The Effect of Akt Isoforms in Tumorigenesis and Glucose Homeostasis in Vivo

By

WAN-NI YU
B.S., National Taiwan University, Taipei, Taiwan, 2000
M.S., National Taiwan University, Taipei, Taiwan, 2002

Thesis

Submitted as partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry and Molecular Genetics in the Graduate College of the University of Illinois at Chicago, 2013

Chicago, Illinois

Defense Committee:

Dr. Nissim Hay, Chair and Advisor
Dr. Pradip Raychaudhuri
Dr. Lester F. Lau
Dr. Bradley Merrill
Dr. Jennifer Schmidt, Biological Science
ACKNOWLEDGMENTS

I would like to thank my thesis advisor Dr. Nissim Hay for his support and guidance during my Graduate work. I would also like to thank my thesis committee members Dr. Lester F. Lau, Dr. Pradip Raychaudhuri, Dr. Bradley Merrill, and Dr. Jennifer Schmidt for their valuable suggestions and helpful discussions. I would like to acknowledge past and present members of the Hay laboratory, especially Dr. Veronique Nogueira and Dr. Chia-Chen Chen for their support in the lab and as well as being good friends over years. I would also like to acknowledge all my friends and colleagues in the department for their priceless help and sharing reagents or equipment, especially Ya-Ting Chang for her support in the department and as well as being a good friend since senior high school. I would like to thank my friends for their never-ending support, especially Pei-Yi Lu and Meishan Lin.

Lastly, I would like to thank my parents and my brother for their never-ending support and encouragement. Without all of them, my PhD life would not have been smoother.

Wan-Ni
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>A. The PI3K – Akt signaling pathway</td>
<td>1</td>
</tr>
<tr>
<td>B. The serine/threonine Kinase Akt and its function</td>
<td>3</td>
</tr>
<tr>
<td>1. Cell survival/ apoptosis inhibition</td>
<td>7</td>
</tr>
<tr>
<td>2. Cell growth</td>
<td>8</td>
</tr>
<tr>
<td>3. Cell proliferation</td>
<td>8</td>
</tr>
<tr>
<td>4. Cellular metabolism and homeostasis</td>
<td>9</td>
</tr>
<tr>
<td>C. The downstream effectors of Akt in energy homeostasis</td>
<td>10</td>
</tr>
<tr>
<td>1. mTOR (mammalian Target of Rapamycin)</td>
<td>11</td>
</tr>
<tr>
<td>2. FoxO (Forkhead box transcription factors, O subfamily)</td>
<td>13</td>
</tr>
<tr>
<td>3. Glycogen Synthase Kinase 3 (GSK-3)</td>
<td>16</td>
</tr>
<tr>
<td>D. The role of Akt isoforms in Cancer</td>
<td>18</td>
</tr>
<tr>
<td>E. The role of Akt isoforms in glucose homeostasis and diabetes</td>
<td>21</td>
</tr>
<tr>
<td>II. The effect of Akt1 ablation on tumorigenesis at the cellular and organismal levels</td>
<td>24</td>
</tr>
<tr>
<td>A. Introduction</td>
<td>24</td>
</tr>
<tr>
<td>B. Materials and Method</td>
<td>27</td>
</tr>
<tr>
<td>1. Reagent and antibodies</td>
<td>27</td>
</tr>
<tr>
<td>2. Experimental animals</td>
<td>27</td>
</tr>
<tr>
<td>3. Genotyping</td>
<td>28</td>
</tr>
<tr>
<td>4. Isolation of primary thymocytes and establishment of p53 null thymic lymphoma cell lines</td>
<td>28</td>
</tr>
<tr>
<td>5. Western Blot analysis</td>
<td>28</td>
</tr>
<tr>
<td>6. RNA interference and Nucleofection</td>
<td>29</td>
</tr>
<tr>
<td>7. Retrovirus, lentivirus transfection and infection</td>
<td>29</td>
</tr>
<tr>
<td>8. Flow Cytometry, Cell cycle analysis and BrdU incorporation</td>
<td>29</td>
</tr>
<tr>
<td>9. Xenograft assay</td>
<td>30</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. Results</td>
<td>30</td>
</tr>
<tr>
<td>1. Systemic deletion of Akt1 increased the lifespan of p53⁻/⁻ mice</td>
<td>30</td>
</tr>
<tr>
<td>2. The deletion of Akt1 impairs the growth of lymphoma cell lines in vitro and tumor growth in vivo</td>
<td>35</td>
</tr>
<tr>
<td>3. Akt inhibitor can also block the growth of lymphoma cell lines and the growth of tumors in xenograft mice</td>
<td>47</td>
</tr>
<tr>
<td>4. The mechanisms of Akt1 deletion and inhibition of Akt activity inhibits proliferation and induces cell death in p53⁻/⁻ thymic lymphoma</td>
<td>52</td>
</tr>
<tr>
<td>D. Discussion</td>
<td>54</td>
</tr>
</tbody>
</table>

## III. The effect of combined Akt1 and Akt2 deletion on mouse mortality, HCC, and glucose homeostasis

| A. Introduction | 56 |
| B. Materials and Methods | 58 |
| 1. Mice | 58 |
| 2. Measurement of glucose, insulin, IGF-1, TNFα, IL-6 and leptin levels | 59 |
| 3. Liver Enzyme AST and ALT measurement | 59 |
| 4. GTT and ITT | 59 |
| 5. Histopathology, immunostaining, and immunocytochemistry | 59 |
| 6. Immunoblotting | 60 |
| 7. Liver perfusion and real-time quantitative PCR | 60 |
| 8. RNA Sequence and analysis | 62 |
| C. Results | 63 |
| 1. Systemic Akt1 deletion in adult Akt2⁻/⁻ mice induces rapid mortality | 63 |
| 2. Liver-specific deletion of Akt1 in Akt2⁻/⁻ mice leads to viable but severely diabetic mice | 67 |
| 3. Liver-specific deletion of Akt1 in Akt2⁻/⁻ mice develop Hepatocellular Carcinoma (HCC) | 72 |
TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. Liver Injury and inflammation could be the cause of HCC development in $Akt_1^{hep-/-}Akt_2^{+/}$ mice</td>
<td>73</td>
</tr>
<tr>
<td>5. HCC in $Akt_1^{hep-/-}Akt_2^{+/-}$ mice might be FoxO dependent</td>
<td>82</td>
</tr>
<tr>
<td>D. Discussion</td>
<td>91</td>
</tr>
</tbody>
</table>

IV. APPENDICES .................................................................................................96

V. CITED LITERATURE .........................................................................................99

VI. VITA ..............................................................................................................113
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1 : Regulation of Akt activity</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2 : Akt protein structure and target protein sequences</td>
<td>23</td>
</tr>
<tr>
<td>Figure 3 : The phenotypes of combined Akt knockout mice</td>
<td>37</td>
</tr>
<tr>
<td>Figure 4 : Schematic illustration of the floxed Akt1 gene locus</td>
<td>42</td>
</tr>
<tr>
<td>Figure 5 : Mice breeding scheme used for p53−/− thymic lymphoma model and RNA expression levels of Akt isoforms</td>
<td>48</td>
</tr>
<tr>
<td>Figure 6 : Akt1F/F and Akt1F/F R26CreERT2 mice before and after induction of deletion of Akt1</td>
<td>53</td>
</tr>
<tr>
<td>Figure 7 : Lifespan of Akt1 deleted p53−/− mice</td>
<td>58</td>
</tr>
<tr>
<td>Figure 8 : The deletion of Akt1 impairs the growth of lymphoma cell lines derived from p53−/− AKT1F/F R26CreERT2 mice</td>
<td>63</td>
</tr>
<tr>
<td>Figure 9 : The deletion of Akt1 impairs the growth of lymphoma cell lines by inducing apoptosis and hindering proliferation</td>
<td>68</td>
</tr>
<tr>
<td>Figure 10 : Overexpression of mAkt in Akt1 deficient lymphoma cells rescues the cell proliferation defect</td>
<td>73</td>
</tr>
<tr>
<td>Figure 11 : Inducible deletion of Akt1 in p53−/− lymphoma tumors hindered tumor growth</td>
<td>78</td>
</tr>
<tr>
<td>Figure 12 : Lymphoma cells treated with Akt inhibitor, MK2206, impaired its growth</td>
<td>83</td>
</tr>
<tr>
<td>Figure 13 : 2 independent Lymphoma cell lines treated with MK2206 induced cell death possibly by affecting mTORC1 activity</td>
<td>88</td>
</tr>
<tr>
<td>Figure 14 : Akt inhibitor hindered p53−/− lymphoma cell growth</td>
<td>93</td>
</tr>
<tr>
<td>Figure 15 : Systemic Akt1 deletion in adult Akt2−/− mice induces rapid mortality</td>
<td>98</td>
</tr>
<tr>
<td>Figure 16 : Systemic Akt1 deletion in adult Akt2−/− mice induces rapid weight lost in combination with low blood glucose</td>
<td>103</td>
</tr>
<tr>
<td>Figure 17 : The phenotype of Akt1hep−/Akt2−/ mice</td>
<td>108</td>
</tr>
<tr>
<td>Figure 18 : Akt1hep−/Akt2−/ display glucose intolerance, insulin tolerance, high levels of blood insulin and glucose</td>
<td>113</td>
</tr>
<tr>
<td>Figure 19 : The deletion of Akt1 in the liver of Akt2−/− mice induces HCC</td>
<td>118</td>
</tr>
<tr>
<td>Figure 20 : Serum levels of ALT and AST in 3 month and 6-month-old Akt1hep−/Akt2−/−Akt1hep−/Akt2−/− mice</td>
<td>123</td>
</tr>
<tr>
<td>Figure 21 : Serum levels of IL-6 in Akt1hep−/Akt2−/− and Akt2−/− mice</td>
<td>128</td>
</tr>
<tr>
<td>Figure 22 : q-PCR analysis of gene expression in livers of Akt2−/− and Akt1hep−/Akt2−/− 6-month-old mice</td>
<td>133</td>
</tr>
<tr>
<td>Figure 23 : Akt1hep−/Akt2−/− mice displayed high levels of pStat3 in non-tumor and tumor tissue compared to Akt1hep−/Akt2−/− and Akt1hep−/Akt2−/− mice</td>
<td>138</td>
</tr>
<tr>
<td>Figure 24 RNA sequencing analysis of gene expression in livers of Akt2−/− and Akt1hep−/Akt2−/− 6-month-old mice</td>
<td>143</td>
</tr>
<tr>
<td>Figure 25 Signature features of FoxO target genes by RNA sequence analysis</td>
<td>148</td>
</tr>
<tr>
<td>Figure 26 : Hypothesis</td>
<td>153</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>RTKs</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IRS2</td>
<td>Insulin receptor substrate 2</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphotidyl inositol-4,5 bi phosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidyl inositol -3, 4, 5 tri phosphate</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PH domain</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>HM</td>
<td>Hydrophobic motif</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>Rictor</td>
<td>Rapamycin insensitive companion of mTOR</td>
</tr>
<tr>
<td>Bad</td>
<td>BCL-2 antagonist of cell death</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>FoxO</td>
<td>Forkhead box transcriptional factors, O subfamily</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology domain 3</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline-rich Akt substrate of 40kDa</td>
</tr>
<tr>
<td>Symbol</td>
<td>Name</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Skp2</td>
<td>S-phase kinase-associated protein 2</td>
</tr>
<tr>
<td>eIF4E</td>
<td>eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>Glut4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>GK</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding proteins</td>
</tr>
<tr>
<td>Raptor</td>
<td>Regulatory of associated protein of mTOR</td>
</tr>
<tr>
<td>mLST8/GβL</td>
<td>Lethal with sec thirteen/G protein β subunit like protein</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>S6K1</td>
<td>S6 kinase 1</td>
</tr>
<tr>
<td>4EBP</td>
<td>eIF4E (eukaryotic translation initiation factor 4E) binding protein</td>
</tr>
<tr>
<td>InsR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxidative species</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose 6 phosphatase</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute medium-1640</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>Tam</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
</tbody>
</table>
mAkt  myristoylated Akt
MSCV  Murine Stem Cell Virus
AST  Aspartate aminotransferase
ALT  Alanine aminotransferase
GTT  Glucose tolerance test
ITT  Insulin tolerance test
PBS  Phosphate-buffered saline
GH  Growth Hormone
HCC  Hepatocellular carcinoma
TNFα  Tumor necrosis factor alpha
HGF  Hepatocyte growth factor
IL-6  Interleukin 6
IL-1  Interleukin 1
q-PCR  Quantitative real-time polymerase chain reaction
Lepr  Leptin receptor
Sgk1  Serum-glucocorticoid regulated kinase 1
Eif4ebp3  Eukaryotic translation initiation factor 4E-binding protein 3
i.p.  intraperitoneally
Summary

Germ line Akt1-deficient mice are resistant to cancer development which is driven by hyper-activation of PI3K-Akt signaling in mouse model. However, it is not clear whether Akt1 is required for tumor maintenance, and whether systemic Akt1 deficiency can block tumor progression in the mouse. It is also not known whether Akt1 deficiency could hinder tumorigenesis which is not driven by hyper-activation of PI3K-Akt signaling. Here, we use Akt1^{f/f}Rosa26CRE^{ERT2} mice, in which Akt1 could be systemically deleted after tamoxifen administration. These mice were crossed with p53^-/-mice which are tumor prone. Systemic whole body Akt1-deficient mice substantially increased survival of these mice. Thymic lymphoma cell lines isolated from the mice undergone cell death and cell cycle arrest following by Akt1 deletion. Xenograft tumors of these mice are inhibited after Akt1 deletion.

PI3K-Akt signaling is frequently activated in human cancer. Therefore, Akt is a popular target for cancer therapy. However, the long-term effect is not well known since most of tests were done in the xenograft model. Here we used Akt1^{f/f}; Akt2^-/-; Rosa26CRE^{ERT2} mice and Akt1^{f/f}; Akt2^-/-; Albumin-Cre mice to generate combined Akt1 and Akt2 deleted adult mice and liver-specific deleted mice in order to mimic drug therapy condition. Akt1^{f/f}; Akt2^-/-; Rosa26CRE^{ERT2} adult mice could not survive after tam administration and Akt1^{hep^-/-}Akt2^-/- mice developed severe diabetes and hepatocellular carcinoma (HCC). Thus, using Akt inhibitors that target both Akt1 and Akt2 need to be aware of the side effects.
I. INTRODUCTION

A. The PI3K – Akt signaling pathway

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that is activated by insulin and various growth factors such as Insulin-like growth factor (IGF-1) (1). PI3K consists of two subunits, the p85 regulatory subunit and the p110 catalytic subunit. The p85 regulatory subunit contains SH2 and SH3 domains. The SH2 domain binds preferentially to activated (phosphorylated) tyrosine residues and mediates the association of the p110 catalytic unit to the plasma membrane. Upon growth factor binding to receptor tyrosine kinase (RTKs), PI3K can become activated either by direct protein-protein interaction with RTKs or indirectly through other intermediates such as insulin receptor substrate (IRS) (2). Activated PI3K catalyses the conversion of phosphotidyl inositol-4,5 bi phosphate (PIP2) to phosphatidyl inositol - 3, 4, 5 tri phosphate (PIP3) at the plasma membrane to increase cytosolic PIP3 levels (Fig 1). Akt is recruited by PIP3 to the plasma membrane by binding to its pleckstrin homology (PH) domain. At the plasma membrane, Akt gets fully activated by two phosphorylations, one at threonine 308 and one at serine 473. The function of PI3K is negatively regulated by phosphatase and tesin homolog (PTEN), a lipid phosphatase. PTEN converts PIP3 to PIP2, reducing the PIP3 levels in the cytosol, thus diminishing the PI3K signaling transduction pathway (3). Oncogenic Ras can also activate the PI3K-AKT signaling pathway by directly activating the catalytic subunit of PI3K, p110 (4).
Figure 1: Regulation of Akt activity

Upon growth factors binding to insulin receptors, the receptors are activated. p85 regulatory subunit of PI3K is recruited to phosphotyrosines. The p110 catalytic subunit of PI3K is activated to generate PIP3. Following binding to PIP3, Akt translocates to the plasma membrane for its full activation via phosphorylation of threonine 308 and serine 473. Thr308 is phosphorylated by PDK1 and Ser473 is phosphorylated by mTORC2. Following activation, Akt activates multiple downstream effectors, including FoxO and mTORC1. Akt activity is also negatively regulated by PTEN and by a negative feedback loop induced by mTORC1 activation. The figure depicted the regulation of Akt by insulin receptor which activates PI3K through insulin receptor substrate proteins (IRS) on the intracellular domains of receptors.
Aberrant activation of the PI3K-AKT signaling pathway has been widely implicated in human cancers. Hyperactivation of this pathway has been observed to occur through a number of modalities, including gain of function gene mutations, gene amplification and loss of function gene mutations of a negative regulator such as PTEN. Since Akt is an important downstream effector of PI3K, the role of Akt and its downstream substrates are extensively studied in cancer research. Akt was shown to mediate cell survival, cell proliferation and energy homeostasis via regulating a variety of substrates. These Akt mediated characteristics are advantageous for tumor cell survival and propagation.

B. The serine/threonine Kinase Akt and its function

Akt, also known as protein kinase B, is a serine/threonine protein kinase that phosphorylates many targets that regulate energy homeostasis, cell differentiation, proliferation, and apoptosis. (5-8). Akt is the most important signaling mediator in the phosphoinositide 3-kinase (PI3K) pathway (9-11). There are three isoforms of Akt in mammals, termed Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, encoded by three distinct genes. These isoforms contain more than 80% protein sequence identity and share the same structural organization (Fig 2). Akts are composed of three important domains including an N-terminal pleckstrin homology domain (PH domain), a central catalytic domain and a carboxy-terminal regulatory domain that contains the hydrophobic motif (HM). Phospholipids such as PIP3 can bind to the PH domain of Akt, leading to a conformational change (12,13) and subsequent translocation of Akt to plasma membrane. Akt is phosphorylated at two conserved sites, Threonine 308 and Serine 473. Threonine 308 is located in catalytic domain
Figure 2: Akt protein structure and target protein sequences

Akt1 (PKBα), Akt2 (PKBβ), Akt3 (PKBγ)

Akt proteins are composed of three important domains including an N-terminal pleckstrin homology domain (PH domain), a central catalytic domain and a carboxy-terminal regulatory domain that contains the hydrophobic motif (HM). Akt is phosphorylated at two conserved sites, Threonine 308 and Serine 473. Phosphorylation at both S473 and T308 residues is required for generating a fully activated Akt. Akt will phosphorylate its various target proteins which contain the consensus motif, Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr Hyd, where Xaa, Yaa and Zaa is any amino acid other than glycine and alanine.
phosphorylated by phosphoinositide-dependent kinase 1 (PDK1). PDK1 also contains a PH domain, thus it also translocates to the plasma membrane through PIP3. The PH domain of PDK1 has a higher affinity to PIP3, therefore PDK1 is recruited to plasma membrane prior to Akt (14). Serine 473, located in the regulatory domain of Akt, is phosphorylated by mammalian target of rapamycin complex 2 (mTORC2). mTORC2 is formed of rapamycin insensitive companion of mTOR (Rictor), mLST8, mSin1 as well as accessory factors Deptor and Protor-1 (15,16).

Phosphorylation at both S473 and T308 residues is required for generating a fully activated Akt (17), which then dissociates from the plasma membrane and returns to the cytoplasm and nucleus to access its substrates. Akt will phosphorylate its various target proteins which contain the consensus motif, Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr, where Xaa, Yaa and Zaa is any amino acid other than glycine and alanine (Figure 2) and regulate a wide variety of cellular processes including energy homeostasis, cell differentiation, proliferation, and apoptosis (5-8). For example, Akt mediates the anti-apoptotic effect by negatively regulating BCL-2 antagonist of cell death (Bad) (18,19), caspase-9, and forkhead box-O transcriptional factor (FoxO) (20), whereas Akt mediates cell cycle progression through tuberous sclerosis complex (TSC), FoxO, MDM2, p21, and p27 (21). Akt also regulates glucose metabolism through glycogen synthase kinase-3 (GSK3) and FoxO (22,23). Particularly important in skeletal muscle and liver, AKt stimulates glycogen synthesis through negatively regulating GSK3 and prevents GSK3 from phosphorylating and inhibiting its substrate glycogen synthase. Moreover, Akt
negatively regulates FoxO activity, which has been shown to promote the expression of gluconeogenic enzymes in hepatocytes.

In the mouse, there are also three separate genes encoding the three different isoforms of Akt, Akt1, Akt2, and Akt3. The three isoforms of human (mouse) are located at chromosomes 14q32 (12F1-2), 19q13 (7B1), and 1q44 (1H4-6). The physiological functions of individual isoform are studied by generating Akt isoform-deficient mice. Akt1 is the predominant isoform and is highly expressed in most tissues (24-26). On the other hand, Akt2 is mainly expressed in insulin-responsive tissues including in the liver, skeletal muscle and adipose tissue (26,27). Akt3 is expressed at the lowest level in all adult tissues with the exception of the brain and testes, where it is expressed at the highest levels (28). Previous analysis of mice lacking either individual Akt isoforms or different combinations of Akt isoforms has indicated that the Akt1 isoform plays a dominant role in embryonic development, fetal growth and survival, whereas Akt2 and Akt3 have non-redundant functions in glucose homoeostasis and postnatal brain development, respectively (see chapter III). For example, Akt1 knockout mice display mild growth retardation and Akt2 knockout mice exhibit a diabetic phenotype (29,30). Compound mice display more pronounced physiological consequences. For instance, Akt1 and Akt2 double knockout mice are neonatal lethal, exhibit severe growth deficiencies and impaired development of various organs including brain, muscle, and adipose tissue(8). Akt2 and Akt3 double knockout mice are viable but display impaired glucose homeostasis and growth deficiencies (31).

The functions of Akt are listed as follows:
1. **Cell survival/ apoptosis inhibition**

Akt enhances cell survival by blocking the function and expression of pro-apoptotic proteins and processes. For example, Akt negatively regulates, directly or indirectly, the function or expression of several Bcl-2 homology domain 3 (BH3) only proteins such as Bcl-2 interacting mediator of cell death (Bim), which can bind to prosurvival Bcl-2 and Bcl-X family proteins and inhibit their anti-proapoptotic potential. For example, Akt inhibits the BH3-only protein, BAD, by directly phosphorylating it on Serine 136(18,19). Akt also inhibits the expression of BH-3 only proteins through effects on transcription factors such as FoxO. FoxO was shown to promote transcription of BH3-only protein BIM, a pro-apoptotic protein (20), and Fas ligand (FasL) (32,33). Recently, Akt was shown to regulate pro-survival Bcl-2 family protein, MCL-1, by inhibiting GSK3 (34).

In addition, Akt was shown to elevate mitochondrial hexokinase (mtHK) association and activity at the mitochondria and require active mtHK to inhibit apoptosis by antagonism of BH3 interacting domain death agonist (Bid)-mediated Bax and Bak activation at the mitochondria (35). However, Akt has been shown that it is unable to prevent apoptosis following microinjection of cytochrome c into cytosol (36).

In addition, it is possible that Akt can regulate cell-survival effects through crosstalk with other pathways or through effects on cellular metabolism. For example, PI3K-Akt pathway was shown to activate NF-κB survival signaling (37,38).
2. **Cell growth**

Akt promotes cell growth (i.e., an increase in cell mass) through activation mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a protein complex that functions as a nutrient/energy sensor and controls protein synthesis (mRNA translation) for cell growth. Akt activates mTORC1 indirectly by phosphorylating and inhibiting tuberous sclerosis complex2 (TSC2), thereby allowing Rheb-GTP to activate mTORC1 signaling. The proline-rich Akt substrate of 40kDa (PRAS40) is another Akt substrate recently found to negatively regulate mTORC1 signaling.

3. **Cell proliferation**

Akt activation can also stimulate proliferation through several downstream targets involved in cell-cycle progression and cell-cycle entry. For cell-cycle progression, Akt phosphorylates p27 and p21, cyclin-dependent kinase inhibitors, and prevents their localization to the nucleus, thus attenuating their cell-cycle inhibitory effects. Akt also inhibits p27 expression through phosphorylation and inhibition of FoxO transcription factors. Akt also mediates degradation of p27 through S-phase kinase-associated protein 2 (Skp2). Skp2 is an E3 ubiquitin ligase which targets specific protein for degradation by the ubiquitin-proteasome system. Akt was shown to mediate Skp2 mRNA translation through mTORC1 and the eukaryotic translation initiation factor 4E (eIF4E) (39). In addition, for cell-cycle entry, GSK3 was shown to mediate phosphorylation of the G1 cyclins, cyclin D and E, and transcription factors, c-jun and myc, which play a central role in G1-to-S-phase cell-cycle transition, and
target them for proteasomal degradation. Akt enhances the stability of these proteins through negative regulation of GSK3 activity.

4. Cellular metabolism and homeostasis

In response to growth factors, Akt signaling regulates nutrient uptake and metabolism through a variety of downstream targets. Growth factor stimulation activates Akt through a PI3K-dependent process (40). All three isoforms are relatively inactive under absence of growth factors. Akt2 is the highly expressed isoform in insulin-responsive tissues and has been shown to be required for glucose transporter type 4 (Glut4) translocation to the plasma membrane, facilitating glucose uptake in response to insulin (41,42). In most cell types, Glut1 is the main glucose transporter and it can be regulated by altering expression levels. It was shown that Akt can contribute to both HIF-1α-dependent transcription of glucose transporter type 1 (Glut1) and cap-dependent translation of Glut1 mRNA by activation of mTORC1, through Akt-mediated phosphorylation of TSC2 and PRAS40 (43,44).

For metabolism, Akt activation is also involved in regulating glucose and lipid metabolism. Upon glucose entry into cells, glucose is converted to glucose-6-phosphate (G6P), an active form, by glucokinases (GK) and hexokinases (HK). G6P can be stored by conversion to glycogen or utilized to produce cellular energy through glycolysis, and Akt signaling was shown to regulate both processes. For glycogen synthesis, Akt can activate glycogen synthase by phosphorylation and inhibition of GSK3, which is negatively regulated glycogen
synthase. For glycolysis, Akt was shown to promote the expression of glycolytic enzymes through HIF1α (45).

Akt also contributes to glucose homeostasis. As described earlier, Akt not only regulates glycogen synthesis and glycolysis, but can also inhibit hepatic glucose production via phosphorylation and inhibition of FoxO1. In the hepatocyte, Akt can inhibit gluconeogenesis and fatty acid oxidation through FoxO activation. FoxO has been shown to promote the expression of the gluconeogenic enzymes in hepatocytes (23).

Akt can regulate lipid metabolism by phosphorylation and inhibition of GSK3, which in turn promotes sterol regulatory element-binding proteins (SREBPs) stability and enhanced lipid production. GSK3 has been shown to promote degradation of SREBPs, which are transcription factors that regulate cholesterol and fatty acid biosynthesis (46,47).

In summary, PI3K-Akt pathway is important for regulation of metabolism. The role of Akt signaling in metabolic disease and tumor metabolism has been enhanced via genetic knockout animal studies.

C. The downstream effectors of Akt in energy homeostasis

The indispensable roles of the Akt in development and the genetic models help elucidate the importance of Akt in regulating a variety of normal physiological functions. The most evolutionarily conserved function of Akt is to maintain energy homeostasis at cellular and organismal levels. As described earlier, Akt is an important downstream effector of the Insulin-PI3K pathway. In response to insulin, Akt is activated in insulin-responsive tissue and stimulates glucose uptake, thereby
promoting the maintenance of normal blood glucose levels. Moreover, Akt activity is also required to mediate intracellular energy levels. To perform the proper regulation of the cellular energy homeostasis, Akt activation is required to regulate its downstream effectors such as mTORC1, FoxO and GSK3.

1. mTOR (mammalian Target of Rapamycin)

The ability for all the living organisms to maintain their energy homeostasis is important for survival. To survive, an organism must be able to both generate and utilize energy efficiently to execute essential biological processes, which are dependent on protein synthesis, the most energy consuming biological process.

TOR kinase is a key regulator of protein synthesis. In mammals, TOR forms two distinct complexes, mTORC1 and mTORC2. These two complexes have very different physiological functions and are composed of different proteins. The rapamycin sensitive mTOR complex, mTORC1, consists of mTOR, regulatory associated protein of mTOR (Raptor), which is the predominant determinant of its activity, mLST8/GβL (lethal with sec thirteen/G protein β subunit like protein)(48), as well as the accessory factor PRAS40 (proline-rich Akt substrate 40kDa) (49,50) and Deptor(16). Although many mTOR associated proteins have been identified, Raptor is still the most important component in the mTORC1 complex. The interaction between Raptor and mTOR are dynamic. Raptor acts as a scaffolding protein for recruitment of substrates for mTOR. All mTOR components except mLST8 have been shown to interact with Raptor rather than mTOR itself. One mechanism for Akt to activate mTOR is through direct phosphorylation of the tuberous sclerosis complex 2 (TSC2), which
inhibits mTORC1 activity. However, when intracellular levels of ATP are reduced and AMPK activity is elevated, TSC2 can be activated. Therefore, another mechanism for Akt to activate mTORC1 is to maintain intracellular ATP levels and reduce AMP-activated protein kinase (AMPK) activity (51). AMPK acts as an intracellular energy sensor and is activated upon a reduction in intracellular ATP/AMP levels. High AMP or a reduction in ATP/AMP ratio is an indicator of low-energy status, thereby promoting the activation of AMPK by phosphorylating its kinase domain (52). AMPK can directly phosphorylate TSC2 and unlike Akt, phosphorylating TSC2 by AMPK induces inhibition of mTORC1. Additionally, AMPK inhibits mTORC1 through direct phosphorylation of Raptor (53). Tuberous sclerosis complex (TSC1) and TSC2 form heterodimer as a GAP (GTPase-activating protein) to Rheb, a small GTPase required for mTOR activation.

The other mTOR complex, mTORC2, is composed of Rapamycin insensitive companion of mTOR (Rictor), mLST8, and Sin1 as well as accessory factors Deptor and Protor-1. mTORC2 acts as the carboxy-terminus hydrophobic motif (HM) kinase for Akt, it phosphorylates Akt at Serine 473.

The function of mTORC1 is to increase mRNA translation and ribosomal biosynthesis via the activation of S6 kinase 1(S6K1) and the inhibition of the eIF4E (eukaryotic translation initiation factor 4E) binding protein (4E-BP), a repressor of mRNA translation (54). mTORC1 phosphorylates and thereby activates S6K1. Active S6K1 modulates protein translation by phosphorylating targets involved in protein synthesis such as ribosomal protein S6 and 4EBP.
4E-BP inhibits cap-dependent mRNA translation by binding to eIF4E. mTORC1 phosphorylates 4E-BP and then releases it from eIF4E, thereby relieving the inhibition on translation.

2. FoxO (Forkhead box transcription factors, O subfamily)

FoxO transcriptional factors (FoxOs) regulate many cellular and physiological processes, including energy metabolism, detoxification, aging, cell cycle progression and cell survival. Four mammalian FoxO proteins have been identified: FoxO1, FoxO3, FoxO4 and FoxO6. FoxO6 is less related to other FoxOs. All the FoxO proteins are inhibited by growth factors and insulin signaling. FoxO proteins contain a conserved N terminal DNA-binding domain (forkhead box), a conserved C terminal transactivation domain and three highly conserved Akt recognition motifs. The DNA binding domain is important for DNA target consensus sequence recognition and DNA binding, and the transactivation domain is crucial for recruitment of other transcription co-factors. FoxO activity is dependent on its cellular location. FoxO proteins contain both a nuclear localization and nuclear export signal, thus FoxOs can be imported into and exported from the nucleus via the nuclear pore. In the nucleus, FoxO is active and is able to activate or repress its targets via transcription. In the cytosol, FoxO is inactive and it is targeted for degradation by the ubiquitin-proteasomal pathway. Upon Akt activation, FoxO proteins are phosphorylated on three conserved residues, creating a docking site for 14-3-3 proteins binding and mediated extrusion to the cytoplasm, thereby inhibiting FoxO activity.
FoxOs are transcriptional factors involved in many cellular and physiological processes through its transcriptional activity. Many FoxO targets have been identified, including those involved in cell cycle arrest (p27), apoptosis (FasL, Bim and TRAIL), and DNA repair (GADD45). FoxO activation induces apoptosis or cell cycle arrest depending on cell type. Moreover, FoxO can arrest cell cycle progression by repression of positive cell cycle regulators such as cyclin D1 and D2 and FoxO can induce apoptosis by promoting transcription of FasL and Bim, pro-apoptotic proteins. As described earlier, FoxO also plays an important role in maintaining glucose homeostasis in response to insulin (22,23). In a fasting status, active FoxOs in the liver promote the expression of gluconeogenic enzymes, G6Pase and PEPCK to increase blood glucose levels. In the fed state, insulin promotes glucose uptake and FoxO inactivation, thereby suppressing the expression of gluconeogenic genes. In addition, activation of FoxO inhibits adipocyte, pancreatic β-cell and myocyte differentiation (22,59-61) and induces skeletal muscle atrophy, cells important for maintaining insulin sensitivity. Haplodeficiency of FoxO1 was shown to inhibit insulin resistance in \textit{InsR}^{+/--} (insulin receptor) mice, in part by reducing hepatic glucose output. Mice expressing constitutively active FoxO1 in the liver develop diabetes, which is similar to phenotype of \textit{Akt1}^{+/--}\textit{Akt2}^{--/--} mice, apparently as a result of increased glucose production (62). Haplodeficiency of FoxO1 was also shown to rescue diabetes in \textit{IRS2}^{--} (insulin receptor substrate 2) mice that display β-cell dysfunction. IRS2 is an adaptor protein between diverse receptor tyrosine kinases and downstream effectors. Recent studies reveal a distinct mechanism
for FoxO to maintain glucose homeostasis. Constitutively active FoxO increases phosphorylation of Akt on both T308 and S473 in vitro and in vivo. Active FoxO was shown to induce expression of Insulin/IGF1 receptors (63), IRS2 (64), and Rictor (65) expression to activate Akt, a positive feedback loop (66). More recently, it has been shown that FOXO elevates HER2/HER3-RTK expression in several cancer cell lines (67), in addition to InsR and IGF1R. The elevation of RTK by FOXO establishes feedback mechanisms that amplify growth factor signaling and restrict prolonged FOXO activation in order to maintain glucose homeostasis.

Another highly conserved role of the PI3K-Akt-FoxO signaling is its role in regulating lifespan and aging (68). In C. elegans, mutations that attenuate the activities of the PI3K ortholog, AGE-1, and the insulin receptor ortholog, DAF-2, inhibit Akt activity and can also extend the lifespan. The increase in longevity by the inhibition of PI3K and Akt activities is a consequence of DAF-16 activation, which is the C. elegans ortholog of FOXO. Loss of function of DAF-16 reverts the lifespan extension phenotype (69). Furthermore, studies in other animal models such as Drosophila and mice suggest that the function of FoxO to prolong lifespan is evolutionarily conserved in all species (70). The regulation of longevity in multicellular organisms is dependent on maintainance of tissue homeostasis. This conserved function of FoxO suggests that FoxO serves as a key mediator downstream of Insulin-PI3K-Akt signaling to maintain homeostasis in all tissues of the body. Aging, on the other hand, is attributed in large part to damage induced and accrued by reactive oxidative species (ROS), and
disruption of tissue homeostasis. ROS increases oxidative stress and induces cellular damage. FoxO was shown to reduce oxidative stress through inducing expression of detoxification enzymes. In response to oxidative stress, FoxOs activate a set of downstream targets such as manganese superoxide dismutase (MnSOD), Catalase and Sestrins, which serve as scavenger enzymes to decrease ROS levels in cells. The functions of MnSOD and Catalase are well characterized and have been shown to directly decrease reactive free radicals by converting them to H$_2$O$_2$ and subsequently to H$_2$O, respectively. There are three mammalian Sestrins, Sestrin1, Sestrin2 and Sestrin3. The function of Sestrins, especially Sestrin3, plays a dual activity downstream of FoxO(65). In its role as a scavenger of ROS, Sestrin 3 mediates ROS detoxification via FoxO and inhibits cellular senescence. As an activator of AMPK, it inhibits mTORC1 activity by phosphorylating TSC2. Sestrin1 and Sestrin2 also can activate AMPK and inhibit mTORC1 activity. Moreover, FoxO itself is regulated depending on the energy status of cells, as AMPK was shown to phosphorylate FoxO and facilitate its nuclear localization. Thus, these studies provide a connection between FoxO and mTORC1 through AMPK and Sestrin.

3. **Glycogen Synthase Kinase 3 (GSK-3)**

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine protein kinase that functions as a regulatory kinase in glycogen synthesis. In mammals, GSK3 exists as two major isoforms, GSK3α and GSK3β, and are encoded by distinct genes. Overall, GSK3α and GSK3β share 98% amino acid sequence identity within kinase domains (71). GSK3 is actively involved in a variety of central
intracellular signaling pathways, including cell proliferation, migration, inflammation and immune responses, glucose regulation, and apoptosis, by phosphorylation and inhibition of its targets, thus dysregulation of GSK3 has been shown to be involved in the initiation or progression of many diseases, including diabetes, Alzheimer's disease, bipolar disorder, and cancer. Phosphorylation of substrates by GSK3 often targets them for proteasomal degradation.

GSK3 was first discovered as a regulatory kinase in regulating glycogen synthesis. In addition, GSK3 has been implicated in other aspects of glucose homeostasis including phosphorylation of insulin receptor IRS1 (72), and of the gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6 phosphatase (G6Pase) but the detailed mechanisms need to be further elucidated (73).

GSK-3 was shown to regulate immune and migratory processes. GSK3 plays a pivotal role in regulating the production of pro- and anti-inflammatory cytokines in innate and adaptive response. The inactivation of GSK3β affects the adaptive immune response by inducing cytokine production and proliferation in naïve and memory CD4+ T cells (74). In cellular migration, the inhibition of GSK-3 has been reported to play conflicting roles and the detail mechanism need to be confirmed.

Other functions of GSK3 include regulation of cell proliferation and apoptosis. GSK3 is a part of the canonical Beta-catenin/Wnt pathway. In response to Wnt signaling, β-catenin level is increased in the nucleus and cytoplasm, therefore,
initiating transcriptional activation of proteins such as c-myc and cyclin D1, which control the G1 to S phase transition in the cell cycle (75). G1-S transition is responsible for cell proliferation. The role of GSK3 in regulating apoptosis is still controversial. Some research shows that GSK3 can regulate apoptosis by both activating pro-apoptotic factors such as p53 and inactivating survival-promoting factors such as CREB and MCI-1 (76, 77) but the details of this regulation need further study.

**D. The role of Akt isoforms in Cancer**

Akt is one of the most frequently activated oncoproteins in human cancers and exerts its tumorigenic effects by inhibiting apoptosis and by accelerating proliferation and growth (as described above). Akt is the major effector downstream of the PI3K pathway. Therefore, any mutation or amplification of the PI3K-Akt signaling pathway results in activation of Akt to promote tumorigenesis. There are three isoforms of Akt, Akt1, Akt2 and Akt3, encoded by three different genes. These three isoforms contain over 85% protein homology and it is not clear whether these three isoforms have different substrate specificities and whether these three isoforms play different roles in cancer development. One recent study showed that Akt isoforms might have distinct subcellular localizations (78). For example, Akt1 is mainly distributed in the cytoplasm, Akt2 is present both in the cytoplasm (mainly in mitochondria) and nucleus, and Akt3 is mainly in the nucleus (78). Animal studies have shown that different isoforms are functionally different in vivo. For instance, Akt2 is highly expressed in insulin-responsive tissues, with Akt2-/- mice developing a diabetic phenotype. In comparison, Akt3 is mainly expressed in the brain, with
Akt3-/- mice displaying smaller brain size. Akt1 is expressed ubiquitously and it was found that Akt1 is the most important isoform for embryonic development. Therefore, the functional difference of each Akt isoform might be due to their subcellular localization and hence availability for their substrates. Another reason for differential phenotypes between each isoform can be attributed to the relative expression levels of the different isoforms in different organs.

Although the mechanisms of the distinct roles of each Akt isoform in tumor formation, development and invasiveness are still unclear, several studies have started to emphasize the differential roles of Akt1 and Akt2 in the regulation of cell cycle progression and cell invasion. In those studies, Akt1 activation was found to decrease mammary epithelial cell migration, and prevent epithelial-to-mesenchymal transition, a process that is required for metastasis (79). Akt1 was shown to mediate the degradation of nuclear factor of activated T cells (NFAT), a pro-migratory and pro-invasive transcription factor in breast cancer cells (80). However, unlike Akt1, overexpression of Akt2 promoted adhesion and invasion in human breast cancer cell lines with upregulation of β1-integrin, which is required for invasion. Among transgenic mice studies, overexpression of activated Akt1 and ErbB2 in the mammary gland of transgenic mice accelerated the mammary gland tumor development but decreased the metastatic lesions (81). However, coexpression of Akt2 with ErbB2 increased the incidence of lung metastases (82,83). These data suggest that Akt1 attenuates while Akt2 enhances cancer cell migration.

Besides a difference in cell migration regulation, a distinct role for individual Akt isoforms in cell cycle progression has also been reported. In non-transformed
mammalian cells, silencing of Akt1 resulted in reduction of cyclin A levels and S-phase entry, while overexpression of Akt2 prevented cell cycle progression in the M-G1 transition with increased p21 levels in the nucleus (84). During early stages of oncogenesis, mammary epithelial cells express low levels of cyclin D1 in PyMT/Akt1\textsuperscript{-/-} mice but high levels of cyclin D1 are found in the lesion of PyMT/Akt2\textsuperscript{-/-} mice in compared to PyMT/wild type mice (83). These results reveal differences in the regulation of cell cycle progression by each Akt isoform.

Although recent many studies are focused on studying the specific targets and distinct functions of each Akt isoform, the role of each Akt isoform in tumor development and therapy is still the most popular topic. Most results show that Akt1 deficient mice are resistant to Pten deletion induced tumorigenesis (85), skin carcinogenesis (5), mammary and salivary gland tumorigenesis (86) and lung carcinogenesis (87), while Akt2 deficient mice are not (83,88). Together, these data suggest that Akt1 is the predominant isoform in the regulation of cancer development. However, it is not known whether systemic deletion of Akt1 after tumor onset is therapeutic. Prior data shows that Akt1-deficient mice do not have adverse physiological consequences, other than size defects. Moreover, it is not clear whether systemic deletion of Akt1 is therapeutic for cancers that were not initiated by activation of PI3K-Akt signaling. This question will be discussed in Chapter II.

Since Akt plays a central role in the development of a wide range of tumors, it is an important therapeutic target for the treatment of many different cancers. Many different types of Akt inhibitors have been generated including ATP-competitive
protein kinase inhibitors, allosteric inhibitors, and inhibitors of PIP3 binding. However, the physiological consequences of Akt ablation are not fully understood. Moreover, it is not clear whether Akt-isof orm specific targeting is preferred against pan-Akt inhibition. So far, most of the Akt inhibitors are being tested in xenograft mouse models but the long-term effects are usually not exhaustively studied. This is true in particular when the side effects are manifested after a prolonged period following treatment. This question will be addressed in the Chapter III.

E. The role of Akt isoforms in glucose homeostasis and diabetes

So far, the regulation of glucose homeostasis is one of the most well-characterized Akt-mediated processes with respect to isoform specificity. Insulin regulates glucose homeostasis by inducing glucose uptake, inhibiting hepatic glucose production, and hindering gluconeogenesis, all of them are under regulation of Akt signaling (see above). Akt2 is the major isoform in regulating glucose homeostasis and dysregulation of Akt2, unlike Akt1(29,89) and Akt3(90-92)(Fig. 3), results in a diabetic phenotype. This is manifested by high blood insulin levels and an impaired ability to reduce blood glucose levels (30,93). This diabetic phenotype is related to the differential expression of Akt isoforms in tissues. In mammals, Akt2 is expressed at the highest level in insulin-responsive tissues, Akt3 is mainly expressed in the brain and Akt1 is the most ubiquitous and abundantly expressed isoform in most tissues. The contributions of the three Akt isoforms to glucose homeostasis were assessed in compound Akt knockout mice (Fig 3). Akt1+/Akt2−/− mice convert the pre-diabetic state of Akt2−/− mice to overt type 2 diabetes, manifested by severe hyperglycemia and lower insulin levels compared to
Akt2\textsuperscript{-/-} mice (94). A recent study showed that Akt1 is the major downstream effector of IRS2 in β cells, which can respond to changes of blood glucose concentrations by secreting its insulin stores and producing insulin (95). Akt2\textsuperscript{-/-}Akt3\textsuperscript{-/-} and Akt1\textsuperscript{+/-} Akt2\textsuperscript{-/-} Akt3\textsuperscript{-/-} mice showed a diabetic phenotype similar to the one observed in Akt2\textsuperscript{-/-} and Akt1\textsuperscript{+/-}Akt2\textsuperscript{-/-} mice, suggesting that Akt3 does not play an important role in the regulation of glucose homeostasis. Interestingly, Akt1\textsuperscript{-/-}Akt2\textsuperscript{+/-} mice do not have a diabetic phenotype, suggesting that the pre-diabetic state is mainly mediated by Akt2 deficiency. Moreover, haplodeficiency of Pten in Akt2\textsuperscript{-/-} and Akt1\textsuperscript{+/-}Akt2\textsuperscript{-/-} mice was sufficient to ease the symptoms of the diabetic phenotype by activation of other Akt isoforms(94). This is also shown in haplodeficiency of Pten in IRS2\textsuperscript{-/-} mice which also displayed a minor diabetic phenotype with elevated Akt activity in comparison to IRS2\textsuperscript{-/-} mice(96). Taken together, these results suggest that activated Akt1, and possibly Akt3, can compensate for Akt2 deficiency with respect to glucose homeostasis and diabetes.

Akt is frequently activated in many human cancers and is therefore a popular target for cancer therapy. Many Akt inhibitors are currently being developed but the physiological consequence of Akt inactivation is not clear. According to compound knockout mice studies, mice with low Akt activity will develop diabetes. In order to determine whether diabetes is a potential side effect of Akt inactivation in the adult organism, we address this question with whole body knockout of Akt1 and Akt2 to mimic drug therapy in the adult animal using an inducible knockout of Akt in chapter III.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt1^{−/−}</td>
<td>Viable</td>
</tr>
<tr>
<td></td>
<td>Mild growth retardation &amp; increased apoptosis</td>
</tr>
<tr>
<td>Akt2^{−/−}</td>
<td>Viable</td>
</tr>
<tr>
<td></td>
<td>Insulin resistance (diabetic phenotype)</td>
</tr>
<tr>
<td>Akt3^{−/−}</td>
<td>Viable</td>
</tr>
<tr>
<td></td>
<td>Smaller size brain</td>
</tr>
<tr>
<td>Akt1^{+/−} Akt2^{−/−}</td>
<td>Viable</td>
</tr>
<tr>
<td></td>
<td>Severe diabetes(hyperglycemia); leptin deficiency</td>
</tr>
<tr>
<td>Akt1^{−/−} Akt2^{+/−}</td>
<td>No diabetic phenotype</td>
</tr>
<tr>
<td>Akt1^{−/−} Akt2^{−/−}</td>
<td>Neonatal Lethal</td>
</tr>
<tr>
<td></td>
<td>50% smaller; skeletal muscle atrophy; Impaired adipogenesis</td>
</tr>
<tr>
<td>Akt1^{+/−} Akt3^{−/−}</td>
<td>Smaller size brain</td>
</tr>
<tr>
<td>Akt1^{−/−} Akt3^{+/−}</td>
<td>Neonatal Lethal (Some Survive)</td>
</tr>
<tr>
<td>Akt2^{−/−} Akt3^{−/−}</td>
<td>Viable</td>
</tr>
<tr>
<td></td>
<td>Diabetic phenotype; reduced level of circulating leptin; smaller brain</td>
</tr>
<tr>
<td>Akt1^{−/−} Akt3^{−/−}</td>
<td>Embryonic Lethal (E11.5)</td>
</tr>
<tr>
<td></td>
<td>Die in uterus at midgestation; placental and vasculature defects</td>
</tr>
<tr>
<td>Akt1^{+/−} Akt2^{−/−} Akt3^{−/−}</td>
<td>Viable</td>
</tr>
<tr>
<td></td>
<td>50% smaller; severe diabetes(hyperglycemia); leptin deficiency; smaller size brain</td>
</tr>
<tr>
<td>Akt1^{−/−} Akt3^{−/−} R26RCreER</td>
<td>Survived after systemic Akt1 deletion in Akt3^{−/−} mice</td>
</tr>
</tbody>
</table>

Figure 3: The phenotypes of combined Akt knockout mice
II. The effect of *Akt1* ablation on tumorigenesis at the cellular and organismal levels

A. Introduction

The serine/threonine kinase Akt is one of the most frequently activated oncoproteins in many human cancers and Akt is the major downstream effector of growth factor/insulin receptor-PI3K signaling pathway. Among three isoforms of Akt, Akt1 is the predominant isoform in regulating development of cancer. Germ line deletion of Akt1 was shown to inhibit tumor development in mouse models of cancer driven by oncogenic signal transduction pathways (5,83,87) and does not have adverse physiological consequences. However, it is not clear whether Akt1 is required for tumor maintenance, and whether Akt1 deficiency could inhibit tumorigenesis that was not initiated by Akt activation and other upstream signaling. In order to uncover the importance of Akt1 for tumor maintenance and to emulate drug therapy, here, we generated *Akt1*^{f/f} *Rosa26Cre^{ERT2}* mice to establish an inducible Akt1 deletion system to mediate systemic Akt1 deletion after tumor formation. *Akt1*^{f/f} *Rosa26Cre^{ERT2}* mice carry a conditional cre recombinase (Cre-ERT2) allele targeted to the ubiquitously expressed ROSA26 locus. The estrogen receptor T2 (ERT2) moiety fused to Cre retains the recombinase in the cytosol until tamoxifen administration releases this inhibition, thus permitting inducible recombination of Akt1 sites where exon 4 of Akt1 is floxed by 2 loxp sites (Fig 4).
Figure 4: Schematic illustration of the floxed Akt1 gene locus

LoxP sites were inserted in order to delete exon 4. The neo cassette was flanked by flp sites (FRT) and was deleted after breeding with mice expressing flp-recombinase.
p53 is tumor suppressor and plays a role in apoptosis, genomic stability, and inhibition of angiogenesis. In its anti-cancer role, p53 works through several mechanisms: 1) It can activate DNA repair proteins under DNA damage, 2) It can induce cell cycle arrest by arresting the cell cycle at the G1/S regulation point, 3) It can initiate apoptosis, programmed cell death. p53 mutations can be found in 50% of human cancers and their penetrance is highly heterogeneous. p53 was shown to negatively regulate PI3K signaling pathway through transactivating PTEN (97). Mice homozygous for the null p53 allele appear normal but are prone to spontaneous development of a variety of neoplasms by 6 months of age (98). Around 30 to 40% of $p53^{-/-}$ mice develop lymphomas and over three-quarters of the lymphomas were of thymic origin (99,100). The high incidence of early-onset thymic lymphomas in $p53^{-/-}$ mice makes these animals a good thymic lymphoma model. By generating $Akt^{+/f} \ p53^{-/-} \ Rosa26Cre^{ERT2}$ mice, we are able to study the role of Akt1 in tumor maintenance and answer whether it is possible to inhibit tumorigenesis, in which the cancer is not initiated by activation of PI3K-Akt signaling. This will be discussed in the following section.

Many pan-Akt inhibitors have been generated and are currently in clinical trials. So far, MK2206 is the most effective inhibitor on the phase II clinical trials. MK-2206 is an allosteric inhibitor and is activated by the pleckstrin homology domain. MK2206 inhibits Akt1/2/3 with IC50 of 8nM, 12nM and 65nM, respectively. The function of MK2206 is to inhibit auto-phosphorylation of both Akt T308 and S473 residues. MK2206 also prevents Akt-mediated phosphorylation of downstream signaling molecules, including TSC2, PRAS40 and ribosomal S6 proteins. In the
following section, we will discuss whether MK2206 can be applied for treating \( p53^- \) thymic lymphoma.

B. Materials and Method

1. Reagent and antibodies

Roswell Park Memorial Institute medium-1640 (RPMI-1640) was purchased from Gibco. Fetal bovine serum (FBS) was purchased from Gemini. MK2206 was purchased from Selleckchem and ChemieTek. Captisol was purchased from Captisol, A Ligand company; antibodies against Akt, phospho-Akt(S473), Akt1, Akt2, S6, phospho-S6 phospho-FoxO3a, actin and Gadph from Cell Signaling; antibodies to actin from Sigma, Skp2 from cell signaling and abcam, FoxO3 from Upstate, p27 from BD bioscience and 4EBP1, Mcl-1 from Santa Cruz Biotechnology. BrdU was purchased from Dako and fluorescein mouse second antibody was from Vector.

2. Experimental animals

The Rosa26CreERT2 strain (Strain 01XAB) was obtained from Tyler Jacks’ laboratory (Massachusetts Institute of Technology, USA). Akt1f/f strain was generated from Ozgene (Australia). The C57Bl/6 p53+/- strain was obtained from the Jackson Laboratories (Bar Harbor, ME). The triple compound R26CreERT2; Akt1f/f; p53-/- mice were generated by mating the three individual strains. NU/NU nude mice were obtained from Charles River Laboratories (Wilmington, MA).
3. **Genotyping**

DNA samples from tail of mice or thymic lymphoma cells were analyzed by PCR using primer sets that can detect wild type and deleted p53 alleles.

4. **Isolation of primary thymocytes and establishment of p53 null thymic lymphoma cell lines**

Primary thymocytes were prepared by gently pressing the thymus between two sterile slides, then washed with PBS twice and resuspended in RPMI medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Thymic lymphoma tissue was isolated from the thymus of mice. Tumors were excised and minced. Thymic lymphoma cells grew in suspension and they were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. No established cell lines were used in this study.

5. **Western Blot analysis**

Cells were collected and lysed in lysis buffer (20 mM HEPES, 1% TX-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA) containing phosphatase inhibitors (10 mM sodium pyrophosphate, 20 mM β-glycero-glycerophosphate, 100 mM NaF, 5 mM IAA, 20 nM OA) and protease inhibitor cocktail (Roche Applied Science). Solubilized proteins were collected by centrifugation and quantified using a protein assay reagent (Bio-Rad). Equal amounts of protein of each sample were resolved by electrophoresis in 8%, 10% or 12% gel and transferred to polyvinylidene difluoride membranes (Bio-Rad).
6. **RNA interference and Nucleofection**

SMARTpool ON-TARGETplus Mouse Skp2 siRNA and control siRNA was purchased from Dharmaco. Thymic lymphoma cells were transfected using Amaza Nucleofector TM II/2b device with Mouse T-Cell Nucleofector kit and program X001. Cells (5X10^6) were spun at 1500rpm for 10 min at room temperature, resuspended in 100ul room temperature Nucleofector Solution, and nucleofected with 30 pmol siRNA. After nucleofection, cells were transferred from cuvette with 500ul pre-equilibrated fully supplemented culture media to 12-well plate containing 1.5 ml pre-equilibrated culture media per well (final volume of 2ml per well).

7. **Retrovirus, lentivirus transfection and infection**

Retroviruses were produced in phoenix-ecotropic cells and lentiviruses were produced in 293FT cells by lipofectamine 2000 transfection. Thymic lymphoma cells were infected by using MagnetoFectionTM. Before infection, viruses mixed with ViroMag R/L and incubated at room temperature for 15 min. Cells mixed with CombiMag then plated in 12-well plate and incubated at magnetic plate for 20 min. After incubation, ViroMag R/L/viruses mixture were added to the CombiMag/cells mixture and incubated for 60 min followed by manufacture’s instructions.

8. **Flow Cytometry, Cell cycle analysis and BrdU incorporation**

Cells were incubated with bromodeoxyuridine (BrdU) (3ug/ml) for 2 hours before the indicated time point for collection. After centrifugation, cells were resuspended in 1ml PBS and fixed by adding 3ml 96% ethanol dropwise. Cells
were stored overnight at 4°C. The day before analysis, cells were pelleted by centrifugation at 1,200 rpm for 5 min. Then cells were denatured by denaturing solution (2N-HCl with 0.5% Triton-X) and neutralized by 0.1M sodium borate, pH8.5. Then cells were stained with anti- BrdU antibody (Dako) overnight at 4°C. Next day, cells were washed and stained with Fluorescein anti-mouse secondary antibody (Vector). Before analysis, the cells were then stained with propidium iodide containing RNase. Flow cytometry was performed using Beckman Coulter EPICS Elite ES (Beckman, Hialeh, FL) and analyzed using Cell lab QuantaTM SC.

9. Xenograft assay

Cells were counted and suspended in cold PBS. 1x10^6 or 2x10^6 cells were injected subcutaneously into rear flank of the nude mice. After palpable tumor formation, mice were randomized into two groups. Either corn oil or tamoxifen (1mg/100ul/per injection) were injected into the nude mice intraperitoneally every other day for two weeks. MK2206 was dissolved in 30% captisol per manufacturer’s instructions and delivered by oral gavage. Tumor sizes were measured with a caliper and calculated by length*height*width*0.5.

C. Results

1. Systemic deletion of Akt1 increased the lifespan of p53^-/- mice

Akt is highly activated in many human cancers. Based on germ-line Akt knockout studies, Akt1 has been shown to be the predominant isoform in regulating cancer development. However, it is not known whether systemic
deletion of Akt1 after tumor onset is therapeutic. In the least, we know that Akt1-deficient mice do not have adverse physiological consequences (Fig 3). Moreover, it is not known whether systemic deletion of Akt1 is therapeutic for cancers that were not initiated by activation of PI3K-Akt signaling. To address this two issues, we first bred Akt1f/f (Fig. 4) mice to ROSA26-CREERT2 (R26CREERT2) mice (101), in which CreERT2 was inserted in the ubiquitously expressed ROSA26 locus, to generate inducible Akt1 knockout mice. Next, we bred Akt1f/f; R26CREERT2 mice to p53−/− mice to generate Akt1f/f; p53−/−; R26CREERT2 mice (Fig 5A), a thymic lymphoma mouse model; p53−/−; R26CREERT2 mice were used as control. Mice with disrupted germline p53 alleles have been shown to have enhanced susceptibility to spontaneous tumors of various types by 6 months of age (98). These mice develop mostly thymic lymphomas and to some extent, sarcomas. In addition, Akt1 was shown to be important for thymic development (102). Our results suggest that both Akt1 and Akt2 are expressed to a similar level in the thymus, but in thymocytes and especially thymic lymphoma cells, Akt1 is the predominant expressed isoform (Fig 5B, Fig 5C).

To test Akt1 deletion efficiency by the inducible system, we administrated tamoxifen by Intraperitoneal injection in two different groups of mice. Only Akt1f/f; R26CREERT2 mice, after tamoxifen treatment, exhibited the Akt1 deletion band by PCR analysis and showed non-detectable Akt1 protein levels in various organs when compared to Akt1f/f mice (Fig 6).
Figure 5: Mice breeding scheme used for $p53^{-/-}$ thymic lymphoma model and RNA expression levels of Akt isoforms.

A. Schematic showing the generation of $Akt1^{F/F} R26Cre^{ERT2} p53^{-/-}$ mice.

B. The expression levels of Akt isoforms in 6 different WT thymuses, quantified by semi-quantitative PCR. $Gadph$ was used as control for efficiency of PCR reactions.

C. The expression levels of Akt isoforms in 3 different primary thymocytes and 3 thymic lymphoma cell lines. T. Thymocyte. L. Lymphoma.
Figure 6: Akt1^{F/F} and Akt1^{F/F} R26Cre^{ERT2} mice before and after induction of deletion of Akt1

Akt1 gene can be deleted by i.p. Tamoxifen (1mg/mouse/day for 7 consecutive days) in Akt1^{F/F} R26Cre^{ERT2} mice.

A. Akt1 deletion can be detected by PCR.

B. Western blot demonstrates effective systemic deletion of Akt1 after Tamoxifen injection.
To determine whether systemic Akt1 deletion affects tumor progression in a p53\(^{-/-}\) mouse model for cancer not initiated by Akt activation, we generated Akt1\(^{ff}\); p53\(^{-/-}\); R26CRE\(^{ERT2}\) mice and p53\(^{-/-}\); R26CRE\(^{ERT2}\) mice as control. These two groups of mice were injected with tamoxifen at dose 1mg/day for 7 consecutive days, six weeks after birth when they start accumulating mutations for tumor development. As shown in Fig 7, the systemic deletion of Akt1 substantially increased the median lifespan of p53\(^{-/-}\) mice from 4 months to 7 months. We did not see a significant change in the lifespan of control p53\(^{-/-}\); R26CRE\(^{ERT2}\) mice (Appendix A). This data suggests that systemic Akt1 deletion prolongs lifespan of p53\(^{-/-}\) mice, possibly by inhibiting tumor progression.

2. The deletion of Akt1 impairs the growth of lymphoma cell lines in vitro and tumor growth in vivo

Primary lymphoma cell lines were isolated from Akt1\(^{ff}\); p53\(^{-/-}\); R26CRE\(^{ERT2}\) and p53\(^{-/-}\); R26CRE\(^{ERT2}\) mice and were exposed to 4-hydroxytamoxifen (4-OHT) \textit{in vitro} to induce Akt1 deletion. The deletion of Akt1 in these lymphoma cell lines profoundly inhibited the growth of the cells (Fig 8A, Appendix B) and Akt1 deletion was sufficient to significantly impair total Akt activity as measured by the phosphorylation of its downstream effectors (Fig 8B, Fig 8C, Fig 9B). This effect of Akt1 deletion on p53\(^{-/-}\) thymic lymphoma is due to induction of apoptosis and a decrease in proliferation as measured by BrdU incorporation (Fig 9A). Lymphoma cells were collected for apoptosis and BrdU incorporation assay at each indicated time point after release from 3 days 4-OHT.
**Figure 7**: Lifespan of Akt1 deleted p53−/− mice

Tamoxifen injection of 6-week-old Akt1<sup>fl/fl</sup>; p53−/−; Cre<sup>ERT2</sup> mice increase their lifespans substantially. Median life span of Akt1<sup>−/−</sup>p53<sup>−/−</sup> mice versus Akt1<sup>WT</sup>p53<sup>−/−</sup> mice is 7 months versus 4 months, respectively.
Figure 8: The deletion of Akt1 impairs the growth of lymphoma cell lines derived from $p53^{-/-} AKT1^{fl/fl} R26Cre^{ERT2}$ mice.

A. The deletion of Akt1 by 4-OHT inhibits the growth of 2 independent cell lines.

B. Western blot shows the protein expression levels in day2 and day 3 after 4-OHT treatment.

C. Western blot shows the protein expression levels at day 4 and day 5 after treatment with 4-OHT. Akt1 is deleted and Akt downstream target FoxO is less phosphorylated.
Figure 9: The deletion of Akt1 impairs the growth of lymphoma cell lines by inducing apoptosis and hindering proliferation.

A. Cells from the 2 cell lines were subjected to BrdU (Bromodeoxyuridine) incorporation, apoptosis assays and proliferation assays through PI (Propidium iodide) and BrdU staining, using flow cytometry, at the indicated time points after release from 3 days of pre-exposure to 4-OHT. The effect was compared to p53−/− R26CreERT2 Cells.

B. Western blot shows the protein expression levels at the indicated time points after release from 3 days pre-treatment with 4-OHT. The result was compared to p53+/− R26CreERT2 Cells.
pretreatment. Interestingly, restoration of p53 expression in thymic lymphoma cell lines derived from $p53^{-/-}$ mice only increased apoptosis, with no effect on cell cycle or proliferation (103). Our results showed that Akt1 deletion is sufficient to induce both cell death and cell cycle arrest in thymic lymphoma cells.

Usually, Akt1 deletion by itself has only a cytostatic effect and can only render cells more susceptible to cell death in presence of other apoptotic stimuli. Moreover, we overexpressed myristoylated Akt (myr-Akt, mAkt) in $Akt1^{f/f}; p53^{-/-}; R26CRE^{ERT2}$ thymic lymphoma cell line and induced deletion of Akt1 by 4OHT. We found that overexpressed mAkt can compensate for deletion of Akt1 and rescue the cell proliferation defect (Figure 10). Finally, we tested whether deletion of Akt1 can block tumor growth in tumor xenografts in mice by intraperitoneal injection of lymphoma cells to nude mice with subsequent monitoring of tumor growth. When tumor size reached 60mm$^3$ in volume, mice were treated with tamoxifen, 1mg/day for 7 consecutive days. As shown in Fig.11A, tumor growth in Akt1 deleted mice was almost completely halted when compared to tumor growth of Akt1 non-deleted mice. In the control lymphoma cells ($p53^{-/-}; R26CRE^{ERT2}$) injected group, there was no notable difference between the vehicle and tamoxifen treatment groups (Fig 11A). The difference of tumor size is shown in Fig 11B with Akt1 deleted in those tumors (Fig 11C). In histological analysis, we can detect more cleaved caspase 3 in the experimental lymphoma cell ($Akt1^{f/f}; p53^{-/-}; R26CRE^{ERT2}$) injected group after tamoxifen treatment to induce Akt1 deletion but there is no difference in control lymphoma cells injected group with or without tamoxifen treatment (Fig 11D).
Figure 10: Overexpression of mAkt in Akt1 deficient lymphoma cells rescues the cell proliferation defect

A. Lymphoma cells were infected with MSCV-GFP and MSCV-mAKT-GFP and treated with either EtOH as control or 300nM 4-hydroxytamoxifen to induce deletion of Akt1.

B. Western Blot showed the protein expression levels after 4 days of 4OHT treatment.
A

Lymphoma cells

![Graph showing cell numbers (x10^7) across different treatments](image)

- Control ETOH
- Control 4OHT
- MSCV-GFP ETOH
- MSCV-GFP 4OHT
- MSCV-mAkt 4OHT

B

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>MSCV-GFP</th>
<th>MSCV-mAkt</th>
</tr>
</thead>
<tbody>
<tr>
<td>4OHT</td>
<td>None</td>
<td>Ethanol</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Ethanol</td>
<td>None</td>
<td>4OHT</td>
<td>4OHT</td>
</tr>
</tbody>
</table>

![Western blot images for Akt1 and Total Akt](image)
Figure 11: Inducible deletion of Akt1 in p53⁻/⁻ lymphoma tumors hindered tumor growth.

A. The isolated lymphoma cells were subjected to a xenograft assay. When the tumor size reached 60mm³, mice were treated either with vehicle or tamoxifen, and the growth of tumors was monitored. Control lymphoma line: p53⁻/⁻; R26CREERT², Lymphoma line A, B: Akt1⁻/--; p53⁻/--; R26CREERT²

B. Tumors isolated from xenograft mice.

C. Western blot analysis showed Akt1 is deleted in the Tamoxifen treated tumors.

D. Sections from control and tamoxifen treated tumors were stained for anti-cleaved caspase 3.
More cleaved caspase 3 staining indicated an increased in cell death in the Akt1 deleted lymphoma tumors. These *in vitro* and *in vivo* studies suggest that Akt1 deficiency can block tumor progression by inducing apoptosis and blocking cell proliferation.

3. Akt inhibitor can also block the growth of lymphoma cell lines and the growth of tumors in xenograft mice

Akt inhibitor, MK2206, is an allosteric inhibitor of Akt activity. MK2206 inhibits Akt1/2/3 with IC50 of 8nM, 12nM and 65nM, respectively. To determine whether MK2206 has the same effect on lymphoma cells, we exposed the thymic lymphoma cells to MK2206 and compared the results to the effect induced by Akt1 deletion. Interestingly, MK2206 also blocked the growth of lymphoma cells *in vitro* and *in vivo* (Fig 12, Fig 14) and we found similar effects of MK2206 on BrdU incorporation and apoptosis to the observation after induction of Akt1 deletion in two independent cell lines (Fig 12A, Fig 12B). As shown on Fig 12C, MK2206 can completely inhibit phosphorylation of Akt after 6 hours of treatment in thymic lymphoma cell lines and can also affect phosphorylation of downstream effectors of Akt, S6 4EBP and FoxO. Moreover, Mcl-1, a pro-survival Bcl-2 family protein, is decreased and cleaved caspase 3 is increased in 2 independent thymic lymphoma cell lines after MK2206 treatment (Fig 13). *In vivo*, lymphoma cells were injected subcutaneously into the rear flank of nude mice. After palpable tumor formation, mice were randomly separated into two groups and treated with either MK2206 or vehicle orally. In the MK2206
**Figure 12**: Lymphoma cells treated with Akt inhibitor, MK2206, impaired its growth.

A. MK2206 treatment inhibits the growth of 2 independent cell lines.

B. Cells from 2 cell lines were subjected to BrdU incorporation and analyzed for apoptosis and BrdU incorporation, using flow cytometry, at the indicated time point after exposure to MK2206.

C. Western blot showed the Akt activity decreased after MK2206 treatment. Also, phosphorylation of downstream effector, FoxO is reduced.
Figure 13: 2 independent Lymphoma cell lines treated with MK2206 induced cell death possibly by affecting mTORC1 activity.
Figure 14: Akt inhibitor hindered p53⁻/⁻ lymphoma cell growth

Isolated lymphoma cells were subcutaneously injected into nude mice. Once mice developed palpable tumors, they were then treated either with vehicle or MK2206 (120 mg/kg, 3 times a week for 2 weeks).
treatment group, lymphoma progression is much slower than the vehicle treatment group. This data suggests that inhibition of Akt is therapeutic for thymic lymphoma that was not initiated by activation of PI3K-Akt signaling.

4. The mechanisms of Akt1 deletion and inhibition of Akt activity inhibits proliferation and induces cell death in p53\textsuperscript{−/−} thymic lymphoma

Previous studies showed that mTORC1 is the most critical downstream effector of Akt, required for cell proliferation and tumorigenesis (5). Our results showed that upon inhibition of Akt activity by MK2206, there was a marked reduction in mTORC1 activity as measured by p-S6 (Fig 12C) and enhanced cell arrest (Fig 12B). In addition, we found that the cyclin kinase inhibitor p27 is elevated following inhibition of Akt activity by MK2206, with concomitant reduction in p-FoxO (Fig 12C). p27, a Cyclin-dependent kinase inhibitor 1B, is an enzyme inhibitor that controls cell cycle progression at the G1 checkpoint. Akt has been reported to suppress p27(Kip1) promoter activity through FoxO in different kinds of cells (104,105). The reduction in FoxO phosphorylation by Akt, which in turn promotes its transcriptional activity, could induce the transcriptional upregulation of p27 (106). Thus, this data suggests that elevation of p27 may mediate G1 cell cycle arrest upon inhibition of Akt activity by MK2206 in thymic lymphoma cells. However, there was no significant difference in p27 levels upon deletion of Akt1 by 4OHT (data not shown) but there was a marked reduction in mTORC1 activity as measured by p-S6 (Fig 8B, Fig 9B), with concomitant reduction in p-FoxO, meaning FoxO is activated (Fig 8C), similar to inhibition of Akt activity by MK2206. Interestingly, Skp2 was found to be markedly reduced in
**Akt1**-deficient lymphoma cells (Fig 8C). Skp2 is an F-box protein that mediates the degradation of p27 and other cell cycle inhibitory proteins (39,107,108). Previous results showed that Skp2 mRNA translation is dependent on mTORC1 and the eukaryotic translation initiation factor 4E (eIF4E) (39). This data suggested that Skp2 might regulate other cell cycle proteins to induce cell cycle arrest in **Akt1**-deficient thymic lymphoma cells.

Previous studies showed that Akt1 deletion by itself has only cytostatic effect and can only render cells more susceptible to cell death by other apoptotic stimuli. Although the exact mechanism of cell death is not fully understood, we found that Akt1 deletion is sufficient to induce cell death in p53\(^{-/-}\) thymic lymphoma cells as measured by cleaved caspase 3 and PI staining (Fig 9A, Fig 11, Fig 12A, Fig 13). Our results showed that there was a reduction in Mcl-1, an anti-apoptotic BCL-2 family protein, upon inhibition of Akt activity by MK2206. Mcl-1 is a short lived protein and its expression is highly dependent on mRNA translation which can be explained by a mobility shift in 4EBP1 protein in both treatment conditions (MK2206 treatment or 4-OHT treatment) (Fig 8B, Fig 13). 4EBP is an inhibitory binding protein of Eukaryotic translation initiation factor 4E (eIF4E), thus regulating mRNA translation (109,110). Further, we try to explore other apoptotic pathway in Akt1-deleted and Akt-inactivated condition. As we showed earlier, FoxO3a activity is elevated in both conditions (Fig12C, Fig8C). There are three putative FoxO binding elements in the Fas ligand (FasL) promoter region. In human Jurkat T cell lymphoma cell lines, FoxO3a induces apoptosis by activating FasL expression and the Fas-FasL apoptotic pathway
Another proapoptotic BH3-only protein Bim, which plays an important role in Bax/Bak-mediated cytochrome c release and apoptosis, was shown to be upregulated by FoxO (111). Thus, Akt might induce apoptosis through FoxO activation in $p53^{-/-}$ thymic lymphoma cells. Indeed, our preliminary data showed marked increase in FoxO targets, FasL and Bim (preliminary data, Appendix C).

**D. Discussion**

In this study, we use genetic manipulation of the mouse genome to understand the importance of Akt1 in $p53^{-/-}$ thymic lymphoma and test whether inhibition of Akt is therapeutic for thymic lymphoma that was not initiated by activation of PI3K-Akt signaling. Our data showed that systemic deletion of Akt1 can prolong lifespan of $p53^{-/-}$ mice, which developed various types of tumors and died by 6 months of age. *In vitro* and *in vivo* studies showed that Akt1 deficiency or inhibition of Akt activity by Akt inhibitor, MK2206, can inhibit cell growth and tumor growth by inducing apoptosis and cell cycle arrest in $p53^{-/-}$ thymic lymphoma. The possible mechanism might be through regulation of two major downstream effectors, mTORC1 and FoxO. mTORC1 is required for cell proliferation and tumorigenesis and inhibition of mTORC1 by rapamycin can block tumor progression (112,113). Our results showed that there was a reduction in mTORC1 activity as measured by p-S6 and by 4EPB1 (Fig 8B, Fig 12C), an inhibitory protein of eIF4E. Both are important in regulating mRNA translation of proteins which are involved in cell proliferation and cell survival such as Skp2, cyclin D1 and Mcl-1. As we showed, Skp2 is downregulated after
deletion of Akt1 by 4OHT. Another downstream effector of Akt, FoxO, was shown to regulate cell proliferation and apoptosis transcriptionally. As we showed earlier, deletion of Akt1 or inhibition of Akt activity upregulated FoxO3 activity (Fig 8C, Fig 12C). We speculated that reduced Mcl-1 expression together with the induced FoxO targets, FasL and Bim, contribute to cell death induced by Akt1 deletion.

In summary, our data showed that inhibition of Akt activity is therapeutic for p53−/− thymic lymphoma and can be applied for treating other types of cancer which is not initiated by activation of PI3K-Akt signaling. Akt is important for tumor maintenance. Without functional Akt, the apoptotic pathway will be activated.
III. The effect of combined Akt1 and Akt2 deletion on mouse mortality, HCC, and glucose homeostasis

A. Introduction

The serine/threonine kinase Akt is frequently activated in many human cancers. Akt has been shown to regulate energy homeostasis, protein synthesis, cell proliferation and apoptosis by phosphorylating different targets such as mTOR and FoxO. Akt exerts its tumorigenic effects by accelerating proliferation and inhibiting apoptosis. Therefore, the pathways leading to Akt activation and Akt itself are being targeted for cancer therapy. However, the physiological consequences of Akt isoforms ablation are not well understood.

Akt isoforms exhibit over 80% protein sequence identity and share the same protein structure. In in-vitro kinase assay studies, it has not been possible to distinguish substrate specificity between the three Akt isoforms. However, in vivo, it is possible that different isoforms have functional differences. To understand the physiological roles of the individual Akt isoforms, animal models deficient in Akt1, Akt2, or Akt3 have been generated. Akt1 is the most abundant expressed isoform in many mammalian tissues, Akt2 is expressed at the highest level in insulin-responsive tissues, and Akt3 is expressed at the highest level in the brain (Table1). Germ line deletion of Akt1 in mice exhibits mild growth retardation and increased spontaneous apoptosis in the testes and thymus (29)(Fig 3). However, there is no
difference at the lifespan of these mice. Mice lacking Akt2, display normal growth but have a diabetic phenotype including elevated fasting glucose levels and insulin resistance (30). Akt3-deficient mice have a smaller brain, resulting from a decrease in both cell size and cell number but maintain normal glucose homeostasis and body weight (31,92). These observations indicate that the three Akt isoforms have some differential and non-redundant physiological functions. The different phenotype and viability of mice lacking individual Akt isoforms also suggest that the Akt isoforms are able to compensate for each other. Among compound mice, the germ line deletion of both Akt1 and Akt2 causes neonatal lethality and multiple developmental defects. They exhibit severe growth deficiency, skeletal muscle atrophy, impaired skin development, induced adipogenesis and delayed osteogenesis. Germ line deletion of both Akt1 and Akt3 leads to embryonic lethality (day12), with severe impairments in growth, cardiovascular development and organization of the nervous system (90). However, Akt2−/Akt3− mice are viable but show impaired glucose metabolism, insulin resistance, and have a 25% reduction in body weight. Interestingly, \( \text{Akt1}^{+/\text{-}} \text{Akt2}^{\text{-}/\text{-}}, \text{Akt1}^{+/\text{-}} \text{Akt3}^{\text{-}/\text{-}} \) and \( \text{Akt1}^{+/\text{-}} \text{Akt2}^{\text{-}/\text{-}} \text{Akt3}^{\text{-}/\text{-}} \) mice are also viable with combined phenotypes. Among those live Akt compound mice, \( \text{Akt1}^{+/\text{-}} \text{Akt2}^{\text{-}/\text{-}} \) mice develop severe diabetes while \( \text{Akt2}^{\text{-}/\text{-}} \) mice display only insulin resistance, indicating a complementary role of Akt1 in the genesis of type 2 diabetes initiated by Akt2 deletion. The mice have leptin deficiency due to impaired adiopogenesis, and data shows that restoring leptin levels is sufficient to restore normal glucose and insulin levels and then cure diabetes in these mice (8,94,114). Taken together, the results suggest that Akt1 is the most important, while Akt2 is
the least important, isoform for embryonic development. Akt2 and Akt3 are dispensable for adult mouse viability and only one allele of Akt1 is sufficient for embryonic development and adult mouse viability. The studies described above were done with germ line deletion of Akt isoforms. Some of the combinations of germ line deletion are either embryonic or neonatal lethal. The purpose of the studies in this section is to determine the phenotypes of combined deletion of Akt1 and Akt2 in adult mice. By using \( \text{Akt1}^{\text{ff}}; \text{Akt2}^{\text{-/-}}; \text{Rosa26Cre}^{\text{ERT2}} \) mice and \( \text{Akt1}^{\text{ff}}; \text{Akt2}^{\text{-/-}}; \text{Albumin-Cre} \) mice to generate conditional whole body DKO mice and liver-specific DKO mice, we are able to uncover the phenotypes of these mice and to mimic drug therapy conditions. The results of the studies would have important implications for cancer therapy, using Akt inhibitors that target both Akt1 and Akt2.

**B. Materials and Methods**

1. **Mice**

   The generation of \( \text{Akt1}^{\text{-/-}} \) and \( \text{Akt2}^{\text{-/-}} \) mice was previously described. The \( \text{R26RCreERT2} \) strain (Strain 01XAB) was obtained from Tyler Jacks’ laboratory (Massachusetts Institute of Technology, USA). The Albumin-CRE Strain was obtained from the Jackson Laboratory (Bar Harbor, Maine). \( \text{Akt1}^{\text{ff}} \) mice were generated by Ozgene (Australia) and \( \text{Akt2}^{\text{ff}} \) mice were a gift from U Penn. Mice were intercrossed to generate the various genotype.
2. **Measurement of glucose, insulin, IGF-1, TNFα, IL-6 and leptin levels**

Glucose levels were determined by using an automatic glucometer (Precision Xtra; Abbott Laboratories). Growth hormone and insulin levels were measured by enzyme-linked immunosorbent assay (ELISA; Millipore) according to the manufacturer's instructions. TNFα, IL-6, IGF-1, Insulin and leptin levels were determined by Milliplex immunoassay (Millipore) according to the manufacturer's instructions.

3. **Liver Enzyme AST and ALT measurement**

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by the UIC Biological Resources Laboratory.

4. **GTT and ITT**

A glucose tolerance test (GTT) was carried out after 16 hours of fasting and intraperitoneal injection of glucose (2g/Kg of body weight). Glucose levels were determined at 0 min before the injection of glucose and at 15, 30, 60, and 120 min after glucose injection. Insulin tolerance test (ITT) was carried out with non-fasted mice and IP injection (0.75 IU/Kg of body weight) of human Insulin (Eli Lilly). Glucose levels were determined at 0 min before the injection of insulin and at 15, 30, 60, and 120 min after insulin injection.

5. **Histopathology, immunostaining, and immunocytochemistry**

Livers were removed from mice and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight.
6. **Immunoblotting**

Liver tissue were homogenized and lysed in lysis buffer (20 mM HEPES, 1% TX-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA) containing phosphatase inhibitors (10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 100 mM NaF, 5 mM IAA, 20 nM OA) and protease inhibitor cocktail (Roche Applied Science). Solubilized proteins were collected by centrifugation and quantified using a protein assay reagent (Bio-Rad). Equal amounts of protein of each sample were resolved by electrophoresis in 8%, 10% or 12% gel and transferred to polyvinylidene difluoride membranes (Bio-Rad). Immunoblotting was performed using anti-p-p70S6K (T389), anti-p-FOXO1/3a (T24/T32), anti-p70S6K, anti-FOXO1 (Cell Signaling), anti-β-actin (Sigma), anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase (Invitrogen) antibodies.

7. **Liver perfusion and real-time quantitative PCR**

Mice were under deep Ketamine/Xylazine (150 mg/kg; 10mg/kg) anesthesia, a 24GX3/4” catheter was carefully inserted into the portal vein. The liver was perfused with 20 ml PBS (pH 7.0), and then removed to a petri dish for tissue collection. Total RNA was extracted from liver tissue at the indicated age by using TRIzol reagent (Invitrogen) followed by RNeasy mini kit (Quiagen) to obtain pure, quality, RNA, and first strand cDNA was produced with SuperScript III reverse transcriptase (Invitrogen). Quantitative Real-time PCR was performed with iQ SYBR green supermix (BIO-RAD) with the iQ5 real-time PCR detecting system. Relative levels of mRNA were compared by using cyclophilin A or
Gapdh primers to normalize for total RNA input. The reaction conditions were as follows: 5 min of denaturation at 95°C, followed by 45 cycles of denaturation for 15 s at 95°C, and annealing and elongation for 1 min at 55°C. The mouse primer sequences were as follows:

Bim, 5'-CGACAGTCTCAGGAGGAACC-3' (forward) and 5' - CCTTCTCCATACCAGACGGA-3' (reverse);

ErbB2, 5' - GACTGTCCTCCTGTGTCG-3' (forward) and 5' - CTCGGACATGGTCCAGAAGGC-3' (reverse);

FasL, 5' - TCCGTGAGTTCCACCAACAA-3' (forward) and 5' - GGGGCTCCCTGTAAATGGG-3' (reverse);

Met, 5' - CAGTGCACTCCACCCTCATT-3' (forward) and 5' - ACCCATGTCTAGCTTGCCC-3' (reverse);

HGF, 5' - GAGCTCCAGCTTCCAAATTGC-3' (forward) and 5' - GAGTTTGGTCCCCACATCA-3' (reverse);

IL-6, 5' - GACAACTTTTGCCATTGTGG (forward) and 5' - ATGCAGGGATGATGTTCTG-3' (reverse);

TNFa, 5' - TGGGAGTAGACAAGGTACAACCC-3' (forward) and 5' - CATCTTCTCAAATTGAGTGACA-3' (reverse);

Cycophilin A, 5' - TTCACAAAACCACAATGGGCACAGGG-3' (forward) and 5' - TGCCGTCCAGCCATCTGTCTTAT-3' (reverse);

Gadph, 5' - AGGTCGTTGGAACGGATTTCG-3' (forward) and 5' - TGTAGACCATGTAGTTGAGGTCA-3' (reverse).
8. **RNA Sequence and analysis**

RNA libraries were generated and multiplex RNA library sequencing was done by University of Chicago Genomics Core. Bioinformatics analysis was done by UIC RRC bioinformatics.
C. Results

1. Systemic Akt1 deletion in adult Akt2/− mice induces rapid mortality

To test whether both Akt1 and Akt2 activity are required for adult mice survival, we generated Akt1f/f; Akt2−/−; R26CreERT2 mice. In Akt1f/f; Akt2−/−; R26CreERT2 mice, Akt2 is a germ line deletion and Akt1 deletion can be introduced by i.p. injections of Tamoxifen (1mg/100ul/25g mouse) in corn oil for 2 weeks, every other day. Surprisingly, we found that even the induction of whole body deletion of Akt1 in Akt2−/− adult 2-month-old mice causes a rapid mortality within 2 months after the induction of systemic Akt1 deletion (Fig. 15). Systemic Akt1 deletion in adult Akt2/− mice also induces rapid weight lost in combination with low blood glucose in certain double deficient mice (Fig.16A, Fig. 16B). Adult double deficient mice lost 5 to 20 % of weight during Tamoxifen administration depending on their genotype. Before death, these mice displayed a weight loss average of 20-30% from their original body weight along with severe fat mass reduction. The mice demonstrated gradual worsening of inability to move along with extremely low blood glucose levels as pertained by glucometer. Some of the readings were below the detection ability of the glucometer. Thus, complete ablation of Akt1 and Akt2 activity cannot be tolerated to sustain life in adult mice. Akt activity is required for adult mouse survival and inhibition of both Akt1 and Akt2 activity in adult mice might be lethal if used as a cancer therapy target.
Figure 15: Systemic Akt1 deletion in adult Akt2−/− mice induces rapid mortality.
Figure 16: Systemic Akt1 deletion in adult Akt2−/− mice induces rapid weight loss in combination with low blood glucose.

A, Akt1f/f; Akt2−/−; R26CREERT2 mice lost 20 to 30% of the total body weight at one month after inducing deletion of Akt1 or before death.

B, Blood glucose level is elevated one week after tam treatment in Akt1f/f; Akt2−/−; R26CREERT2 mice at the fed status, however, mice display hypoglycemia at one month after tam treatment or before death.
2. Liver-specific deletion of Akt1 in Akt2⁻/⁻ mice leads to viable but severely diabetic mice

Complete ablation of Akt1 and Akt2 activity cannot be tolerated in adult mice. Therefore, Akt1 is important for not only embryonic development but also postnatal survival. Inhibition of whole body Akt1 and Akt2 activity in adult mice is lethal but we wanted to explore whether it is possible to inhibit Akt1 and Akt2 activity in a specific organ, such as liver. In mouse liver, Akt2 accounts for 84% of the total Akt protein, with the remaining being Akt1; there is no detectable amount of Akt3 in mouse liver (91). To test whether it is possible to inhibit both Akt1 and Akt2 activity in the liver, we bred Akt1<sup>fl/fl</sup>; Akt2<sup>-/-</sup> mice to Albumin-Cre mice, which express the Cre recombinase specifically in the liver, starting from embryonic day15.5, to generate mice with systemic Akt2 deletion and hepatocyte-specific Akt1 deletion. Surprisingly, Akt1<sup>hep⁻/-Akt2⁻/-</sup> mice are alive but are relatively small in body size and weight (Fig. 17A, Fig. 17B). We further confirmed whether the small body size is due to low growth hormone (GH) secretion. Surprisingly, the growth hormone level is normal in Akt1<sup>hep⁻/-Akt2⁻/-</sup> mice as compared to Akt1<sup>hep⁺/-Akt2⁻/-</sup> and Akt1<sup>hep⁻/-Akt2⁺/-</sup> mice (data not shown). Next, we examined the levels of Insulin-like growth factor 1 (IGF-1) level, a primary mediator of the effects of growth hormone (GH). GH is made in the anterior pituitary gland, released into the blood stream to stimulate liver production of IGF-1. IGF-1 then stimulates systemic body growth, and has growth-promoting effects on almost every cell in the body (Fig. 17C). Interestingly, IGF-1 level was extremely low in Akt1<sup>hep⁻/-Akt2⁻/-</sup> mice (Fig. 17D),
Figure 17: The phenotype of $Akt1^{hep-/}Akt2^{-/-}$ mice

A.B. $Akt1^{hep-/}Akt2^{-/-}$ mice have significantly reduced body weight and size.

C. Growth Hormone (GH) and Insulin-like Growth Factor (IGF-1) system

D. Serum levels of IGF-1 in 3 month and 6-month-old $Akt1^{hep-/}Akt2^{-/-}$, $Akt1^{hep+/}Akt2^{-/-}$, $Akt1^{hep-/}Akt2^{+/-}$ and $Akt1^{hep-/}Akt2^{+/+}$ mice.
The GH/IGF-1 System

- Brain
- Pituitary
- GH
- Liver
- Insulin
- Akt
- Growth
- IGF-1
- Target tissue
- IGFBPs
- IGFBPs
suggesting that this might be the underlying cause of growth retardation in these mice. We concluded that, in addition to GH, Akt is required for the induction of IGF-1 expression.

Akt2\(^{-/-}\) mice have a mild diabetic phenotype and Akt1\(^{+/+}\)Akt2\(^{-/-}\) mice display severe diabetes. Therefore, we wanted to know if Akt1\(^{hep/-}\)Akt2\(^{-/-}\) mice would develop severe diabetes. We showed that Akt1\(^{hep/-}\)Akt2\(^{-/-}\) mice also led to severe hyperglycemia as compared to wild-type mice (data not shown), Akt1\(^{WT}\)Akt2\(^{-/-}\) mice and Akt1\(^{hep^{+/+}}\)Akt2\(^{-/-}\) mice (Fig.18D). Akt1\(^{hep/-}\)Akt2\(^{-/-}\) mice display glucose intolerance as compared to Akt2\(^{-/-}\) mice (Fig.18A), insulin tolerance as compared to Akt1\(^{hep^{-/-}}\) and Akt2\(^{-/-}\) mice (Fig.18B), and a high level of blood insulin (Fig.18C). Taken together, Akt1 deletion in the liver of Akt2\(^{-/-}\) mice is sufficient to convert insulin resistance in Akt2\(^{-/-}\) mice to overt diabetes with hyperglycemia and glucose intolerance. This result indicates that Akt1 in the liver, in addition to Akt2, is important to glucose homeostasis.

3. Liver-specific deletion of Akt1 in Akt2\(^{-/-}\) mice develop Hepatocellular Carcinoma (HCC)

Akt is frequently activated in many human cancers. Therefore, inhibition of Akt activity has been an attractive target of cancer therapy. However, we found that Akt1 deletion in the liver of Akt2\(^{-/-}\) mice developed hepatocellular carcinoma (HCC) at 6-7 months of age for male mice and with a complete penetrance (Fig.19A,Fig. 19B). Female Akt1\(^{hep/-}\)Akt2\(^{-/-}\) mice also developed HCC but much later than male mice (data now shown). Interestingly, HCC was not observed when only one allele of Akt1 was deleted in the liver of Akt2\(^{-/-}\) mice as compared
with other genotype (Fig.19B). By histological analysis, \(Akt^{\text{hep}/-}\)\(Akt^{2/-}\) mice were diagnosed with poorly differentiated HCC, clear cell type (Fig.19C). \(Akt^{\text{hep}/-}\)\(Akt^{2/-}\) and \(Akt^{2/-}\) mice are either normal or show dysplasia, but do not develop HCC. Importantly, the hepatocarcinogenesis observed in \(Akt^{\text{hep}/-}\)\(Akt^{2/-}\) mice is a relatively fast process when compared to most mouse models of hepatocarcinogenesis (115,116). We found that most of \(Akt^{\text{hep}/-}\)\(Akt^{2/-}\) male mice start having these lesions at 3 months of age and female mice start having these lesions at 5 months of age. These unexpected and counterintuitive results may potentially have a major impact on the use of Akt inhibition for cancer therapy.

4. Liver Injury and inflammation could be the cause of HCC development in \(Akt^{\text{hep}/-}\)\(Akt^{2/-}\) mice

Liver carcinogenesis is a multistep and long process: the presence of specific risk factors promotes gene damage, which leads to a cascade of molecular and cellular deregulations that ultimately result in transformation of hepatocytes. The initiation of and predisposition to liver carcinogenesis is often associated with liver injury and inflammation. Alanine transaminase (ALT) and Aspartate transaminase (AST) are two important enzymes in amino acid metabolism. Both enzymes are associated with liver parenchymal cells and are indicators of liver inflammation and damage. ALT is a more specific indicator of liver inflammation than AST. We found that serum levels of the liver enzymes AST and ALT are markedly elevated in \(Akt^{\text{hep}/-}\)\(Akt^{2/-}\) mice as compared to \(Akt^{\text{hep}/+}\)\(Akt^{2/-}\).
Figure 18: Akt1\textsuperscript{hep-/-} Akt2\textsuperscript{-/-} display glucose intolerance, insulin tolerance, high levels of blood insulin and glucose.

A. Glucose Tolerance Test (GTT), Akt1\textsuperscript{hep-/-} Akt2\textsuperscript{-/-} mice shows glucose intolerance as compared to Akt2\textsuperscript{-/-} mice.

B. Insulin Tolerance Test (ITT), Akt1\textsuperscript{hep-/-} Akt2\textsuperscript{-/-} mice displays insulin intolerance as compared to Akt2\textsuperscript{-/-} and Akt1\textsuperscript{hep-/-} mice.

C. Insulin levels at fed status are high in Akt1\textsuperscript{hep-/-} Akt2\textsuperscript{-/-} mice as compared to wild-type and Akt2\textsuperscript{-/-} mice.

D. Fasting and fed glucose levels are extremely high in Akt1\textsuperscript{hep-/-} Akt2\textsuperscript{-/-} mice.

(*, P<0.05, **, P<0.01, \n, P<0.05, \n\n, P<0.01)
C

Insulin levels at Fed status

WT
Akt1hep/- Akt2/-
Akt2/-

Genotype

Insulin (ng/dl)

1-3 month
4-6 month
Fed and Fasting glucose level

Graph showing glucose levels for different genotypes: 
- **Fasting Glucose**
- **Fed Glucose**

Genotypes: 
- Akt1<sup>hep/-</sup> Akt2<sup>-/-</sup> 
- Akt1<sup>hep+/-</sup> Akt2<sup>-/-</sup> 
- Akt1<sup>WT</sup> Akt2<sup>-/-</sup>
Figure 19: The deletion of Akt1 in the liver of Akt2<sup>+/−</sup> mice induces HCC

A, Akt<sup>hep−/−</sup>Akt2<sup>−/−</sup> mice developed HCC and there is no HCC development in control mice, Akt<sup>hep−/−</sup>Akt2<sup>+/−</sup> and Akt<sup>hep+/−</sup>Akt2<sup>−/−</sup> mice at 6.5 month-old. Akt single mice included Akt<sup>hep−/−</sup> and Akt2<sup>−/−</sup> mice.

B, Akt<sup>hep−/−</sup>Akt2<sup>−/−</sup> mice developed HCC with 100% penetrance and none of control mice developed HCC.

C, Akt<sup>hep−/−</sup>Akt2<sup>−/−</sup> mice developed different types of HCC as analyzing by histological analysis.
Figure 19A. The deletion of Akt1 in the liver of Akt2 \textsuperscript{−/−} mice induces HCC.
Figure 19B. The deletion of Akt1 in the liver of Akt2 -/- mice highly induces HCC
Figure 19C. The deletion of Akt1 in the liver of Akt2−/− mice high induces HCC
and Akt2°/° mice (Fig.20), indicating liver damage. Moreover, serum Interleukin 6 (IL-6) levels are highly induced in Akt1°/°Akt2°/° mice (Fig.21). Interleukin 6 (IL-6) is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine and is secreted by T cells and macrophages to stimulate immune response especially tissue damage leading to inflammation. In addition, the high level of IL-6 leads to the hyperactivation of STAT3 in hepatocyte, which was shown to be a major contributor to hepatic tumor development in many models of hepatocarcinogenesis (115,116). Consistently we found that pY-Stat3 is elevated in the Akt1°/°Akt2°/° livers (Fig. 23). We also observed that in the tumors, Akt1 was not completely deleted, suggesting that the tumors were originated from cells that escaped the deletion. (Fig.23). Also, HGF transcription is upregulated by inflammatory modulators such as tumor necrosis factor alpha (TNFα), IL-1 and IL-6. We also found hepatocyte growth factor (HGF) levels are induced (Fig. 22) and c-Met levels, receptor of HGF, are also induced, which are consistent with other models of hepatocarcinogenesis (116). HGF-cMET pathway play a critical role in hepatic tissue injury repair (117). Together these results strongly indicate that the deletion of Akt1 in the liver of Akt2°/° mice induces liver damage followed by inflammation, which are typically prerequisites for HCC development.

5. HCC in Akt1°/°Akt2°/° mice might be FoxO dependent

Forkhead box protein O1 (FOXO1) is a transcription factor that plays important roles in regulation of gluconeogenesis and glycogenolysis by insulin signaling in the liver, and is negatively regulated by Akt as a direct target. From
Figure 20: Serum levels of ALT and AST in 3 month and 6-month-old
Akt1hep−/− Akt2−/−, Akt1hep+/− Akt2−/− and Akt1hep+/− Akt2+/− mice.

A. ALT is higher in Akt1hep−/− Akt2−/− mice compared to Akt1hep+/− Akt2−/− and
Akt1hep+/− Akt2+/− mice

B. AST is higher in Akt1hep−/− Akt2−/− mice compared to Akt1hep+/− Akt2−/− and
Akt1hep+/− Akt2+/− mice (*, P<0.05, **, P<0.01; ▽, P<0.05, ▽▽, P<0.01)
Figure 21: Serum levels of IL-6 in Akt$^{1\text{hep}/-}\ Akt2^{/-}$ and Akt$^{2/-}$ mice.

Akt$^{1\text{hep}/-}\ Akt2^{/-}$ mice display high levels of IL-6 compared to Akt$^{2/-}$ mice.
Figure 22: q-PCR analysis of gene expression in livers of Akt2<sup>−/−</sup> and Akt1<sup>hep−/−/Akt2−/−</sup> 6 month-old mice

RNA samples were isolated from 4 different of mice from each group and analyzed by real-time PCR.
Figure 23: Akt<sup>1<sub>hep</sub>-/-Akt<sub>2</sub>-/- mice displayed high levels of pStat3 in non-tumor and tumor tissue compared to Akt<sup>1hep</sub> +/-Akt<sub>2</sub>-/- and Akt<sup>1hep</sup> +/-Akt<sub>2</sub>-/- mice.

pstat3 is elevated in Akt<sup>1hep</sup>-/-Akt<sub>2</sub>-/- liver and Akt1 protein levels can still be detected in Akt<sup>1hep</sup>-/-Akt<sub>2</sub>-/- tumor part. (NT, non-tumor; T, tumor, from same liver)
previous findings by another group (118), Akt\textsuperscript{hep-/-Akt2\textsuperscript{-/-}} mice (deletion of Akt1 is induced by Cre recombinase driven by α-fetoprotein) were glucose intolerant, insulin resistant and had higher concentrations of Insulin-like growth factor binding protein 1 (Igfbp) protein in both their liver and serum than Akt2\textsuperscript{-/-} mice (118). Igfbp1 is a major target gene of FoxO1, suggesting that FoxO1 was activated in the livers from Akt1\textsuperscript{hep-/-Akt2\textsuperscript{-/-}} mice (Afp-Cre). Deletion of FoxO1 in Akt1\textsuperscript{hep-/-Akt2\textsuperscript{-/-}} mice reversed the glucose intolerance, fasting hyperinsulinemia and reduced liver injury (118). Consistently, we found that the liver of Akt1\textsuperscript{hep-/-Akt2\textsuperscript{-/-}} Akt2\textsuperscript{-/-} mice expressed relatively high levels of FasL, Bim and TNFα, which are established targets of FoxO that induce apoptosis (Fig. 22). FoxO itself was shown to activate upstream signaling through the induction of insulin receptor, IRS protein, and other tyrosine kinase receptors expression (119). Indeed, we found that ErbB2 is elevated (Fig. 22). To understand the gene expression profile in the liver, we subjected mRNA from Akt1\textsuperscript{hep-/-Akt2\textsuperscript{-/-}} and Akt2\textsuperscript{-/-} liver to a genome-wide expression analysis (Fig.24, Figure 25). Consistently, several FoxO1 target genes were found to be increased more than 2-fold by RNA sequencing such as leptin receptor (Lepr) (5.75 log\textsubscript{2}-fold), serum-glucocorticoid regulated kinase 1 (Sgk1) (3.9 log\textsubscript{2}-fold), Igfbp1 (6.95 log\textsubscript{2}-fold) and eukaryotic translation initiation factor 4E-binding protein 3 (Eif4ebp3) (2.03 log\textsubscript{2}-fold) (Fig. 25). In addition, we found that Irs2 is increased (2.45 log\textsubscript{2}-fold) (Fig. 25). Taken together, these data strongly indicate that activation of FoxO1 is a key consequence of deleting Akt from the liver. Thus, the mechanism behind why
Akt1^{hep-/-} Akt2^{/-} mice develop HCC might be through FoxO1 activation and subsequent elevation of pro-tumorigenic cytokines (IL-6 and HGF).
Figure 24 RNA sequencing analysis of gene expression in livers of $Akt2^{-/-}$ and $Akt1^{hep-/-} Akt2^{-/-}$ 6 month-old mice

RNA samples were isolated from 4 different mice from each group and cDNA libraries were established for RNA deep sequencing.
<table>
<thead>
<tr>
<th>Genes</th>
<th>Names</th>
<th>Log₂ (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepr</td>
<td>Leptin receptor</td>
<td>5.75196</td>
</tr>
<tr>
<td>Igfbp1</td>
<td>Insulin-like growth factor-binding protein 1</td>
<td>6.95078</td>
</tr>
<tr>
<td>Sggk1</td>
<td>Serum-glucocorticoid regulated kinase 1</td>
<td>3.90277</td>
</tr>
<tr>
<td>Eif4ebp3</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 3</td>
<td>2.03615</td>
</tr>
<tr>
<td>Irs2</td>
<td>Insulin receptor substrate 2</td>
<td>2.44756</td>
</tr>
<tr>
<td>Rbp1</td>
<td>Retinol binding protein 1</td>
<td>2.00828</td>
</tr>
<tr>
<td>Dhrs9</td>
<td>Dehydrogenase/ Reductase (SDR family) member 9</td>
<td>2.39898</td>
</tr>
<tr>
<td>Pdk4</td>
<td>Pyruvate dehydrogenase kinase, isoenzyme 4</td>
<td>1.92468</td>
</tr>
</tbody>
</table>

**Figure 25** Signature features of FoxO target genes by RNA sequence analysis
D. Discussion

In this study, we used genetic manipulation of the mouse genome to understand the importance of Akt’s role in maintaining mouse survival in order to mimic Akt inhibitor treatment for cancer therapy. As we know, germ line deletion of Akt in mice, such as $Akt1^{+/−} \ Akt2^{+/−}$, $Akt1^{+/-}Akt3^{-/-}$, $Akt2^{-/-}Akt3^{-/-}$, $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ are viable, but germ line deletion mice in $Akt1^{-/-} Akt2^{-/-}$ and $Akt1^{-/-}Akt3^{-/-}$ genotype are lethal (8, 90). Only a single functional allele of Akt1 is sufficient for embryonic development and postnatal survival (31). We found that a complete ablation of Akt1 and Akt2 activity cannot be tolerated in adult mice and the induction of whole body deletion of Akt1 in $Akt2^{-/-}$ adult mice causes rapid mortality within 2 months. $Akt1^{-/-} Akt2^{-/-}$ mice show a gradual worsening of their ability to move and extremely low blood glucose levels in combination with loss of fat mass (data not shown), suggesting that inhibition of Akt1 and Akt2 activity in adult mice disrupts glucose consumption/metabolism and mice might use β oxidation to burn fat as their energy source. This phenotype is very similar to cachexia in patients. Cachexia is a highly complicated metabolic disorder involving features of muscle atrophy, lipolysis and insulin resistance which were seen in patients with chronic illness including type I diabetes, multiple sclerosis, HIV, and cancer. The exact mechanism in which these diseases cause cachexia is poorly understood and currently, there are no widely accepted drugs to treat cachexia. Recently, many studies showed that the Zinc-α2-glycoprotein (ZAG) is elevated in cachexia patients (120). ZAG was shown to stimulate lipolysis, induced reduction of body fat in mice. Since $Akt1^{-/-} Akt2^{-/-}$ mice display a similar cachexia phenotype, it would be interesting to verify whether ZAG
is elevated in the serum of Akt1<sup>−/−</sup> Akt2<sup>−/−</sup> mice to understand the reason behind rapid mortality upon inducing whole body knockout of Akt1 and Akt2. The phenotype of Akt1<sup>−/−</sup> Akt2<sup>−/−</sup> mice suggests that a single functional allele of Akt1 is important not only during embryonic development but also adult survival. Thus, long-term systemic inhibition of Akt1 and Akt2 activity for cancer therapy may not be feasible.

Although mice cannot survive after induction of whole body deletion of Akt1 in Akt2<sup>−/−</sup> background, they survived in hepatocyte-specific Akt1 deletion in combination with systemic Akt2 deletion. However, these mice developed HCC at 6-7 month of age with a complete penetrance and also these mice developed severe diabetes. These unexpected results have significant impact on cancer therapy because in a systemic drug administration, usually the liver is the organ that absorbs the highest dose of the drug and metabolizes it. Liver carcinogenesis is a long process that is usually manifested after a long period post initiation. Therefore, these undesired consequences could be neglected in pre-clinical and clinical trials using inhibitors that mainly inhibit both Akt1 and Akt2.

The course of HCC development is a multistep process involving liver damage, hepatocyte death, inflammation, and cycles of necrosis and regeneration (115). We found that in Akt<sup>hep−/−</sup> Akt2<sup>−/−</sup> livers showed various characteristics of liver damage and inflammation, including the appearance dysplastic hepatocytes (data not shown), increased serum concentrations of the liver enzymes ALT and AST (Fig. 20) and elevated TNF, IL-6 with increased in STAT3 phosphorylation (Fig. 21, Fig.22). We propose that deletion of Akt1 and Akt2 reduces total Akt activity to a level that compromises hepatocyte cell survival but hepatocytes become more susceptible to
cell death induced by FoxO activation, at least in part. Supportively, we found that Akt1<sup>hep/-</sup> Akt2<sup>/-</sup> livers express relatively higher levels of FasL and Bim than Akt2<sup>/-</sup> livers (Fig.22), which are two established targets of FoxO that induce apoptosis. Also, it was showed that deletion of FoxO1 in Akt1/2 DKO liver reduces liver injury (Morris Birnbaum, personal communication), and the deletion of FoxO1 in mice with hepatic deletion of IRS1/2 inhibits liver neoplasia observed in these mice (Morris white, personal communication). Our analysis of Akt1<sup>hep/-</sup> Akt2<sup>/-</sup> livers versus Akt2<sup>/-</sup> livers showed that Akt1<sup>hep/-</sup> Akt2<sup>/-</sup> tissues still expresses Akt1 and that p-Akt is still relatively high in the tumor compared to normal tissue (Fig. 23). Therefore, we assume that hepatocytes, which escape the full deletion of Akt1, give rise to HCC development. In addition, the hyperactivation of STAT3 in the surviving hepatocytes was shown to be a major contributor to HCC development (121) and the elevated cytokines, IL-6, TNF and HGF, were also shown to help induce hepatocyte proliferation and initiation of HCC (122) (Fig.26). Consistently, we found that p-Stat3 is elevated in the Akt1<sup>hep/-</sup> Akt2<sup>/-</sup> livers and that IL-6, TNF, and HGF are elevated as well. Thus, it is possible that rapid and robust induction of hepatocarcinogenesis is occurring as a consequence of elevation of cytokines in the liver and feedback mechanisms induced by FoxO. To further confirm whether HCC development is through regulation of FoxO1, we obtained Akt1<sup>fl/fl</sup>; Akt2<sup>fl/fl</sup>; FoxO1<sup>fl/fl</sup> and Akt1<sup>fl/fl</sup>; Akt2<sup>fl/fl</sup> mice from Morris Birnbaum at U. Penn to see whether it is possible to block HCC development in Akt1<sup>hep/-</sup> Akt2<sup>hep/-</sup> FoxO1<sup>hep/-</sup> mice as compared to Akt1<sup>hep/-</sup> Akt2<sup>hep/-</sup> mice.
Although the induced HCC by Akt1/2 deletion is surprising, it was previously reported that hepatic deletion of tyrosine phosphatase Shp2, which is considered an oncprotein, also induced HCC (123). Similar to our observation, it was shown that both IL-6 and TNFα (Fig.21, Fig.22) are induced, and Stat3 (Fig.23) is activated. In our case, Akt1<sup>hep−/−</sup> Akt2<sup>−/−</sup> (Alb-CRE) mice do not only develop HCC but also severe diabetes (hyperglycemia and hyperinsulinemia). Consistently, Akt1 deletion in the liver of Akt2<sup>−/−</sup> mice, induced by AFP-CRE, also display hyperglycemia (118) and Akt1<sup>−/−</sup> Akt2<sup>−/−</sup> mice show diabetes as well (8). It is possible that the high level of insulin and glucose in Akt1<sup>hep−/−</sup> Akt2<sup>−/−</sup> mice facilitate HCC development. We found that the levels of glucose and insulin in Akt1<sup>hep−/−</sup> Akt2<sup>−/−</sup> mice are higher than or similar to Akt2<sup>−/−</sup> mice, but there is no HCC development in Akt2<sup>−/−</sup> mice. In order to further test the possibility, we generated the reciprocal genotype (Akt1<sup>−/−</sup> Akt2<sup>hep−/−</sup> mice) and the preliminary results support that high levels of insulin and glucose are potential contributors to HCC development. There is neither HCC development nor diabetes in the Akt1<sup>−/−</sup> Akt2<sup>hep−/−</sup> mice.

Our results have important therapeutic implications for cancer therapy by inhibition of Akt activity. Many Akt inhibitors are now in pre-clinical and clinical trials for cancer therapy. Thus our findings suggest that those inhibitors may lead to diabetes and/or HCC during long-term treatment. In summary, our results and results from other groups suggest that if diabetes and HCC are side effects of Akt ablation therapy, it may be reversed by inhibition of FoxO activity.
A complete hepatic deletion of Akt1 and Akt2 induces cell death in individual hepatocytes, at least in part through activation of FOXO mediated cytokine release, which in turn is recognized by Kupffer cells. In turn Kupffer cells release IL-6 and HGF to stimulate the proliferation of surviving hepatocytes with partial deletion of Akt1.

**Figure 26: Hypothesis**
Appendix A: Lifespan of $p53^{-/-} \text{ R26Cre}^{ERT2}$ mice.

Tamoxifen injection of 6-week-old $p53^{-/-} \text{ R26Cre}^{ERT2}$ mice doesn't change much of lifespan as compared to $Akt1^{+/+} p53^{-/-} \text{ R26Cre}^{ERT2}$ mice. Median lifespan of tam treatment and non-tam treatment is 5.5 months versus 4.8 months, respectively.
Appendix B: Tamoxifen treatment doesn’t affect lymphoma cells proliferation.

Lymphoma cells isolated from $p53^{-/-} \text{R26Cre}^{ERT2}$ mice were treated with Tamoxifen to test the effect of Cre recombinase as a control experiment. There is not many difference on cell proliferation after activation of Cre recombinase.
Appendix C: q-PCR analysis of gene expression in lymphoma cells with or without MK2206 treatment.
V. CITED LITERATURE

Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**, 727-736


contains the regulatory serine phosphorylation site. *Biochemical and biophysical research communications* **257**, 906-910


83. Maroulakou, I. G., Oemler, W., Naber, S. P., and Tsichlis, P. N. (2007) Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV) - ErbB2/ neu and MMTV-polyoma middle T transgenic mice. Cancer research 67, 167-177


90. Yang, Z. Z., Tschopp, O., Di-Poi, N., Bruder, E., Baudry, A., Dummler, B., Wahli, W., and Hemmings, B. A. (2005) Dosage dependent effects of Akt1/protein kinase Balpha (PKBalpha) and Akt3/PKBgamma on thymus, skin, and
cardiovascular and nervous system development in mice. *Molecular and cellular biology* 25, 10407-10418


inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. *Molecular and cellular biology* **22**, 7842-7852


VI. VITA

NAME: Wan-Ni Yu

EDUCATION: B.Sc., Medical Technology, College of Medicine, National Taiwan University, Taipei, Taiwan, R.O.C., 2000

M.Sc., Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan, R.O.C., 2002


LICENSE: Registered Medical technologist license, Ministry of Health and Welfare, Taiwan, R.O.C., 2000