7, 8].
It is estimated that the majority of patients with myocarditis recover uneventfully. However, in young patients less than 40 years of age, viral myocarditis accounts for approximately 20% of sudden unexpected death [7, 9]. In addition, acute viral myocarditis can develop into dilated cardiomyopathy (DCM), a chronic end-stage heart disease characterized by cardiomyocyte enlargement and ventricular dilation [10, 11]. Of patients with DCM, roughly half eventually require heart transplantation due to congestive heart failure [11].

**Diagnosis**

The clinical presentation of myocarditis is often associated with diverse, nonspecific signs and symptoms including shortness of breath, chest pain, life-threatening arrhythmias and cardiogenic shock. Particularly patients with viral myocarditis often experience severe flu-like symptoms.

Currently, right ventricular endomyocardial biopsy remains the gold standard diagnostic procedure for myocarditis. Pathological review should include a thorough assessment of myocyte cell death, inflammation and fibrosis following guidelines set out in the Dallas Criteria [12] as well as classification implemented by the WHO/World Heart Federation Task Force [13]. Dallas criteria myocarditis is characterized by an inflammatory infiltrate and associated myocyte necrosis or damage in the absence of an ischemic event. Chronically, myocarditis may be associated with myocyte hypertrophy, interstitial fibrosis and focal mononuclear inflammation. However, due to the nonspecific and variable symptoms of myocarditis, patients are often ineligible for the biopsy procedure. Also, there are many issues with the sampling procedure and interpretation, which result in misdiagnosis. Other clinical diagnostic approaches may include chest
radiography, electrocardiogram, and echocardiography. Traditional etiology identification includes viral serology and fecal isolation of virus or genome.

Recently molecular diagnostic biological techniques (reverse-transcriptase PCR [RT-PCR] and in situ hybridization [ISH]), biomarker assessment (troponin I and T), and noninvasive imaging techniques have been combined with traditional methods to enhance the diagnosis of myocarditis [14-16]. For instance, multiple molecular methods are now available for detection of CVB3 RNA in myocardial tissue, including dot blot, Northern blot, in situ hybridization and RT-PCR [14, 17]. One simple, yet specific and sensitive method to detect viral genomic material is RT-PCR. Hilton et al [18] utilized both in situ hybridization and RT-PCR to investigate the presence of coxsackievirus RNA in childhood myocarditis using formalin fixed tissue.

**Etiology**

Myocarditis can be caused by infections of viruses, bacteria, rickettsia and fungi, as well as conditions of hypersensitivity, autoimmunity and toxins [1] (Table 1). More than a dozen viruses are associated with myocarditis, including picornaviruses, influenza viruses, HIV, herpesviruses and adenoviruses [19]. However, epidemiological studies indicated that nearly 50% of North American clinical myocarditis cases are attributable to enterovirus infection, with the coxsackievirus B serotype 3 making up the most significant portion of such infections [20, 21].

Coxsackievirus was first isolated during a paralytic outbreak in 1948 [22], and coxsackievirus group B was isolated in two patients with aseptic meningitis the following year [23]. Since the mid 1950s, the association of CVB3 with human myocarditis has been widely demonstrated and recently reiterated [1, 11, 24]. Martino
Table 1. Etiology of myocarditis.

<table>
<thead>
<tr>
<th>Viral causes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>coxsackievirus B, parvovirus, herpes simplex virus, adenovirus, hepatitis C, human immunodeficiency virus</strong>, encephalomyocarditis virus, echovirus, cytomegalovirus, poliovirus, influenza, Epstein-Barr virus, herpes zoster, dengue virus, lymphocytic choriomeningitis virus, rubella virus, mumps virus, respiratory syncytial virus, rabies virus, vaccinia virus, varicella-zoster, yellow fever virus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other infectious causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria: <em>Corynebacterium diphtheriae, Staphylococcus aureus</em>. brucella, actinomyces, meningococcus, mycobacterium, pneumococcus, salmonella, tuberculosis, enterococcus, streptococcus</td>
</tr>
<tr>
<td>Protozoa: <em>Trypanosoma cruzi</em> (chagas’ disease), <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>Spirochetes: <em>Borrelia burgdorferi</em> (lyme disease), syphilis, leptospirosis</td>
</tr>
<tr>
<td>Rickettsia: <em>Coxiella burnetii, Rickettsia rickettsii, Rickettsia tsutsugamushi</em></td>
</tr>
<tr>
<td>Fungi: aspergillus, blastomyces, candida, coccidioides, cryptococcus</td>
</tr>
<tr>
<td>Parasite: <em>Echinococcus granulosus, Trichinella spiralis</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Immune disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoantigens: scleroderma, systemic lupus erythematosus, rheumatoid arthritis, systemic vasculitis, myosities, antiphospholipid syndrome, giant cell myocarditis, inflammatory bowel disease, Kawasaki’s disease</td>
</tr>
<tr>
<td>Allergen: penicillins, sulfonamides, cocaine, tricyclic antidepressants</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Toxins and injuries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical: anthracyclines, cyclophosphamide</td>
</tr>
<tr>
<td>Antiretroviral: ddI, AZT, ribaviran</td>
</tr>
<tr>
<td>Heavy metals: lead, mercury, arsenic</td>
</tr>
<tr>
<td>Natural toxins: snake bite, wasp sting, spider bite, scorpion sting</td>
</tr>
<tr>
<td>Physical: radiation, heat shock, hypothermia</td>
</tr>
</tbody>
</table>

* The relative common causes are shown in boldface type.
et al [11] performed an extensive study of the literature and found that 38-50% of myocarditis cases are CVB3 positive as determined by serological assessment of IgM or neutralizing antibodies. Epidemiological records show that as a predominate cause of myocarditis, CVB3 genomes were consistently detected in autopsy and biopsy samples as well as explanted hearts [25, 26].

**General treatment**

To date, no specific and effective therapy is available for myocarditis. Current treatment strategies for patients with myocarditis are primarily supportive and include avoidance of exercise, fever reduction, electrocardiographic monitoring for arrhythmias, and treatment of heart failure. For clinical symptoms of heart failure or arrhythmias, basic medications such as angiotensin-converting enzyme inhibitors or angiotensin-receptor blocking agents, diuretics, beta-blockers, and anti-arrhythmic drugs should be administered. For patients with end-stage cardiac failure, heart transplantation still represents the only definitive therapeutic option [15].

If the onset and course of myocarditis is unclear, supportive management of myocarditis is not sufficient to prevent long term cardiac damage. Good use of diagnostic tools is required to differentiate the different types of myocarditis and tailor the treatment to each form. For patients with viral myocarditis, particularly CVB3-induced myocarditis, antiviral agents eliminating the offending virus would help control the disease development (anti-viral agents discussed in section 1.2). Meanwhile, immunomodulatory therapy such as immunosuppression, IFN treatment, and immunoglobulin administration may not only be suitable for patients with autoimmune-mediated disease, such as giant cell myocarditis, but has also been shown to be beneficial for patients with viral
myocarditis.

Although the administration of immunosuppressive agents such as prednisolone, FK-506, cyclophosphamide, or azathiaprine in animal model or clinical trials has shown little success in ameliorating myocarditis [27, 28], FTY720 has become the first immunosuppressive agent reported to have beneficial effect in treating acute viral myocarditis in a murine model [29]. Several clinical trials showed that FTY720 was well tolerated, and effective when used in an immunosuppressive regimen with cyclosporine and corticosteroids [30, 31]. The benefit of FTY720 over other immunosuppressive treatments may lie in its particular ability to accelerate sequestration of circulating mature lymphocytes into lymph nodes, and decrease their infiltration into target tissues [32, 33].

In contrast, targeted immunomodulation using interferon (IFN-α, IFN-β, or IFN-γ) and immunoglobulin (IG) appears to have more promise in treating myocarditis. High-dose intravenous IG therapy has been shown to be effective in managing myocarditis in animal models and achieved moderate success in some clinical trials [34-36]. Its protective mechanisms may include direct inhibition of virus replication [35] and reduced production of pro-inflammatory TNF-α coupled with increased anti-inflammatory interleukins [37]. High-dose intravenous IG improved recovery of left ventricular function and increased the survival during the first year in pediatric patients with myocarditis [38]. In a recent study, immunoglobulin has also been shown to improve heart function in adults with myocarditis and DCM [39]. More placebo-controlled studies with large cohorts of myocarditis patients are warranted.

Many studies have shown that the interferon anti-viral system can significantly
decrease viral replication and dissemination \textit{in vitro} and \textit{in vivo}, and can be beneficial to patients with viral myocarditis [40-43]. IFN-α was successfully used to treat a group of patients with acute enterovirus-induced myocarditis [44], and both IFN-α and IFN-β have been shown to improve the prognosis for patients with viral myocarditis or DCM [45, 46]. Notably, expression of IFN-γ by recombinant CVB3/IFN-γ vector protected mice against infection of lethal CVB3H3 variant by decreasing the viral load and tissue destruction [47]. Novel IFN amplification using poly I:C (polyinosinic:polycytidylic acid), IFN-α 2b, pegylated IFN-α 2b, and ampligen have proved to be successful in blocking virus infection [48]. These encouraging results support the need for larger, randomized, and placebo-controlled studies with longer follow-up timepoints.
1.2 Overview of CVB3 infection

1.2.1 General characteristics of coxsackievirus B3

Coxsackieviruses are divided into two main groups based on the distinct pathology they cause in murine models. Group A Coxsackieviruses (serotypes 1-22,24) infect mostly striated muscles causing flaccid paralysis [22], while the group B (serotypes 1-6) viruses cause local myositis as well as meningitis, pancreatitis, hepatitis and myocarditis [49]. CVB is a ubiquitous agent and can spread rapidly within the community causing small epidemics. Though a high proportion of infections are subclinical, presentation may range from mild, undifferentiated febrile illness or respiratory symptoms to a severe and sometimes fatal disease of neonates. Besides myocarditis, CVB infections also cause pericarditis, neonatal systemic disorders, aseptic meningitis, meningoencephalitis, acute flaccid paralysis, type 1 diabetes, etc [49, 50]. Fecal-oral transmission of the CVB3 is the primary mode of transmission, and the site of entry for CVB3 is believed to be the alimentary tract. Virus multiplication at the portal of entry may be followed by viremia and further proliferation in the reticuloendothelial system resulting in the dissemination of virus into the target organs [51].

CVB3 is an enterovirus in the family of Picornaviridae. The Enterovirus genus also includes echovirus, poliovirus, etc (Table 2). Picornaviridae is a family of small, icosahedra, non-enveloped viruses with a single-stranded RNA genome of positive polarity (Figure 1). The mature virions of CVB3 are approximately 30nm in diameter with a hexagonal external structure and the interior of the capsid shell is packed with the viral RNA. Similar to other enteroviruses, the genome of CVB3 contains a single open reading frame (ORF) flanked by the 5’ and 3’ untranslated regions (UTRs)
Table 2. The family of Picornaviruses: genera, representative species and the diseases that they cause

<table>
<thead>
<tr>
<th>Genus</th>
<th>Representative species</th>
<th>Receptor</th>
<th>Disease(s)</th>
<th>Natural host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterovirus</td>
<td>Coxsackievirus</td>
<td>CAR, DAF</td>
<td>Myocarditis, Pancreatitis</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>Poliovirus</td>
<td>CD155</td>
<td>Poliomyelitis</td>
<td>Human</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>Rhinovirus</td>
<td>ICAM-1, LDLR</td>
<td>Common cold</td>
<td>Human</td>
</tr>
<tr>
<td>Cardiovirus</td>
<td>Theiler's murine encephalomyelitis virus</td>
<td>n.d.</td>
<td>Encephalomyelitis</td>
<td>Mouse</td>
</tr>
<tr>
<td>Aphthovirus</td>
<td>Foot-and-mouth-disease virus</td>
<td>Integrins</td>
<td>Foot-and-mouth-disease</td>
<td>Cloven-hooved ungulates</td>
</tr>
<tr>
<td>Erbovirus</td>
<td>Equine rhinitis B virus</td>
<td>n.d.</td>
<td>Acute respiratory disease</td>
<td>Horses</td>
</tr>
<tr>
<td>Kobuvirus</td>
<td>Aichi virus</td>
<td>n.d.</td>
<td>Gastroenteritis</td>
<td>Human</td>
</tr>
<tr>
<td>Teschovirus</td>
<td>Porcine teschovirus</td>
<td>n.d.</td>
<td>Encephalomyelitis</td>
<td>Pigs</td>
</tr>
<tr>
<td>Hepatovirus</td>
<td>Hepatitis A virus</td>
<td>HAVcr-1</td>
<td>Hepatitis</td>
<td>Human</td>
</tr>
<tr>
<td>Parechovirus</td>
<td>Human parechovirus</td>
<td>Integrins</td>
<td>Gastroenteritis, Respiratory disease</td>
<td>Human</td>
</tr>
</tbody>
</table>

*CAR, coxsackievirus and adenovirus receptor; DAF, decay accelerating factor; ICAM-1, intracellular adhesion molecule 1; LDLR, low-density-lipoprotein receptor

Figure 1. Images of coxsackievirus: transmission electron micrograph (left) and pictorial representation (right) of a single viral particle of coxsackievirus.
with a total length of around 7.4 kilobases. The ORF encodes four capsid proteins VP1, VP2, VP3, and VP4, and seven nonstructural proteins (2A-2C and 3A-3D) including proteinase 2A, 3C and RNA-dependent RNA polymerase 3D.

Instead of a cap structure 7-methyl guanosine triphosphate group, the 5' end of the genome is linked to a viral encoded protein VPg (3B), and a polyadenylated tail is attached to the 3' end of the genome. The lengthy 5’ UTR of CVB3 forms a highly ordered secondary structure and contains the internal ribosomal entry sequence (IRES) which allows for viral cap-independent translation initiation. Different CVB3 isolates from humans demonstrated a range of virulence phenotypes in mice, from avirulent to the ability to induce severe myocarditis [52]. The 5’UTR was identified as a major determinant of the cardiovirulence phenotype from two clinical CVB3 isolates. The introduction of mutations at the nucleotides 473 and 475 within the CVB3 IRES has resulted in reduced cardiovirulence [53, 54]. Further mutational studies by Yang et al [55] demonstrated the importance of the stem loops G and H within the IRES and the putative host protein-RNA interactions in viral replication. In addition, capsid protein VP2 was also found to be a cardiovirulence factor [56, 57], suggesting that cardiovirulence may have multiple determinants throughout the virus genome.

An early and important biological stage of the virus infectious cycle is host cell receptor attachment. CVB3 enters the cytoplasm through the coxsackievirus and adenovirus receptor (CAR) [58] with the assistance of the decay accelerating factor (DAF) co-receptor [59]. CAR is a member of the immunoglobulin superfamily [60] and has been shown to interact with Zonula-Occludens-1, an epithelial tight junctional complex protein and another factor required for CVB3 entry [61]. Attachment to the DAF
(CD55) receptor likely facilitates initial virus attachment and increases CAR-binding efficiency to the canyon of the virus protein coat [62, 63]. A rapid replicative cycle of virus takes place in the cytoplasm following the virus entry, uncoating, and release of viral RNA. The genomic RNA can act directly as an mRNA template for translation of a single polyprotein that is post-translationally cleaved primarily by CVB3-encoded proteinases 2A and 3C to produce individual structural and non-structural proteins for viral replication [64] (Figure 2). Then, the minus-strand RNA is synthesized to act as an essential template for transcription of complementary viral progeny plus-strand RNA genomes. The nonstructural proteins, particularly the RNA-dependent RNA polymerase 3D, are responsible for viral RNA replication, which takes place with rapid kinetics in the small membranous vesicles of the cytoplasm. Subsequently, the structural proteins are packaged with viral genome RNA to form progeny virions (Figure 3). The entire replication cycle of CVB3 from entry of the host cell to the release of progeny virus takes approximately 6-8 hours. In the late stage of viral replication cycle, apoptotic cascades are triggered to facilitate viral release from host cells [65]. Although CVB3 usually lacks a well characterized latent infection, evidence suggests that CVB3 RNA can persist in target cells for a long period of time as a double-stranded intermediate in some patients [66, 67].

As mentioned above, viral nonstructural proteins such as proteases 2A, 3C, and conceivably 3CD play crucial roles in the viral life cycle and viral pathogenesis. The viral proteases are mostly responsible for “reprogramming” the cellular environment to achieve goals such as: exclusive translation of the viral genome RNA; inhibition of the majority of host protein translation via direct cleavage of cellular factors involved in host
Figure 2. Genome organization and proteolytic cleavage of viral polyprotein. The CVB3 RNA genome consists of the 5'UTR, a polyprotein coding region, the 3'UTR and a poly(A) tail. The viral polyprotein is cleaved into three precursors, P1, P2, and P3 by 2A and 3C protease. Autocatalytic cleavage by 2A<sub>pro</sub> of the polyprotein at the P1-P2 junction separates the structural proteins from the non-structural proteins. Subsequently 3C<sub>pro</sub> cleavages at multiple sites release the rest of the proteases/capsid proteins.
Figure 3. Overview of CVB3 life cycle. (1) CVB3 binds to the CAR receptor with the assistance of co-receptor DAF for cell entry and (2) uncoating. Viral genome RNA is released into cytoplasm following uncoating, and acts as a template for both 3) polyprotein translation and 4) replication in membrane vesicles primarily from ER. The polyprotein is processed into individual structural and non-structural proteins as illustrated in previous figure. In replication, the first step is to synthesize the (-) strand RNA intermediate (yellow line) from the (+) genome RNA (red line). Then the (-) strand RNA intermediate works as template to produce additional (+) strand progeny RNA. Newly synthesized (+) strand RNA is translated to produce additional viral proteins. Lastly, 5) viral proteins assemble with (+) strand RNA to form infectious virus, and 6) newly synthesized virus particles are released by cell lysis.
transcription and translation; replication of viral genome RNA and the subsequent packaging of viral RNA into infectious virions [68, 69]. Besides these proteinases, other nonstructural proteins have also been shown to play important roles in pathogenic events during infection. For instance, the 2B protein modifies the permeability of ER membranes and disrupts the Ca\textsuperscript{2+} concentrations in ER, and this modification may facilitate the release of viral progeny from infected cells [70, 71].

1.22 The pathogenesis of CVB3-induced myocarditis: a triphasic disease

The pathogenesis of CVB3-induced myocarditis is complicated and poorly understood, but evidence suggests that both direct virus-mediated injury and subsequent inflammatory immune response contribute to the damage of cardiomyocytes [3, 72, 73]. Histological analysis of infected murine hearts showed that death and destruction of cardiomyocytes in early viral infection are common before the infiltration of immune cells [3, 73, 74]. However, the host inflammatory response may further cause myocardial injuries [72, 75]. Recent analysis [75] of available data has suggested that the disease can be conceptualized in the following three phases: 1) direct viral injury, or early myocyte death due to viral replication, followed by 2) inflammation with further damage due to the triggered immune response and autoimmune injuries, and later by 3) reparative and ultimate remodeling (Figure 4). In the mouse models of CVB3-induced myocarditis [76], the first 4 days post-infection (pi) are characterized by virus infection and direct damage of cardiomyocytes prior to visible immune cell infiltration. The massive influx of mononuclear cells occurs between 5-14 days pi, accompanied by destruction of infected myocytes and stromal collapse. By day 15 pi, healing advances
Figure 4. Viral myocarditis: a triphasic disease. CVB3-induced myocarditis is a disease composed of three distinct stages, including viremic injury, immune cell infiltration and reclamation. The first phase is characterized by viral infection and direct damage of cardiomyocytes in the absence of visible host immune response. The immune phase of infection includes mobilization of innate and adaptive defenses and is characterized by mononuclear inflammatory cell infiltration. The overdue inflammatory response and autoimmune response may contribute further to the myocardial injury. The final phase of myocardium remodeling consists of healing with fibrotic reparation and eventually cardiac dilation.
with various degrees of fibrotic reparation and cardiac dilation.

Virus-induced direct damage prior to inflammatory infiltration is a major determinant of the prognosis of viral myocarditis. During phase I, the virus infects ventricular cardiomyocytes, replicates and causes direct cellular injury rapidly, resulting in cell lysis and viral dissemination. The observed cytopathic effects include nuclear shrinkage, condensation of chromatin, cell rounding, acidophilic cytoplasm, nuclear pyknosis, and fragmentation of DNA, eventually leading to cell death [77]. CVB3 is able to induce cell apoptosis and is responsible for the direct cytopathic effect in cardiomyocytes [65]. The majority of extensive apoptotic phenotypic alterations in cardiomyocytes are co-localized to the viral replication site as demonstrated by in situ hybridization of both positive and negative strand viral RNA [74, 78]. Our laboratory has demonstrated the activation of caspase-3 and subsequent induction of cytopathic effects by CVB3 infection in vitro, which also highlighted the significance of direct virus-mediated damage [77]. Moreover, experiments using mice with severe combined immunodeficiency further confirmed the CVB3-induced lytic damage to cardiomyocytes [3]. Besides, the serum level of troponin, a sensitive marker of myocardial injury, was found to be closely and specifically correlated with virus titers in the heart, implying the major role of virus-mediated injury during the acute virus infection [79].

In response to viral infection and injury to the heart, the innate and adaptive immune responses are initiated to eliminate viruses, resulting in inflammatory infiltration in Phase II. The first wave of inflammatory processes is initiated by an influx of mostly macrophages and natural killer (NK) cells [27]. The next wave includes the antigen-specific T-lymphocytes and humoral neutralizing antibody response [80, 81].
The innate immune cells first aid in clearance of the virus in infected cells and they also mediate cytolysis of infected cells. The T-lymphocytes are activated to produce CD8+ or cytotoxic lymphocytes (CTL) and CD4+ helper cells in order to further eliminate infected cells and to produce anti-virus antibodies respectively. In most cases, myocardial inflammation promptly resolved. However, immunological damage to myocytes can also arise as a consequence of an overdue or inappropriate response to infection [82-84], by ongoing production of cytokines and chemokines such as TNF-α, IL-1β, IFN-γ, and IL-6, some of which are highly pro-apoptotic [85, 86]. Thus, if this initial immune response fails to clear the viral agent, the persistence of virus or ongoing immune processes directed against the myocardium also contributes to the pathogenesis of dilated cardiomyopathy (DCM) and the ultimate heart failure.

Autoimmune-mediated myocardial injury has been implicated in the development of DCM in the late phase of myocarditis. The post-viral autoimmunity and antigenic mimicry hypotheses have been proposed for cardiomyocyte damage following CVB3 infection. The autoimmunity is supported by the presence of autoantibodies against myosin, actin and other heart-specific antigens in patients with myocarditis [87, 88]. The antigenic mimicry has been supported by the sequence homology and cross-reaction to the monoclonal antibodies between streptococcal M protein, human cardiac myosin and CVB3 proteins [88, 89]. However, more studies are needed to fully validate the autoimmune pathogenesis in viral myocarditis.

If the patient survives the acute myocarditis, the inflammatory lesions may wither and subside with the proceeding of myocardium reparation and resolution at phase III. Due to the extensive myocyte loss, progressive replacement fibrosis occurs, which is
characterized by abundant collagen accumulation within the myocyte dropout regions, as well as interstitial or reactive fibrosis, which extends to areas of normal viable tissue. The extensive fibrosis can lead to ineffective contraction and improper electrical signal conduction, while degradation of the interstitial collagen network may result in the loss of structural support and lead to wall thinning and left ventricle dilation. Thus, cellular alterations and reparative fibrosis in this process can ultimately lead to dilated cardiac hypertrophy [90]. The exact mechanisms of this reparation and resolution process are still elusive. Several factors influence this remodeling process: the degree of injury or amount of remaining viable tissue, persistence of virus and inflammation, and the balance of matrix regulators [91, 92].

1.23 Treatment of CVB3 infection

In recent years, owing to the dynamic development of anti-viral therapeutics, several promising classes of drug candidates have emerged and exhibited the potential for clinical use to treat CVB3-induced myocarditis. Based on the stages of viral life cycle that they are targeting, these drug candidates can be divided into three groups: i) molecules that specially target the virus entry such as pleconaril, WIN 54954 and CAR-Fc; ii) nucleic acid-based antiviral agents that inhibit viral translation and/or transcription such as antisense oligodeoxynucleotide (AODN) and short interfering RNA (siRNA); and iii) inhibitors targeting signal transduction pathways required for viral replication (Figure 5).

Pleconaril is a broad range antiviral molecule which acts by binding to the conserved hydrophobic pocket of the picornaviral capsid, thereby altering its
Figure 5. Therapeutic strategies for CVB3 infection. Representation of the therapeutic targets in the virus life cycle and subsequent inflammatory response. AODN: Antisense oligodeoxynucleotide; siRNA: Short interfering RNA.
conformation and the subsequent receptor attachment. It is currently administered on a compassionate basis for life-threatening meningitis, encephalitis and myocarditis. Data from compassionate use in neonates with aggravated hepatitis as well as the pharmacokinetic evidence from adult infections showed favorable responses [93, 94]. The WIN class of compounds has also shown broad anti-picornaviral activity and been considered for the treatment of enteroviral myocarditis. The WIN-picornavirus interaction has previously been shown to occur beneath the canyon floor of the picornaviral capsid [95], and WIN compounds may destabilize virus through a blockage in the flexibility necessary for uncoating. Currently, WIN 54954 is the most promising compound in this class for the treatment of experimental enteroviral heart disease [96].

In addition, in our laboratory, the efficacy of soluble virus receptors has also been studied in the setting of CVB3 myocarditis [97, 98]. CAR-Fc and DAF-Fc were able to block infection of CVB3 to some degree in cell culture and ameliorate pancreas injury and inflammation in vivo [98]. Recently, Pinkert et al [99] also showed that inducible expression of CAR-Fc prevents cardiac dysfunction in CVB3 myocarditis under therapeutic conditions.

Another anti-CVB3 strategy is to target the viral genome using nucleic acid-based gene-silencing molecules, which are synthetic DNA or RNA of ~20 nucleotides (nts) in length including AODNs and siRNAs. These molecules function through sequence-specific targeting of the viral genome, leading to the substrate blockage and/or cleavage of transcription and/or translation initiation of viral RNA. In recent years, these molecules have become promising candidates in antiviral drug development. The applications of antisense AODN and siRNA in the treatment of CVB3 infection have
been reported in the past decade [100-103]. In our laboratory, the AODNs designed targeting the proximal end of the 3’UTR [102] and siRNA targeting the viral protease 2A have been shown to fundamentally suppress the viral replication and protein expression [101]. However, breakthroughs in finding appropriate methods to deliver the drug to specific organ or tissues need to be made before these molecules can be clinically used.

1.24 CVB3-induced apoptosis and cell signaling

Viral infection can eventually cause host cell death through overwhelming the cellular transcriptional and translational machinery and compromising of membrane integrity. Coxsackieviruses have been shown to cause apoptosis in transformed cells, and the cell death they induced in vivo consists of both apoptotic and necrotic phenotypes [77, 104]. The contribution of CVB3-induced apoptosis to cardiomyocyte loss during myocarditis has been confirmed from both the myocardial tissue of patients and that of infected mice [105-107]. In contrast to necrosis which is the equivalent of accidental cell death, apoptosis is a regulated and programmed cell death process involved in physiological and pathological processes [108]. Morphological changes during apoptosis include blebbing, loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and budding of apoptotic bodies. Apoptosis is essentially regulated and executed by a family of cysteine proteases, known as caspases. Once the caspases cascade is activated, the initiator caspases cleave inactive pro-forms of effector caspases such as caspase 3, thereby activating them. Effector caspases in turn cleave and degrade a variety of cellular structural and signaling proteins leading to the cellular morphological changes.
mentioned above. Apoptosis can be described as primarily a receptor- or mitochondrial-mediated mechanism. In the latter mechanism, intracellular insults to the mitochondria membrane cause the release of cytochrome c from the mitochondria, which further associate with apoptosis activating factor-1 (Apaf-1) and pro-caspase-9 [109]. The activated caspase-9 in turn activates the executioner caspase-3 initiating the downstream irreversible process.

It has been known that eukaryotic translation initiation factor 4GI (eIF4GI) is cleaved by viral 2A and 3C protein during CVB3 infection [110, 111]. Cleavage of such translation initiation factor abolishes cellular cap-dependent protein synthesis, while allowing cap-independent translation of viral RNA [112]. In addition, other viral proteins such as 2B can modify the permeability of the plasma membrane, mitochondria and endoplasmic reticulum [70, 71]. All such cleavage of host structural and transcriptional proteins as well as disruption of intracellular membranes ultimately trigger host cell apoptosis [111, 113] (Figure 6). It has been demonstrated that the mitochondria-mediated apoptosis during the late phase of virus infection facilitates viral progeny release [65]. Overexpression of anti-apoptotic molecules Bcl-2 or BclxL, or use of the caspase inhibitor delayed the decrease in host cell viability and reduced viral progeny release following infection [65]. Therefore, the struggles between host cells and invading CVB3 to dominate the apoptotic process ultimately determine the infection outcome and development of viral myocarditis.

In addition to manipulating the apoptotic events, like other viruses, CVB3 also reforms the cellular environment after entry to foster its own life cycle. All these are achieved through taking over or modulation of various host signaling pathways by the
Figure 6. Host protein cleavage and apoptotic effect of viral proteases 2A and 3C. Besides processing viral own polyproteins, the CVB3 proteases also cleave a number of host proteins reforming cellular environment and leading to cell apoptosis. Cytoskeleton proteins such as dystrophin and cytokeratin 8 can be cleaved by 2A protease. The cleavage of cell translational factors including eIF4G, PABP, La, PTB suppress majority of host cell translation, while facilitate viral IRES-mediated translation. The host gene expression is also inhibited at transcriptional level by the cleavage of transcriptional factors such as TBP, Oct-1, TFIIIC2, and CREB. All these known or other unknown mechanisms lead to cell apoptosis. Therefore transient expression of viral proteases alone in the cell has shown to result in activation of caspase, cleavage of PARP and apoptotic response.
virus. To date, knowledge characterizing host signaling pathways invoked post infection has accumulated from both in vitro and in vivo studies. For example, additional mechanisms of CVB3-induced apoptosis have been found: i) direct interaction of CVB3 with pro-apoptotic proteins such as Siva; ii) direct cleavage of IκBα leading to inhibition of NF-κB transactivation and downregulation of survival genes; iii) increase expression of the matrix protein Cyr61, leading to JNK-mediated activation of apoptosis [65, 114-116].

One of the extensively studied intracellular signal transduction pathways is mitogen-activated protein kinases (MAPKs), which are serine/threonine protein kinases activated in response to a wide variety of extracellular stimuli including viral infection [117, 118]. Our laboratory has shown that CVB3 infection induces a biphasic activation of the MAPK extracellular signal-regulated kinase (ERK) [119]. The first phase of activation correlates with CAR-mediated virus entry, while the later activation of ERK appears to be associated with CVB3 replication [119-121]. Selective inhibition of ERK blocks CVB3 progeny release, decreases virus protein production and circumvents apoptosis [122]. Opavsky et al [123] also showed ERK activation in isolated cardiac myocytes and in the hearts of A/J mice with CVB3-induced myocarditis. Recently, Marchant et al [124] found that the activation of ERK mediates Arf6-dependent trafficking of virus in the cytoplasm after virus entry. Similarly, other MAPKs such as p38 MAPK were also found to be activated during CVB3 infection and benefit viral replication [125].

Recently, studies from our laboratory have also revealed a regulatory role of the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) pathway during CVB3
infection [126-128]. PI3K/Akt and its potential downstream effectors such as nuclear factor kappa B (NF-κB) have long been recognized as a major survival pathway. Esfandiarei et al [128] showed that Akt regulates CVB3 replication through a mechanism which is not caspase dependent and that inhibition of Akt enhanced the apoptosis in the infected cell and decreased viral replication. Similar decrease of cell survival was also observed by inhibition of NF-κB during CVB3 infection [126].

Cell signaling studies can not only improve our understanding of the viral pathogenesis, but also provide a number of potential targets for therapeutic intervention (Figure 5). For example, in the above-mentioned ERK studies, inhibition of ERK by pharmacological inhibitor U0126 blocked viral replication and preserved cell survival [119]. A new generation of such MEK inhibitor, PD184352, has been in phase II clinical trial for assessing antitumor activity and safety [129]. In addition, Si et al [130] have shown in vitro that a natural compound, curcumin, inhibited CVB3 replication and protected cells from virus-induced cytopathic effect by disruption of the ubiquitin-proteasome system. Hence, clinical use of signaling intervention in viral myocarditis holds promise in the visible future.
1.3 Interferon-γ inducible GTPase

1.3.1 Innate immune response and IFN-γ

Replication of CVB3 in the heart and presentation of viral antigens on cardiomyocytes can initiate the immune response directed towards the elimination of infectious agents. The innate immune response of the host represents the first line of defense against the virus infection, and also serves to initiate and regulate subsequent adaptive immune responses. Early during infection, NK cell and macrophage migration, and interferon production by infected cells are principal mechanisms of innate responses against viruses.

The early infiltration of NK cells can induce MHC (major histocompatibility complex) antigen expression on cardiomyocytes by releasing cytokines, and also directly lyse infected cardiomyocytes to clear virus through perforin activity. Animal models depleted of NK cells developed a more severe form of myocarditis as a result of increased viral replication and release [131, 132]. Macrophages account for another major immune cell population that appears early in infection. They may help eliminate virus and virus-damaged tissue through inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO) that inactivates CVB3 proteases 2A and 3C [133, 134]. Also, like NK cells, they are responsible for production of certain pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6. Other subtypes of the innate immune system such as the granulocytes also contribute to the secretion of cytokines, chemokines, growth factors, and proteases, which recruit, activate, and induce proliferation of immune cells [135].

Thus, a number of cytokines are released upon CVB3 infection: IL-1α, IL-1β, IL-6,
TNF-α, and TNF-β are expressed throughout the early phase; IL-2, IL-3, IL-4, IL-10, and IFN-γ are mainly expressed by the infiltrating cells [136, 137]. Certain cytokines such as TNF-α and IFN-γ have been extensively studied owing to their great importance. TNF-α expression in the heart during viral myocarditis has been shown to aggravate the condition and lead to decreased myocardial contractile efficiency and biventricular dilation [137]. TNF-α or TNFR Knockout mice had significantly less myocarditis and fewer CD4+ IFN-γ+ cells than the controls [138].

Compared to cytokines like TNF-α, which can be highly pro-apoptotic [85, 86], IFN-γ induces responsive gene expression and triggers survival pathways which may subsequently contribute to cardiomyocyte hypertrophy [139]. IFNs are critical cytokines released in response to the presence of pathogens, particularly viruses. They are classified into two types according to their receptor: type I (such as IFN-α, IFN-β) and type II (IFN-γ) [140]. In general, all IFNs are antiviral agents. Nevertheless, IFNα/β are usually associated with stimulation of an antiviral state within the infected cell in an autocrine and paracrine manner, while IFN-γ modulates both the innate and the adaptive responses through stimulation of innate cells, enhancing antigen presentation to lymphocytes, and inducing the resistance of host cells to viral infections. Pancreatic overexpression of IFN-γ protected mice from lethal myocarditis [141]. Expression of recombinant IFN-γ protected CVB3-infected mice by decreasing the viral load and tissue destruction [47]. IFN-γ-deficient BALB/c mice had increased levels of viral replication in the heart [142, 143]. These findings illustrate the contribution of IFN-γ in protecting the heart against CVB3 replication.
1.32 p47 GTPases and IGTP

The object of my study, IGTP (interferon-γ inducible GTPase, also named as IRGM3), is a recently emerged gene in a growing 47-kDa small GTPase family. Our previous studies have identified IGTP as one of a few significantly up-regulated genes in CVB3-infected mice hearts, indicating its important role in CVB3 infection [144]. Our later in vitro study showed that IGTP expression could confer cell survival during CVB3 infection, and that IGTP inhibited CVB3-induced apoptosis through the activation of the PI3K/Akt pathway and inhibition of viral replication [145].

IFN-γ is known to exert its effects largely by activation of IFN-γ responsive genes, of which over 200 have been identified [146, 147]. Only a few of these genes encode proteins of well characterized antimicrobial activity, and among them are inducible nitric oxide synthase 2 (NOS2), phagocyte oxidase (phox), RNA-dependent protein kinase (PKR) and indoleamine-2,3-dioxygenase (IDO) [148, 149]. However, little is known for the remainder. Emerging from this poorly characterized group are three IFN-γ inducible GTPase families including the p47, p65 guanylate-binding protein (GBP), and Mx. Particularly, the newly discovered p47 GTPase family has been proven to be key mediators of IFN-regulated host resistance to intracellular pathogens.

The p47 GTPase family is a group of 47-kDa proteins that are produced in response to IFN-γ. At present, the family includes six characterized proteins: IRG-47, LRG-47, TGTP, IGTP, IIGP1 and GTPI (Figure 7). Their amino acid identities range from 26–63% [150], and their orthologs are present in at least 12 host species including humans, monkeys, mice, rats, and fish [148]. Six members of the p47 GTPase family were discovered piecemeal until 1998. Based on phylogenetic principles, a new naming
scheme for the p47 GTPases was introduced in 2005 to allow indefinite extension to homologous genes [151]. This p47 GTPase family was newly named as immunity-related GTPases (IRG) family, and the name of IRGM3 was given to IGTP. **Table 3** gives a parallel nomenclature relating the common names of the six genes and their products to the new names. However, since the original name system is still prevalently adopted in most references, I will keep using the common names such as “IGTP” in this dissertation.

**Figure 7. Dendrogram of the p47 GTPase family.** Primary amino-acid sequence comparisons indicate potential evolutionary relationships between the proteins. Indicated in dark purple are proteins whose roles have been studied in knockout mouse model. Note that the subfamily that contains IGTP and LRG47 appears to have a more dominant role in resistance to intracellular pathogens (Adapted from Taylor GA, *Nat Rev Immunol*. 2004).
The p47 GTPases are expressed at very low level in absence of infection, but following pathogen infections, their protein expressions are induced in various cells and accumulate rapidly to high levels in response to IFN-γ. In the case of IGTP, mRNA levels increase within an hour of exposure to IFN-γ, peak at three hours and remain constant to 48 hours [152]. As many other IFN-γ-activated genes, IGTP induction in response to IFN-γ is dependent on signal transducer and activator of transcription 1 (Stat1) [153]. IGTP can be expressed at high levels in many IFN-γ stimulated cells, including immune cells such as macrophages, T cells, and B cells, and nonimmune cells, such as fibroblasts and hepatocytes [152]. It has been demonstrated previously that IGTP regulates IFN-γ-induced *Toxoplasma gondii* killing in astrocytes [154], suggesting that the effects of the p47 GTPases are not specific to macrophages and may well extend to multiple cell types as a ubiquitous cell-autonomous defense mechanism.

The p47 GTPases are intracellular proteins that are membrane bound. They are distributed among specific membrane compartments in the cytosol. Both LRG-47 [155] and IIGP1 [156] are associated with Golgi membranes and/or the endoplasmic reticulum (ER), whereas IGTP is more than 90% membrane bound and localizes predominantly to the ER [156, 157] (Table 3). The majority of IGTP is associated with the cytoplasmic surface of ER membranes and with distinct globular structure located in the periphery of the ER [157]. Its ER localization is not altered by either IFN-γ stimulation of the cells, or by modulating the amount of GTP bound to the protein. However, unlike LRG-47 and IIGP1, association of IGTP with the ER can be disrupted by high salt concentrations, suggesting that protein–protein interactions may help mediate its binding [157]. Recently, in dendritic cells, a small portion of IGTP was found to also distribute to lipid
bodies (LBs), which are thought to originate from ER membrane and may be involved in the antigen cross-presentation [158]. The character of its localization may have strong implication in the function of IGTP. As many other GTPases associated with the ER have been found to be involved in protein processing or trafficking, it has been proposed that IGTP may also regulate protein processing or vesicle trafficking within the cells [157] [159].

Table 3. Nomenclatures and cellular localization of the p47 GTPases.

<table>
<thead>
<tr>
<th>Common names</th>
<th>New names</th>
<th>Compartment</th>
<th>% Membrane Bound</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGTP</td>
<td>Irgm3</td>
<td>ER</td>
<td>&gt;90%</td>
<td>C-terminal helix</td>
</tr>
<tr>
<td>LRG-47</td>
<td>Irgm1</td>
<td>Golgi, ER</td>
<td>&gt;90%</td>
<td>C-terminal helix</td>
</tr>
<tr>
<td>IIGP/IIGP1</td>
<td>Irga6</td>
<td>ER, Golgi</td>
<td>60-70%</td>
<td>N-terminal myristoylation</td>
</tr>
<tr>
<td>TGTP</td>
<td>Irgb6</td>
<td>n.d.</td>
<td>20-30%</td>
<td>n.d.</td>
</tr>
<tr>
<td>IRG-47</td>
<td>Irgd</td>
<td>n.d.</td>
<td>&lt;10%</td>
<td>n.d.</td>
</tr>
<tr>
<td>GTPI</td>
<td>Irgm2</td>
<td>Golgi</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Common names are those originally given to the genes or gene products in the literature. The new nomenclature for p47 GTPase was introduced by Bekpen et al (2005) to allow indefinite extension to homologous genes in various species.
Recent studies using loss- or gain-of-function approaches have strongly suggested that the p47 GTPases mediate cell-autonomous resistance against bacterial, viral and protozoan pathogens. The function of these p47 GTPases *in vivo* were first defined by creating p47 deficient mice and assessing their response to infections. The knockout mice that lack IGTP, LRG47 and IRG47 show no overt disease and have normal immune-cell development; however, they show remarkably reduced resistance to intracellular pathogens that varies in a gene-specific and pathogen-specific manner. In other words, each of the p47 member confers selective protection against a specific group of infectious agents that mice were challenged with, which include *Toxoplasma, Leishmania, Trypanosoma, Mycobacteria, Salmonella, Listeria*, etc [150]. For example, IGTP-deficient mice are susceptible to *T. gondii*, but not to *Listeria*. The susceptibilities of each p47 GTPase knockout mice to these pathogens are summarized in Table 4.

**Table 4. Decreased host resistance to different intracellular pathogens in mice lacking p47 GTPases.**

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Wild type</th>
<th>IFN-$\gamma$ KO</th>
<th>IGTP KO</th>
<th>LRG-47 KO</th>
<th>IRG-47 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protozoa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Leishmania major</em></td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>n.d</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><em>Mycobacterium avium</em></td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>n.d</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>n.d</td>
<td>n.d</td>
</tr>
</tbody>
</table>

* The responses of the mice to the indicated infections are scored as normal resistance (R) or increased susceptibility (S). n.d., not tested and determined. KO, knockout. (Adapted from Taylor, GA. *Microbes Infect.* 2007 and Martens S. *Annu Rev Cell Dev Biol.* 2006)
How these p47 GTPases mediate host resistance to distinct pathogens still remains elusive, but studies have indicated that multiple mechanisms or tasks may be involved in their antimicrobial activity. The p47 GTPases were initially studied intensively on protozoan infections, Martens et al [160] found that five of the known p47 GTPases including IRG-47 and IGTP are concentrated at the parasitophorous vacuolar membrane in IFN-γ-induced, T. gondii-infected primary astrocytes, and are associated with disruption of vacuolar membranes. But no evidence has been found that they play a direct role in the disruption itself or in the associated membrane vesiculation [161]. Further, the study of LRG-47-mediated bacteria resistance showed that the intraphagosomal residence is not a prerequisite for p47 GTPase function [162]. Instead, LRG-47 may regulate host resistance to bacteria by modulating the pathogen-infected lymphocyte survival and/or production [163].

1.33 IGTP in CVB3 infection

The role of p47 GTPases in resistance to viruses has not been explored to the same extent as their roles in resistance to protozoa and bacteria. Inoculation of IGTP-, LRG-47- and IRG-47-deficient mice with the double-stranded DNA herpes virus, murine cytomegalovirus, did not lead to enhanced susceptibility [159, 162]. In other experiments, IGTP-deficient mice also showed normal resistance to Ebola virus [159]. Despite these results which may reflect the pathogen-specific nature of p47 GTPase-mediated resistance, studies from our laboratory and others did support a role of p47 GTPase in viral resistance. Ectopic expression of TGTP in fibroblast was shown to decrease the susceptibility of the cells to vesicular stomatitis virus-induced lysis [164].
Later, using differential mRNA display, our laboratory identified IGTP as one of two significantly upregulated IFN-γ-inducible genes in CVB3-infected mouse hearts [144]. To address the implication of this finding, Zhang et al further investigated the effect of IGTP on CVB3 infection in a Tet-On inducible HeLa cell line overexpressing IGTP [145]. IGTP expression was found to inhibit viral replication and delay CVB3-induced cleavage of eukaryotic translation initiation factor 4G. More importantly, Zhang et al found that PI3K/Akt, a major cellular survival pathway to prevent apoptotic processes, was strongly activated following IGTP expression, and thus delayed the activation of caspase-9 and caspase-3 in CVB3-induced apoptosis. The ability of IGTP to promote cell survival during CVB3 infection was attenuated by the PI3K inhibitor or a dominant negative Akt construct. These data indicate that IGTP expression confers cell survival in CVB3-infected cells.

The finding of the anti-apoptotic pathway activated by IGTP sheds light on the mechanisms of IGTP-mediated antimicrobial responses in the context of viral myocarditis. In this notable pathway, it has been known that PI3K is a lipid kinase that synthesizes the second messenger PtdIns(3,4,5)P3, which leads to the recruitment and activation of Akt, a serine/threonine kinase (Figure 8). Once activated, Akt may protect cells from death by several mechanisms, such as directly phosphorylating and inactivating the proapoptotic protein BAD, or inhibiting pro-apoptotic genes such as BIM [165, 166]. Akt can also increase the transcription of survival genes by activating the transcription factor NF-κB [166, 167]. However, in regard to the IGTP-induced PI3K/Akt pathway, the exact picture of this signaling, including the mediator upstream of PI3K and downstream effectors, still remains unclear.
Figure 8. The PI3K/Akt survival pathway. The PI3Ks consists of a p110 catalytic subunit and a p85 regulatory adaptor subunit, and can be activated through ligation of RTKs, GPCR, or integrin receptors. Activation of these receptors results in the recruitment of PI3K to plasma membrane, where the p110 subunit catalyses the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to PIP3, which in turn helps to recruit Akt to the plasma membrane through direct contact of its pleckstrin-homology (PH) domain. There, Akt is subsequently phosphorylated by the PDK-1 (phosphoinositide-dependent kinase-1) at T308 (Thr308), by PDK2 at S473 (Ser473) respectively, and fully activated. Activated Akt may protect cells from death through multiple mechanisms. For example, it can directly phosphorylate and inactivate the proapoptotic protein BAD, which is a Bcl2 family member. Akt prevents transcription factors of Forkhead family from nuclear translocation and activation, which regulate expression of several pro-apoptotic genes such as BIM & FAS ligand. Akt can also increase the transcription of survival genes by activating CREB transcription factors and NF-κB. In unstimulated cells, NF-κB complex is inactivated and localized in the cytoplasm by binding to inhibitory proteins known as IκB, so it is unable to regulate transcription. IκB kinase (IKK) is known to phosphorylate and induce the degradation of IκB, which in turn releases NF-κB, allowing it to translocate to the nucleus and induce transcription of antiapoptotic genes. Akt has been reported to phosphorylate and activate IKK. Thus, Akt may inhibit apoptosis by activating NF-κB.
1.4 Endoplasmic reticulum stress response

1.41 Introduction of ER stress response

As mentioned above, the major cellular localization of IGTP is endoplasmic reticulum. As a separate compartment of eukaryotic cells, ER is a continuous membrane system that forms a series of flattened sacs within the cytoplasm. It is the production site for all parts of cell membranes, secretory proteins as well as lipids, and sterol. In the lumen of ER, the nascent secretory and transmembrane proteins further fold into their native conformation, and meanwhile with the aid of molecular chaperones they undergo a series of posttranslational modifications, most notably the glycosylation and the disulfide bond formation. Quality control mechanisms ensure that only correctly folded proteins exit the ER before their transport to Golgi apparatus for further modification. Hence, homeostasis of protein folding in the ER is the balance between the influx of newly synthesized unfolded polypeptide chains and the sum of the effluxes of correctly folded proteins to the Golgi complex and unfolded proteins targeted for proteasomal degradation.

ER is an organelle sensitive to a variety of cellular stresses conditions, physiologic or pathological, such as increased secretory load, the presence of mutated proteins and infections, which can disrupt the balance between the demand and the capacity of the ER for protein folding. These stresses interfere with ER function and cause the accumulation of misfolded or unfolded proteins, and thus initiates a group of coordinated signal transduction pathways known collectively as the ER stress response (ESR, also called the unfolded protein response, UPR) [168-170]. As an effort of the cells to restore ER homeostasis, the ER stress response may consist of four mechanisms:
attenuation of protein synthesis to prevent further accumulation of unfolded proteins; transcriptional induction of ER chaperone genes to increase folding capacity; transcriptional induction of protein degradation enzymes for proteasomal degradation; induction of apoptosis to dispose of injured cells and ensure the organism survival.

Three ER transmembrane proteins, the protein kinase-like ER resident kinase (PERK), the activating transcription factor 6 (ATF6) and the inositol-requiring enzyme 1 (IRE1), are mediators of the ER stress response. In normal conditions, these three ER stress sensors are maintained in an inactive state through their association with the glucose-regulated protein 78 (GRP78, also known as binding protein, Bip), the most abundant chaperone in ER.

Under stress conditions when GRP78 is sequestered to misfolded proteins, the PERK, ATF6, and IRE1 are released and activated sequentially through either autophosphorylation or translocation [168] (Figure 9). PERK activates its cytosolic kinase domain after homodimerization and autophosphorylation, and the only known target of PERK kinase activity is the eukaryotic initiation factor 2α (eIF2α), a ubiquitous cofactor required for the assembly of 80S ribosomes at the initiation codon of mRNAs to commence protein synthesis [171]. Thus, activation of PERK leads to attenuation of protein synthesis via phosphorylation of eIF2α, reducing protein load to ER. In response to ER stress, ATF-6α traffics from the ER to the Golgi, where specific proteases cleave the transmembrane domain and liberate the cytosolic portion of ATF-6α [172]. This severed active fragment then translocates to the nucleus, where it stimulates the transcription of chaperone genes to enhance the protein folding capability. Activated IRE1 removes an intron from the mRNA of X box-binding protein 1 (XBP1), and then
Figure 9. A schematic of mammalian ER stress response signaling. Protein kinase-like ER resident kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) reside at the ER membrane and are mediators of the ER stress response. In normal conditions, the luminal domains of these molecules associate with GRP78 to keep them in an inactive state. On aggregation of unfolded proteins, GRP78 is sequestered to the unfolded proteins, allowing the release and subsequent activation of PERK, ATF6, and IRE1 through either autophosphorylation or translocation. The activation of these mediators occurs sequentially, with PERK being the first, rapidly followed by ATF6, whereas IRE1 is activated lastly. Activated PERK inhibits general protein synthesis by phosphorylating eukaryotic initiation factor 2α (eIF2α). Activation of IRE1 leads to the transcription induction of protein degradation enzymes, which remove and degrade misfolded protein in ER. Together with PERK and IRE1, activated ATF6 stimulates the transcription of chaperone genes for protein folding, and meanwhile induces the expression of proapoptotic genes such as C/EBP homologous protein (CHOP), eventually leading to cell apoptosis.
the spliced XBPI translocates to the nucleus and controls the transcription of genes involved in protein degradation.

### 1.42 ER stress response and apoptosis

However, if such concerted and complex cellular responses fail to restore normal ER function, they also initiate apoptosis through enhancing transcription of proapoptotic genes such as CHOP and GADD34 [169, 170, 173, 174]. One well-studied mechanism is through the activating transcription factor 4 (ATF4) in the PERK pathway. As mentioned above, phosphorylation of eIF2α inhibits most cellular protein synthesis; however it paradoxically enhances the translation of ATF4. Because ATF4 mRNA contains multiple upstream open reading frames (uORFs) that precede the ATF4 ORF, only when ribosomal assembly is impaired, those decoy uORFs can be bypassed in favor of translation initiation at ATF4 start codon [175]. ATF4 as a transcription factor stimulates the expression of C/EBP homologous protein (CHOP), as well as growth arrest and DNA damage-inducible protein 34 (GADD34). CHOP, also known as growth-arrest- and DNA-damage-inducible gene 153 (GADD153), was originally identified in response to DNA damage and proved to be a critical factor that facilitates apoptosis. During ER stress, all three arms of the ER stress responses induce transcription of CHOP. However, the PERK–eIF2α–ATF4 branch is most essential for induction of CHOP expression [175]. CHOP may induce apoptosis through suppression of Bcl2 and through upregulation of GADD34 and Tribbles-related protein 3 (TRB3) [176]. Expression of GADD34 correlates with apoptosis induced by various signals, and its overexpression can initiate or enhance apoptosis [175]. Thus, this cascade eventually leads to irreversible activation
of executioner caspases for apoptosis (Figure 9).

1.43 ER stress response and CVB3 infection

ER stress response has been implicated in the pathogenesis of myriad human diseases, such as diabetes, neurodegenerative and cardiovascular diseases, and this cellular machinery has also been shown to be exploited by many viruses for their own advantage [177, 178]. ER undertakes the cellular activities essential for survival, and meanwhile it is also a host organelle utilized by many viruses for replication and maturation [177, 178]. Various viruses have been shown to induce ER stress response either through overwhelming the ER folding capability, or directly disrupting ER integrity and function during their productive infections. [179-183] (Figure 10). Many of such virus-induced ER stress responses have been reported to be beneficial to the viral replication and pathogenesis [182-185], and accumulating evidence supports the involvement of the PERK and ATF6 pathway in various viral replication events [186-189]. For example, the induced eIF2α phosphorylation and ATF4 expression have been found to aid reovirus replication [182]. Replication of hepatitis C virus has been shown to stimulate the ATF6 pathway selectively to promote its own propagation [180]. It has been shown that picornaviruses require ER membranes for viral protein biogenesis and anchoring of the replication complexes [190-192]. Pharmacological inhibition of ER chaperone such as Hsp90 impaired the replication of poliovirus, rhinovirus, and coxsackievirus in cell culture [193]. In addition, certain coxsackievirus proteins, such as 2B, 2C, and 3A, localize to endoplasmic reticulum, and are implicated in disrupting ER integrity and functions [70, 192, 194]. Recently, our group has demonstrated that CVB3 infection triggers ER stress response as indicated by upregulation of GRP78 upon
infection in both HeLa cells and cardiomyocytes [195]. Zhang et al [195] found that CVB3 infection activates different UPR pathways and induces ER stress-mediated apoptosis through suppression of P58IPK and induction of CHOP, SREBP1 and caspase-12.

Figure 10. Virus induced ER stress response. ER is an essential organelle for replication and maturation of many viruses. Viruses may utilize ER as site of protein translation and accumulate viral protein in ER. Also, some virus-encoded proteins are able to directly disrupt ER integrity and function. To date, increasing number of viruses such as influenza, RSV, HCV, HSV have been shown to disrupt ER function and cause ER stress. The induced ER stress response has been shown to be beneficial to survival and replication of many viruses such as SV40 and HBV. Some viruses, such as HSV and CMV, have also evolved mechanisms to antagonize or overcome the negative effects of ER stress response such as the translational attenuation caused by phosph-eIF2α. The induced ER stress responses also dictate viral replication and pathogenesis through the impact on cell apoptosis and survival.
CHAPTER 2: Rationale, Hypothesis, Specific Aims and Experimental Design

2.1 Rationale

CVB3-induced myocarditis is a common heart disease, particularly in children and young adults. Pathology of viral myocarditis can be described as early myocyte injury or death associated with viral infection, with or without inflammation, and ultimate myocardial remodeling leading to ventricular dilation and heart failure. Our laboratory and others’ have shown that CVB3-induced apoptosis contributes to the significant cell loss that occurs in early infection and is a critical determinant of disease severity and development [27, 77, 196]. Previous functional studies of the differentially expressed genes during CVB3 infection have further revealed the importance of cell death/survival pathway in the host response to CVB3 infection and pathogenesis of viral myocarditis [144].

IGTP is one such significantly up-regulated gene identified in CVB3-infected mouse hearts [144]. Interferon-γ regulates the host response to a variety of infectious agents, and exerts its effects largely by activation of IFN-γ responsive genes [146, 147]. IGTP belongs to a newly emerging family of IFN-γ-inducible 47-kDa GTPases [149, 152]. It localizes mainly in the endoplasmic reticulum [157] and has been shown to be essential for host resistance to acute infection by the protozoans *Toxoplasma gondii* [154] and *Leishmania major* [149]. But its antimicrobial mechanism, particularly in viral infection, is still unclear. Our laboratory has explored the role of IGTP in CVB3 infection and found that IGTP expression confers cell survival during infection, and that IGTP inhibits CVB3-induced apoptosis through the activation of the PI3K/Akt pathway [145]. However, many pertinent questions regarding the prosurvival mechanism of IGTP arise: i)
how does IGTP transduce signals to PI3K and cause the cascade activation, or in other words, what is the mediator upstream of PI3K and downstream effectors? ii) is PI3K/Akt pathway the only mechanism of IGTP to inhibit apoptosis, or do additional prosurvival effects or crosstalk pathways exist? iii) what is the implication of IGTP’s ER localization, and is there any impact of IGTP expression on ER stress response? My thesis study aims to address these questions.

In regard to the first question, it is known that the PI3K can be activated by multiple mechanisms such as through ligand activation of receptor tyrosine kinases, G protein-coupled receptors, or integrin receptors. Among the signaling molecules possibly involved in the activation of PI3K is focal adhesion kinase (FAK). FAK, a 125-kDa cytoplasmic protein-tyrosine kinase, is a member of the focal adhesion family that mediates integrin-initiated signal transduction leading to a variety of cellular responses, including cell proliferation, migration, and adhesion. Upon stimulation by external factors, FAK is phosphorylated at tyrosine 397, which can create a binding site for the SH2 domain of the p85 subunit of PI3K. Phosphorylation of p85 by FAK may activate the p110 subunit and thus the PI3K/Akt signaling pathway. Once activated, Akt may protect cells from death by multiple mechanisms. Particularly, Akt may exert a positive effect on transcription factor NF-κB function by phosphorylation and subsequent degradation of I-κB, an inhibitor of NF-κB, and NF-κB has been long recognized as a critical antiapoptotic molecule activating transcription of survival genes [197, 198]. Here, we hypothesize that FAK is a mediator between IGTP and activated PI3K/Akt, while NF-κB is the key downstream effector of this IGTP-PI3K/Akt pathway.

In regard to the latter questions, it is notable that an increasing number of viruses
have been shown to exploit the ER for their replication and maturation, disrupting ER homeostasis and thereby trigger ER stress response, a group of coordinated signal transduction pathways. In turn, such activated ER stress response can be utilized by many viruses for their own advantage and lead to cell apoptosis. Recently, it has also been demonstrated by our laboratory that CVB3 triggers ER stress response and induces apoptosis through suppression of P58\textsuperscript{IPK} and induction of proapoptotic genes in this pathway. Here, we hypothesize that IGTP relieves the ER stress responses induced by CVB3 infection, and thereby contributes to cell survival and inhibits viral replication.

2.2 Overall hypothesis

The overall hypothesis is that IGTP protects cells from CVB3-induced apoptosis through multiple coordinated mechanisms: 1. IGTP activates the PI3K/Akt survival pathway through focal adhesion kinase and further activates NF-\(\kappa\)B. 2. IGTP relieves the ER stress responses induced by CVB3 infection and thereby promotes cell survival and inhibits viral replication.

2.3. Specific aims

1. To identify the signaling mediator and downstream effector of the IGTP-induced PI3K/Akt survival pathway

2. To examine the effect of IGTP expression on the ER stress responses and ER stress-induced apoptosis during CVB3 infection.
2.4 Experimental design

**Experimental design for aim 1**

In inducible Tet-On HeLa cells overexpressing IGTP (Figure 11), the activation of FAK signaling as indicated by phosphorylation of FAK and paxillin following ectopic expression of IGTP was examined by Western blot analysis. FRNK, a dominant negative FAK, was transfected to examine whether the activation of PI3K/Akt by IGTP depends on FAK. The nuclear translocation and activity of NF-κB following IGTP expression were examined by Western blot, luciferase assay, and gel shift assay. To determine the position of NF-κB in the IGTP-induced PI3K/Akt survival cascade, the IGTP-induced activity of NF-κB was examined after the suppression of FAK and Akt by dominant negative plasmid or specific inhibitor. To further clarify the role of NF-κB in this pathway, the phosphorylation of FAK and Akt after silencing of NF-κB with siRNAs was measured by Western blot. Further, the role of FAK or NF-κB for the pro-survival function of IGTP during viral infection was demonstrated by the MTS cell viability assay and viral plaque assay after blockage of FAK with FRNK or NF-κB with specific siRNAs. The endogenous activation of FAK and Akt by IGTP during CVB3 infection was also demonstrated by Western blot analysis of CVB3 infected A/J mouse hearts. To address whether expression of endogenous IGTP is required for activation of FAK and Akt in murine cardiomyocytes, the activation of these signals was examined using IFN-γ-treated HL-1 cardiomyocytes transfected with siRNA targeting IGTP (Figure 12).

**Experimental design for aim 2**

To examine the effect of IGTP expression on ER stress response, chemical inducers including tunicamycin and Brefeldin A were used to induce ER stress response in parallel
to that induced by CVB3 infection. To measure the activation of ER stress response in Tet-On/IGTP HeLa cells, the upregulation of GRP78 caused by chemical agents or CVB3 infection was monitored following IGTP expression by Western blot analysis. To examine the activation of PERK pathway, the phosphorylation of PERK and eIF2α and the expression of ATF4 were examined by Western blot. To examine the activation of ATF6 pathway, cell fractions were prepared, and the proform and the cleaved form of ATF6 were probed in the cytosol and the nucleus respectively. To examine the effect of IGTP on ER stress-induced apoptosis, the expression of proapoptotic genes CHOP and GADD34 and the cleavage of caspase 3 and apoptosis marker PARP were measured by Western blot. The cell viability during ER stress was measured by MTS assay, and viral release and protein synthesis were examined by viral plaque assay and VP1 expression respectively. To further confirm the effect of IGTP, CVB3-induced ER stress response was also examined in IFN-γ-treated IGTP knockout mouse embryonic fibroblast (MEF) cells in comparison to wildtype MEF. To explore the mechanism by which IGTP relieves ER stress response, a series of inhibitors or dominant negative plasmids for potential crosstalk pathways (JNK, ERK, p38, Akt) were applied to determine whether the relief effect of IGTP is dependent on any of these signaling pathways (Figure 12).
Figure 11. Tet-On IGTP inducible HeLa cell line. The left panel is a schematic of gene regulation in the Tet-On Systems. In this system, HeLa cells were stably transfected with two plasmids: Responsive plasmid contains repeats of tet operator sequence and the gene of interest; Regulatory plasmid encodes regulatory protein termed rTA, which only binds to tet operator sequences and works as transactivator on the gene of interest in the presence of Doxycycline. Thus, in Tet-On IGTP inducible HeLa Cell (right panel), when the Doxycycline was added to the culture medium, the expression of our interested gene, IGTP, was successfully induced from very low background.
Aim 1.

CVB3 infection

IGTP

FAK

PI3K/Akt

NF-κB

Cell survival & viral replication

Doxycycline

FRNK

DN-Akt & inhibitor

siRNA of NF-kB

Aim 2.

CVB3 infection

Chemical agents

IGTP

Doxycycline

Signaling inhibitors

ER stress response

PERK pathway

GRP78

ATF6 pathway

Proapoptotic genes

Cell survival & viral replication

Figure 12. Schematic of the experimental design. The cell viability and viral replication were determined as outcome following various treatments and IGTP induction. MTS assay, morphological change, Western blotting for apoptotic markers were used in combination to analyze cell apoptosis and viability. Viral infectivity was evaluated by viral plaque assay and Western blotting for VP1 protein synthesis.
CHAPTER 3: Materials and Methods

3.1 Cells, animals and virus.

CVB3 was routinely propagated in HeLa cells (ATCC). The virus supernatant was obtained by three cycles of freeze-thaw and centrifugation to remove cell debris and stored at –80 °C. Virus titers were determined by plaque assay prior to infection. A previously established double-stable Tet-On/IGTP HeLa cell line was used in this study. Tet-On/IGTP HeLa cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM HEPES, and 10% Clontech approved fetal bovine serum specially prepared for Tet-On system in the presence of both 100 µg/ml G418 and 100µg/ml hygromycin. To induce the expression of IGTP, cells were cultured in above medium containing 1 µg/ml doxycycline. Cell cultures prepared for detection of cell signals were serum-starved overnight before performing the experiments. MEF cells were propagated in Eagle's medium supplemented with 10% fetal bovine, and 2 mM L-glutamine. HL-1 cells were grown in Claycomb medium (JRH Biosciences) containing 10% FBS, 0.1 mmol/L norepinephrine (Sigma-Aldrich), and 2 mM L-glutamine. Male A/J mice (Jackson Laboratories) at five-week age were injected intraperitoneally with $10^5$ plaque-forming unit (PFU) of CVB3 or phosphate-buffered saline (PBS), and were sacrificed at days 7 or 9 pi (n=5 per time point per group). Heart tissue was homogenized, and cell lysates were collected.

3.2 Antibodies, plasmids and reagents.

In aim 1: Antibodies against phosphorylated Akt (p-Akt), total Akt, and
phosphorylated ERK (p-ERK) as well as the PI3K inhibitor LY294002 were purchased from Cell Signaling Technology. Antibodies against phosphorylated FAK (pFAK) at Tyr397, pFAK at Tyr925, total FAK (tFAK), and NF-κB p65 and p50 subunits were purchased from Santa Cruz Biotechnology. Goat antibodies against mouse IgG and rabbit IgG conjugated to horseradish peroxidase were obtained from BD Biosciences and Santa Cruz Biotechnology, respectively. Rabbit polyclonal antibody against HA-tag was purchased from Covance Research Product. The pHK-FRNK (FAK-related non-kinase) construct containing a HA- His tag at the 3' end of its open reading frame was obtained from Dr. Junlin Guo, University of Michigan. NF-κB siRNA p50 was purchased from Ambion.

In aim 2: Antibodies against GRP78, p58IPK p-eIF2a, PARP, Caspase 3, CHOP, total eIF2a, IGTP, were purchased from Cell Signaling Technology. Antibodies against p-PERK, ATF6, SREBP1, ATF4, GADD34, total PERK, and Histone 1 were purchased from Santa Cruz Biotechnology. The monoclonal anti-VP1 antibody was purchased from DakoCytomation. Antibody against β-actin was purchased from Sigma-Aldrich. Goat antibodies against mouse IgG and rabbit polyclonal antibody were obtained from BD Biosciences and Santa Cruz Biotechnology, respectively. The ER stress inducers tunicamycin and Brefeldin A were purchased from Sigma-Aldrich and eBioscience respectively. Recombinant IFN-gamma was purchased from Cell Sciences. U0126 was purchased from Promega. SP600125 and SB203580 were purchased from Calbiochem. The dominant negative mutant Akt1 construct (encoding an alanine instead of a serine and a threonine) was originally from Upstate Biotechnology, Inc.
3.3 Western blot analysis.

Cells either untreated or treated with different experimental reagents were washed twice with ice-cold PBS containing 5% phosphatase inhibitor (Active Motiff Company) and cell lysates were prepared as described previously [145]. The protein concentration was determined by the Bradford assay (Bio-Rad). Twenty to 80 micrograms of extracted proteins were fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech), and blocked with PBS containing 0.1% Tween 20 and 5% nonfat dry milk for 40 min. Afterward, the membrane was incubated with the specific primary antibody overnight at 4°C, followed by the secondary antibody for 1 h at room temperature. The immunoblots were visualized with an enhanced chemiluminescence detection system according to the protocol of the manufacturer (Amersham Pharmacia Biotech). Densitometry analysis was performed by using the Genetools software from Syngene. Density values for proteins were normalized to the level of control groups (arbitrarily set to 1.0-fold).

3.4 Luciferase assay.

The NF-κB-dependent luciferase reporter gene construct containing the synthetic sequence with four copies of connective NF-κB-binding elements (p-NF-κB-luc) and the pCMV-β-galactosidase control plasmid were obtained from Clontech and used for transfection. The cells were collected at 30 h after transfection using Lipofectamine 2000 (Invitrogen Life Technologies), and the values of luciferase activity from individual transfection were measured using Luciferase Assay System from Clontech. The experiments were conducted in triplicate and the data was normalized by division with
corresponding β-galactosidase activity.

3.5 Electrophoretic mobility Shift Assay (EMSA).

Nuclear proteins were prepared following the manufacturer’s instructions (Invitrogen), and the protein contents were measured using the Bradford method. The double-stranded oligonucleotides containing a consensus NF-κB binding site (QIAGEN) were end labeled with $[\gamma-^{32}\text{P}]\text{ATP}$ using T$_4$ polynucleotide kinase (Promega). For gel mobility shift assay, $^{32}\text{P}$-labeled oligonucleotides (30,000 cpm) and 10 μg of nuclear proteins were incubated in a total volume of 20 μl in the presence of 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% glycerol, and 1 μl of poly(dI·dC). The binding reactions were allowed to proceed at room temperature for 20 min. Two μl of bromphenol blue (0.1% in water) were added, and protein-DNA complexes were resolved by performing electrophoresis on non-denaturing 5% polyacrylamide gels and visualized by autoradiography.

3.6 Cell fractionation.

In aim 2, NE-PER® nuclear and cytoplasmic extraction reagents from Pierce Biotechnology were used to separate nuclear and cytosol proteins. Cell lysates from each fraction were prepared following the manufacturer’s instructions, and the protein contents were measured using the Bradford method.
3.7 Cell viability assay.

Cell viability following virus infection was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay reagents, according to the manufacturer's instructions (Promega). Briefly, cells were incubated with MTS solution for 2 h and absorbance was measured at 492 nm using an enzyme-linked immunosorbent assay plate reader (Tecan, spectra fluoro plus). The absorbance of sham-infected cells was defined as a value of 100% survival and the remaining data were converted to the ratio of the sham-infected sample. Morphological changes of cells following CVB3 infection were evaluated by phase contrast microscopy.

3.8 Viral plaque assay.

Cells either untreated or treated with different reagents were infected with CVB3 for 1 h at multiplicity of infection (MOI) of 10 (Tet-On/IGTP HeLa cells) or 40 (HL-1 cells) Cells were washed and replenished with serum-free DMEM for 9 h. The supernatants were collected to determine viral titer on HeLa cell monolayers in triplicate following the standard procedures described previously [145]. Agar overlays were fixed and cells were stained to visualize plaques at day 3 after infection. The virus titer was calculated as PFU per milliliter.

3.9 Statistical analysis.

Two-way analysis of variance with multiple comparisons and paired Student's t tests was performed. Values shown are the mean ± standard error. A P value of <0.05
was considered statistically significant.
CHAPTER 4: The Mechanism of IGTP-induced PI3K/AKT Pathway

4.1 Results

IGTP expression promotes phosphorylation of FAK and paxillin

Our previous study has demonstrated that IGTP overexpression can activate the PI3K/Akt signaling pathway in the doxycycline (Dox)-inducible Tet-On/IGTP HeLa cell [145]. Here I aimed to identify the signaling protein that mediates IGTP-induced activation of the PI3K/Akt pathway. FAK becomes a good candidate since it has been known that FAK can directly activate PI3K [199-201]. The correlation between IGTP expression and FAK phosphorylation was first examined in abovementioned Tet-On/IGTP cell line by Western blotting. During the 48-hour period following the IGTP expression, the phosphorylation of FAK at both the 397 and 925 sites gradually increased, evident as early as 12 h post induction (Figure 13). Tyrosines 397 and 925 are both primary phosphorylation-sites of FAK. In particular, phosphorylation at Tyr397 correlates with increased catalytic activity of FAK, and is critical for activation of other focal-adhesion-associated proteins such as the FAK-binding protein paxillin [202, 203]. Tyrosine phosphorylation of paxillin occurs in a FAK-dependent manner [204]. As shown in Figure 13, phosphorylation of paxillin at the major Tyr118 site occurred concomitantly with FAK in a similar linear increase pattern, which further confirmed the activation of FAK. These data suggest that IGTP expression can activate FAK.

IGTP-induced activation of Akt is FAK dependent

To confirm the involvement of and define the role of FAK in the IGTP-PI3K/Akt survival pathway, Tet-On/IGTP HeLa cells were transfected with FRNK, a dominant
Figure 13. Over-expression of IGTP induces up-regulation of p-FAK (Tyr397, Tyr925) and p-Paxillin (Tyr-118).

IGTP expression was induced in Tet-On/IGTP HeLa cells with Dox at 1 µg/ml and whole cell lysates were prepared at indicated time points and subjected to immunoblot analysis using indicated antibodies. The membrane was finally stripped and probed for detection of total FAK, which was used as the equal loading control. Results were quantitated by densitometric analyses of the bands and normalized by the relative ratio of p-FAK or p-Paxillin to total FAK. The relative ratio at time 0 h is arbitrarily set to 1.0. Fold increase is the ratio of the amount of each time point to that of time 0 h. These data are representative of three independent experiments. Values are means ± SE. *p<0.05.
negative FAK called FAK-related non-kinase, to examine the regulatory effect of FAK in this pathway. FRNK is a truncated 41-kD protein identical to the C-terminal domain of FAK for targeting (Figure 14). Without catalytic activity, FRNK may act as a competitive inhibitor of FAK in the cells, preventing the localization of FAK to focal adhesions and thereby suppressing its phosphorylation [199, 205].

Figure 14. Schematic of FAK-related non-kinase (FRNK). FAK protein can be subdivided into three functional domains: an NH2-terminal integrin-binding domain, a center kinase domain, and a COOH-terminal domain containing a focal adhesion targeting (FAT) sequence required for localization of FAK. FRNK is a truncated 41-kD protein identical to the COOH carboxyl-terminal domain of FAK, so it can localize to focal adhesions through FAT sequence.

As FRNK affects normal focal adhesion turnover, it may be toxic at high concentrations and cause detrimental cytoskeleton changes and cell death [205, 206]. This prediction is consistent with our observation of the cell rounding and loss after 48 hours of transfection with 2μg/ml FRNK (Figure 15.A). However, induction of IGTP with Dox appeared to decrease this effect, suggesting that IGTP expression can partially rescue the FRNK effect, and thereby IGTP and FRNK might converge in the survival
Figure 15. FRNK overexpression inhibits IGTP-induced upregulation of p-Akt.  

A. Tet-On/IGTP HeLa cells were transfected with 2 µg/ml FRNK, and induced with 1 µg/ml Dox at 16 h post transfection. Cell morphology was observed under a phase-contrast microscope 48 h post induction.  

B. Tet-On/IGTP HeLa cells at 80% confluence were transfected with 1 µg/ml FRNK using lipofectamine 2000, induced with 1 µg/ml Dox at 16 h post transfection, cell lysates were harvested 12 h post induction and subjected to immunoblot analysis with indicated antibodies, among which the β-actin was used as the loading control.  

C. Results were quantitated by densitometric analyses and normalized as the relative ratio of p-FAK, p-Paxillin or p-Akt to β-actin. These data are representative of three independent experiments. Values are means ± SE. *p<0.05.
Figure 15.

A.  

mock  vector (+)  FRNK (+)  FRNK (+)  
Dox (-)  Dox (-)  Dox (+)  

B.  

<table>
<thead>
<tr>
<th>Transfection</th>
<th>Lane 1</th>
<th>Lane 2</th>
<th>Lane 3</th>
<th>Lane 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dox (-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vector</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vector</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRNK (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FrNK-  
-41 kDa  
p-FAK(397)-  
-125  
p-Paxillin(118)-  
-68  
p-Akt(473)-  
-60  
β-actin-  
-42

C.  

Phosphorylated protein expression (fold change)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lane 1</th>
<th>Lane 2</th>
<th>Lane 3</th>
<th>Lane 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-FAK(397)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Paxillin(118)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Akt(473)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*
pathway. I then transfected Tet-On/IGTP HeLa cells with 1μg/ml HA-tagged FRNK, and examined the signaling change by phospho-specific immunoblot analysis. Phosphorylation of Akt at Ser473 has been used as a marker for PI3K/Akt activation [166]. Data in Figure 15.B shows that Dox induction increased the phosphorylation of Akt, agreeing with our previous results on IGTP-induced Akt activation [145]. Compared to the vector transfected control, FRNK transfection totally blocked the Dox-induced increase of p-FAK (Tyr397) and p-Paxillin (Tyr118). With the inhibition of FAK activity, the upregulation of p-Akt (Ser473) after Dox induction was also dramatically reduced. These data strongly suggest that FAK plays a regulatory role for IGTP-induced activation of PI3K/Akt and is the mediator upstream of PI3K/Akt.

**IGTP induces nuclear translocation and activation of NF-κB**

NF-κB is a key anti-apoptotic transcription factor that can be activated by a variety of signals. To determine whether the IGTP-induced PI3K/Akt pathway can lead to activation of NF-κB, Western blot analyses were performed using nuclear and cytosolic fractions prepared at time points post Dox induction as indicated in Figure 16. The p65-p50 heterodimer is the most common active NF-κB complex, so p65 in the nuclear extract and IκBα in cytosol were probed. A significant downregulation of IκBα due to the degradation was observed at 12 h post induction, a time point corresponding to the translocation of NF-κB to the nucleus (Figure 16.A). Interestingly, when NF-κB was accumulated in nuclear, IκBα began to recover 24 h post induction, which may be due to re-synthesis under a potential feedback mechanism of NF-κB activation [207]. This activation pattern of NF-κB was further confirmed by luciferase assay showing the
Figure 16. Expression of IGTP promotes translocation and activity of nuclear transcription factor NF-κB (p65). A. Tet-On/IGTP HeLa cells were induced with 1 μg/ml Dox, whole cell lysates and nuclear lysates were prepared at indicated time points. NF-κB and I-κBα were detected by Western blot. B. Luciferase assay of IGTP-induced NF-κB activity. (a) Schematic structure of plasmid pNF-κB-Luc. LUC: firefly luciferase gene; κB4: four copies of the NF-κB consensus enhancer sequence fused to a TATA-like promoter. (b) Luciferase activity assay. Tet-On/IGTP HeLa cells were cotransfected with pNF-κB-luc and pCMV-β-gal control vector, induced with Dox and harvested at indicated time points. Control cells were not induced but cultured for 48 h. Assay reaction and data normalization were performed as described in Experimental Procedures. Data from three independent experiments are presented as a fold increase. Values are means ± SE. *p<0.05. C. Expression of IGTP increases sequence-specific DNA-binding activity of NF-κB. Tet-On/IGTP HeLa cells were induced with Dox and the nuclear proteins were prepared at indicated time points. A gel mobility shift assay was performed using nuclear proteins and 32p-end-labeled oligonucleotides containing a consensus NF-κB binding site.
transcriptional activity of NF-κB (Figure 16.B), and by gel shift assay demonstrating the association of NF-κB with its DNA binding sequence (Figure 16.C).

**Inhibition of FAK and Akt by FRNK and LY294002, respectively, blocks the IGTP-induced activation of NF-κB**

To determine the position of NF-κB in the IGTP-induced PI3K/Akt survival cascade, I further examined the IGTP-induced activity of NF-κB under the suppression of FAK or Akt. LY294002, a specific inhibitor of PI3K that competitively inhibits its catalytic activity, was added to examine the effect of PI3K/Akt suppression on NF-κB nuclear translocation. After the inhibitor treatment and Dox induction, fractionated cellular proteins were collected, and NF-κB (p50) was probed by Western blotting. Here, histone-1 was used as a nuclear protein loading control to further validate the fractionation technique. The amount of NF-κB in cytosolic and nuclear extracts exhibited a complementary pattern in all groups, clearly showing the translocation from cytosol to nucleus in the Dox-treated samples (lane 2 from the left, Figure 17.A). Compared to DMSO control, LY294002 specifically abrogated this IGTP-induced translocation of NF-κB. The quantification of NF-κB was performed by densitometric analyses (lower panel). To suppress FAK activity, FRNK was transfected into the cells with the vector-transfected cell as a control. Again, the FRNK transfection almost completely blocked the IGTP-induced increase of NF-κB translocation (lane 4, Figure 17.B). The vector control showed slightly inhibited translocation, as was the cases in later experiments, which is probably due to the detrimental impact of the transfection reagent itself. However, the change was not comparable to the effect of FRNK. I then examined
Figure 17. FRNK or PI3K inhibitor LY294002 blocks the IGTP-induced NF-κB translocation and activity.  A. Tet-On/IGTP HeLa cells at 70% confluence were treated with 10 μM LY294002 or same volume of DMSO and induced with 1 μg/ml Dox 1 h post treatment. Nuclear lysates and cytosolic lysates were prepared 10 h post induction and subjected to immunoblot analysis with indicated antibodies, among which the β-actin was used as the cytosol loading control and the histone-1 as nuclear protein loading control. Results were quantitated by densitometric analyses and normalized as the relative ratio of nuclear p50 to histone-1 and cytosol p50 to β-actin, respectively. The relative ratio from untreated cell lysates is arbitrarily set to 1.0. Fold increase is presented as described in Fig 1. The values are representative of three independent experiments.*p<0.05. B. Tet-On/IGTP HeLa cells at 70% confluence were transfected with 1 µg/ml FRNK using lipofectamine 2000 and induced with 1 µg/ml Dox 24 h post transfection. Nuclear lysates and cytosolic lysates were harvested and separated 10 h post induction and subjected to immunoblot analysis with indicated antibodies. Loading control and densitometric analyses are as described above. *p<0.05. C. Tet-On/IGTP HeLa cells were cotransfected with pNF-κB-luc and pCMV-β-gal control plasmid, and treated with PI3K inhibitor or transfected with FRNK as described above. The cells were then induced with Dox and harvested at 10 h post induction. Control cells were not Dox-induced but cultured for 48 h. TNF-α and Bay 11-7085 were applied as positive and negative controls, respectively. Luciferase assay reaction and data normalization were performed as described above in the preceding figure. Data represents values obtained from three independent experiments. Values are means ± SE. *p<0.05.
Figure 17.

A.

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>Dox</th>
<th>LY294002</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB(p50)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Histone 1</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>Dox</th>
<th>transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB(p50)</td>
<td>-</td>
<td>vector</td>
</tr>
<tr>
<td>Histone 1</td>
<td>-</td>
<td>FRNK</td>
</tr>
</tbody>
</table>

Nuclearcytosol

* * *
Figure 17 (continue)

Luciferase Activity (fold increase)

- control
- TNF α
- bay11-7085+Dox
- control +Dox
- LY294002+Dox
- control +Dox
- Vector+Dox
- FRNK+Dox

* indicates significant difference from control group.
the NF-κB transcriptional activation by luciferase reporter assay after the same treatments as that in Western blotting experiment. TNF-α, a well recognized stimulator of NF-κB, and NF-κB inhibitor Bay11-7085 were used as the positive and negative control respectively. The results correlated well with the change of NF-κB translocation. Both FRNK transfection and LY294002 treatment substantially blocked IGTP-induced increase of NF-κB transcriptional activity, although to a lesser extent by FRNK than by LY294002 in this assay. Taken together, inhibition of FAK or Akt blocks the IGTP-induced activation of NF-κB.

**Suppression of NF-κB expression blocks the IGTP-induced activation of both FAK and Akt**

The regulatory network for NF-κB is complex, and multiple feedback mechanisms have been suggested to be associated with NF-κB [208]. In order to further elucidate the role of NF-κB in this pathway, I examined whether NF-κB has influence on FAK and Akt activity. The phosphorylation of FAK and Akt after silencing of NF-κB with validated siRNAs targeting NF-κB1 p50/p105 was measured by Western blot analysis. P105 serves as the precursor of p50, and is constitutively processed to p50 in cells. The data show that 70 nM NF-κB1 siRNA effectively silenced the expression of both p105 and p50 (lane 4 from the left, Figure 18). Specificity of this silencing was confirmed by the fact that no inhibition was observed with the scrambled control siRNA (lane 2 and 3, Figure 18) and that expression of other proteins, such as β-actin, was not significantly affected. With the suppression of NF-κB, the IGTP-induced upregulation of p-FAK and p-Akt was dramatically reduced, compared with the cells induced with Dox but
Figure 18. NF-κB p50 siRNA inhibits the IGTP-induced upregulation of p-FAK and p-Akt. Tet-On/IGTP HeLa cells at 60% confluence were transfected with NF-κB p50 siRNA at a final concentration of 70 nM using Oligofectimine. Scrambled siRNAs at the same concentration were transfected as the control, and 1 µg/ml Dox was added 6 h post transfection. Cell lysates were harvested 30 h later and subjected to immunoblot analysis with indicated antibodies, among which the β-actin was used as the loading control. The experiments were repeated three times.
transfected with scrambled siRNA (lane 3 and 4, Figure 18). However, the expression levels of total FAK and Akt were unaltered after the silencing of NF-κB expression. These data suggest that NF-κB is required for the IGTP-induced activation of FAK and Akt.

**Blockage of FAK or NF-κB attenuates IGTP-induced cell survival and decreases virus replication**

We have shown previously the prosurvival effect of the IGTP-PI3K/Akt pathway during the CVB3 infection [145]. Now, both FAK and NF-κB were found to be mediators of this pro-survival cascade. Thus, I investigated the involvement of these molecules in mediating the pro-survival effect of IGTP during CVB3 infection.

To examine the effect of FAK on cell survival, Tet-On/IGTP HeLa cells were transfected with FRNK or vector control, and induced with Dox before CVB3 infection. Cell viability was determined by the MTS assay 9 h post-CVB3 infection. Compared to non-induced cells, Dox-induced cells exhibited a much higher survival rate (Figure 19.A). However, compared to vector-transfected cells, FRNK transfection significantly aboragated the protective effect of the IGTP-induced survival pathway, decreasing cell viability almost to the levels of non-Dox-induced cells. This observation was further corroborated by morphological assessment of the cultured cells (Figure 19.B), which showed a nearly similar degree of cell loss and detachment in both non-induced/non-transfected cells and Dox-induced/FRNK- transfected cells. To examine the effect of FRNK on viral replication and viral protein synthesis, I performed viral plaque assay to measure viral titer, and Western blotting to measure CVB3 capsid protein
Figure 19. FRNK blocks the IGTP-induced cell survival, and decreases viral protein synthesis and viral release. Tet-On/IGTP HeLa cells at 70% confluence were transfected with 1 µg/ml FRNK 24 h prior to infection, and induced with 1 µg/ml Dox 3 h prior to infection. Then the cells were infected with CVB3 at 10 MOI. A. Cell viability was measured by MTS assay 9 h post infection. Absorbance values were corrected with background reading. B. Cell morphology was observed under a phase-contrast microscope 8 h post infection. C. Cell lysates were collected 9 h post infection and Western blotting was performed to probe the viral capsid protein VP1. β-actin was used as the loading control. D. Supernatants from cell cultures were collected 9 h post infection, and the pfu/ml was determined by plaque assay on HeLa cell monolayers. Data represents values obtained from three independent experiments, *p<0.05.
VP1. In both experiments, FRNK transfection further reduced viral VP1 protein synthesis and viral replication (Figure 19.C and D).

To examine the effect of NF-κB on cell survival and CVB3 infection, NF-κB siRNA or scrambled siRNA was transfected into Tet-On/IGTP HeLa cells, and followed by Dox induction and then CVB3 infection. The same set of assays was performed to examine the cell viability and viral replication as described above. As shown in Figure 20, the suppression of NF-κB also substantially reduced the IGTP-induced pro-survival effect and inhibited viral replication. The reduction of virus titer by NF-κB siRNAs was even greater in magnitude than that by FRNK. All these data further confirm that both FAK and NF-κB are essential for the pro-survival effect of the IGTP-induced PI3K/Akt pathway during CVB3 infection.

Expression of endogenous IGTP is required for activation of FAK and Akt in murine cardiomyocytes

To demonstrate the endogenous activation of FAK and Akt by IGTP during CVB3 infection, I performed in vivo CVB3 infection of A/J mice. Heart lysates were prepared at day 7 and 9 post infection (pi), when inflammatory T-cells have infiltrated into the myocardium and secreted a large amount of cytokines including IFN-γ. As shown in Figure 21.A, the IGTP expression was gradually and significantly upregulated after CVB3 infection, and such up-regulation of endogenous IGTP was remarkably correlated with phosphorylation of both FAK and Akt. I further examined whether such endogenous IGTP expression occurs in cardiomyocytes and is required for FAK and Akt activation by treating murine HL-1 cardiomyocytes with recombinant mouse IFN-γ. The data shown in Figure 21.B demonstrates that upregulation of endogenous IGTP levels correlated with increased phosphorylation of FAK and Akt after IFN-γ treatment, which resembles the expression pattern observed in the above experiment with CVB3-infected murine hearts. This data was further confirmed by applying specific
Figure 20. NF-kB siRNA blocks the IGTP-induced cell survival, and decreases viral protein synthesis and viral release. Tet-On IGTP HeLa cells at 60% confluence were transfected with NF-κB p50 siRNA at a final concentration of 70 nM using Oligofectamine. Scrambled siRNAs at the same concentration were transfected as the control. Cells were induced with 1 μg/ml Dox 3 h prior to infection and then the cells were infected by CVB3 at 10 MOI 24 h post transfection. A. Cell viability was measured by MTS assay 9 h post infection. Absorbance values were corrected with the reading of sham-transfected control. B. Cell morphology was observed under a phase-contrast microscope 8 h post infection. C. Cell lysates were collected 9 h post infection and Western blotting was performed to probe the viral capsid protein VP1, and β-actin was used as the loading control. D. Supernatants from cell cultures were collected 9 h post infection, and the pfu/ml was determined by plaque assay on HeLa cell monolayers. Data represents values obtained from three independent experiments.
Figure 21. Expression of endogenous IGTP is required for activation of FAK and Akt in murine cardiomyocytes. A. Induction of endogenous IGTP was accompanied by activation of FAK and Akt in CVB3 infected murine hearts. A/J mice were injected intraperitoneally with $10^5$ PFU of CVB3 or PBS, and sacrificed at days 7 and 9 pi. Hearts were collected, and cell lysates were subjected to immunoblot analysis using indicated antibodies, among which the $\beta$-actin was used as the loading control. B. HL-1 cardiomyocytes at 80% confluence were treated with IFN-$\gamma$ at a final concentration of 1 $\mu$g/ml for an indicated period of time. Cells were then harvested for Western blot analysis using indicated antibodies. C. HL-1 cells at 60% confluence were transfected with IGTP siRNA at a final concentration of 50 nM and 100 nM, respectively. Scrambled siRNAs at 100 nM of concentration were transfected as a control, and 1 $\mu$g/ml IFN-$\gamma$ was added 24 h post transfection to treat the cells. Cell lysates were harvested 10 h post induction and subjected to immunoblot analyses with indicated antibodies. The data are representative of three independent experiments.
siRNAs to knockdown IGTP expression. As shown in Figure 21.C, increasing concentrations of siRNAs targeting IGTP (especially at 100 nM) significantly suppressed endogenous IGTP expression compared to the scrambled control. The change was paralleled by the suppression of p-FAK and p-Akt after IFN-γ treatment.

4.2 Discussion

Since the discovery of the p47 GTPases, data accumulated in the past decade have clearly defined this protein family as key regulators of cell autonomous resistance to intracellular pathogens [209]. The critical but differential roles for the p47 proteins in host resistance against bacteria, protozoa or viruses have been illustrated in many recent studies, including our previous report on IGTP in CVB3 infections [144, 145]. The study has also revealed that besides limiting viral replication in vitro, this p47 GTPase also functions as a “survival factor” for the host cell, activating the PI3K/Akt survival pathway. However, many aspects of this signaling pathway remain unclear, including the potential mediators and the downstream effectors. In this chapter, using a Tet-On/IGTP HeLa cell line and cardiomyocytes, I found that FAK mediates the activation of the PI3K/Akt pathway by IGTP, and that NF-κB serves as a downstream effector. Moreover, the data show that NF-κB can provide positive feedback for the activation of this FAK-PI3K/Akt pathway.

In view that a physical association between IGTP and PI3K was not detected by coimmunoprecipitation (data not shown), IGTP may activate PI3K/Akt indirectly through certain mediator(s). Growing evidence has shown that after autophosphorylation, activated FAK can form a complex with the p85 subunit of PI3K [199-201]. The
FAK-PI3K/Akt survival pathway has emerged as an important pathway conferring protection against apoptosis induced by various stimuli such as integrin [206] and growth factor [210]. However, it has also been suggested that integrin signaling activates the PI3K and FAK pathways in parallel [211], and in the study of IGF-I pro-survival effect, PI3K even regulates FAK activity indirectly, casting into doubt the order and directionality of molecules within the pathway.

In the present study, I examined FAK and found it was significantly phosphorylated following IGTP expression. The sustained phosphorylation at both primary sites of FAK and at the site of its binding partner paxillin verified the full activation of FAK. IGTP expression protected the cell from apoptosis resulting from disruption of FAK signaling. Expression of exogenous dominant negative FAK completely blocked the activation of Akt by IGTP overexpression. Therefore, these data suggest that FAK is not only involved in this IGTP signaling, but actually positioned upstream of PI3K/Akt in this pathway. To further validate the role of endogenous IGTP in activating this pathway, CVB3-infected murine hearts as well as IFN-γ-treated murine HL-1 cardiomyocytes were examined. Results from both model systems support the notion that endogenous IGTP expression is responsible for the activation of FAK and Akt.

NF-κB is a central player in the complex signal transduction network and acts by regulating expression of immune, stress response, and inflammation related genes [197, 198, 208]. In the majority of systems, NF-κB activation provides a survival-promoting signal [212, 213]. PI3K/Akt signaling stimulated by TNF, PDGF, interleukin-1 and interferons has been reported to suppress apoptosis by activating NF-κB [214-216].
Activation of NF-κB was also observed in transgenic mice that express constitutively activated Akt [217]. The mechanisms through which PI3K/Akt signaling activates NF-κB appear to be pleiotropic and cell type-specific [218, 219]. Contrary to the above studies, inhibition of PI3K was found to enhance LPS activation of NF-κB in monocytic cells [220]. Moreover, results from two recent studies placed Akt as a downstream target of NF-κB in the TNF and EGF transducing pathways [221, 222], which added to the complexity of the relationship between Akt and NF-κB. Additionally, studies on anti-apoptotic role of FAK provide evidence of a link between FAK and NF-κB. FAK overexpression enhanced NF-κB activity and inhibited apoptosis in an NF-κB dependent manner [205, 223]. All these led us to examine whether NF-κB is involved, and its putative position in this pathway. The data show that IGTP expression activates NF-κB by downregulating IκB, allowing its translocation to the nucleus, which is consistent with the mechanism whereby Akt leads to IκB degradation via IKK [224-226]. However, both IGTP-induced nuclear translocation and increased activity of NF-κB were totally blocked with the transfection of dominant negative FAK or treatment of the specific PI3K inhibitor. These data suggest that IGTP-induced activation of NF-κB is regulated by both FAK and PI3K, and that NF-κB functions as a downstream effector in this IGTP survival pathway.

To further define the role of NF-κB in this pathway, I examined the effects of NF-κB knockdown on the activation of FAK and Akt. Interestingly, with the inhibition of NF-κB expression with siRNAs, the phosphorylation of FAK and Akt following IGTP expression was almost completely blocked, without alteration of their total protein expression. This result demonstrates that NF-κB is required for IGTP-induced activation
of FAK and Akt, thereby suggesting that a positive feedback loop exists here in this FAK-PI3K/Akt-NF-κB pathway. Positive and negative feedback mechanisms regulating NF-κB activity within the NF-κB and IκB family have been intensively studied, and exemplified respectively by RelA positive feedback for high-affinity NF-κB complexes [227], and upregulation of IκB [228, 229]. Remarkably, new evidence has emerged suggesting that, in addition to autocrine loop for TNF [230], NF-κB activation can also stimulate its upstream kinases, and thus form positive feedback loops [231-233] or negative feedback mechanisms [208]. Especially, in tumor cell lines, a positive feedback loop was revealed whereby Akt activation of NF-κB further stimulates Akt via down-regulation of the PI3K inhibitor PTEN. These findings fit in line with our observation of the reciprocal inhibition of NF-κB and activity of FAK and Akt, without altering of their protein expression. Therefore, although the promoter region of the human FAK gene was indicated to contain NF-κB binding sites, our data suggest that NF-κB may positively regulate the upstream FAK and Akt activity indirectly through some intermediate, like abovementioned PTEN for Akt [232]. As for FAK, some NF-κB-regulated genes may be prerequisite for FAK activation in focal adhesion [234, 235]. Moreover, a peculiarity of NF-κB is the rapid and transient nature of its activity, as the evident NF-κB increase observed as early as 6 h post induction in our luciferase assay. A significant fraction of genes can be induced at the very early time point of NF-κB activation in a stimulus-specific fashion, demanding only for a limited period of time before shut down, which could influence and contribute to later prolonged activation of NF-κB [207, 236]. Therefore, it is conceivable that early initial activation of NF-κB under IGTP induction could regulate some intermediates that are essential for the full
activation of FAK (and Akt), and the activated FAK-PI3K/Akt pathway further stimulates the sustained activation of NF-κB, thereby amplifying the pro-survival signaling to a necessary level.

The experiments also show that inhibition of FAK and NF-κB significantly suppresses the host cell viability during CVB3 infection, which is consistent with previous observation for inhibition of Akt [145], highlighting the pro-survival role of this IGTP-FAK-PI3K/Akt-NF-κB signaling pathway. During the early phase of virus infection, it would be advantageous for the virus to preserve the cell viability, thereby allowing for efficient production of viral progeny. Therefore, as previous studies have shown [145, 237], inhibition of survival pathway here as FAK and NF-κB in our study, led to inhibition of CVB3 viral progeny production and release. It is noteworthy that two recent studies have addressed the central role of NF-κB activation in CVB3 infection. On one hand, virus may manipulate NF-κB to augment their replication [237]. On the other hand, host may take IκBα cleavage during CVB3 infection as a sensor for host counteracting response [115]. The interactions of virus and host defense response converging at NF-κB further underscore the significance of this survival cascade from IGTP to NF-κB.

In summary, here I further investigated the IGTP-induced PI3K/Akt survival pathway in the context of CVB3 infection. I identified FAK, functioning upstream of PI3K/Akt, as the mediator for IGTP-induced activation of PI3K/Akt pathway, and NF-κB as both downstream effector and positive regulator of FAK and PI3K/Akt activity. These findings add further insights into the prosurvival function of IGTP in response to CVB3 infection.
CHAPTER 5: The Effect of IGTP on Endoplasmic Reticulum Stress Response

5.1 Results

IGTP relieves chemical-induced ER stress response

Previously using a Dox-inducible Tet-On/IGTP HeLa cell line, we have demonstrated that IGTP expression can promote cell survival through activating FAK-PI3K/Akt signaling pathway [145, 238]. In this chapter, I further investigated the influence of IGTP expression on ER stress response using this established IGTP-inducible cell line. To assess whether IGTP expression modulates the ER stress response, classical ER stress inducers, tunicamycin and brefeldin A (BFA), were used to treat the cells that were either induced with Dox or uninduced, and the expression of GRP78 was examined by Western blot analysis. As a major chaperone and stress-sensing protein in ER, the upregulated expression of GRP78 is the outcome and marker of the ER stress response for enhancing folding capability [169, 174]. Tunicamycin is an inhibitor that causes ER stress by inhibiting protein N-linked glycosylation, while BFA causes the accumulation of unfolded proteins by blocking ER to Golgi trafficking. As shown in Figure 22.A, tunicamycin treatment of the Tet-On/IGTP HeLa cells led to the activation of ER stress response, i.e. the gradual increase of GRP78 expression, in a dose-dependent manner. However, when IGTP was induced with Dox, the upregulation of GRP78 expression was significantly inhibited, particularly in the cells treated with a dose of 1μg/ml tunicamycin, as compared to the corresponding uninduced cells. The inhibitory effect of IGTP on ER stress response was further verified by experiments using another pharmacological agent BFA, in which the dose-dependent upregulation of GRP78 by BFA treatment was significantly inhibited by the Dox-induced IGTP expression (Figure
Figure 22. IGTP relieves ER stress response triggered by tunicamycin or BFA. Tet-On/IGTP HeLa cells were induced with 1μg/ml Dox for 16 h and then treated with tunicamycin (Tu) (A) or Brefeldin A (BFA) (B) at the indicated concentrations. Cell lysates were harvested at 20 h or 16 h after cells were treated with tunicamycin or BFA respectively, and then subjected to immunoblotting using indicated antibodies. Data were quantitated by densitometric analyses and normalized to the β-actin expression. Untreated controls were arbitrarily set to 1.0, and expression of GRP78 was calculated as fold increase compared to the control group. Data represents the mean ± S.E. from three independent experiments. (*p<0.05)
22.B). Thus, IGTP relieves the ER stress response triggered by different ER stress inducers.

**IGTP expression inhibits the activation of both PERK and ATF6 pathways of ER stress response**

ER stress response consists of three branches: PERK, ATF6, and IRE1. Among them, the PERK and ATF6 pathways are believed to be activated sequentially in the early phase of stress conditions, playing the determinant roles in cell survival under ER stress [173, 174]. To explore how IGTP expression affects ER stress response, activation of PERK and ATF6 pathways was investigated respectively. Activation of PERK pathway was examined by detection of the phosphorylation of PERK (p-PERK) and its only target, the translational initiation factor eIF2α (p-eIF2α) by Western blot. As shown in Figure 23, PERK phosphorylation was significantly increased at 16 h post tunicamycin treatment at the concentration of 1 μg/ml, and accordingly, eIF2α was also activated through phosphorylation without changes on the total protein level. However, the p-PERK and p-eIF2α were remarkably decreased following the induction of IGTP expression with Dox, almost to the level of the untreated control (Figure 23). Although causing translational attenuation, phosphorylation of eIF2α also paradoxically enhances the expression of ATF4, which is a transcription factor that stimulates the expression of apoptotic genes in ER stress [169]. Expression of ATF4, the downstream gene of the PERK-eIF2α cascade, was further determined. The upregulation of ATF4 level after tunicamycin treatment was strongly inhibited by IGTP expression in parallel with the changes of p-PERK and p-eIF4a (Figure 23), indicating the overall inhibition of the
Figure 23. IGTP inhibits the activation of PERK pathway of ER stress response. Tet-On/IGTP HeLa cells were induced with 1 µg/ml Dox for 16 h and then treated with 1 µg/ml tunicamycin for 16 h or untreated. Cell lysates were subjected to immunoblot analysis with indicated antibodies. Detection of the β-actin was used as the loading control. The experiments were repeated three times.
PERK pathway by IGTP expression.

After dissociation from GRP78 under ER stress, the ATF6 proform (90kDa) translocates to the Golgi where it is cleaved into its active form (50kDa) by proteinases. Active ATF6 then moves to the nucleus and induces transcription of ER chaperon genes [169, 174]. To assess the activation of ATF6, cell fractions were prepared, and the ATF6 proform and the cleavage products were probed in the cytosol and the nucleus respectively by Western blot. The data show that tunicamycin treatment alone caused a very evident loss of ATF6 proform in the cytosol, and an elevated level of the active form in the nucleus, whereas with the IGTP expression, the ATF6 in the cytosol was largely reserved as compared to the uninduced and tunicamycin-treated cells (Figure 24.A). To further determine the effect of IGTP expression on the ATF6 branch of the pathway, the regulation of downstream ER stress responsive genes by activated ATF6 was examined by Western blot analysis. Figure 24.B shows that expression of p58IPK, an ER molecular chaperone downstream of ATF6 pathway, was upregulated by tunicamycin treatment following ATF6 activation (cleavage), however, this p58IPK upregulation was reduced by the induction of IGTP expression with Dox, which is correlated well with the inhibition of ATF6 activation by IGTP expression in Figure 24.A. The transcription factor SREBP-1 was also examined. SREBP-1 is a key regulator of lipid metabolism in ER, and its activation occurs under ER stress in parallel with that of ATF6 by exactly the same mechanism [169]. The observed cleavage of SREBP-1 after tunicamycin treatment and inhibition of such cleavage by IGTP further corroborate what happened to ATF6. Combined together, these results suggest IGTP inhibits the activation of both PERK and ATF6 pathways of ER stress response.
Figure 24. 

A. Tet-On/IGTP HeLa cells were induced with 1 µg/ml Dox for 16 h and then treated with 1 µg/ml tunicamycin for 20 h or untreated before harvest. Cell fractionation was performed, and cytosolic and nuclear fractions were collected separately. Proform and cleaved forms of ATF6 were detected in the cytosolic and nuclear fraction, respectively. Results were quantitated by densitometric analyses and normalized to the β-actin and histone H1 expression, respectively. Untreated controls were arbitrarily set to 1.0, and levels of ATF6 were calculated as fold increase compared to the control group. Data represents the mean ± S.E. from three independent experiments (*p<0.05). 

B. Whole cell lysates from the cells treated as described above were harvested and subjected to immunoblotting to detect the expression of p58IPK and SREBP1. Detection of β-actin was used as the loading control. The experiments were repeated three times.
IGTP attenuates the ER stress-mediated apoptotic response

As aforementioned, if the initial stress responses can not resolve the accumulation of misfolded proteins and restore normal ER function promptly, the signal pathway will eventually shift to programmed cell death. To determine whether IGTP expression can block or attenuate such transition, crucial mediators (CHOP, GADD34, caspase-3) involved in this apoptosis induction were examined by Western blot analysis. The ATF-4 in the PERK branch promotes apoptosis by increasing transcription of the proapoptotic transcription factor CHOP, which in turn increases the amount of GADD34, another well known proapoptotic gene [170, 173]. As shown in Figure 25, CHOP was strongly upregulated after tunicamycin treatment, and this CHOP upregulation correspondingly led to the increased production of GADD34, as compared to the controls. However, the upregulation of these apoptotic mediators was significantly suppressed when IGTP expression was induced with Dox (Figure 25). These data were further solidified by examining the downstream apoptotic events including both the activation of the critical executioner caspase-3 and the cleavage of its substrate, poly-(ADP-ribose) polymerase (PARP) [170], [239]. The data show that at 20 h post tunicamycin treatment, the 19 kDa active form of executioner caspase-3 was dramatically increased and meanwhile so was the 89 kDa cleavage product of PARP in the tunicamycin-treated cells. Nevertheless, these increases were significantly attenuated following the induction of IGTP expression with Dox (Figure 25). Thus, these results suggest that IGTP expression relieves the ER stress-mediated apoptosis through inhibition of the proapoptotic signals emanating from the ER stress response.
Figure 25. IGTP expression inhibits the ER stress-mediated apoptotic response. Tet-On/IGTP HeLa cells were induced with 1 µg/ml Dox for 16 h and then treated or untreated with 1 µg/ml tunicamycin as indicated for 20 h. Cell lysates were harvested and subjected to immunoblot analysis with indicated antibodies, among which the β-actin was used as the loading control. The experiments were repeated three times.
IGTP relieves the ER stress response triggered by CVB3 infection and inhibits viral replication

Many viruses induce ER stress in infected cells either through disrupting ER function or causing ER overload [177, 240]. Recently our laboratory has proved that CVB3 infection also triggers ER stress response, which eventually leads to the apoptosis of infected cell [195]. To further determine the effect of IGTP expression on CVB3-induced ER stress responses, Tet-On/IGTP HeLa cells were infected with CVB3 at 10 MOI or sham-infected, and then the expression of GRP78 as well as other molecular markers in the activated ER stress response pathways were detected by Western blotting. Compared to the sham-infected control, GRP78 expression in the CVB3-infected non-induced cells gradually increased starting from 6 h pi (Figure 26). However, for the cells induced with Dox and infected with CVB3, such upregulation of GRP78 was largely inhibited, suggesting that IGTP can relieve CVB3-induced ER stress. To determine whether this ER stress relief can alter each individual branch of ER stress response, the activation of the PERK and ATF6 pathways in the CVB3-infected Tet-On/IGTP cells was examined. Firstly, on the PERK pathway, CVB3 infection caused a significant increase in both p-PERK and p-eIF2α levels at 12 h pi; but in the Dox-induced cells, such upregulation was hardly detectable (Figure 27.A), indicating that IGTP expression inhibits the CVB3-induced activation of PERK pathway. Secondly, to examine the ATF6 pathway, cell fractions were prepared from the CVB3-infected or sham-infected control cells, and the proform of ATF6 in the cytosolic fraction and the cleaved form in the nuclear fraction was detected respectively. As shown in Figure 27.B, CVB3 infection at 10 MOI for 12 h caused a loss of ATF6 proform in cytosol and a significant
Figure 26. IGTP inhibits the upregulation of GRP78 that is induced by CVB3 infection in Tet-On/IGTP HeLa cells. Tet-On/IGTP HeLa cells were treated with 1µg/ml Dox for 20 h or untreated, and then infected with CVB3 at 10 MOI or sham infected. Cell lysates were collected at the indicated time points post infection (pi) and subjected to immunoblotting. GRP78 expression was quantitated by densitometric analyses and normalized to β-actin expression. Sham-infected controls were arbitrarily set to 1.0, and expression of GRP78 was calculated as fold increase compared to the control group. Data represents the mean ± S.E. from three independent experiments (*p<0.05).
Figure 27. IGTP inhibits the activation of PERK and ATF6 pathway that is triggered by CVB3 infection in Tet-On/IGTP HeLa cells. A. Tet-On/IGTP HeLa cells were treated with 1µg/ml Dox for 20 h or untreated, and then infected with CVB3 at 10 MOI. Cell lysates were collected at the indicated time points post infection (pi) and subject to immunoblot analysis using indicated antibodies. The expression of p-eIF2α were quantitated by densitometric analyses and normalized to the β-actin expression. The value of p-eIF2α for the uninduced control sample at 0 h is arbitrarily set to 1.0, and fold change of other samples were calculated based on the value of control. The data represents the mean ± S.E. from three independent experiments (*p<0.05). B. Cells were induced with Dox for 20 h and then infected with CVB3 at 10 MOI or sham-infected for 12 h. Cell fractions were prepared, and the proform and cleaved form of ATF6 was detected in the cytosolic and nuclear fraction, respectively.
increase of its active form in the nucleus. However, after the induction of IGTP expression with Dox, the translocation of the active ATF6 into nucleus was largely diminished as compared to the corresponding uninduced controls. Taken together, the relief effect of IGTP on chemical-induced ER stress was fully recapitulated in the CVB3-triggered ER stress response.

To further illuminate the significance of such ER stress relief effect of IGTP on CVB3 infection, CVB3 replication efficiency in Tet-On/IGTP cells (induced or uninduced with Dox, treated or untreated with tunicamycin) was determined. Firstly, different extent of preemptive ER stress response was induced by 1 μg/ml tunicamycin treatment for different periods prior to CVB3 infection. Cells and supernatants were collected for Western blot analysis to measure viral VP1 protein and for plaque assay to quantify the produced viral particles. As shown in Figure 28.A, viral VP1 protein synthesis in cells treated with tunicamycin but uninduced with Dox was greatly enhanced, and the degree of increase in VP1 protein production showed close correlation with the extent of ER stress response as indicated by the level of GRP78 expression. Also notably, the attenuation of GRP78 increase by IGTP expression was coupled with its inhibition of VP1 production and viral particle formation in each corresponding group. The plaque assay further shows that the ER stress response induced by tunicamycin treatment for 12 h significantly promoted viral particle production in CVB3-infected cells and this promotion was significantly reduced by Dox-induced IGTP expression (Figure 28.B). Thus, these results suggest that ER stress response can be beneficial to CVB3 infection, and relieving ER stress response by IGTP inhibits CVB3 replication and protein synthesis in infected cells.
Figure 28. Preemptive ER stress response induced by tunicamycin facilitates viral protein synthesis and viral release of CVB3.  

A. Dox induced or uninduced Tet-On/IGTP HeLa cells at 16 h post induction were treated with or without 1µg/ml tunicamycin for indicated hours and then were infected with CVB3 at 10 MOI for 10 h. Cell lysates were collected and subjected to immunoblot analysis using indicated antibodies. CVB3 VP1 protein expression was quantitated by densitometric analyses and normalized to the β-actin expression. VP1 level of untreated cells was arbitrarily set to 1.0, and fold increase of other samples was calculated based on the untreated control. Data represents the mean ± S.E. from three independent experiments (*p<0.05). 

B. Dox induced or uninduced Tet-On/IGTP HeLa cells were pretreated with or without 1µg/ml tunicamycin for 12 h and then infected with CVB3 for 10 h at 10 MOI. Supernatants from the cell cultures were collected, and the pfu/ml was determined by plaque assay on HeLa cell monolayers. Data represents the mean ± S.E from three independent experiments (*p<0.05).
Figure 28.

A.  

<table>
<thead>
<tr>
<th></th>
<th>Tu</th>
<th>-</th>
<th>-</th>
<th>12h</th>
<th>12h</th>
<th>24h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dox</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CVB3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRP78</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

kDa

-78

-37

-42

B.  

<table>
<thead>
<tr>
<th></th>
<th>Tu</th>
<th>-</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dox</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PFU/ml (Log10)

5

5.5

6

6.5

-  -

-  -

-  -

*
IGTP is responsible for IFN-γ-induced relief of ER stress response during CVB3 infection

To further confirm the role of IGTP in relieving ER stress response and in inhibiting CVB3 replication, an IGTP KO MEF cell model was used in this study in addition to the Tet-On/IGTP cell line. As shown in Figure 29.A, as compared to the substantial IGTP expression induced by IFN-γ in the WT MEF, the IGTP KO MEF consistently lacked the IGTP expression even under the IFN-γ stimulation. When MEF was infected with CVB3 at a MOI as high as 40, the viral VP1 protein was detected 12 h pi, and due to the strong antiviral activity of IFN-γ, VP1 protein synthesis in the IFN-γ-treated WT and IGTP KO cells was dramatically reduced as compared to the corresponding cells not stimulated with IFN-γ (Figure 29.B). However, the extent of such repression of CVB3 VP1 protein synthesis was less obvious in the IGTP KO cells than in the WT MEF, which indicates that IGTP, as an IFN-γ inducible gene, plays a major role in IFN-γ-induced anti-CVB3 effect. As it has been reported that IFN-γ modulates ER stress response [241, 242], transient IFN-γ treatment in our experiment significantly diminished the upregulation of GRP78 induced by CVB3 in the WT MEF, as compared to the untreated samples (Figure 29.B). Notably, the extent of such attenuation of GRP78 expression appeared to be largely decreased or lost in the IFN-γ-treated IGTP KO cells, which supports that IGTP is at least one of the major IFN-γ-inducible genes responsible for relieving the CVB3-induced ER stress response in the MEF (Figure 29.B).

The relief of ER stress response by IGTP requires PI3K/Akt activity

The association with various signaling pathways especially the PI3K/Akt and MAPKs has been implicated in the activation or feedback of ER stress response
Figure 29. IGTP relieves CVB3-induced ER stress response in mouse embryonic fibroblasts (MEF). A. WT MEF and IGTP KO MEF were treated with 500 U/ml IFN-γ for indicated hours. Cell lysates were collected after treatment and IGTP expression were probed by immunoblotting. B. WT and KO MEF were infected with sham or CVB3 at 40 MOI or sham-infected in the presence or absence of 500U/ml IFN-γ. Cell lysates were harvested at the 30 h pi and subjected to immunoblotting analysis. Results were quantitated by densitometric analyses and normalized to the β-actin expression. Untreated /sham-infected control from either cell line was arbitrarily set to 1.0, and levels of GRP78 or VP1 were calculated as fold increase compared to their respective control groups. Data represents the mean ± S.E from three independent experiments (*p<0.05).
It is known that IGTP also activates cell prosurvival signaling [145]. Therefore, I further assessed whether any of these signaling pathways are potentially involved in the effect of IGTP on ER stress response. Firstly, specific inhibitors for JNK, ERK, and p38 MAPK and PI3K/Akt pathways as well as the vehicle control at the nontoxic concentration was applied to the IGTP-induced cells prior to tunicamycin treatment. Among all the tested signaling pathways, only the inhibition of PI3K/Akt pathway by LY294002 showed significant effect on GRP78 level. As shown in Figure 30.A, the suppression of GRP78 increase by IGTP was largely reversed when PI3K/Akt was inhibited. However, such reverse did not appear in cells treated with specific inhibitors for ERK, JNK and p38 MAPK. MTS assay was performed to evaluate the cell viability during ER stress, and the data show that IGTP expression rescued tunicamycin-induced cell death significantly as compare to the cells uninduced with Dox (Figure 30.B). When kinase inhibitors were added, again only the inhibition of PI3K/Akt pathway with LY294002 significantly decreased the cell viability, and almost eliminated the rescue effect of IGTP.

To further solidify the data on the involvement of PI3K/Akt pathway in the IGTP-mediated ER stress relief, two plasmids (pDN-Akt and pFRNK) that expresses the dominant negative Akt and FRNK respectively, were used to transfect Tet-On/IGTP cells. Because FAK is positioned upstream of Akt in the IGTP-induced signaling pathway (chapter 4), FRNK would have an analogous effect on knocking down the Akt activity. Resembling that in the inhibitor experiment, the relief effect of IGTP on ER stress response appeared to be blocked by the inhibition of Akt activity. As shown in Figure 30.C, the tunicamycin-induced upregulation of GRP78 was restored by the transfection of
Figure 30. The relief of ER stress response by IGTP depends on the activation of PI3K/Akt pathway. A. Dox induced or uninduced Tet-On/IGTP HeLa cells were treated either with vehicle (DMSO) or with specific inhibitors including PI3K inhibitor LY294002 at 10µM, ERK inhibitor U0126 at 20µM, p38 inhibitor SB203580 at 20µM, and JNK inhibitor SP600125 at 40µM. Cells were then treated with 1 µg/ml tunicamycin for 16 hours. Cell lysates were harvested and subjected to immunoblot analysis. B. After cells were treated with inhibitors and tunicamycin as described above, cell viability was measured by MTS assay. Absorbance values were corrected by subtracting the background reading. The value of MTS assay in untreated cells was defined as 100% survival (control). The cell viability of other samples was expressed relative to the control. Data represents the mean ± S.E from three independent (*p<0.05). C. Tet-On/IGTP HeLa cells were transiently transfected with vector plasmid, or plasmid encoding DN-Akt or FRNK using Lipofectamine 2000. At 24 h post transfection, cells were then treated with 1 µg/ml tunicamycin for 16 h before the assays. Cell lysates were harvested and subjected to immunoblot analysis to detect GRP78 expression. D. Cells treated under the same conditions as in C were also subjected to MTS assay. The value of cell viability of untreated cells was defined as 100% survival (control). The cell viability of other samples was expressed relative to the control. Data represents the mean ± S.E from three independent (*p<0.05).
Figure 30.

A. 

Dox  -  -  -  +  +  +  +  +  +
Tu    -  +  +  +  +  +  +  +  +

Inhibitors for  -  -  DMSO  JNK  p38  ERK  PI3K

kDa

GRP78-  -78
p-Akt (473)-  -60
β -actin-  -42

B. 

% of cell survival (MTS assay)

Tu  -  +  +  +  +  +  +  +  +
Dox -  -  -  +  +  +  +  +  +

Inhibitors for  -  -  DMSO  JNK  p38  ERK  PI3K

C. 

kDa

Tu  -  +  +  +  +  +  +  +  +
Dox -  -  -  +  +  +  +  +  +

transfection  -  -  -  pcDNA3  DN-Akt  FRNK

D. 

% of cell survival (MTS assay)

Tu  -  +  +  +  +  +  +  +  +
Dox -  -  -  +  +  +  +  +  +

transfection  -  -  -  pcDNA3  DN-Akt  FRNK
the cells with either pDN-Akt or pFRNK as compared to the vector-transfected control cells. Furthermore, the enhanced cell survival by IGTP expression was also aboragated specifically by DN-Akt or FRNK as compared to the controls (Figure 30.D). Thus, inhibition of PI3K/Akt pathway by either inhibitor or dominant negative mediators blocked the ER stress relief effect as well as the subsequent prosurvival effect of IGTP. Therefore, these results suggest that the relief effect of IGTP on ER stress response is dependent on the activation of PI3K/Akt pathway.

5.2 Discussion

As shown in the previous chapter, IGTP can inhibit CVB3 infection and preserve cell survival through activating the FAK-PI3K/Akt signaling pathway [145, 238]. In spite of these findings, the prosurvival mechanism of IGTP during viral infection as well as the implication of its ER localization still remains largely unclear. In this chapter, I found that IGTP relieves the ER stress response that is triggered either by pharmacological agents or by CVB3 infection. It is also notable that in physiological conditions when ER stress inducers was absent, Dox-induced IGTP overexpression alone did not alter the expression levels of GRP78, indicating that Dox or IGTP expression does not trigger ER stress or disrupt ER homeostasis. In this study, two types of common ER stress inducers were used, i.e., an inhibitor of protein glycosylation and an inhibitor of vesicle trafficking. The results show that IGTP alleviated the dose-dependent ER stress response triggered by either tunicamycin or BFA (Figure 22). It is noteworthy that the intensity, duration and periodicity of ER stress experienced by cells during virus infection could be quite different from those induced by chemical
agents [173]. For example, CVB3-induced ER stress response occurred earlier (6 h pi) than those induced by tunicamycin or BFA (16-20 h post treatment). However, in our experiments, IGTP overexpression could significantly relieve both types of ER stress response. The IGTP-induced relief of ER stress response in CVB3 infection was further verified by experiments using WT and IGTP KO MEF, in which GRP78 expression levels in WT MEF after IFN-γ stimulation and CVB3 infection was significantly lower than that of the IGTP KO MEF due to the presence of IGTP. Taken together, these results demonstrate that IGTP relieves the ER stress response triggered by various inducers including CVB3.

To further specify the effect of IGTP on individual branches of ER stress response, I examined the activation of PERK and ATF6 pathways that react at the early stage of stress. The results show that IGTP inhibited the activation of both PERK and ATF6 pathways during ER stress. The phosphorylation of PERK has been known as an earliest event during ER stress [246]. Phosphorylation of eIF2α by activated PERK at Ser51 inhibits cap-dependent translation of the majority of cellular mRNAs. However, ATF4 can escape this translational attenuation by multiple uORFs, and the increase of ATF4 further stimulates the expression of CHOP [174]. The data show that IGTP expression inhibited the activation of this entire PERK signaling cascade including the phosphorylation of PERK and eIF2α, and the upregulation of ATF4 expression (Figure 23). ATF-6 pathway responses to ER stress by the cleavage and translocation of the active ATF6 to the nucleus, where it upregulates the transcription of target genes that overlap much with those activated by ATF-4. Herein, IGTP also blocked the activation of ATF6 pathway as demonstrated by the reduced nuclear translocation of the active ATF6
Therefore, the effect of IGTP on ER stress response appears to be global, rather than pathway-exclusive.

Prolonged ER stress is considered to be one of the major triggers of apoptosis and can activate a combination of intrinsic and extrinsic apoptotic pathways [247-249]. Although maybe transiently favorable for cell survival under mild stress, the activation of PERK and ATF6 eventually serves to shift the stress response to pro-apoptotic signaling [170, 173]. The activation of PERK leads to the induction of pro-apoptotic CHOP via ATF4, which is one key element in ER stress-mediated apoptosis [170]. ATF6 functions closely following PERK and also contributes to CHOP induction [177]. The pro-apoptotic function of ATF-6 has been evidenced by the observations that ATF6 was selectively activated in apoptotic myoblast cells during differentiation and that inhibition of ATF-6 enhanced cell survival [250]. Accordingly, the inducers in the present study triggered the eventual shift from ER stress to cell apoptosis, and this transition appears to occur through activation of the widely recognized ATF4-CHOP-GADD34 cascade, which initiated the activation of the executioner caspase-3 and subsequent cleavage of PARP. Nevertheless, this transition here was remarkably suppressed by Dox-induced IGTP expression (Figure 25). Consistently, cell viability was also greatly enhanced thereafter (Figure 30). These data are in accordance with the previous observation that IGTP preserved the cell survival and inhibited CVB3 replication [145]. Thus, these results suggest that IGTP blocks the initiation of the pro-apoptotic pathway and preserves cell survival during ER stress through inhibition of PERK and ATF6 pathways.

ER is an essential organelle for replication and maturation of many viruses, and an increasing number of viruses have been reported to manipulate the ER stress response to
support their own replication in cells [183, 251, 252]. The induced eIF2α phosphorylation and ATF4 expression have been found to aid reovirus replication [182]. Replication of hepatitis C virus has been shown to stimulate the ATF6 pathway selectively to promote its own propagation [180]. Although the detailed mechanism of CVB3-mediated ER stress is not clear yet, both the 2B and 3A protein of CVB3 have been implicated in disrupting ER integrity or increasing membrane permeability [70, 113, 194]. On the other side, CVB3 is capable of synthesizing viral proteins by cap-independent translation initiation of its genomic RNA through the IRES-driven mechanism [253]. Thus, the virus-induced eIF2α phosphorylation in the PERK pathway would restrict the cap-dependent translation of cellular proteins, providing more translational machinery available for CVB3 RNA that does not need a cap structure for translation initiation, and thus enhance the viral protein synthesis [55, 195]. Accordingly, in the present study, with the CVB3-triggered ER stress and subsequent activation of the PERK and ATF6 pathways suppressed by IGTP expression, viral VP1 protein synthesis was shown to be inhibited. Moreover, the preemptive ER stress response appears to aid both the protein synthesis and replication of CVB3, which underscores the significance of ER stress relief effect in controlling CVB3 infection. This connection was further supported by experiments using IGTP KO MEF cell line, in which the abrogation of the IGTP-mediated relief on ER stress response was accompanied by the lessened inhibition of CVB3 protein production under IFN-γ treatment. These results suggest that IGTP, as one of the many IFN-γ-inducible genes in the cells, plays a major role in suppressing ER stress responses and the relief on ER stress response might be an important anti-CVB3 mechanism of IGTP. In this perspective, the present study
further demonstrates the potential of IGTP as an effective anti-CVB3 agent in treatment of CVB3 infection.

The association of various signal pathways with the ER stress response has been increasingly substantiated, and recent reports have illustrated solid connections between ER stress response and the PI3K/Akt, JNK, and p38 MAPK pathways, which comprise mutual regulation and negative feedback [243-245, 254]. In this study, I further investigated whether IGTP relieves the ER stress response via any of the above signaling pathways. In the experiment using various kinase inhibitors to PI3K/Akt and MEK/MAPKs, only the treatment with PI3K-specific inhibitor, LY294002, was found to resuscitate the upregulation of GRP78 during ER stress and abrogate the IGTP-enhanced cell viability, indicating the role of PI3K/Akt in mediating IGTP-induced alleviation of ER stress (Figure 30). The dependence of IGTP on PI3K/Akt pathway to relieve ER stress was further confirmed using DN-Akt- or FRNK-transfected cells, wherein suppression of Akt activity again largely eliminated the relief effect of IGTP. Multiple interactions between PI3K/Akt survival pathways and ER stress responses have been identified previously, such as through eIF2a-CHOP-TRB3 and P58IPK signaling, where Akt pathway was shown to be essential to protect cells from ER stress-induced apoptosis [170, 176, 245, 254]. The results in the present study are also consistent with our previous finding that Akt pathway is pivotal for the prosurvival effect of IGTP during CVB3 infection. Therefore, our results show that the IGTP-mediated relief of ER stress response depends on the activation of PI3K/ Akt pathway, but not the MAPK pathways.

Besides the dependence on Akt pathway, other alternative, but not mutually exclusive mechanisms for the IGTP-mediated relief effect may exist, but have yet to be
identified. For instance, the membrane binding capability of small GTPases and the involvement of p47 GTPases in disruption of pathogen vacuolar membrane both point to the potential of IGTP in structural modification of intracellular membranes [161, 255]. It is plausible that, in this way, IGTP may hamper the ER membrane disruption or vesicle formation by the virus. Also, the 2B and 3A protein of CVB3 are both capable of disrupting ER function through blocking ER-to-Golgi trafficking [113, 194], which is an essential mechanism to clear misfolded proteins from the ER. As the involvement of many GTPases in ER trafficking has been reported [256], it is possible that IGTP may relieve ER stress by promoting the outgoing trafficking from ER. Lastly, the possibility of direct interaction between IGTP with ER chaperones cannot be excluded.

In summary, my study has demonstrated that IGTP can relieve ER stress response triggered by either chemical inducers or CVB3 infection. I also found that IGTP protects cells from ER stress-mediated apoptosis by inhibiting the activation of the PERK and ATF6 branches of ER stress response. The results further suggest that the IGTP-mediated alleviation of ER stress depends on the activation of PI3K/Akt cascade. Moreover, the relief effect of IGTP on ER stress responses not only contributes to ER homeostasis but also helps restrict CVB3 infection, which further highlights that IGTP may serve as a potential candidate in developing anti-CVB3 therapeutics.
CHAPTER 6: Conclusions and Future Directions

6.1 Conclusion

Accumulating evidence has demonstrated the important roles of p47 GTPases in cell autonomous resistance to pathogen infections. Our previous *in vivo* study of gene expression profile and *in vitro* study of cell signaling have addressed the implication of IGTP in CVB3 infection. However, the underlying mechanisms for its prosurvival function remain elusive. In this dissertation, I present my work on further defining the prosurvival role of IGTP in the setting of CVB3 infection. The cellular signal transduction was studied primarily by *in vitro* characterization using Tet-On/IGTP HeLa cells, IGTP knockout MEF, and HL-1 cardiomyocytes. A summary of the highlights from this study is listed below:

1. IGTP protects cells from CVB3-induced apoptosis through multiple coordinated mechanisms rather than a single signaling pathway.
2. IGTP activates and transduces the PI3K/Akt survival signal through focal adhesion kinase, and endogenous IGTP is required for activation of FAK and Akt in murine cardiomyocytes.
3. NF-κB works as both the downstream effector and positive feedback regulator of IGTP-induced PI3K/Akt pathway. Blockage of NF-κB attenuates IGTP-induced cell survival and decreases virus replication.
4. IGTP relieves the endoplasmic reticulum stress response that is induced by CVB3 infection and thereby promotes cell survival and decreases viral replication.
5. IGTP expression inhibits the activation of both the PERK and ATF6 pathways of ER
stress responses as well as the subsequent induction of proapoptotic genes.

6. The relief effect of IGTP on ER stress response is likely dependent on the PI3K/Akt pathway, but not the MAPK pathways.

As one of the few studies on the function of p47 GTPases in response to viral infections, my work has further substantiated the role of p47 GTPases in host anti-viral resistance. Efforts have been made not only to clarify the role of IGTP in activating the PI3K/Akt pathway, a major cell survival pathway in the cell, but also to address the implication of the ER localization in IGTP function through the investigation of ER stress response. The findings of this work may promote the understanding of host-virus interactions in viral myocarditis and help identify potential targets for therapeutic intervention.

6.2 Future directions

To gain a better understanding of the functions and antiviral mechanisms of IGTP in CVB3 infection, I propose additional experiments in the following directions:

1. As shown in the present study, the activated PI3K/Akt pathway can confer cell resistance to apoptosis and promotes cell survival in the phase of direct viral infection. This survival pathway may also subsequently lead to target cell hypertrophy in the inflammatory phase of viral myocarditis via activation of transcription factors and kinases (e.g. mTOR, GSK-3β) involved in regulation of cell growth and via re-awakening of fetal cardiac gene expression. To examine whether IGTP promotes cell growth and
hypertrophy through this survival pathway, an IGTP-stably transfected cardiomyocyte H9c2 cell line will be established. In this cell line, the expression and phosphorylation of the pro-growth downstream effectors of Akt (e.g. NF-κB, GSK-3β, p70S6K, mTOR and TSC2) compared to control cell line can be determined by Western blot, kinase assay and luciferase reporter assay. Fetal gene (e.g. ANF, β-MHC) re-expression can be measured by Q-RT-PCR and the size of cardiomyocytes can be measured. To confirm the above findings in vivo and evaluate the contribution of IGTP pathway to cardiac hypertrophy, IGTP knockout (KO) mouse model and adenovirus/IGTP-transduced mice need to be used. IGTP KO mice are available from our collaborator Dr. G. Taylor in Duke University. The A/J mice overexpressing IGTP can be generated by transduction of recombinant adenoviruses (Ad/IGTP). These mice with wildtype controls will be challenged with CVB3. Using these mice, the previous findings of IGTP-induced signaling activation can be further confirmed in vivo. Also, the changes in fetal gene expression and cardiac hypertrophy indexes such as cardiomyocyte size, organ weight and ventricular wall thickness will be evaluated in these mice to analyse the influence of IGTP expression on the development of cardiac hypertrophy.

2. To further define the mechanism by which IGTP relieves ER stress response, the functional evaluation of IGTP on cellular protein trafficking from ER-to-Golgi is probably worthwhile conducting. It is known that picornaviruses, such as poliovirus and coxsackievirus, can block the ER-to-Golgi trafficking processes in infected cells through viral 3A or 2BC proteins, and cause a rearrangement of ER membranes to form vesicular compartment as the sites of viral replication. Coat complexes, COPI and
COPII, are required for vesicle trafficking between the ER and Golgi, and they can be regulated by small GTPases such as ADP-ribosylation factor 1 (Arf1). It is plausible that IGTP expression may promote the physiological ER trafficking of cellular proteins and prevent the CVB3-induced disruption of ER trafficking (COPI- or COPII-mediated vesicular transport), which thereby contributes to the relief of ER stress and inhibition of viral replication. To determine the effect of IGTP on virus-induced membrane remodeling and ER trafficking, corresponding cellular compartments and transport markers (e.g. calreticulin, p58, Sec31) will be labeled with antibodies in Tet-on/IGTP Hela cells, and the changes such as ER dilation, Golgi fragmentation, and distribution of COP-I and COPII components will be monitored and analyzed by confocal microscopy following CVB3 infection and/or IGTP expression. To further demonstrate the effect of IGTP on vesicle trafficking and protein secretion, a temperature-sensitive vesicular stomatitis virus (VSV) G protein, VSVGts045-GFP, can be transfected into the cells as an indicator. The trafficking of this GFP-labeled VSVG cargo protein following CVB3 infection and/or IGTP expression will be examined by confocal microscopy.
Bibliography

7. Grist NR RD: Epidemiology of viral infections of the heart. ; in J.E.Banatvala (ed) Viral infections of the heart London., Edward Arnold, 1993
112

50 Schmidt NJ, Magoffin RL, Lennette EH: Association of group b coxsackieviruses with cases of pericarditis, myocarditis, or pleurodynia by demonstration of immunoglobulin m antibody. Infect Immun 1973;8:341-348.


Mann DL: Tumor necrosis factor and viral myocarditis: The fine line between innate and inappropriate immune responses in the heart. Circulation 2001;103:626-629.


Huber SA, Cunningham MW: Streptococcal m protein peptide with similarity to myosin induces cd4+ t cell-dependent myocarditis in mrl/++ mice and induces partial tolerance against coxsakieviral myocarditis. J Immunol 1996;156:3528-3534.


133 Saura M, Zaragoza C, McMillan A, Quick RA, Hohenlad C, Lowenstein JM, Lowenstein CJ: An antiviral


147 Kaiser F, Kaufmann SH, Zerrahn J: Igtp, a member of the ifn inducible and microbial defense mediating 47


141 Schlaepfer DD, Mitra SK, Ilic D: Control of motile and invasive cell phenotypes by focal adhesion kinase. Biochim Biophys Acta 2004;1692:77-102.


247 Alwine JC: Modulation of host cell stress responses by human cytomegalovirus. Curr Top Microbiol ...


Appendix: List of Publications, Abstracts and Presentations

A. Publications in refereed journals from Ph.D study

1. **Liu Z**, Zhang HM, Taylor GA, Yang D. Interferon-gamma-inducible GTPase relieves endoplasmic reticulum stress response depending on the activation of PI3K/Akt but not the MAPK pathway. (Manuscript submitted)


B. Abstracts and presentations
