Cellular Responses to Lactic Acidosis in Human Cancers

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Molecular Genetics and Microbiology in the Graduate School
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ABSTRACT

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Abstract

The physiology of the tumor microenvironment is characterized by lower oxygen (hypoxia), higher lactate, extracellular acidosis and glucose starvation. We examined the global, transcriptional cellular responses to each of these microenvironmental stresses *in vitro*, projected them onto clinical breast cancer patients' samples *in vivo*, and returned to perform further *in vitro* experiments to investigate the potential mechanisms involved in these stress responses. The reciprocal exchange of information was critical and advanced our understanding of the potential clinical relevance of cellular responses.

Our expression array result showed that lactic acidosis induces a strong response, distinct from that of hypoxia in human mammalian epithelial cells (HMECs), indicating lactic acidosis is not only a by-product of hypoxia but has a unique role as a stimulant to cells in the tumor microenvironment. Cellular responses to lactosis and acidosis further demonstrated that acidosis was the main driving force in the lactic acidosis response. These responding gene signatures were then statistically projected into clinical breast cancer patients' expression data sets. The hypoxia response, as reported previously, was associated with bad prognosis, where as the lactic acidosis and acidosis responses, were associated with good prognosis. Additionally, the acidosis response could be used to separate breast tumors with high versus low aggressiveness based on its inversed correlation with metastatic character. We further discovered that

lactic acidosis, in contrast to hypoxia, abolished Akt signaling. Moreover, it downregulated glycolysis and shifted energy utilization towards aerobic respiration.

We continued to examine the cellular response to lactic acidosis temporally in MCF7 cells, a breast cancer cell line. The lactic acidosis response of MCF7 cells also showed the prognostic result of better clinical outcomes in datasets of breast cancer patients. Strikingly in MCF7 cells, lactic acidosis and glucose deprivation actually induced similar transcriptional profiles, with only a few genes being oppositely regulated. Furthermore, lactic acidosis, similar to glucose starvation, induced AMPK signaling and abolished mTOR. However, lactic acidosis and glucose deprivation induced opposite other metabolic phenotypes. Lactic acidosis significantly repressed glucose uptake whereas glucose deprivation significantly induced it. Among the genes differentially regulated by these two stresses, thioredoxin-interacting protein (TXNIP) was among the most different. The negative regulatory role of TXNIP on glucose uptake has been demonstrated previously. In the cancer research field, TXNIP is recognized as a tumor suppressor gene. We observed that lactic acidosis induced TXNIP strongly and most importantly, TXNIP played a critical role in regulating glucose uptake in cells under lactic acidosis. Furthermore, MondoA, the transcription factor and glucose sensor previously reported to regulate TXNIP induction upon glucose exposure, was also responsible for regulating TXNIP under lactic acidosis. We demonstrated that TXNIP

not only plays an important role in the lactic acidosis response but also has strong prognostic power to separate breast cancer patients based on survival.

Taken together, we showed the independent role of lactic acidosis as the stimulant in the tumor microenvironment to induce specific cellular responses distinct from hypoxia and held different prognostic values in tumors. Lactic acidosis directed metabolic reprogramming of cancer cells to rely more on oxidative phosphorylation than glycolysis in energy generation. On the other hand, lactic acidosis and glucose deprivation induced similar gene expression profiles but were with different metabolic phenotypes of glucose uptake and a gene, TXNIP, played an important role in regulating the metabolism induced by lactic acidosis. This study offered a new perspective of the stresses in the tumor microenvironment. Several major directions, such as applying this established *in vitro* and *in vivo* system into investigating other stresses, identification of surface receptors sensing lactic acidosis and consolidating metabolic profiling with expression profiles in cells treated with lactic acidosis can be pursued and these will provide us with more information for the development of potential therapeutic strategies with lactic acidosis in human cancers.

Dedication

I would like to dedicate my thesis work to my family. I want to thank my parents for their unconditional support, to my husband for his company and love, to my brother for always standing by my side.

I would also like to dedicate this to cancer patients, survivors and fighters around the world. There is hope and this disease can be treated.

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Chapter 1

Introduction

1.0 The physiology of the tumor microenvironment

The tumor microenvironment is defined by the National Cancer Institute as "the normal cells, molecules, and blood vessels that surround and feed a tumor cell. A tumor can change its microenvironment, and the microenvironment can affect how a tumor grows and spreads". The tumor microenvironment is heterogeneous with different types of cells, various signals and abnormal vasculature. It has an interactive and critical role in tumor development and progression. It is a hostile environment with a physiology very different from that of normal tissues. The physiology of the tumor microenvironment is characterized by depletion of oxygen (hypoxia), decreased glucose and ATP level, accumulated lactate and extracellular acidosis(Vaupel 2004). Poor tissue perfusion, abnormal tumor vasculature, and dysregulated tumor metabolism are among the causes for this tumor physiology. Given the heterogeneous nature of cancer cells, variations reflecting the complexity of the tumor microenvironment are commonly observed in individual tumors. The tumor microenvironment can trigger specific cellular responses, modulate biological processes and cause distinctive phenotypic changes in cells. Among these microenvironment stresses, hypoxia has received most attention. Lactic acidosis, on the other hand, is commonly regarded as an effect of tumor

hypoxia. However, cancer cells prefer glycolysis for energy production, even under ambient oxygen supply, a phenomenon that is termed the Warburg effect. Aerobic glycolysis also contributes to lactate accumulation and thus to the acidification of the tumor microenvironment. In addition to these causes, some cancer cells pump protons out into the extracellular space through the high expression of proton transporters; they also create a reversed pH gradient compared with normal cells, resulting in an acidic extracellular pH (pHe) and an alkaline intracellular pH (pHi)(Cardone, Casavola et al. 2005). Moreover, inefficient removal of lactate in poorly perfused tissue can exacerbate lactic acidosis. Therefore, it is critical to examine hypoxia and lactic acidosis separately to understand their respective contributions to tumors phenotypes.

1.1Hypoxia

1.1.1 The role of the hypoxia-inducible transcription factor 1 (HIF-1)

Hypoxia was the best understood microenvironmental stress in human cancers. Hypoxic tumors were more resistant to radiation therapies and chemotherapies and low O_2 (hypoxia) in tumors was correlated with poor survival and increased metastasis. The transcriptional activation of the hypoxia-inducible transcription factor (HIF-1) is required in cellular responses to hypoxia. HIF-1 is composed of two subunits, HIF-1α and HIF-1β, both of which are required for active transcription. HIF-1β constantly exists in cytoplasm whereas HIF-1α, although able to be synthesized, would be oxidized by

proline hydroxylase at one or two of its proline residues and immediately degraded by von Hippel-Lindau tumor suppressor protein (pVHL) under an environment with ambient oxygen (Semenza and Wang 1992; Semenza 1998; Maxwell, Wiesener et al. 1999; Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001; Semenza 2001)

However, under low oxygen, HIF-1α is not degraded and its protein level increases exponentially as cellular O_2 concentration drops from 20 to 0.5% (corresponding to pO2 values of 140 to 3.5 mmHg). HIF-1αthen binds with HIF-1β to be translocated from the cytoplasm into the nucleus to activate its downstream target genes, the hypoxia-inducible genes(Jiang, Semenza et al. 1996). The downstream target genes induced by HIF-1 are those involved in angiogenesis, erythropoiesis and metabolism, including VEGF, PDGF, EPO, GLUT1 and most of the core genes in glycolysis(Semenza, Roth et al. 1994; Semenza 2003).

1.1.2 Hypoxia response induced by factors other than low oxygen

HIF-1α protein stabilization is not enabled only by lower oxygen level. The HIF-1α stabilization and resulting hypoxia response actually can happen under a sufficient supply of oxygen by many genetic events seen in human cancers. These factors include the loss of VHL(Motzer, Bander et al. 1996), p53(Blagosklonny, An et al. 1998), PTEN(Zundel, Schindler et al. 2000), or, on the other hand, activation of phosphatidylinositol 3-kinase/Akt signaling (Zhong, Chiles et al. 2000) can all lead to

oxygen-independent hypoxia responses. Oxygen-independent HIF1α stabilization was observed in patients with the von Hippel-Lindau disease. Patients with this disease had one copy of the VHL gene inactivated or lost and when the remaining normal copy of this gene was lost or inactivated, HIF1α protein became stabilized without the requirement of lower oxygen. This attributed to the development of multiple neoplasms in patients with von Hippel-Lindau disease(Semenza 2002). Recently, it was discovered that loss or inhibition of fumurate hydratase (FH) and succinate dehydrogenase (SDH), enzymes critical for the functional tricarboxylic acid (TCA) cycle, would stabilize HIF1α, activate hypoxia pathways and cause tumor formation(Isaacs, Jung et al. 2005; Selak, Armour et al. 2005; Pollard, Spencer-Dene et al. 2007). These statuses represented "pseudo-hypoxia" and opened a new field of hypoxia pathways being regulated by genetic changes instead of oxygen.

1.1.3 The relationship between hypoxia and lactic acidosis

Normal tissues in the microenvironment with ambient supply of oxygen typically rely on aerobic metabolism for energy production. Glucose is the main source of energy and it enters cells through glucose transporters to be metabolized into pyruvate, which is then metabolized into CO_2 and H_2O through Krebs cycle to produce 38 moles of ATP per mole of glucose. On the other hand, under anaerobic conditions, pyruvate is reduced to lactate and generates oxidized nicotinamide adenine dinucleotide (NAD^+) from reduced

NADH and produces only 2 moles of ATP per mole of glucose. Aerobic metabolism has a much higher efficiency of glucose under oxygenated conditions whereas glycolysis is upregulated in anaerobic conditions are phenomena referred to as the Pasteur Effect.

A higher concentration of lactate and extracellular acidosis (lactic acidosis), are both commonly regarded as by-products of the hypoxia response because cells grown in low oxygen must rely on glycolysis to compensate for the shortage of ATP caused by decreased oxidative phosphorylation. Increased glycolysis naturally generates increased levels of lactate. Nevertheless, hypoxia and lactic acidosis do not always correlate spatially and temporally. Helmlinger et al., using high- resolution measurements, found that oxygen tension and interstitial pH in human tumor xenografts were not correlated(Helmlinger, Yuan et al. 1997). Oxygen was not able to inhibit glycolysis and acid production in MDA-MB-435 cells, indicating the contribution of endogenous metabolism to tumor acidity(Schornack and Gillies 2003).

1.2 Glucose metabolism and cancer

1.2.1 Warburg effect

Under hypoxia, cells decrease the rate of oxidative pheosphorylation but increase glycolysis. However, Otto Warburg observed an interesting phenomenon that even with sufficient oxygen supply, cancer cells still prefer glycolysis, termed aerobic glycolysis. Dr. Warburg viewed cancer as a mitochondria disease. Cancer cells were

positioned to have dysfunctional mitochondria and thus compensated with increased glycolysis to sustain their energy level. This hypothesis was slowly discredited and replaced by genetic models of cancer in the 1980s while changes in tumor metabolism were viewed as secondary events. However, recently, some evidence has suggested that altered tumor cell metabolism could be caused by tumorigenic mutations. Aerobic glycolysis may confer a growth advantage to cancer cells in the tumor microenvironment. [^{18}F] fluorodeoxyglucose positron emission tomography ([^{18}F] FdG-PET) patient scans demonstrated the majority of more than 90% metastatic tumors were highly glycolytic (Czernin and Phelps 2002). Under normoxia, nonmetastatic cells consumed less glucose and expressed lower level of HIF-1α. In contrast, metastatic cells had increased glycolysis and expressed higher HIF-1α, indicating that dysregulated HIF-1α to induce the Warburg effect in cancer cells (Robey, Lien et al. 2005). Moreover, glucose levels were significantly lower in metastatic carcinomas than in non-metastatic carcinomas with the mean ± SD of 0.3 ± 0.2 umol/g versus 1.0 ± 0.4 umol/g (p-value=0.0020). No patients with the glucose level above 0.9 umol/g had distant metastasis of rectal adenocarcinomas(Walenta, Chau et al. 2003).

1.2.2 Hypoxia-inducible factor (HIF) drives increased glycolysis

Cell-surface glucose transporter GLUT1 and nearly all of the enzymes involved in glycolysis are transcriptionally induced by HIF-1 following hypoxia(Semenza, Roth et

al. 1994). In HIF-1α knockouts, these transcripts are significantly decreased. These results show that the glycolytic switch seen in tumors is not simply due to a limited oxygen supply that blocks oxidative phosphorylation, but it is also through the transcriptional program induced by HIF-1 which upregulates all glycolytic enzymes (Iyer, Kotch et al. 1998; Ryan, Lo et al. 1998; Greijer, van der Groep et al. 2005). In some cases, such as in the renal-cell carcinoma cell line RCC4, constitutively increased HIF-1α levels are associated with constitutively higher rates of glucose consumption. RCC4 cells express constitutively high HIF-1α due to a mutation in the von Hippel-Lindau (VHL) ubiquitine ligase which degrades HIF-1α. Complementing VHL in these cells results in normal level of HIF-1α expression and significantly decreases aerobic glucose consumption (Robey, Lien et al. 2005). Additionally, pyruvate also regulates hypoxia-inducible gene expression independently of hypoxia by stimulating HIF-1α accumulation. This finding supports the role of pyruvate in metabolic signaling and indicates potential mechanism of increased glycolysis to promote the survival and malignant transformation of cancer cells (Lu, Forbes et al. 2002).

1.2.3 Akt drives increased glycolysis

Elstrom et al. demonstrated that Akt alone is sufficient to induce glucose uptake and aerobic glycolysis. Additionally, tumor cells with activated Akt undergo increased cell death when subjected to glucose starvation (Elstrom, Bauer et al. 2004). Loss of ATP

production from cells with repressed glycolysis under low glucose conditions could be compensated for by activators of fatty acid oxidation to generate ATP in mitochondria (Buzzai, Bauer et al. 2005). Ramanathan et al. directed a systematic study using a panel of cells transformed with different oncogenes and measured their metabolites and susceptibilities to inhibitors of glycolysis or oxidative phosphorylation. They found that transformed cancerous cells relied more on glycolysis for energy generation than on aerobic respiration and were more sensitive to glycolysis inhibitors (Ramanathan A 2005).

1.2.4 AMPK as an energy sensor

The AMP-activated protein kinase (AMPK) is a heterotrimer, composed of an α catalytic kinase subunit, β and γ regulatory subunits. AMPK is activated by a decreased intracellular ATP: AMP ratio, which often occurs in cells under hypoxic or nutrient-deprived conditions (Kahn, Alquier et al. 2005). LKB1, a tumor suppressor, previously identified to be mutated in the rare autosomal dominant Peutz-Jeghers syndrome (PJS), was found to be the upstream kinase of AMPK (Alessi, Sakamoto et al. 2006; Hardie, Hawley et al. 2006). In mouse embryonic fibroblasts (MEFs) from LKB1 knockout mice, energy stresses raising AMP, such as glucose deprivation, do not induce AMPK (Hawley, Boudeau et al. 2003; Shaw, Kosmatka et al. 2004). AMPK-mediated suppression of mTOR signaling is important to determine the cellular responses under

glucose deprivation (Inoki, Zhu et al. 2003; Corradetti, Inoki et al. 2004; Shaw, Bardeesy et al. 2004). Activated AMPK phosphorylates many metabolic enzymes which can induce changes in gene expression. 6-phosphofructo-2-kinase (6PF2K) is among the targets of AMPK phosphorylation and induces the activation of fructose-2,6-bisphosphatase (F26BP) which causes phosphofructokinase activation as well as increases glycolysis (Hardie 2000).

1.3 Lactic acidosis

1.3.1 Lactate accumulation in solid tumors

Lactate accumulation and lactacidemia are often regarded as the surrogate phenotype of tumor hypoxia(Stubbs, McSheehy et al. 2000). On the other hand, recent molecular research has demonstrated aerobic lactate production from glycolysis caused by genetic alterations, such as increased expression of oncogenes and decreased expression of tumor suppressors (Dang and Semenza 1999; Semenza 2002). These findings partially confirmed Dr. Warburg's early observations and hypothesis that cancer is a metabolic disease with increased aerobic glycolysis and decreased oxidative phosphorylation in cancer cells. High lactate concentrations have been observed in tumors with more metastatic potential in cervix carcinoma, head and neck cancer, and colorectal adenocarcinoma. Additionally, many studies of tumor lactate levels have revealed that tumors with higher concentrations of lactate are associated with worse overall survival

and disease-free survival while tumors with low lactate concentrations are associated with better overall and disease-free survival in cervix carcinoma and head and neck cancers (Schwickert, Walenta et al. 1995; Brizel, Schroeder et al. 2001; Walenta and Mueller-Klieser 2004). The technique of imaging bioluminescence was applied to measure lactate levels in metastatic and non-metastatic human rectal adenocarcinomas. Conclusively, lactate levels were significantly higher in metastatic carcinomas than non-metastatic carcinomas with the mean ± SD of 13.4 ± 3.8 umol/g versus 6.9 ± 2.5 umol/g (p-value = 0.0046). Moreover, distant metastasis was not observed in patients with tumor lactate levels below the population median of 8.0 umol/g (Walenta, Chau et al. 2003). The increased lactate production is associated with increased expression of LDH-A(Koukourakis, Giatromanolaki et al. 2006) and the monocarboxylate transporter MCT4, which exports lactate from cancer cells(Koukourakis, Giatromanolaki et al. 2007). Lactate had always been regarded as an important metabolic product and recently Sonveaux et al. discovered it could also be used as the fuel for oxygenated tumors (Sonveaux, Vegran et al. 2008). They discovered that tumor cell populations are heterogeneous with oxygenated and hypoxia regions. Hypoxic tumor cells mainly use glucose for glycolysis to produce energy whereas oxygenated tumor cells can use the lactate generated from glycolysis as the energy source. By inhibiting monocarboxylate transporter 1 (MCT1) which blocks lactate uptake by oxygenated tumor cells, these cells are forced to use glucose as the energy source while hypoxia tumor cells die from glucose

starvation(Sonveaux, Vegran et al. 2008). This elegant study provided a therapeutic implication to treat cancer by manipulating lactate in tumor cells.

1.3.2 Acidosis

There are multiple factors and complex mechanisms causing the acidification of the tumor microenvironment. Glycolytic lactate production and release are generally considered the major cause of the acidic tumor microenvironment. Additionally, hypoxia stimulates glycolysis and further enhances pHe acidification(Gatenby and Gillies 2004). Nevertheless, tumors in nude mice derived from cells lacking lactate dehydrogenase are still able to acidify the metabolic microenvironment(Yamagata, Hasuda et al. 1998). Restricting blood flow to murine tumors by vascular clamping has been shown to stop lactate production and release. However, the microenvironment continued to acidify(Parkins, Stratford et al. 1997). These results indicate other mechanisms are involved in extracellular acidification. Transformed and tumor cells, compared with normal cells, had reversed pH homeostasis. Normal cells have resting intracellular pH (pHi) of 6.9-7.1 and extracellular pH (pHe) of 7.2-7.4, whereas transformed and cancer cells have pHi of 7.2-7.7 and pHe of 6.2-6.8(Cardone, Casavola et al. 2005). Cancer cells overexpress the sodium-hydrogen exchanger NHE1 and carbonic anyhdrase 9, functioning to maintain an alkaline intracellular pH with an acidic extracellular pH(Fang, Gillies et al. 2008). Low extracellular pH is correlated with

resistance to chemotherapy(Raghunand and Gillies 2000) and, increased chromosomal instability *in vitro*(Morita, Nagaki et al. 1992; Morita 1995)

1.4 The bridge to link in vitro and in vivo

1.4.1 Studying tumor microenvironment in vivo

The physiology of the tumor microenvironment was able to be directly measured with several tools *in vivo*. EF5, the eppendorf polarographic probe was applied to measure oxygen tension, pH probes to measure acidity, bioluminescence techniques to detect lactate/glucose level ((Kallinowski, Schlenger et al. 1989; Mueller-Klieser and Walenta 1993; Thews, Kelleher et al. 1995; Dewhirst, Klitzman et al. 2000). The results of measurements acquired from various tumors in human and animal models reflected large variations between tumors. These measurements were indicative of tumor behaviors thus predictive of clinical outcomes and could be utilized to help assess clinical outcomes (Mueller-Klieser and Walenta 1993; Schwickert, Walenta et al. 1995; Walenta, Salameh et al. 1997; Walenta, Wetterling et al. 2000; Brizel, Schroeder et al. 2001; Walenta and Mueller-Klieser 2004). The elucidation of molecular mechanisms underlied this complex tumor microenvironmental physiology *in vivo* could advance people's understanding of cancers significantly.

Although measurements of the physiological parameters *in vivo* offered valuable information of tumors, these procedures were relatively inapplicable as a clinical routine

due to their delicate requirements, such as tumor samples being snap frozen in sophisticated laboratory settings and the invasive nature of these measurements. The other obstacle was the lack of molecular information to characterize oncogenic stages and how these differences were associated with the clinical phenotypes.

1.4.2 Studying tumor microenvironment in vitro

The well-established cell culture systems provided us the powerful tools to investigate tumor microenvironments *in vitro*. Complex microenvironmental stresses were analyzed and decomposed before being individually applied to cells to allow the casual relationships of each individual stress to a particular cell behavior being examined. In addition, genetic and pharmacological manipulations could be applied to evaluate the molecular mechanisms.

For instance, the stress of low oxygen (hypoxia) being applied to cells *in vitro* was shown to promote angiogenesis, cellular migration and energy consumption, which provided biological rationales for the potential mechanisms of hypoxia responses to be associated with poor clinical outcomes (Harris 2002; Semenza 2002). High concentration of lactate (lactosis) would modulate NAD/NAD^+ balance, induce hypoxia pathways and increase CD44 expression as well as activate CD44 and hyaluronan expression in fibroblasts when being applied to cells *in vitro*(Lu, Forbes et al. 2002; Stern, Shuster et al. 2002; Formby and Stern 2003; Walenta and Mueller-Klieser 2004). Acidosis was shown to

elevate angiogenesis, cellular migration and tissue remodeling (Xu and Fidler 2000; Fukumura, Xu et al. 2001). Similarly, lactic acidosis treatment to cells *in vitro* triggered calcium signaling(Huang, Swietach et al. 2008), HIF1α stabilization(Mekhail, Gunaratnam et al. 2004), and cell death(Graham, Frazier et al. 2004). Lactic acidosis also had been shown to modulate gene expression in different cell types, including melanoma cells(Moellering, Black et al. 2008), human mesenchymal stem cells(Zieker, Schafer et al. 2008) and renal cells(Nowik, Lecca et al. 2008). These in vitro studies demonstrated the impact of these microenvironmental stresses on tumor progression. However, the relevance of these observations in human tumors *in vivo* was not accessible.

1.4.3 Gene signatures as the bridge to link in vitro and in vivo studies

Microarrays have become one of the most powerful tools to simultaneously examine multiple genes. Expression profiles from microarrays can be used as the surrogate of the molecular phenotype of cellular responses to each applied perturbation *in vitro*. On the other hand, expression profiles acquired from tumor cells provided a lot of genetic and molecular information that would be extremely difficult to get through histology, traditional diagnostics or harsh, invasive techniques. With the help of bioinformatics and statistics, the molecular phenotypes acquired from cell cultures could be projected into the molecular phenotypes of tumors and provide us with prognostic

predictions. This method has been successfully demonstrated in wound healing(Chang, Nuyten et al. 2005), hypoxia(Chi, Wang et al. 2006) and oncogenic signaling(Bild, Yao et al. 2005). These approaches to translate findings obtained with defined cell culture manipulations *in vitro* to the behaviors of human cancers *in vivo* by recognizing similar molecular mechanisms were fundamental to our research.

1.5 Goals of the project

There were several goals I would like to achieve through this project. First, I wanted to examine whether lactic acidosis, as one of the tumor microenvironment stresses, was able to induce a specific lactic acidosis response or had to depend on hypoxia as the main stimulant. I wanted to know the prognostic value of the lactic acidosis signature, which could later be developed as a diagnostic parameter. Most importantly, I wanted to find potential mechanisms responsible for the lactic acidosis response and identify genes critical for this event.

Chapter2

Materials and Methods

Cell culture

Human Mammalian Epithelial Cells (HMEC) were cultured in MEGM media (Cambrex). The prostate carcinoma cell line DU145 was cultured in RPMI1640 with 10%(v/v) FBS, 1% sodium pyruvate, 1% L-glutamine, 1% HEPES and 1% antibiotics (penicillin, 10000 UI/ml; streptomycin, 10000 UI/ml). The colon adenocarcinoma cell line WiDr and the cervix squamous cell carcinoma cell line SiHa were cultured in DMEM with 10% FBS and 1% antibiotics. The breast cancer cell line MCF7 was cultured in DMEM (GIBCO11995) with 10% FBS, 1X non-essential amino acids and 1% antibiotics. Mouse embryonic fibroblast (MEF) cells were cultured in DMEM with 15%FBS.

Conditions mimicking different tumor microenvironmental stresses

Lactic acidosis conditions were created by adding 25 mM lactic acid (Sigma) and the pH was adjusted to 6.7. HEPES (25 mM) was added to DMEM media to stabalize pH. Hypoxia was created by adjusting the incubation chamber with the indicated oxygen levels of 2%, 1% or 0.5% oxygen, depending on the experiments. Lactosis was created by adding 25mM sodium lactate (Sigma). Acidosis was created by titrating with

1M HCl to reach the desired pH values. Glucose deprivation was created by using 0 Glucose/L DMEM media (GIBCO11966).

RNA Isolation and microarray analysis

Cells were washed with ice-cold 1X PBS three times and lysis buffer were applied to them. RNAs were extracted with the miRVana kit (Ambion) following the manufacturer's protocol. Cell lysates were detached by cell scrappers and transferred to eppendorf tubes with the homogenite additive buffer being added in at the one tenth of the amount of lysis buffer. The mixture was incubated on ice for 10 min. Applying equal amount of acid phenol chloroform as the lysis buffer and vortexing for 1-2 min, followed by centrifuge for 5 min at 10,000g at room temperature. RNAs were in the aqueous phase (upper layer) so collecting the supernatant containing the RNAs and transferring them to new eppendorf tubes. 1.25X volume to the supernatant of 100% ethanol was added and mixed thoroughly with it. The lysate/ethanol mixture was passed through filter cartridges by centrifugation. Washing buffer 1, followed by washing buffer 2/3 were applied to the cartridges and discarded. Finally, the elution buffer pre-heated to 95C was used to elute out RNAs, which were then subjected to concentration measurements and quality checks before being hybridized to Affymetrix Hu133plus2 gene chips. The data in CEL files were first normalized by Expression Console (Affymetrix) to generate RMA files, which could then be analyzed with cluster3.0 and

displayed by treeview(Eisen, Spellman et al. 1998). All of the microarray results were deposited in the GEO database with the GEO accession numbers of GSE9649 (HMEC cells) and GSE19123 (MCF7 cells).

Realtime RT-PCR

RNAs were reverse-transcribed to cDNAs with the following protocol. In each reaction, 1ug RNA was used and the total volume of RNA solution was brought to 10ul with the addition of picopure water. 1ul of 0.5ug/ul oligo dT and 1ul dNTP of the concentration of 10mM were added, followed by 65°C, 5 min incubation to denature the secondary structures of RNAs. Samples were put on ice for 2 min for primers to anneal and 4ul 5X first strand buffer, 0.1M DTT, 1ul RNase inhibitor and 1ul Superscript II were added afterwards. Reactions were treated with 42°C for 2 hour, 70°C for 15 min, and 4°C finally. 30ul 1X TE buffer was added to have the final volume of 50ul of each reaction. Quantitative PCR was performed using Power SYBRGreen PCR Mix (Applied Biosytem). The following primers were used: ErbB3 Forward: CAGGGGTGTAAAGGACCAGA, Reverse: CGCCAGTAGAGAAAAGTGCC, CD55 Forward: AGGTCCCACCAACAGTTCAG, Reverse: AAAATGCTTGGTTGTCCTGG, PLAU Forward: TGTGAGATCACTGGCTTTGG, Reverse: ACACAGCATTTTGGTGGTGA, SOD2 Forward: TTTGGGGACTTGTAGGGATG, Reverse: AGAAAGCCGAGTGTTTCCCT , Actin-beta Forward:

CTCTTCCAGCCTTCCTTCCT, Reverse: AGCACTGTGTTGGCGTACAG , B2M Forward: TGCTGTCTCCATGTTTGATGTATCT, Reverse: TCTCTGCTCCCCACCTCTAAGT, TXNIP Forward: CTGGCGTAAGCTTTTCAAGG, Reverse: AGTGCACAAAGGGGAAACAC , ARRDC4 Forward: CCCCCTCCCACATGGTCACA Reverse: TCCCTGGCTCCCTTCCATGTGT

Serum stimulation of Akt activation and western blot analysis

DU145 cells were serum-starved (0.5%FBS) for 24 hrs, then incubated for 24 hr in serum starved (0.5%FBS) media (as the control) or media with 25mM lactic acid. Then 20%, 10%, or 5% FBS were applied for 30 mins to induce Akt activation. Proteins were extracted with a PARIS kit (Ambion) and equal amounts of protein were loaded onto SDS-PAGE gels and detected with pSer473 Akt antibody (Cell Signaling), total Akt antibody, or the β-tubulin antibody (internal loading controls).

Glucose and lactate Measurement

WiDr and SiHa cells were plated in six-well plates at a density of 800,000 cells per well. The next day fresh media for the respective conditions, including control, 25mM lactic acidosis, 25mM sodium lactate, and acidosis of pH6.7, pH6.5 and pH6.0 were applied to cells for 48 hrs under either normoxia or hypoxia (0.5% O_2). After a 48hr incubation, media were collected for glucose (ACCU-CHECK, Roche) and lactate

(ARKRAY) measurements with respective meters and the results were normalized by cell numbers to obtain the glucose consumption and the lactate production amount per million cells. To count cell numbers, cells were trypsinzed with 0.5ml trypsin-EDTA to cover the surface of the well. Once the cells were fully detached and became round in shape, 2.5ml 1X PBS was added to each well and these lysates were resuspended by pipetting up and down for at least seven times. Finally, 100ul of the cell lysates were acquired and put in the counting cup with 10ml 1X PBS for machine counting with the cell coulter (Beckman).

ATP Determination

SiHa cells were plated at a density of $2x10^4$cells/ml. The next day, respective media for control and 25mM lactic acidosis, as well as media containing drug inhibitors, 2-DG and rotenone (Sigma), were applied. Cells were then incubated under normoxia or hypoxia (1% oxygen) respectively. ATP was measured by ATPlite 1 step luminescence ATP detection assay system kit with the protocol provided by the manufacturer after 48 and 72 hours (Perkin Elmer). To prevent the interference caused by different colors of control versus lactic acidosis media, we replaced culture media with PBS right before the addition of substrate solution. An ATP standard curve ranging from the concentrations of 10uM, 1uM, 100nM, 10nM, 1nM to 100pM in triplicate was generated to help assess the accuracy of the ATP measurements.

RNA interference

The best conditions to achieve the highest efficiencies of the siRNA transfections were determined by fluorescent microscopy and flow cytometry using siGLO LaminA/C positive control siRNAs (Dharmacon) at the concentrations of 50nM or 100nM with different volumes of lipofectamine 2000 in the range of 1ul to 2.5ul applied (Invitrogen). The best condition was determined as to use 100nM siRNA with 2.25ul lipofectamine per 100ul of reaction in 24-well with the transfection efficiency over 80%. For the actual experiments, MCF7 cells were plated in 12-well plates at 200,000 cells per well. Once the cells reached 60% confluence, 100nM siRNAs targeting the gene of interest and non-targeting control were transfected using lipofectamine 2000 (Invitrogen). To verify the successful knocking down of the intended transcripts, RNAs were isolated with miRVana kit (Ambion) 24 or 48 hours after transfection. Proteins were collected by lysing the cells with RIPA buffer 48 or 72 hours after transfection and subjected to realtime RT-PCR as well as western blot analyses.

Western blot analysis

Proteins were collected with RIPA buffer containing protease inhibitors and their concentrations were measured with the Bradford assay (Bio-Rad). Equal amounts of proteins were loaded into SDS-PAGE gels for the protein expression analyses. After gel

electrophoresis, proteins were transferred to polyvinyl fluoridine or nitrocellulose membranes, then blocked with 5% skim milk and incubated with antibodies. Primary antibodies against AMPK, S6K (Cell Signaling), TXNIP (MBL) and MondoA were applied following the manufacturers' protocols. Depending on the primary antibodies, secondary antibodies of either goat anti-rabbit IgG or goat anti-mouse IgG were applied. Finally, ECL detecting reagent was applied, andresults were developed on film.

Glucose uptake assays

MCF7 cells were plated in 6-well or 12-well plates at 800,000 or 200,000cells per well. Once cells were more than 75% confluent, they were washed with 1X PBS twice, and then, serum starvation media (0.1%FBS) was applied for 3 hrs. Cells were then treated with varying conditions for the desired time. MEF cells were plated in 12-well plates at 100,000 cells per well. Once they reached more than 70% confluence, the various indicated conditions were applied for 4 hrs. For glucose uptake measurement, cells were then washed with 37°C KRH buffer twice. Then, 500 ul or 200 ul KRH buffer containing 0.5 uCi or 0.2 uCi 2-deoxy-D-glucose (GE Healthcare) was added for 1 hr in a 37°C incubator. Cytochalasin B (20uM) (Sigma) was added for negative controls. After incubation, cells were washed three times with 1 ml or400 ul of ice-cold KRH buffer containing 20 mM glucose and 0.5 mM phloretin(Sigma) to quench the glucose uptake. Finally, cells were lysed with 1 ml or400 ul RIPA buffer and the lysates were subjected to

liquid scintillation counting. Protein concentrations were measured with Bradford assay (Bio-Rad). To measure the glucose uptake of genetically-manipulated cells, glucose uptake was measured 24 or 48 hours after the transfections.

Chapter 3

Genomic Analysis of the Lactic Acidosis Response in Human Cancers

Introduction

We created tumor microenvironment *in vitro* by manipulating the mammalian cell culture conditions that could mimic relevant stresses *in vivo* and examined the effect of individual microenvironment stresses on cells. Drugs and genetic tools were also applied to elucidate the molecular mechanisms and genetic circuitry responsible for these cellular responses. Previous studies show that hypoxia *in vitro* promotes angiogenesis, energy consumption and cellular migration; these relationships, provide the rationale for the association of hypoxia with bad prognosis in cancer patients (Harris 2002; Semenza 2002). Applying lactic acidosis to cell cultures had been shown to induce the angiogenic genes VEGF and IL8 (Xu and Fidler 2000; Fukumura, Xu et al. 2001; Shi, Le et al. 2001), calcium signaling(Huang, Swietach et al. 2008), HIF1α stabilization(Mekhail, Gunaratnam et al. 2004), cell death(Graham, Frazier et al. 2004) and other transcriptional changes (Moellering, Black et al. 2008; Nowik, Lecca et al. 2008; Zieker, Schafer et al. 2008). However, applying these observations acquired from *in vitro* perturbations to refer to *in vivo* cancer phenotypes is relatively indirect. Gene signatures from microarrays helped solve this by providing surrogate molecular phenotypes from

manipulated cultured cells representing defined biological processes *in vitro* that can then be assessed in human cancer samples *in vivo*. This approach has identified the importance of wound healing, hypoxia responses and oncogenic mutations in tumor progression (Chang, Sneddon et al. 2004; Bild, Yao et al. 2006; Chi, Wang et al. 2006). Our study utilizes this strategy to apply lactic acidosis to cells *in vitro* and generate surrogates of the molecular phenotypes with the help of microarrays, to further assess its impact *in vivo* in human cancers. This is followed by more experiments *in vitro* to elucidate the molecular mechanisms of the lactic acidosis response.

Results

Cellular responses to lactic acidosis are distinctive from those of hypoxia

To clarify the impact of hypoxia and lactic acidosis on the cancer cells, human mammalian epithelial cells (HMEC) were growth factor and serum-starved for 24 hours and exposed to regular, neutral pH media (control), 2% oxygen-hypoxia, 25 mM lactic acidosis with pH 6.7 or a combined treatment of hypoxia and lactic acidosis for 24 hours. These conditions are meant to mimic the levels of respective stresses in human cancers. RNAs were isolated and applied to Affymetrix Hu133plus 2 microarrays, which can measure the expression of more than 54,000 probe sets and at least 47,000 transcripts and variants.

Gene expression profiles were normalized by RMA and filtered with the criteria of at least two observations with at least 1.75 fold changes to select 4722 probe sets, mean-centered and hierarchically-clustered(Eisen, Spellman et al. 1998). The clustering result showed different expression patterns of genes induced by hypoxia versus lactic acidosis (Figure 3-1A). EGLN, BNIP3, CAIX and stanniocalcin1, genes that were previously-reported to be induced by hypoxia in studies performed with cDNA arrays(Chi, Wang et al. 2006) showed reproducibly higher expression under hypoxia. However, among these hypoxia-induced genes, EGLN3 and BNIP3 were still upregulated by the combined treatments of hypoxia and lactic acidosis whereas CAIX and stanniocalcin1 were repressed by the combined stresses (Figure 3-1B). ERBB3, CXCL16, PLAUR,MHC I, angiogenin, IL15, and CD55 were among the most significantly induced genes by lactic acidosis. Most of these genes were further upregulated by the combined treatment of lactic acidosis and hypoxia (Fig 3-1B). Additionally, the inductions of ERBB3, CD55 and PLAU by lactic acidosis and their further upregulation under the combined treatment were confirmed by realtime PCR (Fig 3-1D). On the other hand, genes repressed by lactic acidosis were involved in cell cycle, cell proliferation and glucose metabolism (Fig 3-1B). With the help of statisticians and using Bayesian multivariate regression analysis (Lucas, Carvalho et al. 2006; Seo, Goldschmidt-Clermont et al. 2007) to select for genes with significant expression changes (Bayesian significance of 1%), 217 genes were shown to be induced by hypoxia, 1585 genes to be induced by lactic acidosis and 54 to be shared

in common between the two conditions among the 1802 genes (2%) with significant expression changes (Fig 3-1C).

To further investigate the molecular pathways triggered by hypoxia and lactic acidosis, we analyzed the Gene Ontology (GO) enrichment of the genes induced and repressed by hypoxia and lactic acidosis with GATHER(Chang and Nevins 2006). The top GO terms enriched in the hypoxia-induced genes included hexose metabolism, glycolysis, glucose metabolism and catabolism (Table 3-1) whereas the GO terms enriched in the hypoxia-repressed genes included cell cycles and RNA metabolism (Table 3-2). On the other hand, among the genes induced by lactic acidosis, G-protein coupled receptor signaling, antigen processing and presentation, and cellular catabolism were found to be enriched (Table 3-3) while the top GO terms of genes repressed by lactic acidosis were genes involved in cell cycle, RNA metabolism and RNA processing (Table 3-4).

Table 3-1 GO terms enriched in the hypoxia-induced genes

Upregulated in Hypoxia	Annotation
1	GO:0019318 [7]: hexose metabolism
2	GO:0005996 [6]: monosaccharide metabolism
3	GO:0006096 [8]: glycolysis
4	GO:0006006 [8]: glucose metabolism
5	GO:0006007 [9]: glucose catabolism
6	GO:0019320 [8]: hexose catabolism
7	GO:0046164 [6]: alcohol catabolism
8	GO:0046365 [7]: monosaccharide catabolism
9	GO:0019883 [6]: antigen presentation, endogenous antigen
10	GO:0019885 [6]: antigen processing, endogenous antigen via MHC class I
11	GO:0006066 [5]: alcohol metabolism
12	GO:0016052 [6]: carbohydrate catabolism
13	GO:0044275 [7]: cellular carbohydrate catabolism
14	GO:0030333 [5]: antigen processing
15	GO:0006092 [7]: main pathways of carbohydrate metabolism
16	GO:0044262 [6]: cellular carbohydrate metabolism
17	GO:0019882 [5]: antigen presentation
18	GO:0015980 [6]: energy derivation by oxidation of organic compounds
19	GO:0005975 [5]: carbohydrate metabolism
20	GO:0006955 [4]: immune response
21	GO:0006091 [5]: generation of precursor metabolites and energy
22	GO:0006000 [8]: fructose metabolism
23	GO:0006952 [5]: defense response
24	GO:0006094 [8]: gluconeogenesis
25	GO:0051242 [5]: positive regulation of cellular physiological process
26	GO:0043283 [4]: biopolymer metabolism
27	GO:0043065 [7]: positive regulation of apoptosis
28	GO:0019319 [8]: hexose biosynthesis
29	GO:0046165 [6]: alcohol biosynthesis
30	GO:0046364 [7]: monosaccharide biosynthesis

Table 3-2 GO terms enriched in the hypoxia-repressed genes

Downregulated in Hypoxia	Annotation
1	GO:0016072 [6]: rRNA metabolism
2	GO:0000278 [6]: mitotic cell cycle
3	GO:0006457 [7]: protein folding
4	GO:0044238 [4]: primary metabolism
5	GO:0044237 [4]: cellular metabolism
6	GO:0008152 [3]: metabolism
7	GO:0000279 [6]: M phase
8	GO:0031057 [7]: negative regulation of histone modification
9	GO:0035067 [7]: negative regulation of histone acetylation
10	GO:0051129 [6]: negative regulation of cell organization and biogenesis
11	GO:0007049 [5]: cell cycle
12	GO:0051052 [6]: regulation of DNA metabolism
13	GO:0016070 [5]: RNA metabolism
14	GO:0006364 [7]: rRNA processing
15	GO:0031056 [6]: regulation of histone modification
16	GO:0035065 [6]: regulation of histone acetylation
17	GO:0046502 [7]: uroporphyrinogen III metabolism
18	GO:0006090 [7]: pyruvate metabolism
19	GO:0006423 [9]: cysteinyl-tRNA aminoacylation
20	GO:0006780 [8]: uroporphyrinogen III biosynthesis
21	GO:0006520 [6]: amino acid metabolism
22	GO:0043283 [4]: biopolymer metabolism
23	GO:0043170 [4]: macromolecule metabolism
24	GO:0006396 [6]: RNA processing
25	GO:0000085 [7]: G2 phase of mitotic cell cycle
26	GO:0000244 [10]: assembly of spliceosomal tri-snRNP
27	GO:0008535 [8]: cytochrome c oxidase biogenesis
28	GO:0009303 [8]: rRNA transcription
29	GO:0008283 [4]: cell proliferation
30	GO:0007067 [8]: mitosis

Table 3-3 GO terms enriched in the lactic acidosis-induced genes

Upregulated in LA	Annotation
1	GO:0007186 [6]: G-protein coupled receptor protein signaling pathway
2	GO:0019883 [6]: antigen presentation, endogenous antigen
3	GO:0019885 [6]: antigen processing, endogenous antigen via MHC class I
4	GO:0006888 [6]: ER to Golgi transport
5	GO:0009056 [4]: catabolism
6	GO:0044248 [5]: cellular catabolism
7	GO:0007166 [5]: cell surface receptor linked signal transduction
8	GO:0007243 [6]: protein kinase cascade
9	GO:0009057 [5]: macromolecule catabolism
10	GO:0044265 [6]: cellular macromolecule catabolism
11	GO:0044255 [5]: cellular lipid metabolism
12	GO:0048193 [6]: Golgi vesicle transport
13	GO:0050877 [4]: neurophysiological process
14	GO:0019882 [5]: antigen presentation
15	GO:0030163 [6]: protein catabolism
16	GO:0009966 [4]: regulation of signal transduction
17	GO:0006629 [5]: lipid metabolism
18	GO:0043285 [5]: biopolymer catabolism
19	GO:0043122 [5]: regulation of I-kappaB kinase/NF-kappaB cascade
20	GO:0006643 [6]: membrane lipid metabolism
21	GO:0007249 [7]: I-kappaB kinase/NF-kappaB cascade
22	GO:0009581 [5]: detection of external stimulus
23	GO:0007600 [5]: sensory perception
24	GO:0006886 [6]: intracellular protein transport
25	GO:0043170 [4]: macromolecule metabolism
26	GO:0044257 [7]: cellular protein catabolism
27	GO:0006508 [8]: proteolysis and peptidolysis
28	GO:0030333 [5]: antigen processing
29	GO:0046907 [5]: intracellular transport
30	GO:0044260 [5]: cellular macromolecule metabolism

Table 3-4 GO terms enriched in the lactic acidosis-repressed genes

Downregulated in LA	Annotation
1	GO:0007186 [6]: G-protein coupled receptor protein signaling pathway
2	GO:0007049 [5]: cell cycle
3	GO:0008283 [4]: cell proliferation
4	GO:0043283 [4]: biopolymer metabolism
5	GO:0016070 [5]: RNA metabolism
6	GO:0006396 [6]: RNA processing
7	GO:0044237 [4]: cellular metabolism
8	GO:0050875 [3]: cellular physiological process
9	GO:0006259 [5]: DNA metabolism
10	GO:0044238 [4]: primary metabolism
11	GO:0008152 [3]: metabolism
12	GO:0006139 [5]: nucleobase, nucleoside, nucleotide and nucleic acid metabolism
13	GO:0050874 [3]: organismal physiological process
14	GO:0007154 [3]: cell communication
15	GO:0006397 [7]: mRNA processing
16	GO:0007166 [5]: cell surface receptor linked signal transduction
17	GO:0008380 [7]: RNA splicing
18	GO:0000375 [8]: RNA splicing, via transesterification reactions
19	GO:0000377 [9]: RNA splicing, via transesterification reactions with bulged adenosine as nucleophile
20	GO:0000398 [8]: nuclear mRNA splicing, via spliceosome
21	GO:0016071 [6]: mRNA metabolism
22	GO:0000278 [6]: mitotic cell cycle
23	GO:0006260 [6]: DNA replication
24	GO:0050877 [4]: neurophysiological process
25	GO:0007165 [4]: signal transduction
26	GO:0000279 [6]: M phase
27	GO:0000074 [6]: regulation of cell cycle
28	GO:0000087 [7]: M phase of mitotic cell cycle
29	GO:0000067 [6]: DNA replication and chromosome cycle
30	GO:0000075 [7]: cell cycle checkpoint

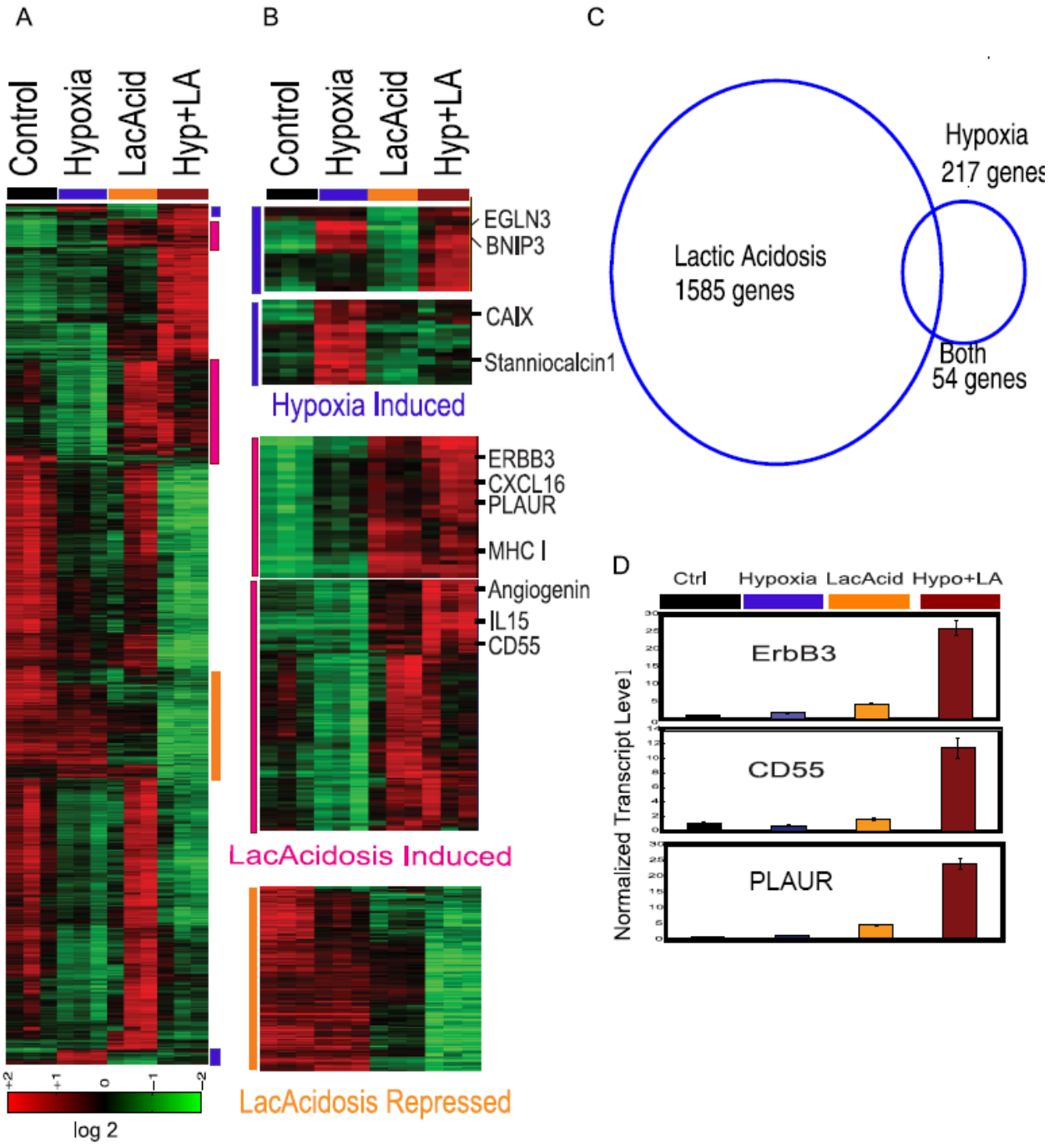

Figure 3-1 Cellular responses to hypoxia and lactic acidosis

Figure 3-1 Cellular responses to hypoxia and lactic acidosis

(A)(B) Gene expression response of HMEC cells exposed to control, hypoxia, lactic acidosis and the combined stresses of hypoxia and lactic acidosis. 4722 genes with expression variations of at least 1.75 fold in two samples were selected and hierarchically clustered. Genes induced by hypoxia (vertical blue bar), lactic acidosis (pink), and repressed by lactic acidosis (orange) are marked and further expanded in (B). (C)Venn diagram showing the number of genes significantly induced by lactic acidosis (1585 genes), hypoxia (217 genes) and both stresses (54 genes). (D) Realtime RT-PCR confirmation of three lactic acidosis-induced genes: ErbB3, CD55 and PLAUR normalized by actin-beta. Their inductions were further enhanced under the combined stresses.

Cellular responses to lactosis and acidosis

High lactate (lactosis) and low pH (acidosis) are both commonlyobserved in tumors; however, they do not necessarily coexist simultaneously. To examine cellular responses to high lactate and acid individually, HMEC cells were exposed to lactosis, created by 25 mM sodium lactate with neutral pH, and acidosis, created by media acidified to pH6.7 with HCl. RNAs were extracted from these cell cultures and normally cultured control HMECs, to be applied to Affymetrix Hu133plus2 microchips for whole human genome expression analysis. Gene expression profiles were normalized by RMA, filtered with the criteria that at least 4 (6 samples in each experimental condition) exhibited at least a 1.75 fold change. Two hundred thirten probes were selected and hierarchically-clustered (Fig 3-2). Acidosis induced a significantly distinct gene expression pattern compared with lactosis (Fig 3-2). Acidosis-induced genes included many genes that were identified to be induced by lactic acidosis whereas lactosis did not have as strong an impact (Fig 3-1B). To determine the relative contributions of acidosis and lactosis to the lactic acidosis response, the gene expression level changes in these three groups were compared. There was a high concordance between transcriptional responses to lactic acidosis and acidosis with similar genes being significantly induced and repressed (Fig 3-3A). On the contrary, this concordance was not seen between the transcriptional responses to lactic acidosis and lactosis. There was also no concordant relation observed between cellular responses to hypoxia and these three other groups (Fig 3-3B). In conclusion, lactic

acidosis (created by lactic acid) and acidosis (created by HCl) stimulated similar genetic responses that were different from the responses stimulated by lactosis (created by sodium lactate) or hypoxia (created by 2% oxygen).

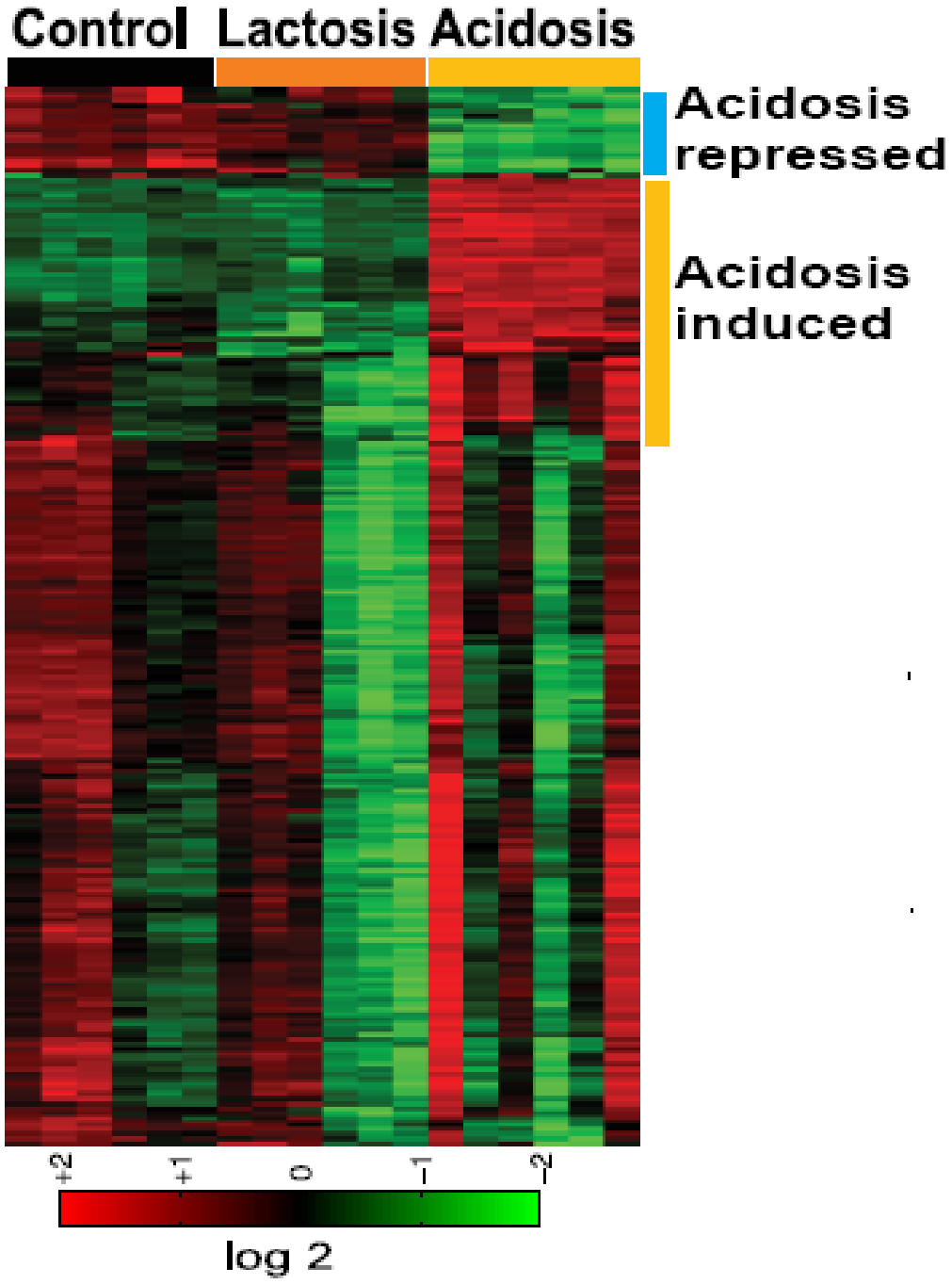

Figure 3-2 Cellular responses to lactosis and acidosis

Figure 3-2 Cellular responses to lactosis and acidosis

HMEC cells were exposed to control, lactosis and acidosis. Filtering criteria with at least 1.75 fold change in 4 samples (from 6 samples) was applied to select for 213 genes. The cluster of gene strongly induced (yellow vertical bar) and repressed (blue vertical bar) by acidosis, in contrast to their expression patterns under control and lactosis.

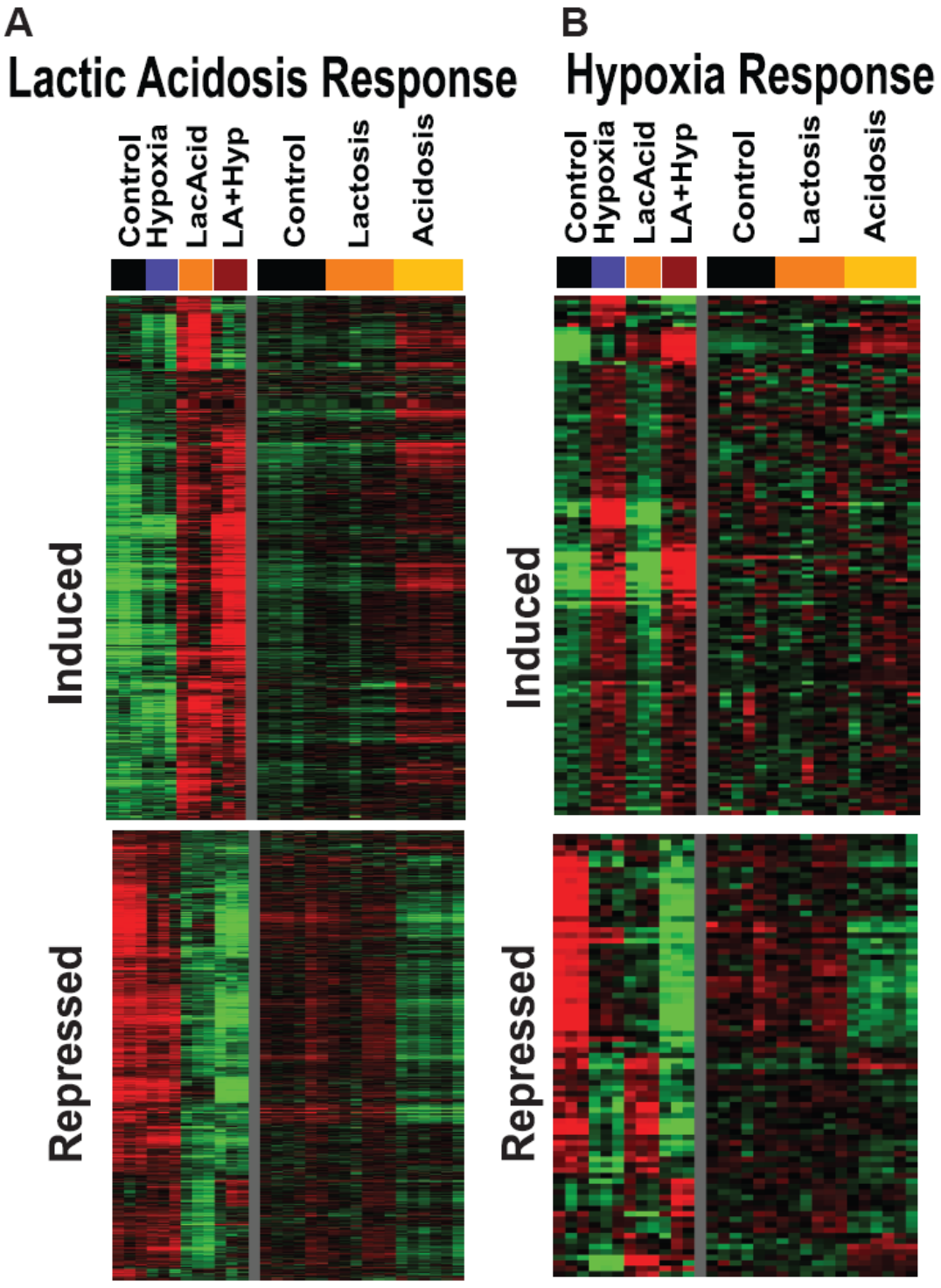

Figure 3-3 Acidosis is the main driving force for lactic acidosis response

Fig 3-3 Acidosis is the main driving force for lactic acidosis response
(A) Genes strongly induced and repressed by lactic acidosis were also being strongly upregulated and downregulated by acidosis. (B) Genes strongly induced/repressed by hypoxia, however, did not show associated regulations by acidosis.

Genomic analysis of hypoxia and lactic acidosis responses in human cancers

It has been previously shown that the hypoxia response elicited from cultured epithelial cells *in vitro* can provide a molecular gauge of the hypoxia response to predict poor clinical outcomes when projected into cancerous human tissues *in vivo*(Chi, Wang et al. 2006).This is done with the principle of projecting the *in vitro* gene signature of the hypoxic "response" or "pathway activity" onto numerical scores of each patient's tumor sample data *in vivo* to acquire the corresponding predictions of values. Similar approaches have been applied in several other studies that used gene signatures generated *in vitro* to predict responses or pathway activities *in vivo*(Huang, Ishida et al. 2003; Chang, Sneddon et al. 2004; Bild, Yao et al. 2005; Chi, Wang et al. 2006; Chi, Rodriguez et al. 2007). Therefore, we acquired gene signatures from cellular responses to different microenvironment stresses and evaluated them in a number of breast cancer patients' datasets via a weighted average of the signature gene set based principal components analysis. Analysis of a Cox survival model showed the hypoxia response gene signature generated here, in accord with previous findings(Chi, Wang et al. 2006), was associated with poor clinical outcomes projected onto breast cancer patients' tumor samples, including the Miller dataset, NKI (Wang) dataset, and the Pawitan and Sotiriou datasets which were two studies of invasive breast carcinoma(Miller, Smeds et al. 2005; Pawitan, Bjohle et al. 2005; Wang, Klijn et al. 2005; Sotiriou, Wirapati et al. 2006) (Fig 3-4A). On the other hand, lactic acidosis signature, when projected onto these clinical

datasets, predicted better clinical outcomes (Fig 3-4B). In the Miller dataset, which includes the p53 status of each tumor(Miller, Smeds et al. 2005), the estimated lactic acidosis pathway activity was higher in tumors with wild-type p53 and lower in those with mutated p53 (Fig 3-4D). Acidosis signature, concordant with the lactic acidosis signature, exhibited better prognosis in these cancer patients *in vivo* (Fig 3-4C). We also used binary logistic regression to examine the probabilities of acidosis with a series of breast cancer cell lines containing different metastatic abilities(Minn, Gupta et al. 2005). Acidosis probabilities were inversely correlated with the aggressiveness of xenografted breast cancer cells. Higher acidosis probability was associated with lesser metastatic potential, and lower acidosis probability was associated with greater metastatic potential (Fig 3-4E).

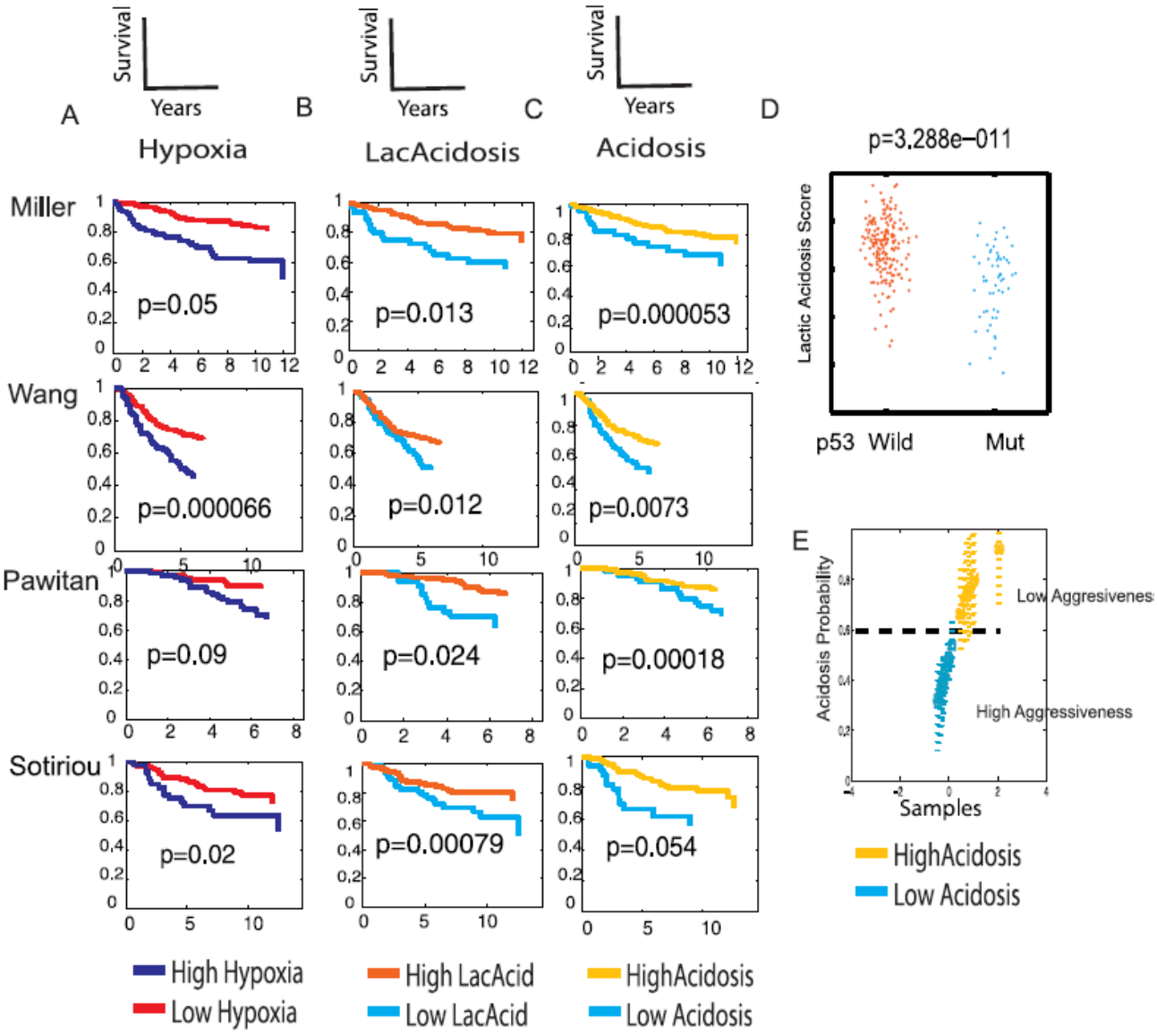

Figure 3-4 The prognostic results from gene signatures of hypoxia, lactic acidosis and acidosis

Figure 3-4 The prognostic results from gene signatures of hypoxia, lactic acidosis and acidosis

(A)Higher hypoxia response would be associated with worse prognosis in four independent breast cancer patients' tumor datasets. (B)Higher lactic acidosis response would be associated with better prognosis, in contrary to that of hypoxia. (C) Acidosis response, in concordance with lactic acidosis response, would be associated with better clinical outcomes. (D)The lactic acidosis response score is significantly higher in tumors with wild-type p53 than those with mutated p53. (E) Acidosis response is reversely-correlated with breast cancer cell lines with different metastatic abilities. Higher acidosis response was associated with lower aggressiveness and vice versa. Figure (A) to (D) were generated by Dr. Joseph Lucas.

Lactic acidosis directs metabolic reprogramming towards aerobic respiration by inhibiting glycolysis

To elucidate the mechanism behind this surprising finding that lactic acidosis projected a better prognosis, pathway composition between tumors with high and low lactic acidosis responses were analyzed with Gene Set Enrichment Analysis (GSEA)(Subramanian, Tamayo et al. 2005). Biological processes involved in the TCA cycle, electron transport and oxidative phosphorylation were enriched in tumors with high lactic acidosis response. Energy in mammalian cell lines is generated from two major pathways, glycolysis and aerobic respiration. Aerobic glycolysis provides a growth advantage to cancer cells, as silencing LDH-A, the gene critical for converting pyruvate to lactate, not only stimulates mitochondrial respiration but also severely diminishes the tumorigenicity of cancer cells and decreases their proliferation under hypoxia(Fantin, St-Pierre et al. 2006). To test the hypothesis that lactic acidosis exerts an anti-Warburg effect *in vitro* to shift energy homeostasis towards utilization of oxidative phosphorylation instead of glycolysis in cancer cells, SiHa cells, cervix carcinoma cells, were treated with rotenone, an inhibitor of mitochondria and aerobic respiration, to examine its impact on ATP production under the control, hypoxia and lactic acidosis conditions. In control conditions without microenvironmental stresses, we observed approximately 35% reduction ATP production from rotenone. This ATP production

sensitivity to rotenone decreased to 16% under hypoxia but increased significantly to 72% under lactic acidosis for 48 hours. This effect was intensified after 72 hours with 82% ATP production sensitivity under lactic acidosis but still 35% under control conditions and 18% under hypoxia (Fig 3-5A). The contribution of ATP production from glycolysis was also tested with the glycolysis inhibitor, 2-DG. Approximately 71% of ATP production was sensitive to 2-DG at 48 hours and this increased to 77% under hypoxia and decreased to 63% after 48 hours under lactic acidosis (Fig 3-5B). These results indicated that under lactic acidosis, ATP production is more reliant on aerobic respiration than on glycolysis, in contrast to hypoxia. Notably, both lactic acidosis and hypoxia reduced ATP production to approximately 50% and 63%, respectively, at in 48 hours and 96 hours and when combined, they dramatically decreased ATP production to ~17.3% of the control level (Fig 3-5C).

To examine the impact of hypoxia and lactic acidosis genetically, genes involved in the metabolic framework were examined. Hypoxia increased the expression of genes involved in glycolysis significantly, including of HK, PFK, ALDO, GAPDH, PGK, PGM, ENO, PK, PDK1 and LDH whereas lactic acidosis, on the contrary, decreased the expressions of all of them (Fig 3-6A). We performed hierarchical clustering to examine the expression of these glycolysis genes under hypoxia, lactic acidosis and the combined stresses and observed that hypoxia significantly induced them. On the other hand, lactic acidosis repressed glycolytic genes both under normoxia and hypoxia (Fig 3-6B). The

mean expression values of these genes induced by hypoxia and repressed by lactic acidosis in these samples (triplicate) were calculated and displayed in Fig 3-6C. When these glycolysis genes were used as the gene signature to predict the prognosis in breast cancer patients' datasets, they recapitulated the prognostic results that high hypoxia response was associated with worse clinical outcomes (Fig 3-7A) but high lactic acidosis response was associated with better clinical outcomes (Fig 3-7B). The correlation of these glycolysis genes induced by hypoxia versus lactic acidosis was negative across all of the breast tumor samples (Fig 3-7C).

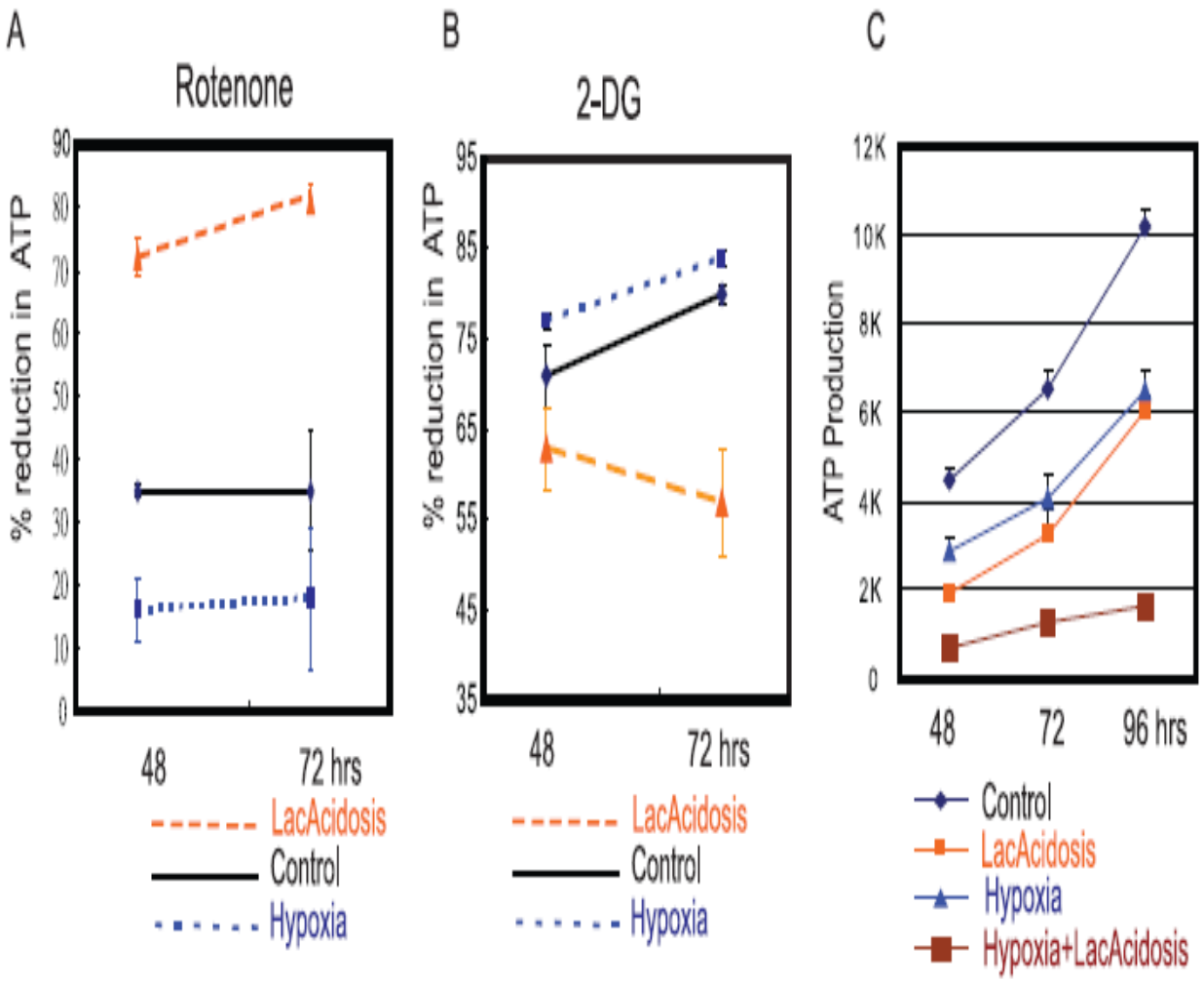

Fig 3-5 Lactic acidosis shifts energy utilization to aerobic respiration by inhibiting glycolysis

Fig 3-5 Lactic acidosis shifts energy utilization to aerobic respiration by inhibiting glycolysis.

(A, B) The contribution to ATP generation by aerobic respiration and glycolysis under conditions of control, lactic acidosis and hypoxia was measured by the degree of inhibition of ATP generation after rotenone (A) and 2-DG (B) treatment. (C)The amount of ATP generation by cells incubated under different conditions at 48, 72 and 96 hours.

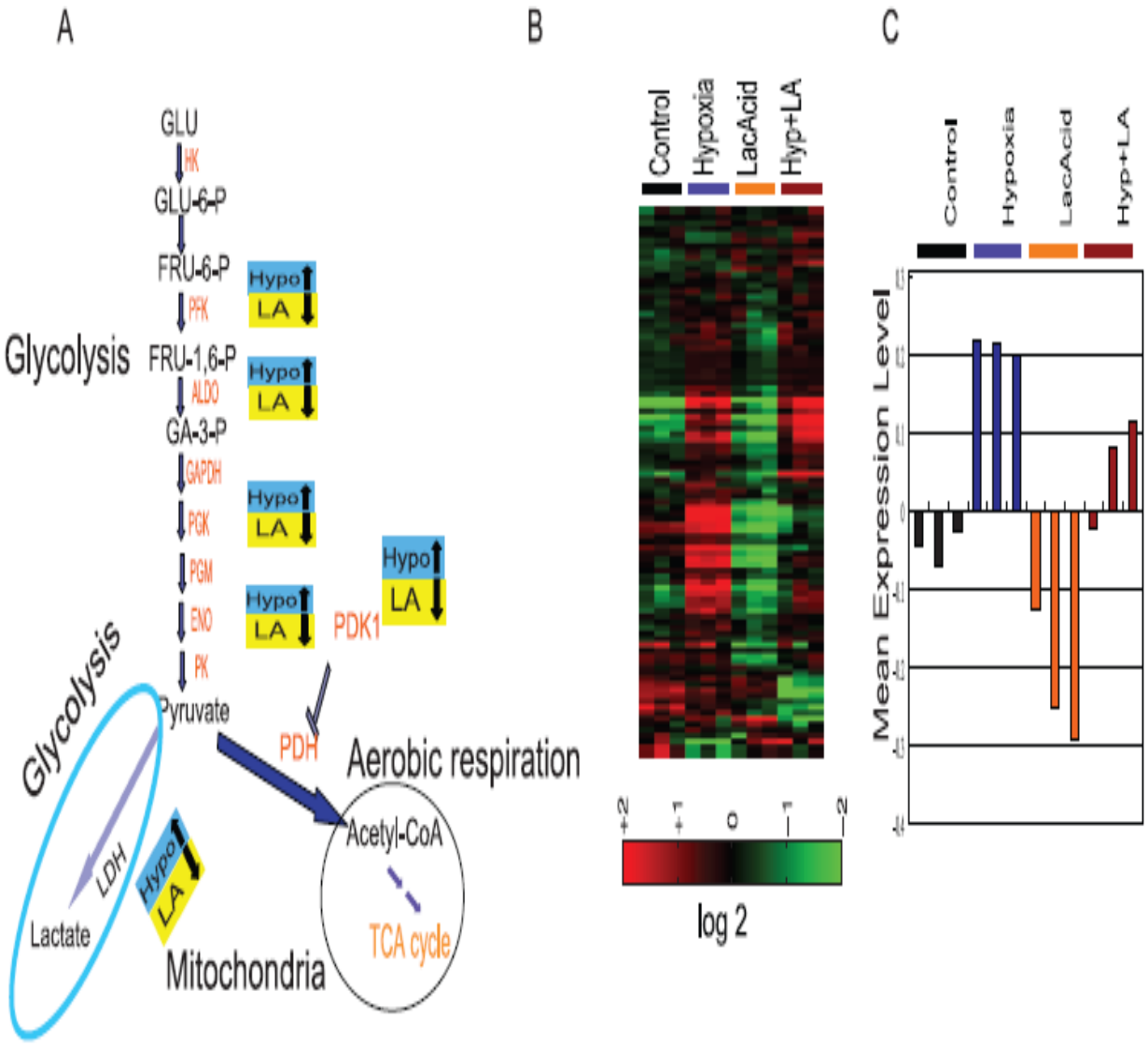

Figure 3-6 Lactic acidosis shifts energy utilization to aerobic respiration by inhibiting glycolysis

Figure 3-6 Lactic acidosis shifts energy utilization to aerobic respiration by inhibiting glycolysis.

(A)Genes regulating the glycolysis pathway were induced by hypoxia and in contrast, repressed by lactic acidosis. (B)Hierarchical clustering result of the genes involved in the glycolytic pathway. (C) The mean expression values of these glycolytic genes.

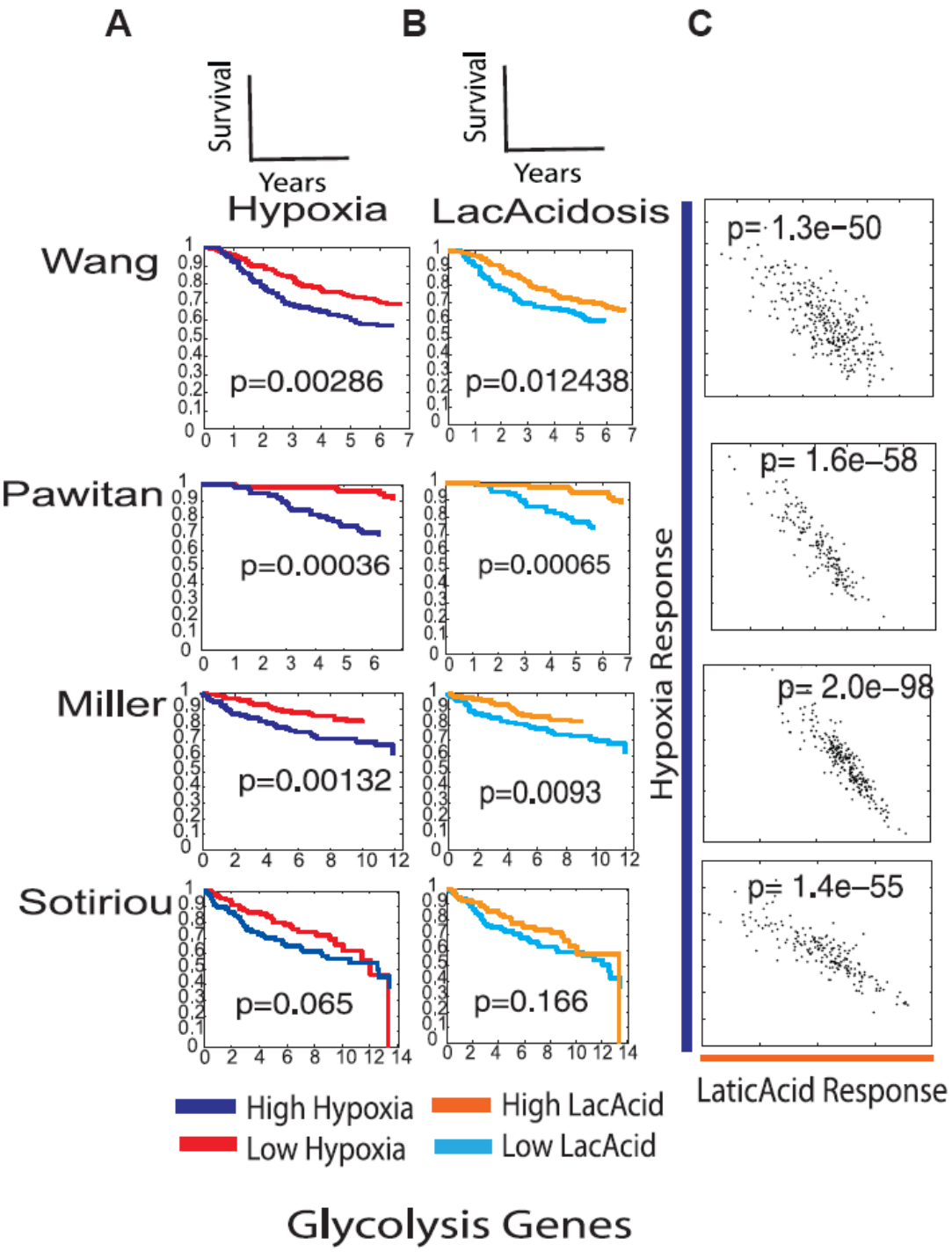

Figure 3-7 Glycolysis genes recapitulated the prognostic power

Figure 3-7 Glycolysis genes recapitulated the prognostic power

(A)(B) The expression of genes in the glycolysis pathway were used to predict pathway activities and stratified the indicated breast cancer samples under hypoxia and lactic acidosis. This small number of genes recapitulated the prognostic results as using the whole hypoxia and lactic acidosis gene signatures. (C) Scatter plots showing the negative correlation between the probability of hypoxia response (Y-axis) and lactic acidosis response (X-axis) of genes in the glycolysis pathway. Each point in the scattered plot represents a single tumor sample. Figure (A) to (C) were generated by Dr. Joseph Lucas.

Lactic acidosis inhibits Akt and the glycolytic phenotypes of cancer cells

To further examine the effect of lactic acidosis in oncogenic signaling, the lactic acidosis signature was queried in the database of connectivity map, composed of gene expression profiles generated from 453 perturbations caused by 164 different small molecules (Lamb, Crawford et al. 2006). The top perturbations positively correlated with the lactic acidosis signature were wortmannin and LY-294002, both of which are inhibitors of phosphoinositide 3-kinase (PI3K) (Fig 3-8A). PI3 kinase phosphorylates phosphoinositides PtdIns(3,4)P2 (PIP2) to PtdIns(3,4,5)P3 (PIP3) and recruits Akt from the cytosol to the cell membrane to trigger its phosphorylation. Akt phosphorylation then activate Akt, which promotes cell proliferation, glucose uptake, and the oncogenic potential of cells(Vivanco and Sawyers 2002). To examine the relationships between lactic acidosis, acidosis and oncogenic signaling, several gene signatures with dysregulation in the oncogenic pathways induced by genetic manipulations were used and a consistent inversed relation between the acidosis and Akt pathway signatures were identified in three breast cancer patients' datasets (Miller, Smeds et al. 2005; Minn, Gupta et al. 2005; Pawitan, Bjohle et al. 2005). Higher acidosis response was correlated to lower Akt activity in tumors and lower acidosis response was correlated with higher Akt activity (Fig 3-8B). This inverse correlation between acidosis response and Akt signaling can be further shown in another independent mouse model study of prostate neoplasia with wild-type and Akt-transgenic mice. There was high acidosis response in

wild-type mice but a low acidosis response in Akt-transgenic mice with characteristics of prostate cancer (Fig 3-8C). As the inverse correlation between lactic acidosis/acidosis responses with Akt signaling was reproducibly shown with bioinformatics analyses, we then decided to test whether lactic acidosis would abolish Akt signaling in a prostate cancer cell line, DU145. When cultured in serum-free conditions, the Akt enzymatic activity of DU145 cells was inhibited, as shown by the absence of phosphorylation of Ser-473. Serum exposure for 30 minutes activated Akt activity by phosphorylating Ser-473. However, prior exposure to lactic acidosis for 24 hours abolished this activation by serum. Even when lactic acidosis was removed and replaced with neutral pH media during serum exposure, this inhibitory effect of lactic acidosis on Akt phosphorylation still persisted (Fig 3-8D). Given the results that lactic acidosis decreased glycolysis and abolished Akt signaling, we further tested its effects in glucose consumption and lactate production. WiDr (colon cancer) and SiHa (cervix cancer) cells were incubated under control, 25mM lactic acidosis, 25mM sodium lactate, and acidosis of three different acidity (pH 6.7, pH 6.5 and pH 6.0) under either normoxia or hypoxia (0.5% oxygen). Sodium lactate had no effects on glucose consumption or lactate production whereas lactic acidosis and acidosis both decreased them. Moreover, increased acidity enhanced the reduction of glucose consumption and lactate production and these effects became even more significant under hypoxic compared with normoxic conditions (Fig 3-9A, Fig 3-9B).

Taken together, our evidence suggests that in the homeostasis of cellular energy production, hypoxia enhances glycolysis and represses aerobic respiration. Lactic acidosis, in contrast, decreases glycolysis and shifts energy utilization towards aerobic respiration (Fig 3-10).

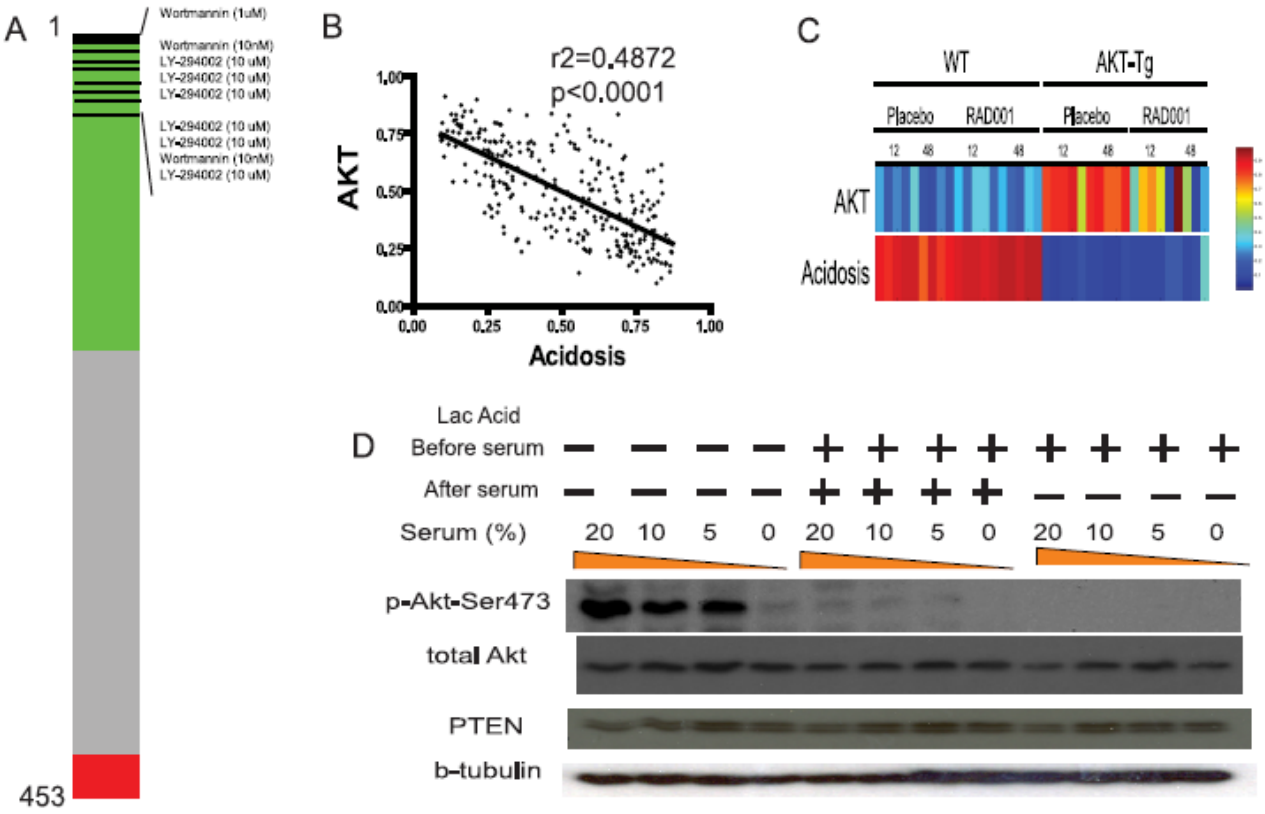

Fig 3-8 Lactic acidosis inhibits Akt of cancer cells

Figure 3-8 Lactic acidosis inhibits Akt of cancer cells.

(A)PI3K inhibitors are highly ranked with lactic acidosis signature in the connectivity map analysis. This "barview" is constructed from 453 horizontal lines, each representing an individual treatment instance, ordered by their corresponding connectivity scores calculated with lactic acidosis signature (+1, top; -1, bottom) with the instances corresponding to wortmannin and LY-294002 shown as black bars. Colors reflecting the sign of their scores (green, positive; gray, null; red, negative).(B)(C)The relationship between the predicted Akt and acidosis response pathway activities in the gene expression pattern of breast cancer (B)and prostate cancer(C) expression studies. The prostate tissue is shown between wildtype (WT) and Akt transgenic mice (AKT-Tg) treated with placebo or mTOR inhibitor, RAD001. (D)The effect of lactic acidosis on Akt phosphorylation in DU145 cells during serum exposure. Serum stimulation was added to DU145 cells placed in 0.2% serum condition for 24 hours without(-) or with (+) 25mM lactic acid. Figure (A) was generated by Dr. Joseph Lucas. Figure (B) and (C) were generated by Dr. Seiichi Mori.

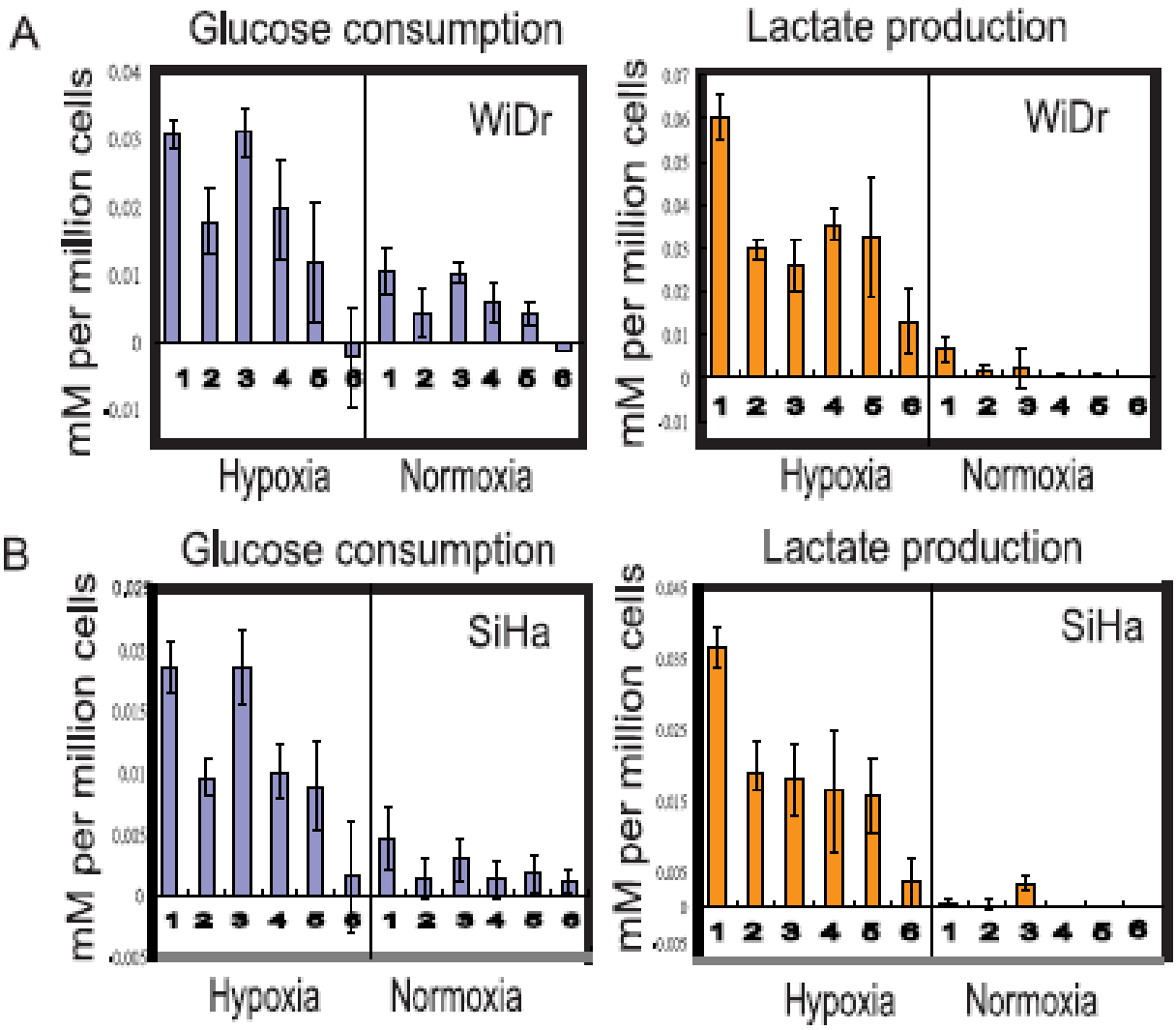

Figure 3-9 Lactic acidosis reduced glucose consumption and lactate production and increased acidity of lactic acidosis enhanced both effects

Fig 3-9 Lactic acidosis reduced glucose consumption and lactate production and increased acidity of lactic acidosis enhanced both effects

(A)(B)The amount (mM)of lactate production (orange) and glucose consumption (blue) in per million cells during 48hours in WiDr(A)and SiHa(B) cells under hypoxia(left) or normoxia(right) with the following media conditions of (1)control, (2)25mM lactic acidosis (pH6.7), (3)25mM sodium lactate, (4)acidosis of pH6.7, (5)acidosis of pH6.5 (6)acidosis of pH6.0.

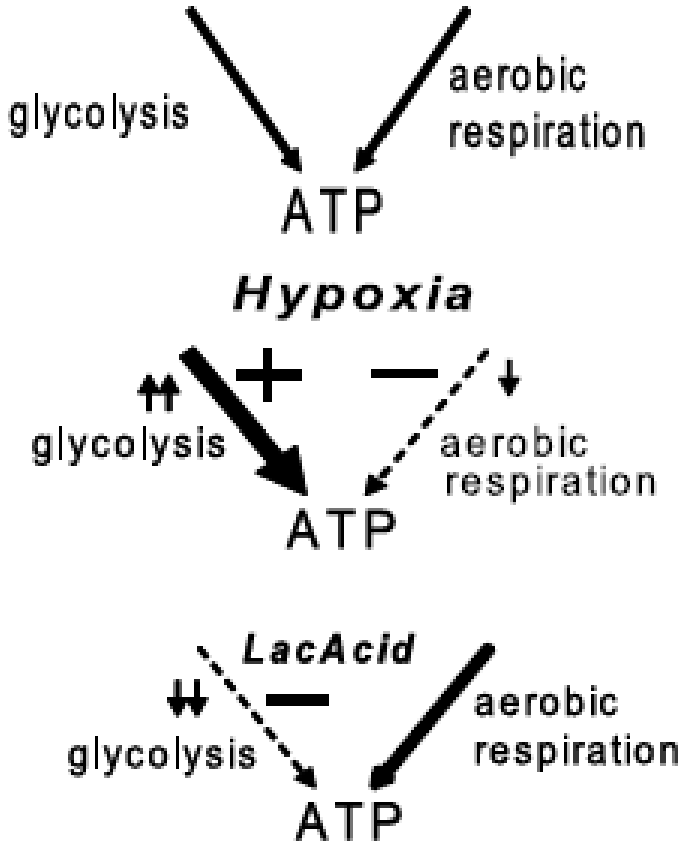

Figure 3-10 Hypoxia and lactic acidosis acted differently on balancing the model of energy homeostasis

Figure 3-10 Hypoxia and lactic acidosis acted differently on balancing the model of energy homeostasis.

Cells rely on glycolysis and aerobic respiration to produce energy. To maintain the energy homeostasis, hypoxia increases glycolysis but represses aerobic respiration. In contrast, lactic acidosis decreases glycolysis but increases aerobic respiration in energy production.

Discussion

Lactic acidosis and hypoxia often exist simultaneously in tumors, and lactic acidosis is commonly regarded as the consequences of tumor hypoxia. This work provides the genomic analysis of lactic acidosis responses *in vitro* and *in vivo* and establishes the independent role of lactic acidosis to stimulate cellular responses. These analyses showed that tumors with strong lactic acidosis and acidosis responses were associated with better clinical outcomes, while a strong hypoxia response was found to be associated with poor clinical outcomes. With various genomic analyses, the association of lactic acidosis and acidosis response with better clinical outcomes may be shown to originate from cell's ability to direct energy utilization towards aerobic respiration by inhibiting glycolysis. Lactic acidosis not only repressed gene expression of glycolytic pathways but also inactivated Akt. In addition to that, lactic acidosis, in contrast to hypoxia, decreased both glucose consumption and lactate production in tumor cells. Tumor glycolysis is an important characteristic of malignancy and confers proliferating advantages during somatic evolution(Gatenby and Gillies 2004). Therefore, the inhibitory effect on glycolysis by lactic acidosis is likely a critical factor to contribute to the anti-Warburg effect and thus to better clinical outcomes in tumors with strong lactic acidosis response phenotypes. The inhibition of Akt and glycolysis might also block the energy shift to anaerobic glycolysis under hypoxia and make cancer cells vulnerable to energy depletion and more susceptible to cell death under hypoxia

(Graham, Frazier et al. 2004). For instance, lactic acidosis represses hypoxia-induced LDH-A expression and LDH-A inhibition by RNAi was previously demonstrated to diminish tumorgenicity(Fantin, St-Pierre et al. 2006).

Recent studies suggested that mutations affecting mitochondria DNA or enzymes regulating TCA cycle could induce the formation and progression of tumors and the Warburg effect. Two enzymes involved in the TCA cycle, succinate dehydrogenase (SDH) and fumarate hydratase (FH) were identified as tumor suppressor genes(Gottlieb and Tomlinson 2005). Cells depending on glycolysis or aerobic respiration for energy production would affect the tumor phenotypes and the clinical outcomes of patients. For instance, hypoxia triggered expression of genes encoding glucose transporters and glycolytic enzymes and repressed functions of mitochondria to drive for glycolysis (Papandreou, Cairns et al. 2006; Zhang, Gao et al. 2007). Additionally, hypoxia induced the expression of PDK1, which inactivated pyruvate dehydrogenase (PDH), the enzyme in charge of converting pyruvate to acetyl-CoA. Moreover, Akt activation led to increased glycolysis since it upregulated glucose transport and rendered cancer cells consuming glucose for their survival(Elstrom, Bauer et al. 2004). Availability of oxygen, NAD/NADH, AMP/ATP ratio and enzyme modification all regulated the cellular rate of glycolysis. On the other hand, regulators directing cells towards aerobic respiration could suppress cancer phenotypes. The tumor suppressor gene, p53, for instance, directed energy utilization towards aerobic

respiration by not only inducing genes for aerobic respiration (Matoba, Kang et al. 2006) but also repressing genes inhibiting glycolysis(Bensaad, Tsuruta et al. 2006).

Since lactic acidosis could be accumulated as the metabolic end product of glycolysis, the inhibitory effect of lactic acidosis may come from the negative feedback mechanism which prevented cells from acidity-induced cell death by regulating the rate of glycolysis based on extracellular pH, attributed by the abundance of different acid substances(Helmlinger, Sckell et al. 2002) and the buffering capacity of the extracellular fluid.

It would be interesting to further elucidate the mechanism how lactic acidosis inhibited Akt and glycolysis, which could possibly lead to novel strategies to treat cancer by modifying tumor behaviors. The lactic acidosis response gene signature could also be applied to identify cancers preferring to use aerobic respiration to produce energy thus might have better responses to chemotherapies targeting mitochondria function(Costantini, Jacotot et al. 2000).

Besides ATP, glycolysis was also responsible for generating acetyl-CoA, the substrate for TCA cycle to carry through aerobic respiration. Lactic acidosis inhibited glycolysis and thus might reduce the amount of acetyl-CoA generated from glycolysis. Therefore, cells might utilize other metabolic pathways, such as β-oxidation of fatty acids, to compensate for this reduction. Akt was previously reported to suppress β-oxidation as well as energy generation from fatty acids (Elstrom, Bauer et al. 2004;

Buzzai, Bauer et al. 2005). Akt inhibition by lactic acidosis, therefore, may increase fatty acid β-oxidation and provide acetyl-CoA for energy generation. Based on the predictions from GSEA analyses, breast tumors with high lactic acidosis response were enriched in gene sets of fatty acid degradation and amino acid metabolism. In conclusion, lactic acidosis response in vitro and in vivo demonstrated the impact of lactic acidosis in cellular metabolism which may play an important role in modulating clinical phenotypes of human tumors.

Chapter 4

Lactic acidosis triggers starvation response with paradoxical induction of TXNIP through MondoA

Introduction

Human cancers are extremely complicated with alterations, such as mutations in oncogenes and tumor suppressor genes in cancer cells, in addition to the heterogeneous tumor microenvironment. The stresses of the tumor microenvironment included tumor hypoxia, extracellular acidosis, accumulation of lactate and depleted glucose, glutamine and nutrients(Vaupel 2004). These stresses could be from poor tissue perfusion, uncontrolled proliferation,abnormal tumor vasculature or dysregulated cancer cell metabolism during the development of cancer. They not only impact the metabolism and physiology of cancer cells but also affect the prognosis of cancer patients clinically. Therefore, in this study, we focused on investigating how these tumor microenvironmental stresses regulate cells in human cancers mechanistically.

It has been challenging to associate the changes caused by microenvironmental stresses *in vitro* to the clinical cancer phenotypes *in vivo*. We overcame this obstacle previously by generating signatures from genes significantly modulated by lactic acidosis *in vitro* as the surrogate of the molecular phenotypes and projecting them onto cancer patients' gene expression data(Chen, Lucas et al. 2008). This approach has also been successfully applied to elucidate the pathways of hypoxia(Chi, Wang et al. 2006)

and vascular injury(Chi, Rodriguez et al. 2007) in human cancers. The reciprocal flow of information from *in vitro* and *in vivo* systems provided us with evidence that lactic acidosis caused metabolic reprogramming to shift energy production of cells to oxidative phosphorylation by inhibiting glycolysis(Chen, Lucas et al. 2008).

In studies utilizing microarrays to link results from cultured cells with human cancers, primary epithelial cells are often chosen due to their intact genetics and signaling circuitry to provide the common responses to designated perturbations (Chang, Sneddon et al. 2004; Bild, Yao et al. 2005; Chi, Wang et al. 2006; Chi, Rodriguez et al. 2007; Chen, Lucas et al. 2008). Nevertheless, it would be relevant to assess gene signatures in cancer cells because we are projecting them to human cancers. Additionally, cancer cell lines are technically easier to culture and genetically-manipulated for mechanistic studies. Therefore, we used a breast cancer cell line, MCF7, to examine the cellular responses to the microenvironmental stresses lactic acidosis, glucose deprivation and hypoxia in this study. Our goal was to not only understand cellular responses but also to identify the key regulators responsible for these responses.

The critical role of hypoxia-inducible factor in regulating hypoxia response has been validated (Harris 2002; Semenza 2002; Giaccia, Simon et al. 2004; Schofield and Ratcliffe 2004). Another stress of the tumor microenvironment, glucose deprivation, has also been characterized for its effect on AMPK signaling which activated TSC1/TSC2 to inhibit the energy sensor mTOR, thus inhibiting ribosomal biogenesis, translation and

cell proliferation (Shaw, Kosmatka et al. 2004). Conversely, regulatory factors responsible for the lactic acidosis gene response program are unknown. In this study, we exposed the breast cancer cell line, MCF7, to hypoxia, lactic acidosis and glucose deprivation in parallel to gain a better understanding of the molecular mechanisms responsible for the phenotypic changes. By comprehensive analyses of the transcriptional programs and metabolic responses, thioredoxin-interacting protein (TXNIP) was identified as the critical gene regulating the lactic acidosis response. Moreover, the transcriptional complex, MondoA:Mlx functioned upstream in sensing lactic acidosis and modulating TXNIP activation. MondoA:Mlx heterodimers normally localize at the outer mitochondria membrane (OMM)(Sans, Satterwhite et al. 2006). Upon glucose exposure, MondoA:Mlx complexes translocate into the nucleus and regulate thioredoxin-interacting protein (TXNIP), previously shown to negatively regulate glucose uptake(Parikh, Carlsson et al. 2007; Stoltzman, Peterson et al. 2008). TXNIP was originally characterized as an inhibitor of thioredoxin *in vitro* (Nishiyama, Matsui et al. 1999; Junn, Han et al. 2000; Yamanaka, Maehira et al. 2000)and now known as a critical regulator of glucose metabolism *in vivo*(Parikh, Carlsson et al. 2007; Chutkow, Patwari et al. 2008; Hui, Andres et al. 2008).

Results

The temporal transcriptional response of MCF7 cells under lactic acidosis

The temporal gene expression patterns from MCF7 breast cancer cells exposed to lactic acidosis (25mM lactic acid with pH6.7) at various time points over 24 hours were examined. Cells were brought to replicating arrest status by serum withdrawal for 24 hours before their exposure to control or lactic acidosis conditions for 1, 4, 12 or 24 hours. Cells from each condition and time point were collected in triplicate. RNAs harvested from these MCF7 cells were applied to Affymetrix Hu133plus2 chips that contained approximately 54,000 probe sets on nearly 47,000 transcripts and variants. The original results in.cel files were deposited in the Gene Expression Omnibus (GSE19123).

Original files from microarrays were first normalized by RMA and, zero-transformed against the average expression levels of the same probe sets of the time-matched control samples. A total of 1761 probe sets were selected with at least two fold changes in at least two samples and hierarchically-clustered by similarities in gene expression patterns (Fig 4-1A). The clustering result showed that lactic acidosis induced significant responses in MCF7 cells with temporal differences reflected. Among the genes induced in MCF7 cells by lactic acidosis were PLAU, (similar to previous observations in HMECs), major histocompatibility complex (MHC) class I, CD44 and REDD1, a p53 transcriptional target following DNA damage (Fig 4-1B) (Ellisen, Ramsayer et al. 2002). CD44 was previously reported to be induced by lactosis in cancer

cells(Stern, Shuster et al. 2002). Among the genes repressed by lactic acidosis in the later time were many genes regulating cell proliferation, such as cyclin D, PCNA, CCRK, E2F3 and E2F6 (Fig 4-1C). Cells may respond to lactic acidosis by repressing proliferation to conserve energy under the stressed microenvironment.

Gene Set Enrichment Analysis (GSEA) was performed to compare the pathway composition in the gene expression of all control vs. lactic acidosis samples of MCF7 cells(Subramanian, Tamayo et al. 2005). Cancer cells exposed to lactic acidosis were s enriched in gene sets representing nutrient deprivation (Peng, Golub et al. 2002), good breast cancer prognosis (van 't Veer, Dai et al. 2002) and exposure to a DNA damaging agent (bleomycin). They were depleted in gene sets representng E2F1 target genes, DNA replication, poor breast cancer prognosis (van 't Veer, Dai et al. 2002), mitotic cycles and RNA processing.

We previously reported that the hypoxia and lactic acidosis response signatures acquired from primary HMECs could provide the molecular surrogates to be projected onto the cancerous human tissues *in vivo* and offer prognostic values (Chen, Lucas et al. 2008). To test the robustness of this approach, we projected lactic acidosis response signatures generated from MCF7 cells onto the same breast cancer dataset (Miller, Smeds et al. 2005). Survival analysis showed that tumor samples with higher levels of lactic acidosis pathway activity defined by 12 hour exposures to lactic acidosis had good prognoses (Fig 4-2A), consistent with ourobservations in previous studies in HMECs

(Chen, Lucas et al. 2008). Additionally, the lactic acidosis response from MCF7 cells and HMEC cells were correlated (Fig 4-2B).

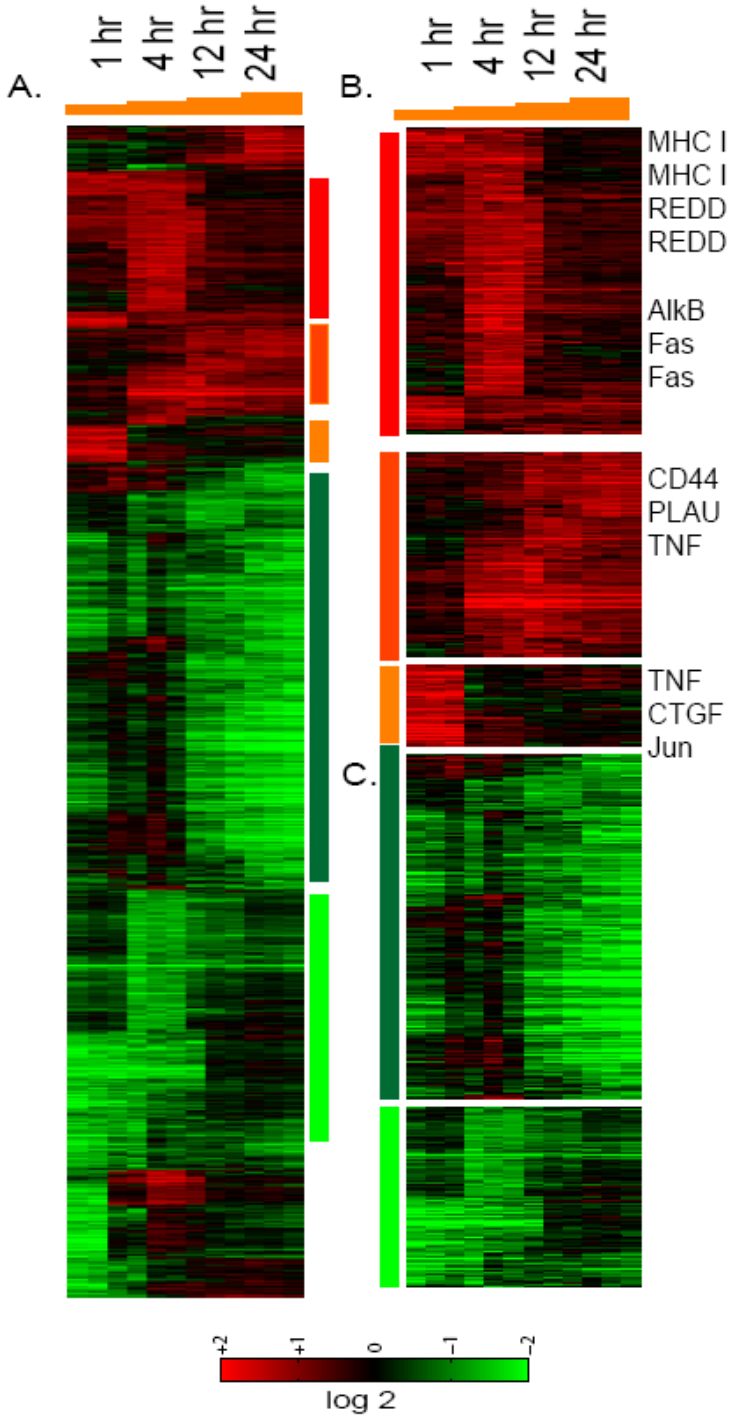

Figure 4-1
Overview of the temporal regulation of lactic acidosis response in MCF7 cells

Figure 4-1 Overview of the temporal regulation of lactic acidosis response in MCF7 cells

(A)The gene expression response of MCF7 to lactic acidosis at indicated time points. Clusters of genes induced and repressed by lactic acidosis at different time points are marked and expanded in (B) and (C).

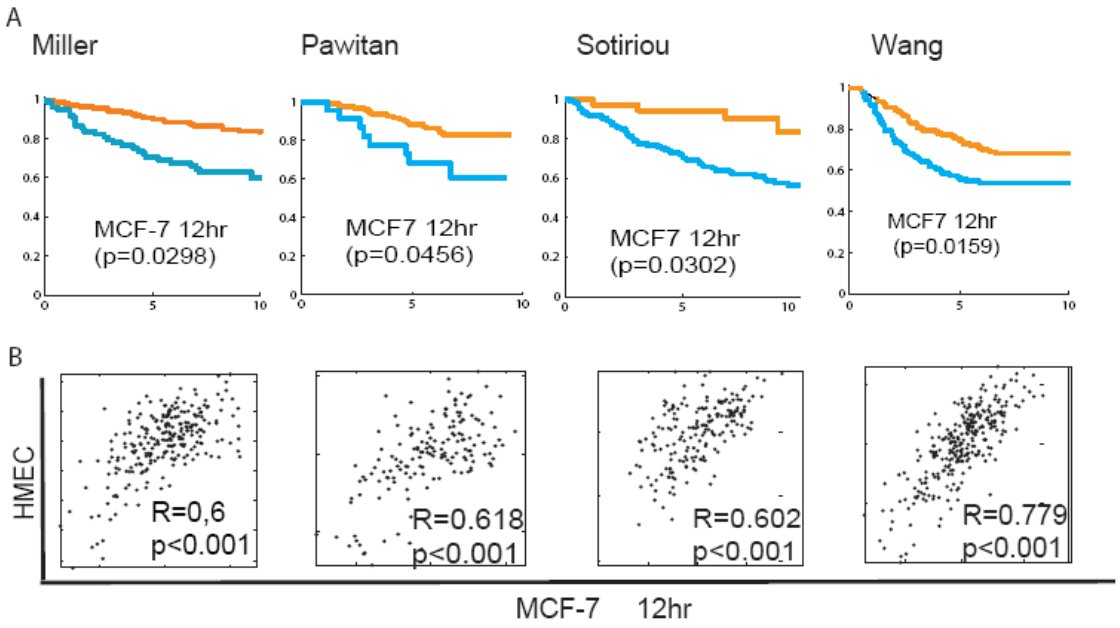

Figure 4-2 The prognostic results of the lactic acidosis response from MCF7 cells

(A)The prognostic significance of lactic acidosis response in MCF7 cells at 12 hours were assessed in the Miller breast cancer expression dataset. Kaplan-Meier survival curves were generated by tumors stratified with the degrees of the responses to link clinical outcomes with lactic acidosis responses. (B) Scattered plots showing the relationship between the levels of lactic acidosis response obtained in MCF7 cells (X-axis) and HMEC (Y-axis) at 12 and 24 hours. Each point in the scatter plot representing a single tumor from the indicated breast cancer datasets. The overall correlation (R) and statistical significane (p-value) of lactic acidosis response in these two types of cells were indicated. Figure (A) and (B) were generated by Dr. Daniel Merl.

Lactic acidosis triggers the starvation response

High enrichment in starvation of glucose and glutamine was predicted by GSEA analysis of MCF7 cells treated by lactic acidosis. We had also previously shown that lactic acidosis decreased ATP production. To elucidate cellular responses to tumor microenvironmental stresses, particularly to lactic acidosis and glucose starvation, we performed a large-scale experiment in quintuplicate by exposing MCF7 cells to control condition, 25 mM lactic acidosis, glucose deprivation or 1% oxygen hypoxia. RNAs were collected at 4 hours to be applied to Affymetrix Hu133 plus2 microarrays. The results were RMA normalized, filtered with twoobservations with of two fold differences to select 3903 probes and hierarchically clustered. Intriguingly, there appeared to be a high similarity in the transcriptional profiles between cellular responses to lactic acidosis and glucose deprivation, distinct from that of hypoxia (Fig 4-3A). Further examination of this transcriptional profile revealed that lactic acidosis and glucose deprivation induced and repressed similar sets of genes, inclusive of MHC class I, DNA repair gene alkyation repair homolog 7 (alkB), CTGF, Jun and TNF (Fig 4-3B). On the other hand, hypoxia induced a different set of genes including many genes of glycolysis and those previously reported to be induced by hypoxia, such as EGLN3, CA9, BNIP3, STC1 and PKM2 (Fig 4-3B). In yeast cells, the induction of many genes upon stresses of any kind has been reported. Nevertheless, we found there was only a small set of this kind of "common stress" genes induced under lactic acidosis, glucose deprivation and hypoxia. Among

these genes were hypoxia-inducible gene (HIG-2) and REDD which were both induced under hypoxia previously (Fig 4-3B). When cells are stressed with glucose starvation, decreased AMP/ATP ratio induces AMP kinase (AMPK) by phosphorylating this kinase at Thr172. In addition, glucose starvation inhibits mTOR signaling, which is detectable by de-phosphorylation of S6K. Although we were surprised by the similar transcriptional responses of MCF7 cells to lactic acidosis and glucose deprivation, we decided to examine whether lactic acidosis caused a pseudo-starvation responses in cells by measuring AMPK phosphorylation and mTOR inhibition under lactic acidosis. Proteins were extracted from cells exposed to lactic acidosis after 2 and 6 hours and subjected to western blotting. The results indicated that lactic acidosis induced AMPK and repressed mTOR, both indicative of cellular responses to glucose starvation (Fig 4-4).

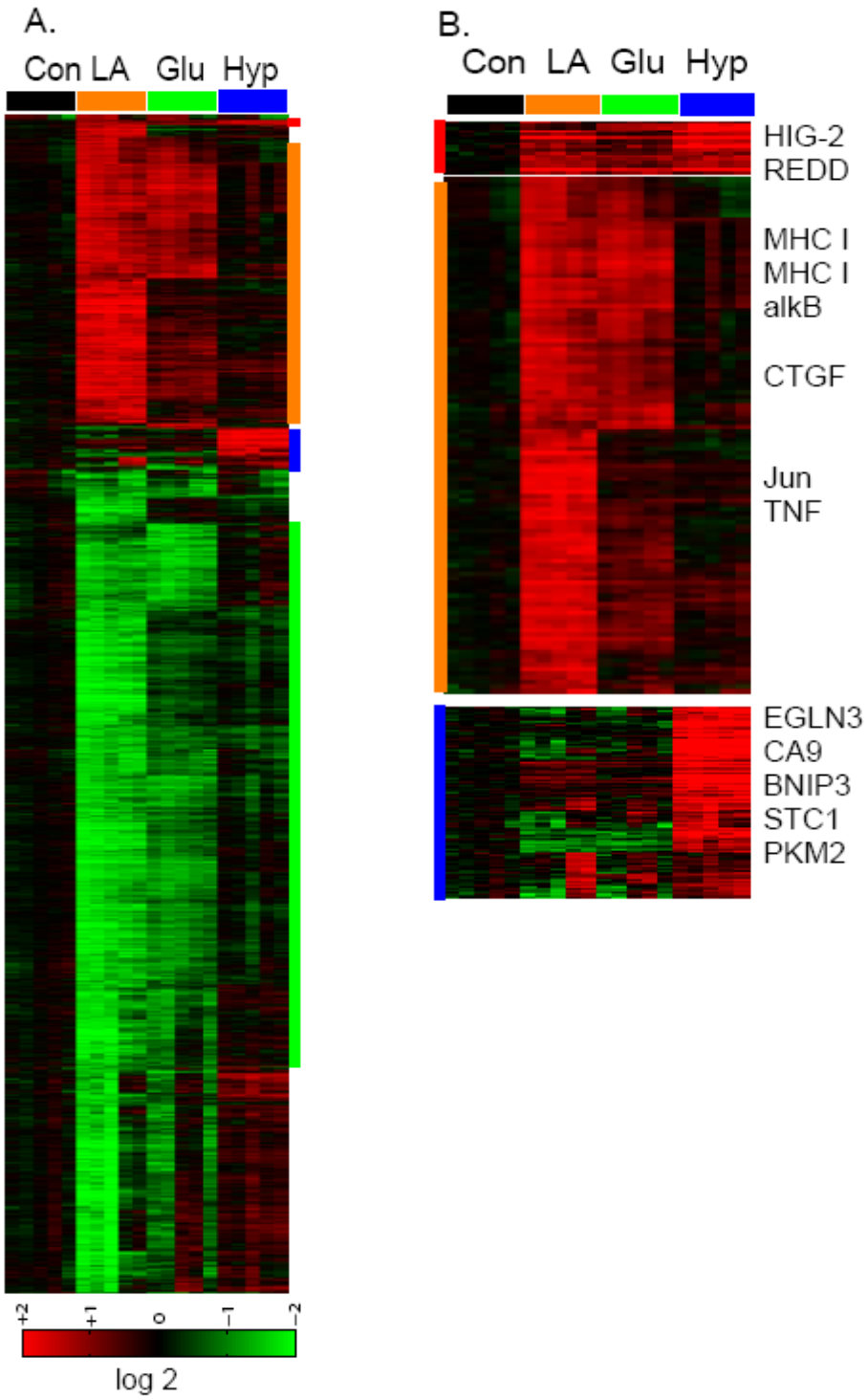

Figure 4-3 Lactic acidosis triggers starvation response

Figure 4-3 Lactic acidosis triggers starvation response

(A)The transcriptional response of MCF7 cells to lactic acidosis, glucose deprivation and hypoxia at four hours. Selected gene clusters which were induced commonly by lactic acidosis, glucose deprivation and hypoxia (red bar), by lactic acidosis and glucose deprivation (orange bar), or by hypoxia alone (blue bar) were highlighted and expanded in (B) with the gene names shown.

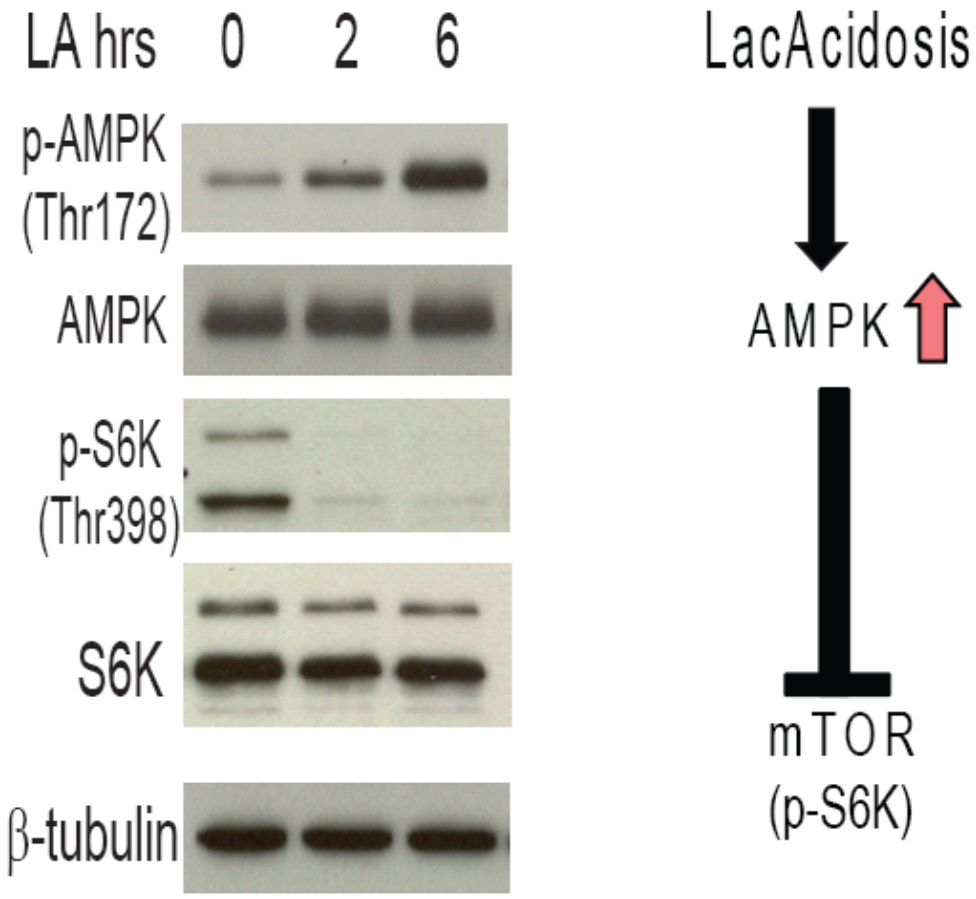

Fig 4-4 Lactic acidosis triggers starvation responses
Lactic acidosis triggers the activation of AMPK (phosphorylation at Thr 172) and inhibits mTORC1 signaling by reducing S6K phosphorylation at Thr 398.

Different metabolic phenotypes of glucose uptake caused by lactic acidosis and glucose deprivation

Lactic acidosis inhibited glucose consumption and lactate production; therefore, we decided to examine its effect on glucose uptake. By using 2-deoxy-D-[2,6-3H]-glucose, it was shown that lactic acidosis reduced glucose uptake by 67%. In contrast, glucose deprivation increased glucose uptake by 84%. Although lactic acidosis and glucose deprivation induced similarly transcriptional responses, increased AMPK signaling and inhibited mTOR signaling, they had opposite phenotypes of glucose uptake (Fig 4-5). To look for genes contributing to this opposite metabolic phenotype, statistical methods were applied to select genes with significant but opposite expression. We selected 115 probe set with 49 genes being strongly upregulated by lactic acidosis and repressed by glucose deprivation and another 66 genes being strongly upregulated by glucose deprivation and repressed by lactic acidosis (Fig 4-6A). Three probes representing the same gene, TXNIP (VDUP1), were the ones most differentially regulated by lactic acidosis and glucose deprivation, being strongly induced by lactic acidosis but repressed by glucose deprivation. It has been previously reported that TXNIP is induced under hypoxia and we observed ~40% induction under hypoxia in our microarray results. The contrasting expressions of TXNIP caused by these two stimuli were further confirmed with realtime PCR (Fig 4-6B). To elucidate the impact of glucose and lactic acidosis on TXNIP, an independent experiment was performed with cells

exposed to 25mM lactic acid only, 4.5 g/L glucose only, or the combined treatments. TXNIP expression was induced by 4-5 folds by only lactic acidosis or glucose. However, synergistic induction of approximately 18 fold was observed when both of these stimulants were applied (Fig 4-7A). Additionally, we found sodium lactate and acidosis induced TXNIP for around 2 fold respectively and lactic acidosis induction was the synergistic effect of these two stresses (Fig 4-7B) This result demonstrates that lactic acidosis and glucose induced TXNIP through potentially different mechanisms. TXNIP was initially identified as a protein interacting with thioredoxin and regulating cellular responses to oxidative stresses. Additionally, it can also regulate glucose uptake and function as an important regulator in metabolic diseases, such as diabetes. To test our hypothesis that TXNIP is responsible for the anti-Warburg effect of lactic acidosis, two independent anti-TXNIP siRNAs were applied to MCF7 cells. Western blot was performed to examine induction of TXNIP by lactic acidosis and the successful knockdown (Figure 4-8A).Lactic acidosis repressed glucose uptake in MCF7 cells treated with negative control siRNAs by 52%. The inhibitory effects of lactic acidosis upon glucose uptake were significantly rescued to 39% and 44% respectively by the two different siTXNIP siRNAs (Fig 4-8B). Moreover, we conducted another experiment using established wild-type MEFs and a pair of TXNIP- knockout MEF lines. The effect increased, with 68% repression of glucose uptake by lactic acidosis in wild-type MEF cells but only 28% repression in TXNIP- knockout MEFs (Fig 4-8C).

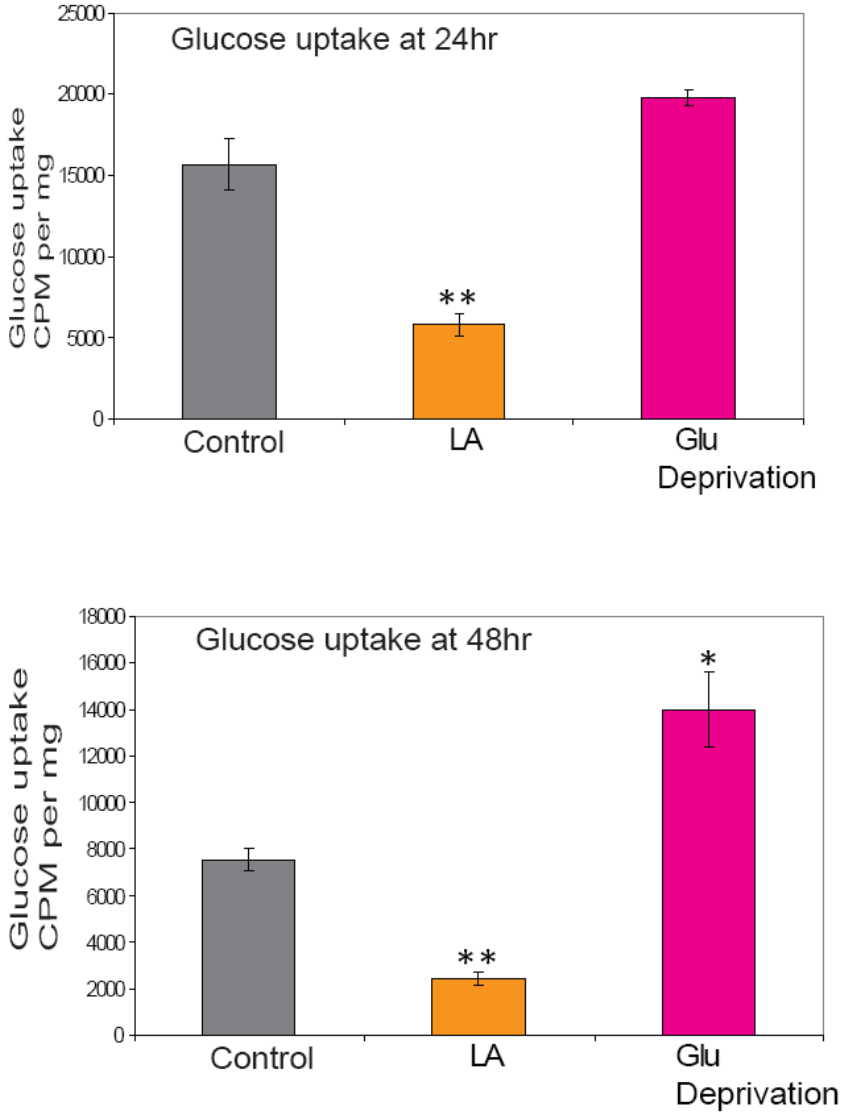

Figure 4-5 Lactic Acidosis and glucose deprivation induced opposite metabolic phenotypes of glucose uptake

The amount of glucose uptake of MCF7 cells under control, lactic acidosis and glucose deprivation conditions.

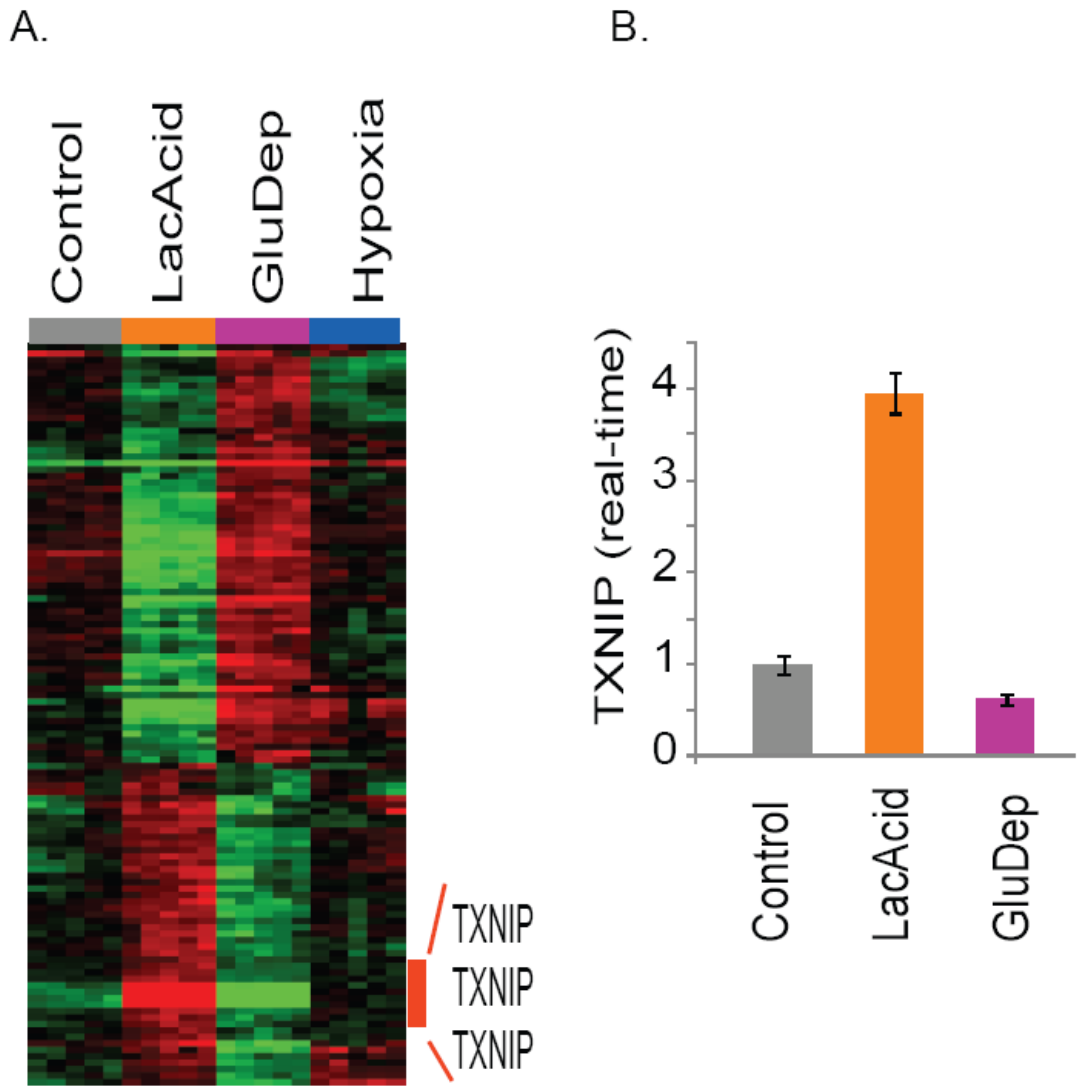

Figure 4-6 TXNIP was the most differentially regulated genes by lactic acidosis and glucose deprivation

(A)The most differentially-regulated 115 genes by lactic acidosis and glucose deprivation with three probes of TXNIP highlighted. TXNIP was strongly induced by lactic acidosis but repressed by glucose deprivation. (B) Real-time PCR confirmation of the TXNIP expression that TXNIP transcript was induced under lactic acidosis but reduced by glucose deprivation.

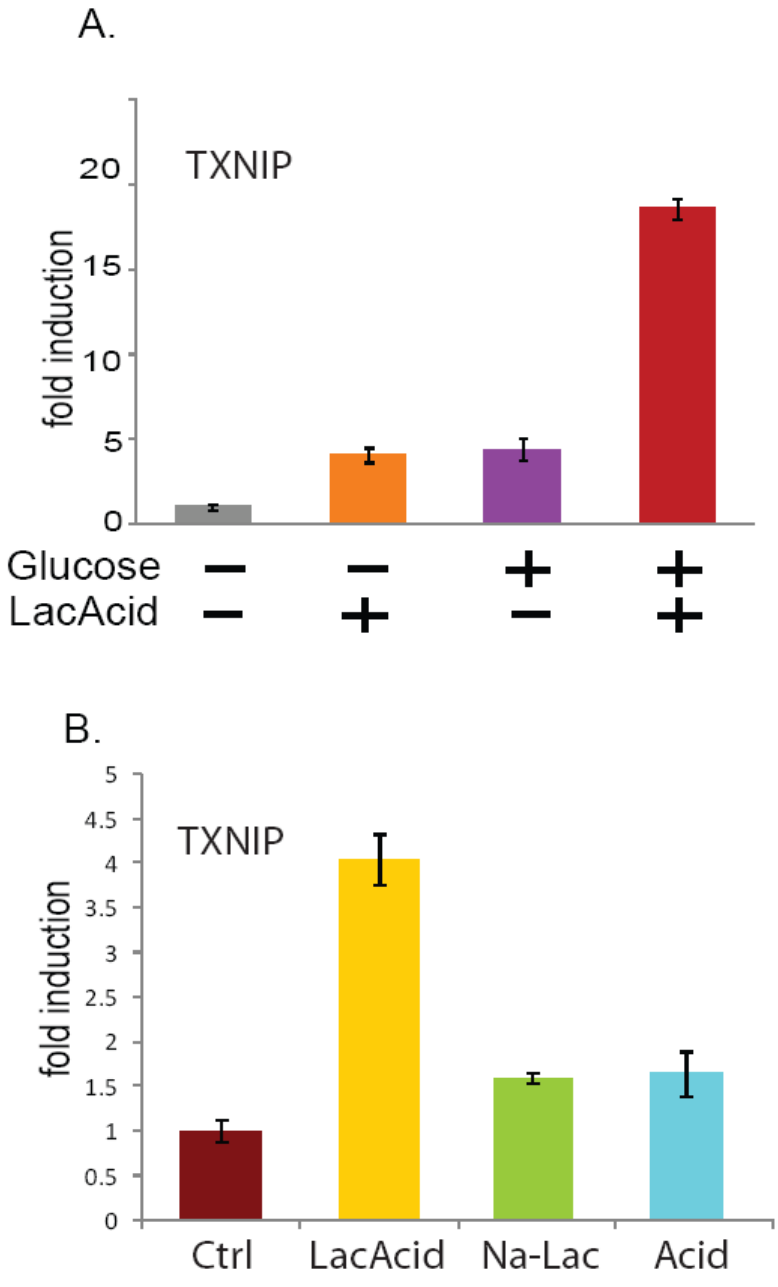

Figure 4-7 TXNIP inductions by glucose, lactic acid, sodium lactate and acid

Figure 4-7 TXNIP inductions by glucose, lactic acid, sodium lactate and acid

(A) Realtime PCR results showing glucose or lactic acidosis can each induce TXNIP expression ~4 fold. Combined treatments with glucose and lactic acidosis together can induce TXNIP expression to more than 16 fold.
(B) Realtime PCR results of lactic acidosis reproducibly showing 4 fold inductions in TXNIP expression. Sodium lactate or acid each induces TXNIP ~1.75 fold.

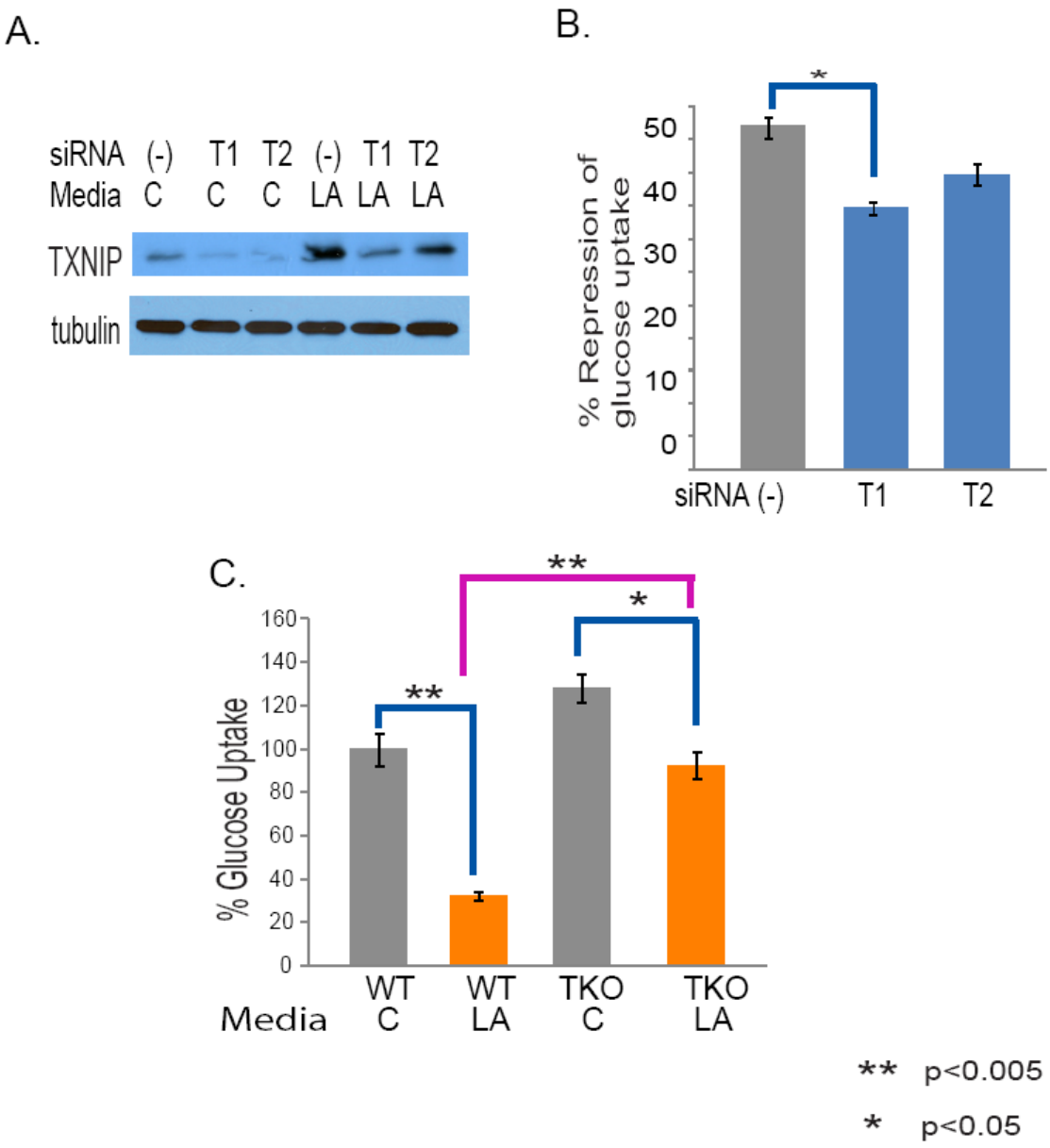

Figure 4-8 Identification of TXNIP as the regulator of lactic acidosis response

Figure 4-8 Identification of TXNIP as the regulator of lactic acidosis response

(A)Western blot result showing the level of TXNIP proteins of MCF7 cells treated with control or siRNAs against TXNIP in control versus lactic acidosis conditions. (B)The normalized percentage of glucose uptake inhibited by lactic acidosis in MCF7 cells treated with the non-targeting siRNAs and siRNAs against TXNIP. Lactic acidosis caused 52% repression of glucose uptake in MCF7 cells transfected with non-targeting siRNAs(-). In cells transfected with two different siTXNIPs (T1, T2), glucose uptake was increased and the repressing effect of lactic acidosis was decreased to 39% and 44% respectively. (G) Lactic acidosis caused 68% repression in wildtype(WT)MEF cells but only 28% repression in TXNIP knockout (TKO) MEF cells. Most importantly, lactic acidosis significantly decreased the glucose uptake of wildtype cells but had little repressing effect on TKO MEF cells.

MondoA: Mlx complex in regulating TXNIP activation by lactic acidosis

The induction of TXNIP by glucose relies on a well-conserved carbohydrate response element (ChoRE), consisting of two E-boxes, in the TXNIP promoter. To examine whether this region plays a critical role in TXNIP induction by lactic acidosis, mutations were created in the ChoRE region of the TXNIP promoter. Lactic acidosis induced the reporter activities of TXNIP and ARRDC4 promoters to more than 6 fold. However, the induction was reduced by 61% in ChoRE, the TXNIP promoter with mutations in the ChoRE element (Fig 4-9A). Glucose induced TXNIP through the binding of either MondoA:Mlx or carbohydrate response elements-binding protein (ChREBP) to two E-boxes of the TXNIP promoter. We hypothesized that the induction of TXNIP by lactic acidosis was through the activation of MondoA:Mlx because higher expression was detected in cells exposed to lactic acidosis. Additionally, TXNIP and its alpha-arrestin-domain paralogue ARRDC4, both were downstream target genes of MondoA, showed simultaneous upregulation by lactic acidosis. Therefore, we hypothesized that MondoA is more relevant in mediating TXNIP upregulation upon lactic acidosis exposure. MondoA is usually localized at the outer mitochondria membrane (OMM), but when activated by glucose, it translocates into the nucleus and associates with its binding partner, Mlx, to bind E-boxes of promoters to induce TXNIP and ARRDC4. To directly test whether MondoA binds TXNIP and ARRDC4 promoters,

chromatin immunoprecipitation was performed with an antibody against MondoA in MCF7 cells treated with different acidities of lactic acidosis. The results showed that lactic acidosis indeed induced MondoA binding to TXNIP and ARRDC4 and the binding was enhanced by increased acidities of lactic acidosis (Fig 4-9B). To further determine the necessity of MondoA in TXNIP induction, two different siRNAs against MondoA were applied to knock down MondoA in MCF7 cells. Lactic acidosis, unlike its inducing effect on TXNIP, did not increase the expression of MondoA. Both siRNAs against MondoA demonstrated successful knockdown of MondoA proteins. In terms of TXNIP, MondoA knockdown significantly abolished lactic acidosis-induced TXNIP (Fig 4-10A). The level of glucose uptake in the MCF7 cells treated with lactic acidosis caused 57% repression when transfected with non-targeting siRNAs. The repression effect of lactic acidosis was decreased to 44% and 40% with MCF7 cells transfected with two different MondoA siRNAs (M1 and M2) (Fig 4-10B). However, siMondoA silencing still left residual MondoA activity due to the transient nature of siRNAs and showed slight induction of TXNIP under lactic acidosis. Therefore, MondoA knockout MEFs created by the cre-loxP system were used. We confirmed the observation that TXNIP induction by lactic acidosis requires MondoA by showing no TXNIP protein expression in MondoA knockout MEFs whereas exogenously expressed full-length MondoA protein successfully rescue TXNIP protein expression and its upregulation by 2DG and lactic acidosis (Fig 4-10C).

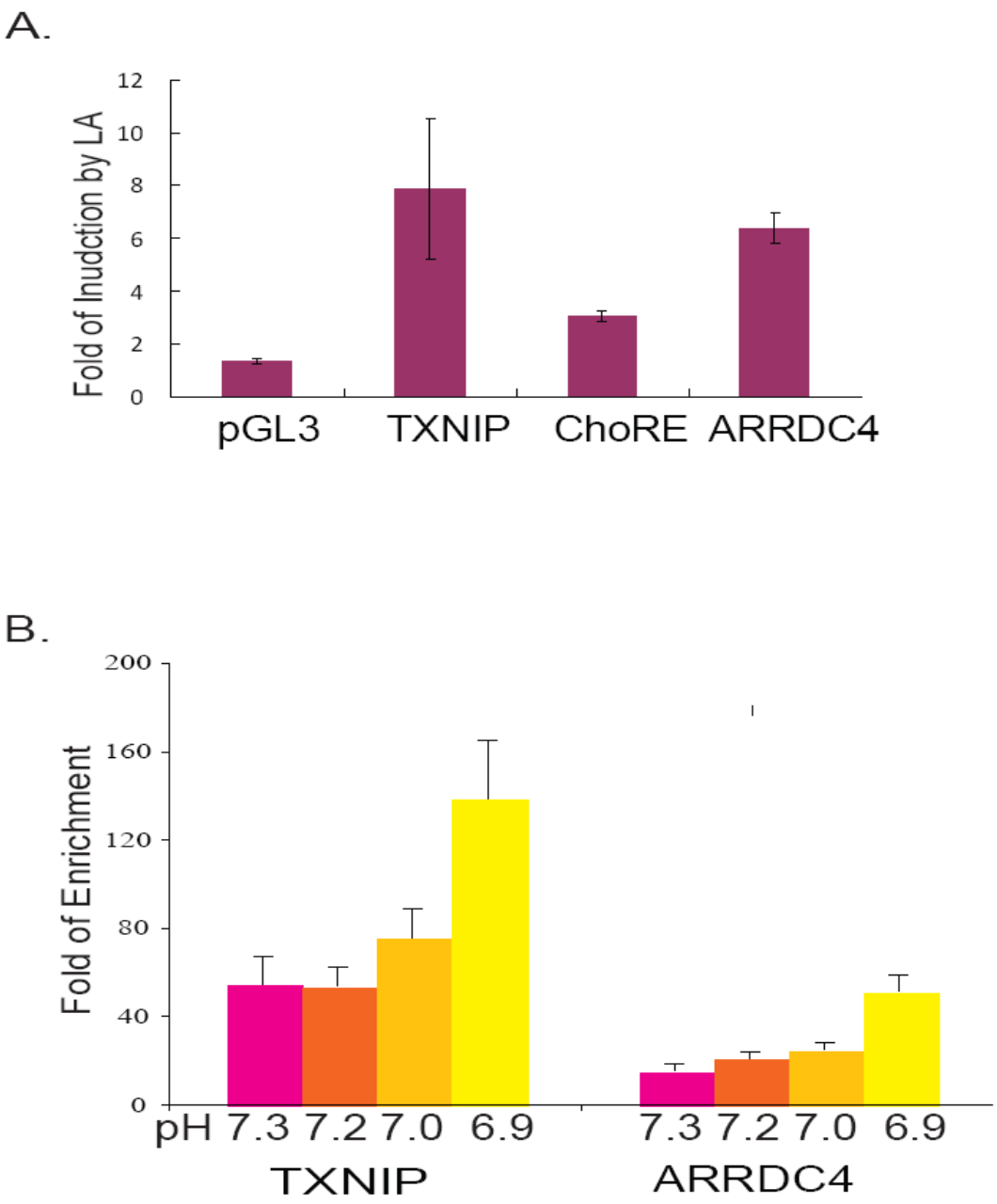

Figure 4-9 MondoA binding is required for TXNIP and ARRDC4 activation

Figure 4-9 MondoA binding is required for TXNIP and ARRDC4 activation

(A)Normalized luciferase activities showing the fold inductions by lactic acidosis for the reporter constructs driven by wild type TXNIP promoter, TXNIP promoter with the ChoRE mutations and ARRDC4 promoter. (B)The physical binding of MondoA to the promoters of TXNIP and ARRDC4 was examined by Chromatin immunoprecipitation (ChIP) for MCF7 cells under lactic acidosis of different acidities. Figure (B) was generated by Dr. Chris Peterson in Dr. Don Ayer's lab in University of Utah.

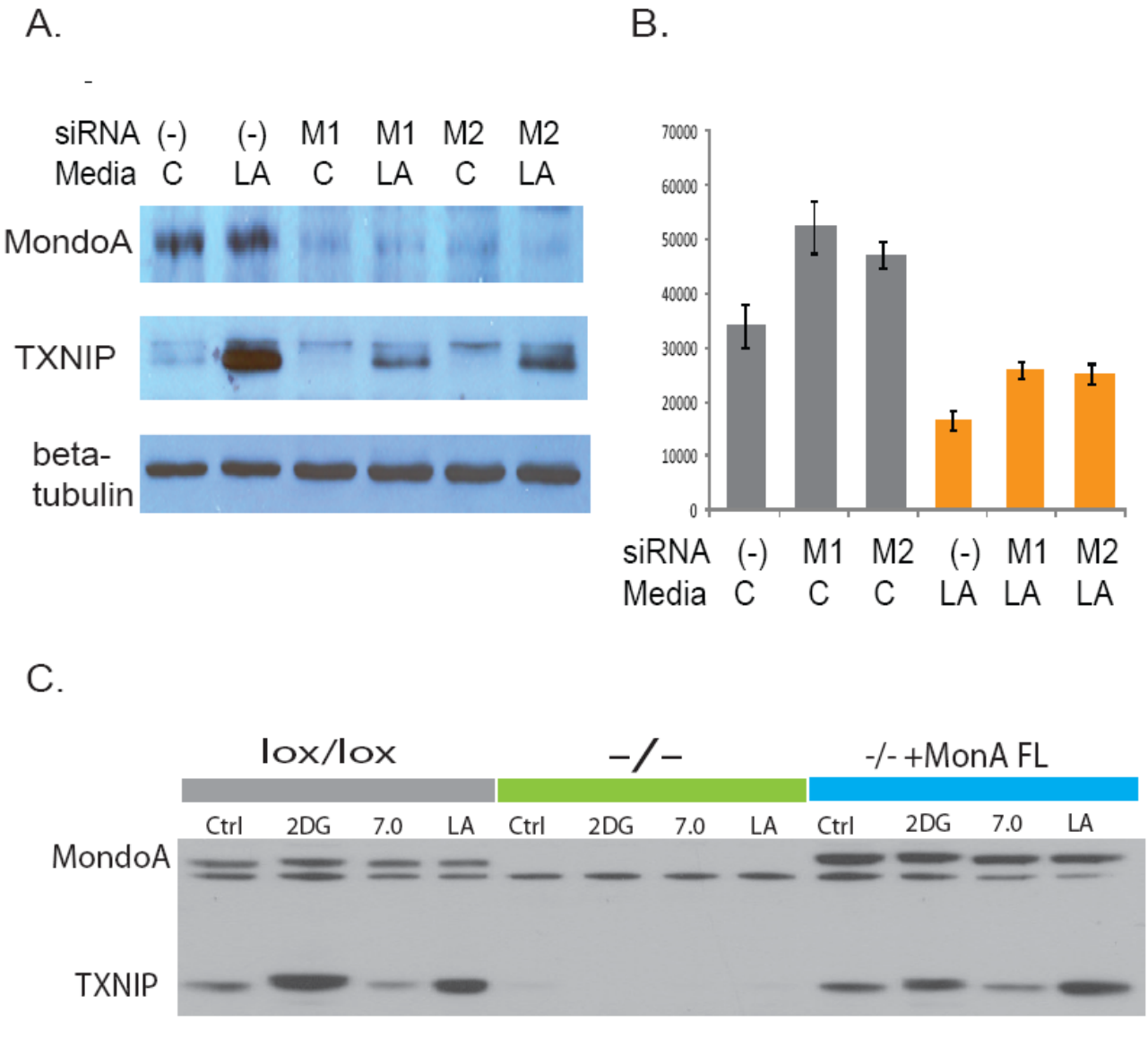

Figure 4-10 MondoA is responsible for TXNIP induction by lactic acidosis

Figure 4-10 MondoA is responsible for TXNIP induction by lactic acidosis

(A)The level of MondoA proteins in MCF7 cells treated with non-targeting siRNAs or two siRNAs against MondoA under control or lactic acidosis conditions. (B)The level of glucose uptake in the MCF7 cells treated with the indicated conditions. Lactic acidosis caused 57% repression in MCF7 cells transfected with non-targeting siRNAs. The repression effect of lactic acidosis was decreased to 44% and 40% with MCF7 cells transfected with two different MondoA siRNAs (M1 and M2). (C)The level of MondoA and TXNIP proteins in lox/lox (MEF with wild type MondoA), -/- (lox/lox MEF with cre overexpression to delete MondoA) and -/- rescued by full-length ModnoA under control, 2-DG, pH7 and lactic acidosis conditions. Figure (C) was generated by Dr. Don Ayer's lab in University of Utah.

TXNIP expression and lactic acidosis pathways in human cancers

To examine the prognostic significance of TXNIP expression in human cancers, breast cancer expression datasets were stratified based on TXNIP expression. Tumors with high TXNIP expression were associated with better clinical outcomes in four breast cancer datasets used in lactic acidosis signature projections (Fig 4-11A). We also found positive correlations between predicted lactic acidosis pathway signatures with the TXNIP expression levels in the breast cancer datasets (Fig 4-11B). ARRDC4, a paralogue of TXNIP with a structure similar to α-arrestin and the downstream target of MondoA, was shown to have prognostic power in two (Miller and Pawitan) of the four breast cancer datasets as well (Fig 4-11C). The expression of ARRDC4 was also correlated with the predicted lactic acidosis pathways in these two breast cancer datasets (Fig 4-11D).

These observations support the previously identified role of TXNIP as a tumor suppressor in several cancer types. TXNIP can suppress oncogenic phenotypes and loss of TXNIP has been associated with tumor development (Goldberg, Miele et al. 2003; Han, Jeon et al. 2003; Sheth, Bodnar et al. 2006). Therefore, TXNIP plays an important role in regulating the lactic acidosis response in human cancers.

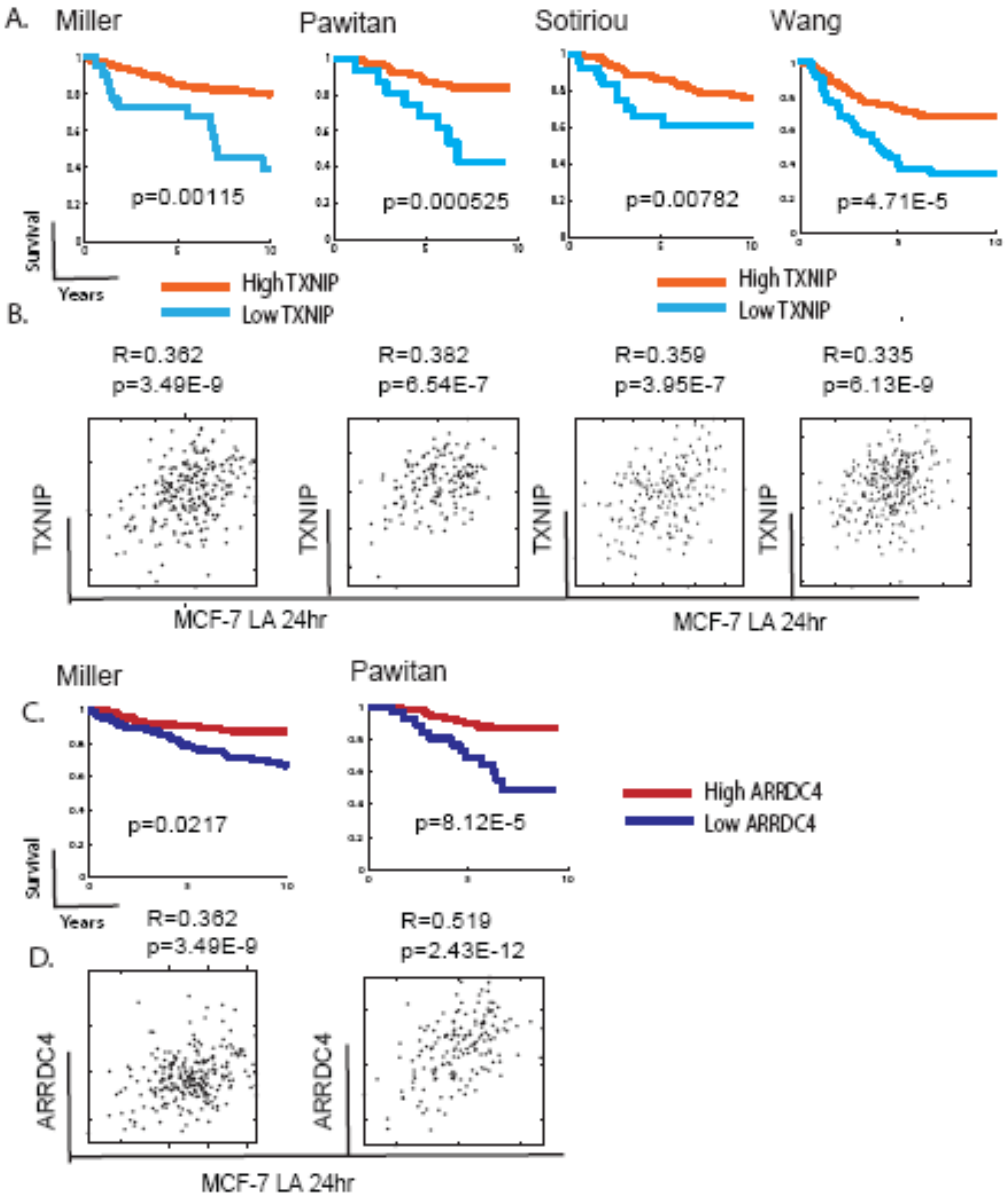

Figure 4-11 The expression of TXNIP, ARRDC4 and lactic acidosis pathway in human cancers

Figure 4-11 The expression of TXNIP, ARRDC4 and lactic acidosis pathways in human cancers

(A)(C) The breast tumor samples from patients in the indicated datasets stratified by differential expression of TXNIP (A) and ARRDC4 (B) were applied to generate Kaplan-Meier survival curves to associate the TXNIP levels with the clinical outcomes.

(B)(D) Scattered plots indicating the relationship between the expression of TXNIP (B) or expression of ARRDC4 (D) (Y-axis) and predicted lactic acidosis pathway activities (X-axis) in the indicated tumor datasets. Each dot in the scatter plots representing a single tumor. The overall correlation (R) and statistical significance/p-value (p) were shown.

Figure (A) to (D) were generated by Dr. Daniel Merl.

Lactic acidosis and glucose deprivation induced similar and different pathways

Lactic acidosis and glucose deprivation induced similar gene expression profiles and AMPK-mTOR signaling pathways. However, they acted upon MondoA-TXNIP oppositely. Lactic acidosis strongly induced TXNIP whereas glucose deprivation inhibited it with opposite metabolic phenotypes of glucose uptake (Fig 4-12).

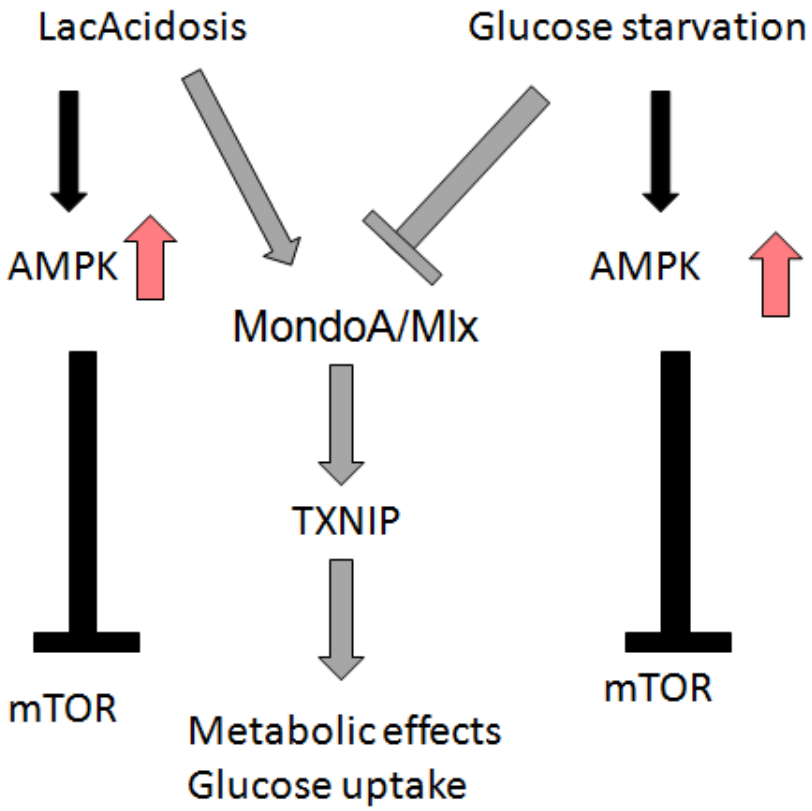

Figure 4-12 Similarities and differences of cellular responses to lactic acidosis and glucose deprivation

Lactic acidosis and glucose deprivation both induced the starvation responses and AMPK-mTOR signaling in MCFs. However, they induced opposite effects of the MondoA/Mlx-TXNIP pathway with different metabolic phenotypes of glucose uptake.

Discussion

Hypoxia, glucose deprivation and lactic acidosis are commonly observed in tumor microenvironment physiology. It has been reported that glucose deprivation causes metabolic stress and induces starvation responses which are detectable by AMPK activation, mTOR inhibition and metabolic changes in cancer cells. Interestingly, we observed that lactic acidosis induced a transcriptional "starvation" response similar to that induced by glucose deprivation, with similar biochemical phenotypes being detected. However, the induction of the starvation response by lactic acidosis occurred in the presence of abundant oxygen and nutrients, so this might be considered "pseudo-starvation".

There are several potential mechanisms responsible for the phenotype of the starvation-like response, including AMPK activation and mTOR inhibition, induced by lactic acidosis. Lactic acidosis reduces ATP generation in cancer cells,(Chen, Lucas et al. 2008) leading to a high AMP/ATP ratio, which to activates AMPK. In addition, extracellular acidosis triggers an increase in cytosolic Ca^{++} which might activate CaMKK, an upstream kinase of AMPK to activate and phosphorylate AMPK (Hawley, Pan et al. 2005; Hurley, Anderson et al. 2005; Woods, Dickerson et al. 2005). AMPK activation may regulate several transcriptional factors and co-activators to affect gene expression (Yang, Hong et al. 2001; Kawaguchi, Osatomi et al. 2002). The anti-tumor activities of AMPK activators, such as metformin and AICAR(Rattan, Giri et al. 2005; Swinnen, Beckers et al.

2005; Zakikhani, Dowling et al. 2006; Buzzai, Jones et al. 2007) may also regulate similar pathways stimulated by lactic acidosis. Lactic acidosis might deplete intracellular nutrient such as glutamine by inhibiting acidosis-sensitive glutamine pumps or reducing glucose uptake in cancer cells. The depletion of intracellular nutrients would then repress the energy sensor mTOR and activities required for cell proliferation.

Both hypoxia and glucose deprivation caused an increase of glucose uptake and glycolysis of cells to provide sufficient energy for cells. However, lactic acidosis created a starvation phenotype but at the same time inhibited glucose uptake and glycolysis in cancer cells. We conclude that another molecular pathway is also involved in the lactic acidosis response of cancer cells, the MondoA-TXNIP pathway. Given that lactic acidosis reduced ATP and metabolic substrate from the glycolysis pathways, metabolic reprogramming might play a role to compensate for the energy depletion.

TXNIP was induced by lactic acidosis and this would inhibit glucose uptake and reduce lactate production, which formed a negative feedback loop. The loss of TXNIP, on the other hand, led to glycolytic phenotypes of cancer cells. Higher TXNIP expression was associated with longer survival, in consistence with its previously-identified role as a tumor suppressor. Deficiency of TXNIP led to occurrences of tumors (Nishinaka, Nishiyama et al. 2004; Sheth, Bodnar et al. 2006). In addition to its roles in regulating lactic acidosis response, TXNIP was known as a negative regulator of thioredoxin expression and function to mediate oxidative stress (Nishiyama, Matsui et al. 1999; Junn,

Han et al. 2000; Yamanaka, Maehira et al. 2000), and as a feedback regulator of S-nitrosylation(Forrester, Seth et al. 2009), which might play important roles in the cellular responses to the tumor microenvironment. TXNIP and its α-arrestin paralogue, ARRDC4, were both transcriptionally regulated by the MondoA:Mlx complex(Stoltzman, Peterson et al. 2008; Kaadige, Looper et al. 2009). We demonstrated that lactic acidosis activated TXNIP through MondoA although it remained unexplained how lactic acidosis stimulated a transcriptional response similar to glucose deprivation.

In conclusion, we demonstrated that lactic acidosis simultaneously triggered two tumor suppressing pathways, AMPK-mTOR and MondoA-TXNIP. It would be worthwhile to search for small molecule compounds which could induce similar responses to be developed therapeutically.

Chapter 5

Conclusions and Future Directions

Conclusions

Our study presents a novel perspective of the impact of lactic acidosis. Higher lactate accumulation and extracellular acidosis are often viewed as by-products of tumor hypoxia or as the result of increased aerobic glycolysis in cancer cells. We reported the study to examine the impact of lactic acidosis as an independent stress to stimulate cellular responses and discovered that lactic acidosis in fact induced a significant transcriptional program very different from that of hypoxia. In terms of the prognostic values, we showed that hypoxia response was associated with bad prognosis, in accord with previously-reported result but lactic acidosis, on the other hand, was associated with better prognosis. We further elucidated the mechanism of these effects and demonstrated that lactic acidosis, in contrast to hypoxia, downregulated genes involved in glycolysis and redirected energy production towards aerobic respiration. In addition, lactic acidosis inhibited Akt signaling, glucose consumption and lactate production. Therefore, we proposed that lactic acidosis creates an anti-Warburg effect physiologically which may have the potential to be developed as an alternative cancer therapy.

We carried this study further by examining the transcriptional responses of a breast cancer cell line, to lactic acidosis, hypoxia, and glucose deprivation after our discovery of the unexpected anti-Warburg effect of lactic acidosis. Interestingly, the transcriptional profile of the lactic acidosis response, once more, was significantly different from that of hypoxia, but it was similar to that of glucose deprivation, with a small number of genes differentially regulated between lactic acidosis and glucose deprivation. Among these differentially regulated genes, one gene was the most differentlyregulated caught our attention, TXNIP. TXNIP has been characterized to negatively regulate glucose uptake in adipocytes and in skeletal muscles (Parikh, Carlsson et al. 2007). Given that we observed opposite glucose-uptake phenotypes in MCF7 cells under lactic acidosis and glucose deprivation, we used RNAi technology to demonstrate the regulatory role of TXNIP in glucose uptake in MCF7 cells under lactic acidosis, which was even more obvious in TXNIP- knockout MEFs. Furthermore, MondoA, the transcription factor and glucose sensor that binds and induces TXNIP upon glucose exposure, was also critical in regulating TXNIP responding to lactic acidosis. The lactic acidosis response of MCF7 cells, when projected onto clinical breast cancer patients' datasets, again showed a correlation with better prognosis, which could be recapitulated by using TXNIP alone.

Most cancer studies *in vivo* are done with mouse models, which have contributed to great advancement in cancer research. However, there are limitations of

the application of mouse model results to human cancer treatments. This study offers an incentive to take advantage of mature microarray technologies and bioinformatics tools to establish bridges to link *in vitro* results with *in vivo* cancer patients' information. This study also highlights the differences between the measurements of physiological parameters and cellular responses and provides a new perspective on the tumor microenvironment.

Future directions

We have examined cellular responses to hypoxia, lactic acidosis, acidosis and glucose deprivation, all of which were important physiological characteristics of the tumor microenvironment. Another stress, oxidative stresses and oxygen radicals in the form of reactive oxygen species (ROS) ,resulting from dysregulated metabolism in cancer cells, infiltration of ROS-producing macrophages and frequent hypoxia-reoxygenation in tumor vascular networks, were particularly high in human tumor cells (Szatrowski and Nathan 1991) and caused persistent oxidative stress in cancers (Toyokuni, Okamoto et al. 1995). ROS induced DNA instability and tumor progression (Szatrowski and Nathan 1991; Wiseman and Halliwell 1996), activated kinase signaling pathway. We can utilize the system established in this project and treat primary cells or cancer cells with oxidative stresses in cell culture *in vitro*, acquire the gene expression profiles with the application of microarrays and project the cellular response signatures

onto the clinical cancer patients' tumors *in vivo* to examine cellular responses to oxidative stresses in human cancers. Additionally, given the intermittent and reversible nature of the tumor microenvironment with these stresses fluctuating with temporal and spatial variations, such as intermittent hypoxia, defined by hypoxia followed by reoxygenation, it would also be interesting if we could apply and withdraw, followed by reapply a stress and examine the impact to cellular responses. We have got some preliminary data on neutralization, created by applying lactic acidosis media to cells followed by withdrawing them and re-applying neutral pH, regular culture media again to cells. There were immediately two different groups of genes separated by the criteria of whether their induced or repressed responses by lactic acidosis were rescued by neutralization. One thing we found very intriguing was that the neutralization treatment induced many interferon response genes which were commonly observed to be induced by viruses. On the other hand, temporal regulations played an important role in this kind of experiments. We have only performed experiments up to 48 hour incubation and we should probably try longer exposure such as 10 or 20 days in the future. One caveat in analyzing data from this sort of intermittent experiment was which groups to be defined as the control group. Assigning the cells cultured in the very original control media as the control or the cells incubated in lactic acidosis media prior to the neutralization treatment as the control would sometimes change our conclusions. The other danger is we may overlook genes which are critical but do not have significant

induction or repression in gene expression. Although there are challenges remained to be overcome, these experiments will offer great opportunities for researchers to further investigate the complex matters of the tumor microenvironment.

The other direction to carry this project forward is to identify the cellular surface receptors responsible for sensing the stress of lactic acidosis and acidosis. Two protein families, G-protein coupled receptors (GPCRs) and acid-sensing ion channels (ASICs) which contained most of the acid-sensing receptors were among the top candidates of our further investigation. However, GPCRs and ASICs responded to quite different ranges of pH with GPCRs sensing acidities around pH6.5 and ASICs around pH5.5. The measured intratumor pH was ranged from pH6.5 to pH6.9 and the lactic acidosis microenvironment we previously created was around pH6.7. Therefore, we expected GPCRs to be more probable candidates in sensing and activating lactic acidosis as well as acidosis responses. The acid-sensing GPCR family proteins included G2A (Murakami, Yokomizo et al. 2004), GPR68 (OGR1, ovarian cancer GPCR)(Ludwig, Vanek et al. 2003), GPR65 (TDAG8, T cell-death associated gene 8)(Wang, Kon et al. 2004) and GPR4(Im 2005; Tomura, Mogi et al. 2005). These receptors responded to both extracellular acidosis and various lysolipids. Several tools and reagents we have in the lab or have easy accesses on can help us investigate this. We have observed TXNIP promoter to be significantly induced by lactic acidosis and acidosis treatment and increased acidity from pH 6.7, pH6.5 to pH6.0 enhanced the inductions. Additionally, TXNIP expression

was also upregulated with more acidic lactic acidosis or acidosis treatments up to pH 6.0. Therefore, we can establish the reporter cell lines of TXNIP to monitor the cellular responses to acidosis. To measure acidosis response, we can utilize the luciferase reporter gene assay system composed of the firefly luciferase reporter with 1.2kb of TXNIP promoter region which can be induced to upregulate the luciferase activities more than 8 folds by acidosis and a renilla luciferase reporter driven by a constitutively active SV40 promoter (pRL-SV40) as an internal control for cell viability to establish the acid-sensing reporter cell lines which stably express these luciferase. Moreover, RNAi silencing can be applied to inactivate candidate acid-sensing receptors, such as G2A, GPR68, GPR65 and GPR4 to examine whether silencing these receptors would affect previously-observed lactic acidosis and acidosis responses of cells. If these procedures can be successfully achieved, this will be a powerful tool allowing us to search for small molecules which can modulate lactic acidosis and acidosis responses and lead to potentially future therapeutic applications.

We found the significant impact of lactic acidosis treatment on cancer cell metabolism. Therefore, further investigating this by examining metabolites responding to these tumor microenvironmental stresses will be another direction worth pursuing. We have acquired preliminary data on metabolomics from MCF7s exposed to lactic acidosis, glucose deprivation and hypoxia. Organic acids, amino acids and acyl carnitines were analyzed. Interestingly, we found that lactic acidosis and hypoxia

induced relatively similar amino acid metabolic profiles mostly in contrast to that of glucose deprivation. Although the mechanisms were still unknown, developments in consolidating our expression profiles with metabolic profiles would be very powerful in elucidating the mechanisms of cancer cell metabolism.

There are multiple directions we can pursue further and many interesting hypotheses remained to be tested. Lactic acidosis is a complex stress and connected with multiple aspects of physiology. It can present us with many opportunities to discoveries.

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Publication

1.**Chen JL**, Merl D, Peterson C, Wu J, Liu P, West M, Ayer D and Chi JT
Lactic acidosis triggers starvation response with paradoxical induction of TXNIP through MondoA. Manuscript submitted and being reviewed by PLoS Genetics

2.Merl D., **Chen JL**, Jen-Tsan Chi, and Mike West.
An intergrative analysis of cancer gene expression studies using Bayesian latent factor modeling. Annals of Applied Statistics. 2009. In Press

3. Lucas J.E., Carvalho C.M., **Chen J.L**., Chi J.T. and West M.
Cross-study projections of genomic biomarkers: An evaluation in cancer genomics. PLoS One 2009;4(2):e4523. Epub 2009 Feb 19.

4.**Chen JL**, Lucas JE, Schroeder T, Mori S, Wu J, Nevins J, Dewhirst M, West M, Chi JT.
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5. Izumiya Y, Lin SF, Ellison T, **Chen LY**, Izumiya C, Luciw P, Kung HJ.
Kaposi's sarcoma-associated herpesvirus K-bZIP is a coregulator of KSHV Rta: physical association and promoter-dependent transcriptional repression. J Virol. 2003 Jan;77(2):1441-51

Scholarships and Awards

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