Gene expression profiling and regulation mechanisms of hepatic antioxidant enzymes by chemopreventive polyphenolic compounds

（機能性ポリフェノール化合物による肝臓抗酸化酵素遺伝子の発現プロファイリングおよび制御機構に関する研究）

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Abbreviations

AKR1C1/2/3, aldo-keto reductase family 1, member C1/2/3

ALDH1L2, aldehyde dehydrogenase 1 family, member L2

ARE, antioxidant response element

CHX, cycloheximide

CYPs, cytochrome P450 superfamily

DPYD, dihydropyrimidine dehydrogenase

DUSP1, dual specificity protein phosphatase 1

EMSA, electrophilic mobility shift assay

GCLC/M, glutamate--cysteine ligase catalytic/modifier subunit

GSR, glutathione reductase

HERPUD1, homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein

HMGCLL1, 3-hydroxymethyl-3-methylglutaryl-CoA lyase-like 1

HO-1, heme oxygenase-1

IPA, ingenuity pathway analysis

Keap1, kelch-like ECH-associated protein 1

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NQO1, NAD(P)H: quinone oxidoreductase 1

Nrf2, nuclear factor-erythroid 2 p45-related factor 2

siRNA, small interfering RNA

SLC family, cystine/glutamate transporter solute carrier family

SQSTM1, sequestosome-1

SRXN1, sulfiredoxin-1

TF, transcriptional factors

TXNIP, thioredoxin-interacting protein

TXNRD1, thioredoxin reductase 1

Abstract

Polyphenolic compounds occurring in many edible plants are the main part of phytochemicals and have been reported to possess multiple bioactivities on chemoprevention of human chronic diseases. Accumulated studies have suggest that consumption of polyphenol-rich vegetables are beneficial in the prevention of chronic diseases such as cancer, stroke, diabetes, neurodegenerative disease and heart disease based on laboratory studies and epidemiological investigations. The chemopreventive mechanisms of polyphenolic compounds have been investigated at molecular levels. However, most of the studies only paid attentions on limited genes or signaling pathways, which were not consistent with the multiple functions of polyphenolic compounds and the complicated pathology of human chronic diseases. Applications of omics tools in nutrigenomics and evidence-based food or traditional medicine researches will meet the requirement for full understanding of the chemopreventive mechanisms of polyphenolic compounds. Of omics, DNA microarray and pathway analysis are powerful tools to study the molecular mechanisms of polyphenolic compounds due to their advantages on analyzing the effect in genome wide.

In the present study, two typical polyphenolic compounds were chosen, myricetin from food such as grape and baicalein from traditional medicine, due to their similar structures which could help analyzing the structure-activity relationship. Besides, myricetin could provide the example to apply microarray in nutrigenomic research, and baicalein could be a model for application of microarray in evidence-based traditional medicine research. Hepatocytes are the main cells for polyphenolic compounds metabolizing with the aid of drug metabolizing enzymes *in vivo*. Thus, HepG2 cells were chosen as the model for its wide use in regulation of drug metabolizing enzymes, ADME (absorption, distribution, metabolism, and excretion), toxicological and other basic researches.

I performed gene expression profiling by DNA microarray to evaluate the chemopreventive function and underlying genes targeted by myricetin and baicalein

in HepG2 cells, and then I used Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) to analyze the huge microarray data that can help better understanding the effects of these polyphenolic compounds on cell biological function in genome wide. Finally, I used real-time PCR to verify the accuracy of some genes of interests. Among total 44K gene probes, myricetin treatment up-regulated the signals of 143 gene probes (0.33% of total probes) and down-regulated signals of 476 gene probes (1.08% of total probes) by ≥2-fold, baicalein treatment up-regulated the signals of 440 gene probes (1.04% of total gene probes) and down-regulated signals of 254 gene probes (0.6% of total gene probes) by ≥2-fold in HepG2 cells. Gene Ontology analysis revealed that drug metabolizing enzymes were significantly influenced by polyphenolic compounds treatment. The network pathways analyses by IPA further revealed top 10 canonical pathways were modulated by each treatment. Of which, an Nrf2 (nuclear factor-erythroid 2 p45-related factor 2)-mediated ARE (antioxidant response element) pathway was involved in both baicalein- and myricetin-induced gene expressions of hepatic metabolic enzymes. The representative enzymes involved in Nrf2-ARE pathway were further confirmed at mRNA level by real time polymerase chain reaction (PCR).

To uncover the molecular mechanism underlying the activation of Nrf2-ARE pathway, I further investigated the effect at both the transcriptional level and the posttranscriptional level of Nrf2 expression by baicalein treatment in HepG2 cells. At the transcriptional level, Nrf2-related network analysis and upstream protein kinases signaling pathways data revealed that baicalein regulated the expression of Nrf2 mRNA by targeting transcription factors (such as NF-κB and AHR) and nuclear proteins (such as c-Src and c-Jun) via phosphorylating the upstream protein kinases MEK, AKT and JNK signaling pathways. At the posttranscriptional level, molecular data revealed that baicalein activated Nrf2-ARE pathway by inhibiting Nrf2 ubiquitination and protein turnover via stimulating Keap1 modification and ubiquitination. Besides, baicalein-induced phosphorylation of protein kinases may lead to Nrf2 phosphorylation, which also allowed Nrf2 escaping from Keap1 inhibition. All of these events finally increased nuclear Nrf2 accumulation, ARE

binding activity and transcription activity to enhance ARE-mediated genes expressions. Furthermore, myricetin was found to exert its chemopreventive effect by the same mechanism due to its similar structure as baicalein. Additionally, treatment with Nrf2 siRNA attenuated both the baicalein-induced and myricetin-induced ARE activity and gene expressions. Based on structure-activity analysis, I found that the basic flavan skeleton seems to be the key structure of polyphenolic compounds to activate Nrf2.

These results provided a comprehensive data for understanding the gene expression, hepatic metabolism and bioactive role of these polyphenolic compounds. The pathway network analysis and molecular data revealed that baicalein and myricetin exert their chemopreventive effects by activation of Nrf2-ARE pathway, which could not only help us to apply microarray in nutrigenomic research and evidence-based traditional medicine discovery, but also guide us to study the interactome and transcriptome of Nrf2 for fully understanding its role in preventing the chronic diseases and improving the human health.

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Chapter 1

Introduction

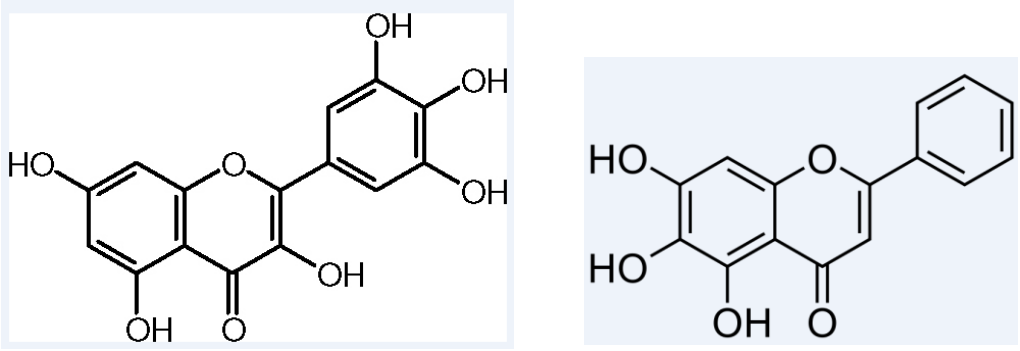
1.1 Phytochemicals

1.1.1 Multi-function in human health

Dietary phytochemicals are biologically active chemical compounds that occur naturally in edible plants, and some molecules of them are responsible for the color and organoleptic properties of fruit and vegetables. Dietary phytochemicals are considered generally to be nonessential nutrients, but they may affect human health due to their chemical properties [1, 2]. There are nearly 10,000 different dietary phytochemicals considered to be beneficial in the prevention of chronic diseases such as cancer, stroke, diabetes, neurodegenerative diseases and heart disease based on laboratory studies and epidemiological investigations [3, 4]. Especially after the failure of clinical trials on proposed chemopreventive essential nutrients such as vitamins and dietary fibres, many recent studies move on to the chemopreventive mechanisms by natural dietary phytochemicals [1, 2].

1.1.2 Polyphenolic compounds

Polyphenolic compounds from natural plants constitute the main part of phytochemicals and have been proved as the most promising chemopreventive compounds for many chronic human diseases prevention. We paid attention on two typical polyphenolic compounds in the present study: a traditional Chinese medicine baicalein from the herb *Scutellaria baicalensi* and a dietary flavonol myricetin from many edible plants (Figure 1.1) due to their similar structures which could help us to analyze the structure-activity relationship. Meanwhile, myricetin could provide an example to apply microarray in nutrigenomics research, and baicalein could be a model for application of omics in evidence-based traditional medicine research.



Myricetin **Baicalein**

Figure 1.1 Molecular structures of myricetin and baicalein.

1.1.2.1 Myricetin

Myricetin is a typical polyphenol with hydroxyl substitutions at the 3,5,7,3',4' and 5' positions and widely distributed in many grapes, berries, fruits, vegetables, herbs as well as red wine with many bioactivities such as antioxidant, anti-inflammation, anticancer, anti-diabetes and anti-mutation (Figure 1.1) [5, 6]. For example, myricetin could inhibit the growth of A549 cells through inhibition of thioredoxin reductase expression [7], neoplastic cell transformation by targeting MEK [8], and UVB-induced skin cancer by targeting Fyn [9]. Myricetin also induced apoptosis in human leukemia HL-60 cells via mitochondrial-dependent way [10], and blocked metastatic process by inhibiting MMP-2 expression [11] and MRPs-mediated resistance to anticancer drug vincristine in transfected MDCKII cells [12]. Moreover, myricetin protected cells by repairing DNA damage [13, 14], reduced oxidative stress [15, 16], inhibited hyperglycemia and glucose uptake [17-19], modulated Ca^{2+} transport activity [20-22] and inhibited inflammation [23-25]. These data suggest that myricetin may influence expressions of many genes to exert its multiple bioactivities.

1.1.2.2 Baicalein

Baicalein (5, 6, 7-trihydroxyflavone) is a flavone originally isolated from the traditional Chinese medicinal herb, the roots of *Scutellaria baicalensis*, one of the most popular and multi-purpose herbs used in China (Figure 1.1). Baicalein has been

clinically used for anti-cardiovascular illness, and antitumor, anti-inflammatory, antioxidant, antiviral, and antibacterial purposes [26]. Accumulated data showed that baicalein had inhibitory effects on the adhesion, migration and invasion of MDA-MB-231 human breast cancer cells [27] and the transcriptional activity of β-catenin/Tcf in HEK293 cells [28]. Baicalein also suppressed the growth of colon cancer cells [29], lung cancer cells [30], and prostate cancer cells [31]. Baicalein had inhibitory effects on LPS-induced NOs production [32], COX-2 gene expression [33], activation of NF-κB [34] and productions of many inflammatory cytokines [35-37]. Moreover, baicalein could serve as a free radical scavenger against hydroxyl radicals, and had the ability to inhibit both lipoxygenase and xanthine oxidase enzymes [38-40]. Baicalein also could mediate P450 system [41, 42] and induce the expression of Nrf2 and downstream phase II genes such as HO1 (heme oxygenase-1) and NQO1 (NAD (P) H: quinone oxidoreductase 1) [43, 44], suggesting that baicalein may be involved in activation of Nrf2-ARE antioxidant pathway.

1.2 DNA microarray

Accumulating studies aimed to reveal the molecular mechanism underlying the effects of polyphenolic compounds on Nrf2-ARE pathway activation. However, most of these researches only targeted the limited genes or signaling pathways, which is limited significance with the complicated pathology of human chronic diseases and cannot provide the global assessments of the multi-function of polyphenolic compounds. Thus, DNA microarray-based nutrigenomics study gets its priority on this field.

1.2.1 DNA microarray introduction

After genome sequencing, DNA microarray analysis has evolved rapidly and become the most widely used source of genome-scale data in the life sciences since its introduction in 1995 [45]. Microarray expression studies are producing massive quantities of gene expression and other functional genomics data, which promise to

provide key insights into gene function and interactions within and across metabolic pathways [46-48]. Thus, DNA microarray is currently widely used on the fields of nutrient-related diseases and predisposition, food safety assessment, individualized medicine and function food.

Although certain limitations of the current technology exist and have become more apparent during the past couple of years [49, 50], the ability of microarrays to monitor the expression of thousands of genes simultaneously is unsurpassed, especially in nutrigenomics and evidenced-based traditional medicine field [51, 52].

1.2.2 Application in nutrigenomics

Nutrigenomics is the study of the effects of foods and food constituents on gene expression and has also been described by the influence of genetic variation on nutrition with a nutrient's absorption, metabolism, elimination or biological effects [53, 54]. During the last decade, the completion of several large genome projects has markedly altered the research agenda by drawing attention to the importance of genes in human nutrition, and has provided a wealth of new genetic information to be explored. Subsequently, scientists find that the nutrients can be potent dietary signals that influence the metabolic programming of cells and have an important role in the control of homeostasis. Recently, nutritionists and clinicians have increasingly started to recognize that genetic predisposition can be an important contributor to the main causes of mortality that are linked to diet, such as cardiovascular disease, diabetes type II and cancers [51, 55, 56]. At present, nutrigenomics has been associated with the idea of personalized nutrition based on genotype and it will ultimately lead to evidence-based dietary intervention strategies for restoring health and fitness and for preventing diet-related disease [51]. Although nutrigenomics is a science still in its infancy, its contribution to public health over the next decade is thought to be one of the major events.

DNA microarray could help us to study the molecular mechanism underlying the pathogenesis of diet associated diseases and provide us comprehensive gene expression profiles and interaction pathway networks.

1.2.3 Application in evidence-based medicine study

Evidence-based medicine is the use of mathematical estimates of the risk of benefit and harm, derived from high-quality research on population samples, to inform clinical decision-making in the diagnosis, investigation or management of individual patients [57]. Evidence-based traditional medicine is multi-component drug from the natural plant with two potential values including easy accessible and low-cost source of medicines for primary health care, and the source for finding novel leads and/or targets for drug development [52]. Especially, the success in application of several traditional medicines at the basis of essential modern drugs such as poppy, belladonna and digitalis, combining with the recent successful treatment of HIV-patients with mixtures of compounds, lead to the emergency need for developing traditional medicine based on evidences by using omics tools [52, 58]. Traditional medicine is often based on personalized medicine which is the dream of modern pharmacy with the ultimate goal of human genetics based tailor made pharmacotherapy.

As the holistic approaches underlying the practice of traditional medicine and new tendencies in modern medicine towards personalized medicine require in-depth knowledge of mechanisms of action and active compounds, the use of omic techniques is crucial for understanding and interpretation of Traditional Chinese Medicine development, especially in view of its expansion in Western countries [59, 60].

DNA microarray could not only help us to improve our understanding and treatment of human chronic diseases, but also provide us a fascinating appropriate way to prevent some predicted diseases based on the identification of the aberrant gene expressions by using personalized medicine.

1.2.4 Ingenuity Pathway analysis (IPA)

DNA microarray technology enables us to simultaneously examine the expression of thousands of genes. However, in biology gene expression changes do not occur as independent events as such lists suggest, but in a highly coordinated and interdependent manner. Understanding the biological meaning of the observed

changes requires elucidating such biological interdependencies.

Recently, many pathway analysis technologies were used for allowing the mapping of gene expression data into relevant pathways based on their functional annotation and known molecular interactions. Among which, one of the most often used and most powerful tool is Ingenuity pathways analysis (Ingenuity® Systems, http://www.ingenuity.com, IPA) system, which is a software that helps researchers to model, analyze, and understand the complex biological and chemical systems at the core of life science research. IPA has been broadly adopted by the life science research community and is cited in thousands of peer-reviewed journal articles. IPA enables biologists and bioinformaticians to identify the biological mechanisms, pathways, and functions most relevant to their experimental datasets or genes of interest [61, 62].

1.3 Chemopreventive Nrf2-mediated ARE pathway

Among the multi functions of polyphenolic compounds, the best studied and the most noticeable one is antioxidant property, which is the main cause involving in the chemopreventive effect of polyphenolic compounds. Most recently, accumulating studies turned their eyes on the critical role of Nrf2-ARE antioxidant pathway.

1.3.1 Discovery

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a bZip transcription factor and a member of the Cap ‘n’ Collar family of regulatory proteins, which was first identified and isolated in 1994 with an acidic activation domain that may participate in the transcriptional stimulation of beta-globin genes [63]. Then, Nrf2 was shown to mediate the cellular responses to electrophiles and oxidants by binding to an enhancer element (antioxidant response element, ARE) in the promoter regions of cytoprotective genes [64, 65]. Furthermore, the most convincing data from Nrf2-knockout mice showed an impaired induction of detoxifying enzymes and redox-balancing proteins [66, 67], demonstrating that Nrf2 is a critical regulator of

AREs. These investigations lead to the discovery of Nrf2-ARE signaling pathway, which was mainly attributed to Masayuki Yamamoto's group who identified Keap1 (Kelch-like associated protein 1), a homologue of Drosophila actin-binding protein called Kelch [68], as a negative regulator of Nrf2. What followed was that many researchers in this field focused on the molecular mechanisms of Nrf2 regulation, following a flurry of discoveries of additional Nrf2-regulated genes including detoxifying enzymes, drug transporters, and cellular redox regulators which could regulate many human chronic diseases [69-72, 73]. Therefore, Nrf2 has emerged as a master regulator of cellular defense mechanism that elicits an adaptive response and promotes cell survival under stress. The regulation of Nrf2-Keap1-ARE pathway seems to provide us a reassuring way to keep health by making our own 'medicines' and perhaps all we need to do is just to help the cells perfect the timing and degree of Nrf2 activation.

1.3.2 Mechanism of Nrf2 pathway activation

The transcription factor Nrf2 controls expressions of genes for antioxidant enzymes, metal-binding proteins, drug-metabolizing enzymes, drug transporters, and molecular chaperones [74]. Despite many models of regulation of Nrf2-Keap1-ARE pathway had been proposed, such as dissociation of Keap1 and Cul3, the hinge and latch model, nucleocytoplasmic shuttling of Keap1, the ubiquitination of Keap1, and Nrf2 directly sensing inducers [75], the most compelling model could be summarized as following (Figure 1.2): Keap1 has been identified to function as a molecular switch to turn on and off the Nrf2-mediated antioxidant response. Under normal homeostatic conditions, Keap1 is in the off position and functions as an E3 ubiquitin ligase, constantly targeting Nrf2 with Cul3-Rbx1 for ubiquitination and proteasomal degradation. However, the switch is turned on when oxidative stress or phytochemicals inhibit the activity of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase through modifying cysteine residues of Keap1 or phosphorylation of Nrf2, resulting in the release of Nrf2 from Keap1 binding. As a consequence, Nrf2 enters into the nucleus where it heterodimerizes with small Maf or other uncertain proteins, binds to ARE element,

and activates its downstream genes. The proposed pathways could be mainly classified into two catalogues including Keap1-dependent and Keap1-independent based on huge number of studies aiming to elaborate the molecular mechanism of Nrf2-ARE pathway during the last decade.

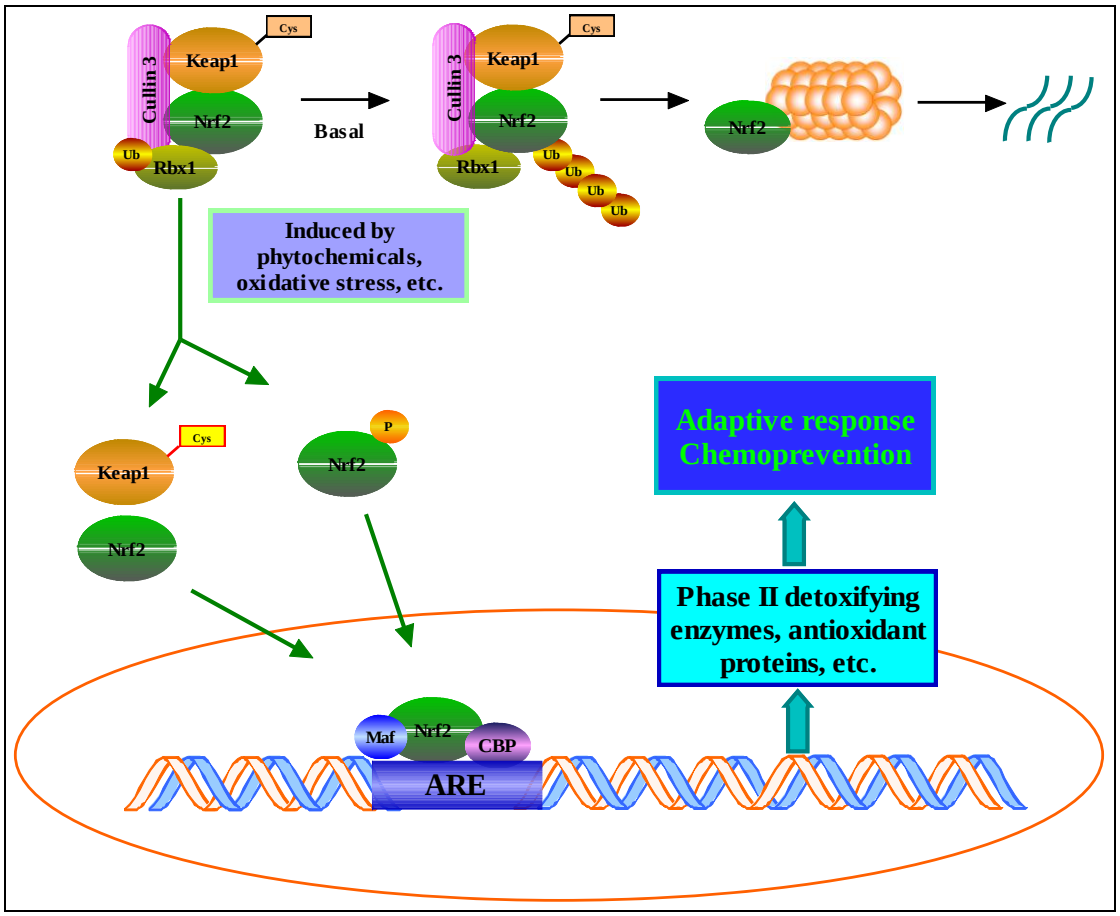


Figure 1.2 The mechanism of Nrf2-Keap1-ARE pathway activation. Under basal conditions, Nrf2 is inhibited by Keap1 binding, and targeted by Cul3-Rbx1 ubiquitination system for proteasomal degradation. Under induced-state, Nrf2 is released from Keap1 by phosphorylation of Nrf2 or Keap1 modification, enters nucleus and subsequently activates Nrf2-ARE pathway.

1.3.3 The effects of dietary phytochemicals on Nrf2-ARE pathway

Due to the critical role of Nrf2 in chemoprevention, it is highly possible that phytochemicals target Nrf2-ARE pathway to exert their multi-function in human health, and many recent studies have verified this possibility. As shown in Figure 1.3, summarized dietary phytochemicals with effect on Nrf2-ARE pathway activation and

their respective chemical structures are classified into 5 groups: fruits & vegetables, spices, teas & coffee, herb medicines and marine products.

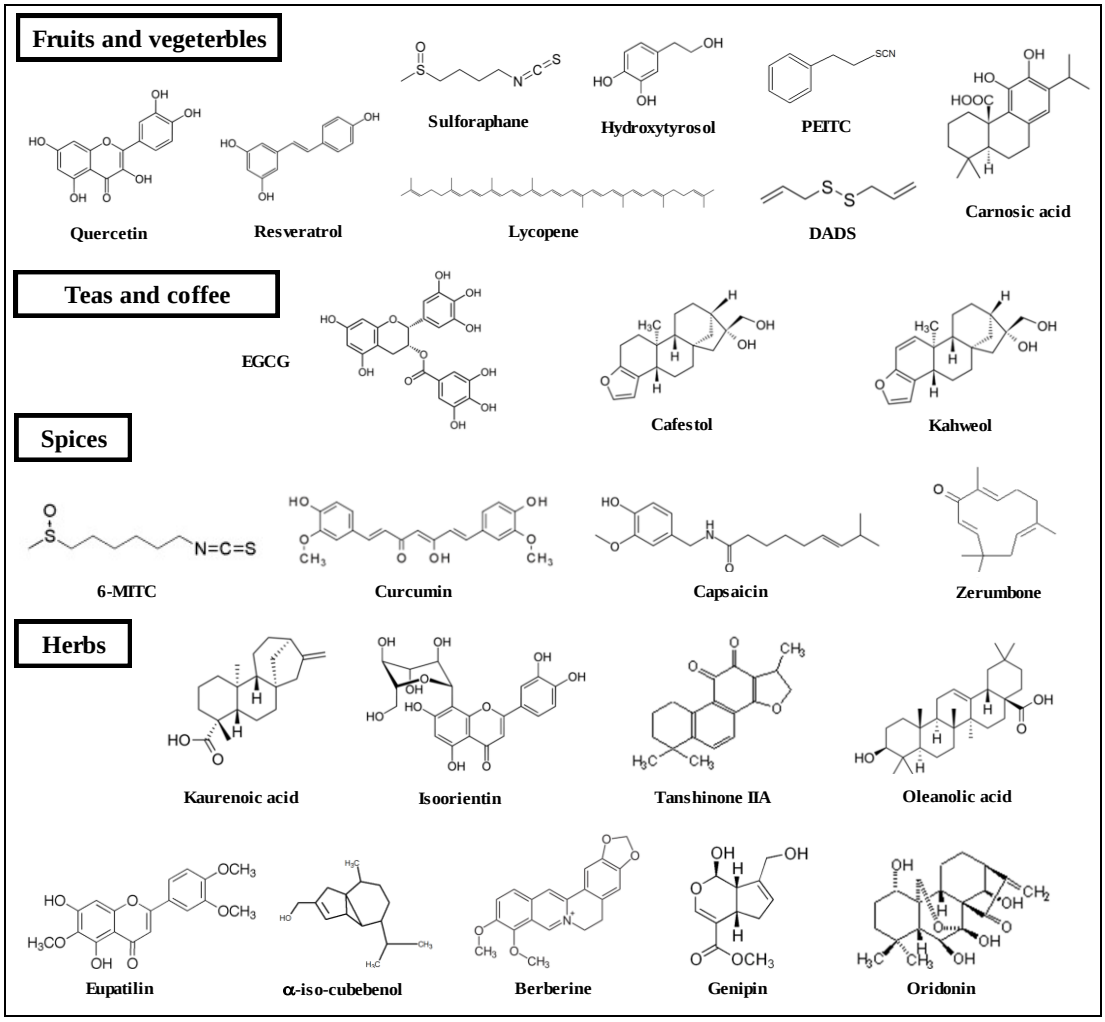


Figure 1.3 Structures of representative phytochemicals potentially activating Nrf2. These phytochemicals are present in many fruits & vegetables, spices, teas & coffee and herb medicines.

For example, isothiocyanates and EGCG are well studied Nrf2-ARE activators. Isothiocyanates including sulforaphane, methylsulfinyl isothiocyanates (6-MSITC) and phenethyl isothiocyanate (PEITC) are present in cruciferous vegetables such as cauliflower, wasabi, cabbage, cress, bok choy, broccoli and similar green leafy vegetables. They act as potent inducers of ARE-driven phase II gene expression with multi mechanisms. Sulforaphane could abrogate toxicity as well as the accumulation of arsenite in cultured murine hepatocytes by activating Nrf2 signaling [76]. Sulforaphane also enhanced the expression of detoxifying enzymes including NQO1,

GST and GCL in the small intestine of *nrf2*-wild-type mice, but not in *nrf2*-null mice [77]. Signaling data revealed that sulforaphane induced Nrf2-driven phase 2 enzyme expressions by stimulating MAP kinases activation [78-81] and by facilitating dissociation of Nrf2 from Keap1 through modification of Keap1cysteine residues [82]. PEITC treatment of PC-3 cells could also activate Nrf2-ARE pathway through the activation of ERK and JNK [83]. Additionally, the indoles such as indole-3-carbinol (I3C) and 3, 3'-diindolylmethane (DIM) from cruciferous vegetables induced JNK-dependent ARE activation [84].

EGCG, a major active catechin component of green tea, was found to be the most potent Nrf2 activator among the green tea polyphenols [85]. EGCG has been reported to activate Nrf2 and induce expression of HO-1 through activation of Akt and ERK1/2 in endothelial cells [86] or p38 MAPK and Akt in B-lymphoblasts [87]. Similar study also observed that EGCG induced activation of ERK1/2 and Akt through phosphorylation in cultured human mammary epithelial MCF-10A cells [88]. Furthermore, EGCG inhibited the growth and liver/pulmonary metastasis of colon tumor implanted orthotopically in the cecum of nude mice, and its anticancer effect was proposed to be partly mediated by activating the Nrf2-UGT1A signal pathway [89]. DNA microarray results from Nrf2-knockout mouse with comparison to the wild type showed that 671 Nrf2-dependent genes and 256 Nrf2-independent genes were modulated by EGCG in liver, whereas 228 Nrf2-dependent genes and 98 Nrf2-independent genes were identified in the small intestine [90].

1.3.4 Metabolism of polyphenolic compounds and drug metabolizing enzymes

Liver is the main place for polyphenolic compounds metabolizing with the aid of drug metabolizing enzymes. Drug metabolizing enzymes are consisted of phase I, phase II metabolizing enzymes and phase III transporters that play central roles in the metabolism, elimination and detoxification of xenobiotics and drugs [91]. Bioavailability of polyphenol is dependent on absorption, metabolism and elimination by the modulation of metabolizing enzymes. Variations in the activity of these enzymes and transporters affect drug activity and elimination, and eventually

increased half-life and toxicity of xenobiotics [92]. Recently, several studies have focused on the mechanisms that regulate the expression of the drug metabolizing enzymes, and Nrf2 has been identified to be the key mediators regulating the gene expressions of phase I, II metabolizing enzymes and phase III transporters in drug-induced changes [93, 94].

Polyphenolic compounds have been implicated as pharmaceutical agents and their metabolism has become more important subject in drug discovery and development [95]. The modulation of chemopreventive enzymes by polyphenolic compound is an important step for human health since these enzymes can inactivate carcinogens, which contributes to the cancer preventive properties of these compounds. However, the whole-view for the influence of polyphenolic compounds on hepatocytes, especially on drug metabolizing enzymes and transporters are still not clear.

1.4 Thesis investigation

Accumulating data reveals that polyphenolic compounds, the main part of phytochemicals, are the most potent and promising compounds with exerting multi-function on chemoprevention of human chronic diseases such as cancer, cardiovascular disease, diabetes, degenerative disease and inflammatory disease. Polyphenolic compounds could exert their chemopreventive functions via induction of Nrf2-ARE pathway, and the bioactivities are depending on their metabolism with the aid of drug metabolizing enzymes in the hepatocytes of the liver. However, the effects of polyphenolic compounds on expressions of drug metabolizing enzymes and the exact molecular mechanism underlying the activation of Nrf2-ARE pathway still remained unknown. Moreover, increasing applications of omics tools in post genome era lead to emergency need for personalized nutrition guides or medicine, making it necessary and urgent to apply omics in nutrigenomics and evidence-based food or traditional medicine discovery, which could improve the quality of our life. Thus, I choose two typical polyphenolic compounds and HepG2 cell model in the present study. Myricetin from food such as grape and baicalein from traditional medicine

have the similar molecular structures which could help us to analyze the structure-activity relationship. Besides, myricetin could provide the example to apply microarray in nutrigenomics research, and baicalein could be a model for application of omics in evidence-based traditional medicine research. HepG2 cells are widely used in regulation of drug metabolizing enzymes, toxicological and other basic researches. I plan to study the multi-function and the molecular mechanism underlying the chemopreventive effects of these polyphenolic compounds based on gene expression profiling and pathway network analysis with efficient analysis tools.

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Chapter 2

Gene expression profiling and pathway network analysis of polyphenolic compounds

2.1 Abstract

Polyphenolic compounds from natural plants have been proved as promising chemopreventive compounds for many chronic human diseases. Two typical polyphenolic compounds were chosen for study in this chapter: a dietary flavonol myricetin from many edible plants and a traditional Chinese medicine baicalein from the herb *Scutellaria baicalensi*. First, gene expression profiling was performed by DNA microarray to evaluate the chemopreventive function and underlying genes targeted by these polyphenolic compounds in HepG2 cells. Next, the huge microarray data was analyzed by Gene Ontology (GO) especially focused on drug metabolizing enzymes, and further by Ingenuity pathway analysis (IPA), a professional analysis software, in the form of generating canonical pathways and networks. Finally, real-time PCR was used to verify the accuracy of some genes of interests. These results could help us better understanding the effects of these polyphenolic compounds on the genome wide.

Among total 44K gene probes, myricetin treatment up-regulated the signals of 143 gene probes (0.33% of total probes) and down-regulated signals of 476 gene probes (1.08% of total probes) by ≥2-fold; baicalein treatment up-regulated the signals of 440 gene probes (1.04% of total gene probes) and down-regulated signals of 254 gene probes (0.6% of total gene probes) by ≥2-fold in HepG2 cells. These genes were categorized into 35 groups and hit for biological processes, molecular functions, and signaling pathways. The result showed that the expressions of drug metabolizing enzymes were significantly affected by these polyphenolic compounds treatments. Further networks and pathways analyses revealed that an Nrf2 (nuclear factor-erythroid 2 p45-related factor 2)-mediated ARE (antioxidant response element)

pathway is involved in baicalein- and myricetin-induced gene expressions of hepatic metabolic enzymes. The representative enzymes involved in Nrf2-ARE pathway were further confirmed at mRNA level by real time polymerase chain reaction (PCR). These data provided us a comprehensive data for understanding the gene expression, hepatic metabolism and bioactive role of these polyphenolic compounds.

2.2 Introduction

Polyphenolic compounds from natural plants have been proved as promising chemopreventive compounds for many chronic human diseases. Baicalein and myricetin are widely distributed typical polyphenolic compounds with multi-function such as anti-inflammatory, antitumor, anti-diabetes and antiviral [1-4]. However, the underlying molecular mechanism of these chemopreventive effects and their metabolizing process remained unknown.

Hepatocytes are the main cells for polyphenolic compounds metabolizing with the aid of drug metabolizing enzymes. Drug metabolizing enzymes are consisted of phase I, phase II metabolizing enzymes and transporters that play central roles in the metabolism, elimination and detoxification of xenobiotics and drugs [5]. Bioavailability of polyphenol is dependent on absorption, metabolism and elimination by the modulation of metabolizing enzymes. In brief, phase I enzymes can modify the structure of the xenobiotics, phase II enzymes then increase aqueous solubility of flavonoids and conjugate them with glutathione or glucuronate, and finally transporters can either take flavonoids up from blood into hepatocytes (such as some SLC family enzymes) or efflux them into bile and blood (such as some ABC transporters). Examples of these enzymes include NQO1, GSR (glutathione reductase), HO-1, SRXN1 (sulfiredoxin-1), GCLM (glutamate cysteine ligase modifier subunit), AKR1C2 (aldo-keto reductase family 1, member C2) and TXNRD1 (Thioredoxin reductase 1) [6]. Variations in the activity of these enzymes and transporters affect drug activity and elimination and eventually increased half-life and toxicity of xenobiotics [7]. Recently, several studies have focused on the mechanisms

that regulate the expression of the drug metabolizing enzymes. Various nuclear factors including the aryl hydrocarbon receptor (AhR) [8, 9], orphan nuclear receptors [10, 11], and Nrf2 have been identified to be the key mediators regulating the gene expressions of phase I/II metabolizing enzymes, antioxidant proteins and transporters in drug-induced changes [8, 12].

Polyphenolic compounds have been implicated as pharmaceutical agents. Therefore, their metabolism has become more important subject in drug discovery and development [13]. The modulation of chemopreventive enzymes by polyphenolic compound is an important step for human health since these enzymes can inactivate carcinogens, which contributes to the cancer preventive properties of these compounds. Once baicalein enters the human body, its bioavailability and bioactivity are also dependent on modulation of the drug metabolizing enzymes. Five major metabolites including baicalein 6-*O*-beta-glucopyranuronoside (M1), 6-*O*-methyl-baicalein 7-*O*- beta-glucopyranuronoside oroxylin A 7-*O*-beta-glucuronide (M2), baicalein 7-*O*-beta- glucopyranuronoside (M3), 6-*O*-beta-glucopyranuronosyl-baicalein 7-*O*-sulfate (M4), and baicalein 6,7-di-*O*-beta-glucopyranuronoside (M5) were found in rats administered with baicalein or baicalin orally [14]. Oral administration of myricetin 3-rhamnoside in rat revealed that intestinal micro-organisms can cleave glycosidic bonds into aglycone myricetin, which resulted in the urinary excretion of 3,5-dihydroxyphenylacetic acid [15]. However, the whole-view for the influence of these polyphenolic compounds on hepatocytes, especially on drug metabolizing enzymes and transporters are still not clear.

DNA microarray technology enables us to simultaneously examine the expression of thousands of genes. Changes in transcript levels are assessed by microarray analysis on an individual basis, essentially resulting in long lists of genes that were found to have significantly changed transcript levels. However, in biology these changes do not occur as independent events as such lists suggest, but in a highly coordinated and interdependent manner. Understanding the biological meaning of the observed changes requires elucidating such biological interdependencies. The most

common way to achieve this is to project the gene lists onto distinct biological processes often represented in the form of gene-ontology (GO) categories or metabolic and regulatory pathways as derived from literature analysis [16]. GO is an expert-curated database assigning genes to various functional categories. Although this currently covers only 38, 137 of the total 44, 000 genes annotated in Entrez Gene, it is a great tool to see which of the genes in a list belong together in terms of one of the GO branches: biological process, molecular function, and cellular component. *p*-Values can be used to rank the GO categories in relationship to the genes found as significantly regulated on the microarray [17]. More recently, pathway analysis technologies allow for the mapping of gene expression data into relevant pathways based on their functional annotation and known molecular interactions. To further examine the molecular functions and genetic networks, the data generated from the microarray was explored using specialized software such as Ingenuity Pathways Analysis (Ingenuity® Systems, http://www.ingenuity.com, IPA), which is a web-delivered application that enables the discovery, visualization, and exploration of molecular interaction networks in gene expression data. IPA enables biologists and bioinformaticians to identify the biological mechanisms, pathways and functions most relevant to their experimental datasets or genes of interest [16, 18-20].

In order to know the influence of baicalein and myricetin on hepatocytes in genome-wide scale, gene expression profiling of HepG2 cells with or without polyphenol treatment was carried out by using the Affymetrix 44K oligonucleotide microarray. Subsequently, the significantly changed genes were classified according to GO categories and the signaling pathway networks were analyzed by using Ingenuity Pathways Knowledge Base. These results revealed that expressions of many hepatic metabolic enzymes genes were regulated by polyphenolic compounds, and an Nrf2-mediated ARE pathway was demonstrated to be involved in both baicalein- and myricetin-induced gene expression of hepatic metabolic enzymes. Some of the metabolic enzymes genes especially the phase II and antioxidant enzyme genes were further confirmed by real time PCR. These data provided a comprehensive data for better understanding the gene expression, hepatic metabolism and bioactive role of

polyphenolic compounds.

2.3 Materials and methods

2.3.1 Materials, cell culture and cytotoxicity

Myricetin purified by HPLC was obtained from Extrasynthese (Lyon Nord, Genay, France). Baicalein was purchased from Sigma (St. Louis, MO, USA). Human hepatoblastoma HepG2 cells were obtained from the Cancer Cell Repository, Tohoku University, Japan, and cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) assay [21] was used to check the cytotoxicity of polyphenols. In brief, HepG2 cells were seeded onto 96-well plates at a density of 10^4 cells of each well and then were pre-incubated for 24 h at 37 ℃. Polyphenolic compounds at the indicated doses (0, 10 μM, 20 μM and 40 μM) were added, and incubated for another 24 h. MTT was then added to the plate (final concentration 0.5 mg/ml), and incubated for additional 4 h. The acidic isopropanol (0.04–0.10 N HCl in isopropanol) was added to dissolve the formazan crystals and the optical density (OD) was measured at 575 nm using a microplate reading spectrophotometer (Thermo Scientific, USA). Viability was determined by comparing the OD of polyphenol-treated cells with those of the untreated cells.

2.3.2 RNA extraction and microarray hybridization

HepG2 cells were pre-cultured in dishes for 24 h and then treated with the concentration of 20 μM for each polyphenol in 0.1% DMSO for 9 h. Total RNA was extracted using an Isogen RNA Kit (Nippon Gene Co., Tokyo, Japan) following the manufacturer's protocol. RNA quality was assessed by automated capillary gel electrophoresis on an Agilent bioanalyzer 2100 (Palo Alto, CA, USA) according to manufacturer's instructions. These total RNA samples were labeled according to the standard one-cycle amplification and labeling protocol developed by Affymatrix (Santa Clara, CA, USA). The cRNAs were labeled at 40°C for 2 h with cyanine 5

(Cy5) for samples and with cyanine 3 (Cy3) for the universal human reference RNA (Affymatrix Technologies). After the amplification and labeling, the yields and dye incorporation efficiencies were determined using a spectrophotometer. Affymatrix Gene Chip Human U133 plus 2.0 Array containing over 44K oligonucleotides were used for this study following microarray processing protocol, the hybridized fluorescence were scanned in Affymatrix scanner.

2.3.3 Microarray data analysis

Microarray results were first classified by Gene Ontology ID (http://www.geneontology.org/), and then were analyzed by Ingenuity Pathway Analysis (IPA) System (http://www.ingenuity.com). The gene ontology project is a major bioinformatics initiative with the aim of standardizing the representation of gene and gene product attributes across species and databases. The Ingenuity Knowledge Base is the largest knowledge base of its kind, with millions of findings created from the full text literature and is updated on weekly basis [22]. Gene accession numbers, the fold change upon baicalein treatment vs. the control cells, and the *t*-test P-value were imported into the IPA software. IPA was carried out with $P<0.002$ as the cutoff point. The genes were categorized according to the molecular functions using the software. The analysis of canonical pathways identified the pathways from the IPA library of canonical pathways that were most significant to the dataset [23, 24].

2.3.4 Confirmation by Real-time PCR

Validation of up-regulated expression was done by real time PCR. The primers specific to the genes used in the present study are shown in Table 2.1, which were designed according to the NCBI sequence database using the software Primer 3. Reverse transcription and real-time PCR were performed with DyNAmo™ SYBR® Green 2-Step qRT-PCR Kit (Finnzymes Oy., Espoo, Finland) according to the

Table 2.1 The primers and Tm values used for real time PCR.

Gene primers	Direction	Sequences	Tm (℃)
HO-1	Fw	cca gcg ggc cag caa caa agt gc	60
	Re	aag cct tca gtg ccc acg gta agg	
AKR1C3	Fw	aag taa agc ttt gga ggt cac a	59
	Re	gga cca act ctg gtc gat gaa	
AKR1C1	Fw	atc cct ccg aga aga acc at	59
	Re	aca cct gca cgt tct gtc tg	
AKR1C2	Fw	gat ccc atc gag aag aac ca	59
	Re	aca cct gca cgt tct gtc tg	
GSR	Fw	gat ccc aag ccc aca ata ga	59
	Re	ctt aga acc cag ggc tga ca	
TXNRD1	Fw	atc agg agg gca gac ttc aa	61
	Re	ccc aca ttc aca cat gtt cc	
DUSP1	Fw	cag ctg ctg cag ttt gag tc	59
	Re	agg tag ctc agc gca ctg tt	
SLC7A11	Fw	gtg tcc acc atc tcc aaa gg	60
	Re	cgt cca gat ggt cag aga ca	
GCLC	Fw	gag ctg gga gga aac caa g	61
	Re	tgg ttt ggg ttt gtc ctt tc	
GCLM	Fw	ggg aac ctg ctg aac tgg	61
	Re	gca tga gat aca gtg cat tcc	
NQO1	Fw	ctg gtt tga gcg agt gtt ca	60
	Re	ttc cat cct tcc agg att tg	
SRXN1	Fw	cat cga tgt cct ctg gat ca	61
	Re	ctg caa gtc tgg tgt gga tg	

manufacturer's manual. Briefly, RNA (200 ng) was reverse-transcribed to cDNA using Oligo dT and M-MuLV RNase at 37˚C for 30 min, and the reaction was then terminated at 85°C for 5 min. The Tm-value of PCR was determined according to each primer sequence (https://www.finnzymes.fi/tm.determination.html). Each PCR reaction contained 250 ng of reverse transcripts, 75 ng of each primer and 10 µl Master mix. The thermal cycling conditions were held at 95˚C for 15 min followed by 55 cycles of 30 sec at 94˚C, 30 sec at Tm-value (Melting temperature), and 30 sec at 72˚C in Rotor-Gene-3000AKAA (Corbett Research Pty., NSW, Australia). The result was represented by the relative expression level normalized with control cells.

2.3.5 Statistical analysis

All the experimental data shown were repeated at least three times, unless otherwise indicated. Differences between treated and control cells were analyzed by the Student's *t*-test. A statistical probability of $P<0.05$ was considered significant.

2.4 Results

2.4.1 Gene expression profiling

According to the results of our initial experiments, HepG2 cells were treated with or without 20 μM of polyphenol for 24 h. Under these conditions, HepG2 cells did not show cytotoxicity in response to treatment with each polyphenol. Cellular mRNA was prepared and processed for hybridization to the human oligonucleotide DNA microarray, as described in Materials and Methods. Comparing the hybridization signals from polyphenol-treated mRNA with those of the control mRNA revealed that (As shown in Table 2.2):

For myricetin, the expressions of 21 genes were changed by greater than or equal to 4 fold, of which, expressions of 11 genes were upregulated while 10 genes were downregulated. The expressions of 598 genes were changed between 2-fold and 4-fold, of which, expressions of 132 genes were upregulated and 466 genes were downregulated. The expressions of 2878 genes were changed between 1.5-fold and 2-fold, of which, expressions of 961 genes were upregulated and 1917 genes were downregulated;

For baicalein, the expressions of 43 genes were changed by greater than or equal to 4 fold, of which, expressions of 38 genes were upregulated while 5 genes were downregulated. The expressions of 651 genes were changed between 2-fold and 4-fold, of which, expressions of 402 genes were upregulated and 249 genes were downregulated. The expressions of 2867 genes were changed between 1.5-fold and 2-fold, of which, expressions of 1382 genes were upregulated and 1485 genes were

downregulated;

Taken together, there were expressions of 3561 genes out of the total 44K genes (8.09%) showing fold changes above 1.5-fold by baicalein treatment, and 3497 genes (7.95%) of that by myricetin treatment (Table 2.2).

Table 2.2 The number of genes that regulated by polyphenolic compounds treatment.

Fold change	Myricetin treatment		Baicalein treatment	
	Numbers	Regulation	Numbers	Regulation
≥ 4	11	up	38	up
	10	down	5	down
2 ≤ ~ < 4	132	up	402	up
	466	down	249	down
1.5 ≤ ~ < 2	961	up	1382	up
	1917	down	1485	down
Total	1104	up	1822	up
	2393	down	1739	down

To validate the accuracy of the microarray data, some representative genes with changes in expression were chosen, and their expression levels were detected by real-time PCR with the same RNA. The real-time PCR results exhibited a similar expression pattern with that of the DNA microarray (Figure 2.1); suggesting the DNA microarray data obtained in the present study is valid.

2.4.2 Expression of hepatic drug metabolizing enzymes by polyphenolic compounds

Gene Ontology database was used to compare the microarray data between the different polyphenolic compound treatment groups based on GO number, and the results showed that after treatment with polyphenolic compounds, the most process is metabolic process, and the most affected molecular functions are transferase

activity, transcription factor activity, catalytic activity and oxidoreductase activity (Supplementary table 2). These results suggested that polyphenolic compound played important role on metabolism in HepG2 cells.

To know the influence of polyphenolic compounds on hepatocytes and to predict its metabolism in liver, I investigated the gene expression changes of drug metabolizing enzymes and transporters in polyphenol-treated cells. As shown in Table 2.3, the gene expression changes of 68 drug metabolizing enzymes and transporters were observed. Among them, 22 genes were associated with phase I such as CYP1A1 (cytochrome P450 superfamily 1A1), CYP24A1 (cytochrome P450 superfamily 24A1), ALDH1L2 (aldehyde dehydrogenase 1 family, member L2), and HERPUD1 (homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein); 23 genes associated with phase II and antioxidant enzymes such as GCLC (glutamate-cysteine ligase catalytic), GCLM, SQSTM1 (Sequestosome-1), and TXNIP (Thioredoxin-interacting protein); 23 genes associated with transporters such as ATP and SLC family:

In myricetin treatment group, the expressions of genes that were upregulated more than 2-fold included ALDH1L2, CYP1A1, CYP24A1, HERPUD1 in phase I, GCLC, GCLM, AKR1C1/2, SQSTM1, HO-1, TXNRD1 in phase II or antioxidant enzymes, and SLC2A14 in transporters. On the other hand, the expression of GSTA1 gene was downregulated. Thus, treatment with 20 μM myricetin upregulated the expressions of hepatic drug metabolizing enzymes, especially phase I/II and antioxidant enzyme genes.

In baicalein treatment group, the expressions of genes that were upregulated more than 2-fold included CYP1A1 in phase I, GCLC, GCLM, AKR1B10, SQSTM1, HO1, TXNIP in phase II or antioxidant enzymes and some SLC family genes in transporters. Thus, treatment with 20 μM baiclaien upregulated the expressions of hepatic drug metabolizing enzymes, especially phase II and antioxidant enzyme genes.

These results indicated that polyphenolic compounds could affect gene expression of many drug metabolizing enzymes especially phase II and antioxidant enzyme genes. However, how these changes are associated with biological function still

remained unknown. Thus, I further analyze the pathways and networks by IPA system.

Table 2.3 The differential expressions of drug metabolizing enzymes by polyphenol treatment.

Gene symbol	Gene description	Accession No.	Myricetin		Baicalein	
Phase I enzymes			Fold	Change	Fold	Change
ALDH1L2	Aldehyde dehydrogenase 1 family, member L2	AI654224	2.31	up	1.27	down
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	NM_000103	1.45	up	1.04	up
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	NM_000781	1.27	up	1.09	up
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	NM_000499	6.00	up	2.81	up
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	NM_000782	2.42	up	1.52	up
CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	NM_000767	1.54	up	1.04	up
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	NM_000780	1.25	up	1.00	down
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	J04449	1.24	up	1.32	up
CYP2C19	cytochrome P450, family 2, subfamily C, polypeptide 19	X65962	1.39	up	1.13	up
CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	U40053	1.33	up	1.21	up
CYP2U1	cytochrome P450, family 2, subfamily U, polypeptide 1	AL359563	1.35	up	1.23	up
CYP4A22	cytochrome P450, family 4, subfamily A, polypeptide 22	AL135960	1.20	up	1.11	down
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	NM_019885	1.32	down	1.12	down
CYP2S1	cytochrome P450, family 2, subfamily S, polypeptide 1	AF335278	1.24	up	1.28	up
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	AC007002	1.23	up	1.33	up
CYP4V2	Cytochrome P450, family 4, subfamily V, polypeptide 2	BE326857	1.22	down	1.39	down
CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1	BC010358	1.94	up	1.06	up
DPYD	dihydropyrimidine dehydrogenase	NM_000110	1.35	down	1.44	down

ESD	esterase D/formylglutathione hydrolase	AA193515	1.42	down	1.41	up
FMO4	flavin containing monooxygenase 4	NM_002022	1.18	up	1.45	up
HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	AF217990	2.64	up	1.02	down
FMO6P	flavin containing monooxygenase 6 pseudogene	AL021026	1.35	up	1.03	down
Phase II enzymes and antioxidant proteins						
GSTA1	glutathione S-transferase A1	NM_000846	2.08	down	1.23	up
GSTM3	glutathione S-transferase M3 (brain)	AI459140	1.35	up	1.59	up
GSTP1	glutathione S-transferase pi	NM_000852	1.24	up	1.37	up
MGST1	Microsomal glutathione S-transferase 1	AV705233	1.42	up	1.54	up
SULT2B1	sulfotransferase family, cytosolic, 2B, member 1	NM_004605	1.21	up	1.35	up
SULT1C2	sulfotransferase family, cytosolic, 1C, member 2	AI307799	1.28	up	1.34	up
HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase (hydroxymethylglutaricaciduria)	NM_000191	1.57	up	1.14	down
GCLC	glutamate-cysteine ligase, catalytic subunit	NM_001498	3.09	up	3.50	up
GCLM	glutamate-cysteine ligase, modifier subunit	AI753488	2.19	up	2.59	up
HMGCLL1	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase-like 1	BC024194	1.45	up	1.58	up
AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	NM_020299	1.25	up	2.29	up
AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	BF508244	2.96	up	1.83	up
AKR1CL2	aldo-keto reductase family 1, member C-like 2	AI243406	1.08	up	1.19	up
AKR1C2	Aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	CA425039	2.79	up	1.17	up
SQSTM1	sequestosome 1	N30649	2.14	up	2.45	up
CBR3	carbonyl reductase 3	NM_001236	1.36	up	1.49	up
HO-1	heme oxygenase (decycling) 1	NM_002133	2.16	up	3.00	up
ATF4	activating transcription factor 4 (tax-responsive enhancer element B67)	NM_001675	1.06	up	1.46	up
NFE2L2	nuclear factor (erythroid-derived 2)-like 2	AF323119	1.12	up	1.59	up

FTH1	ferritin, heavy polypeptide 1	AA083483	1.36	up	1.09	up
SOD1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	NM_000454	1.08	down	1.24	down
SOD3	superoxide dismutase 3, extracellular	NM_003102	1.36	up	1.41	up
SOD2	superoxide dismutase 2, mitochondrial	W46388	1.13	down	1.21	up
TXN	Thioredoxin	AF065241	1.31	up	1.15	up
TXNIP	thioredoxin interacting protein	NM_006472	1.10	up	2.06	up
TXNRD1	thioredoxin reductase 1	NM_003330	2.04	up	1.92	up
GSR	glutathione reductase	AI888037	1.29	up	1.03	up
Transporters						
ATP6V1E2	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E2	NM_080653	1.08	up	2.24	up
LOC100133772 /// SLC16A5	solute carrier family 16, member 5 (monocarboxylic acid transporter 6) /// similar to MCT	NM_004695	1.39	up	2.63	up
SLC16A6	solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	NM_004694	1.89	up	2.27	up
SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	NM_006934	1.08	up	2.36	up
SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	AB040875	1.52	up	3.77	up
SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	AA148507	1.13	up	2.27	up
SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	W72527	1.13	up	3.79	up
SLC2A14 /// SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3 /// solute carrier family 2 (facilitated glucose transporter), member 14	AL110298	2.06	up	2.46	up
SLC2A10	solute carrier family 2 (facilitated glucose transporter), member 10	NM_030777	1.07	down	2.14	up
SLC20A1	Solute carrier family 20 (phosphate transporter), member 1	AI671885	1.49	up	2.06	up
ABCA11	ATP-binding cassette, sub-family A (ABC1), member 11 (pseudogene)	NM_024903	1.09	down	1.70	up
ABCA17P	ATP-binding cassette, sub-family A (ABC1), member 17 (pseudogene)	AI570450	1.11	up	1.23	up
ABCA9	ATP-binding cassette, sub-family A (ABC1), member 9	AI284184	1.23	up	1.23	up
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	NM_005691	1.07	up	1.38	up

ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	AI248055	1.09	down	1.30	up
ATP7A	ATPase, Cu++ transporting, alpha polypeptide (Menkes syndrome)	NM_000052	1.64	down	1.36	up
ATP4B	ATPase, H+/K+ exchanging, beta polypeptide	NM_000705	1.02	down	1.27	up
ATP2B2 /// LOC100134286	ATPase, Ca++ transporting, plasma membrane 2 /// similar to ATPase, Ca++ transporting, plasma membrane 2	M97260	1.77	up	1.71	up
ATP11B	ATPase, class VI, type 11B	AB023173	1.10	down	1.29	up
ATP10A	ATPase, class V, type 10A	N35112	1.18	down	1.29	up
ATP11A	ATPase, class VI, type 11A	AL161996	1.05	down	1.26	up
ATP6V0A2	ATPase, H+ transporting, lysosomal V0 subunit a2	BG106215	1.30	up	1.68	up
ATP6V1E2	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E2	NM_080653	1.08	up	2.24	up

2.4.3 Ingenuity pathway analysis

To further analyze the underlying relationship between significantly changed genes and biological functions, I input microarray data into IPA system, and the system output the analysis report including significantly affected canonical pathways, networks, bio functions, molecules and so on. I just select some of the important issues to analyze in this chapter.

First, canonical pathways affected by polyphenol treatment could help us to find the most promising property of polyphenol. As shown in Table 2.4, top 10 canonical pathways altered by myricetin and baicalein were listed out. Among them, Nrf2-mediated pathway was identified as the third category with the ratio of 5.41E-02 by myricetin treatment and the second category with the ratio of 6.49E-02 by baicalein treatment. The expressions of 10 genes and 12 genes in this pathway were regulated above 2-folds out of the total 185 genes by myricetin and baicalein, respectively. On the other hand, myricetin affected metabolisms of xenobiotics, C21-steroid hormone, glycerophospholipid, glycerolipid and glutamate, while baicalein affected metabolisms of tyrosine, methionine and cysteine, which further confirmed that drug metabolizing enzymes expressions were significantly affected by myricetin and baicalein treatment. Moreover, I also found that both of them can affect

Table 2.4 Top 10 canonical pathways altered by polyphenol treatment

Ingenuity Canonical Pathways	-log(p-value)	Ratio	Molecules
Myricetin treatment			
Metabolism of Xenobiotics by Cytochrome P450	3.55E00	3.83E-02	ADH6, CYP1A1, AKR1C1, AKR1C3, DHRS2 (includes EG:10202), AKR1C2, CYP4F11, ADH4
Bile Acid Biosynthesis	3.04E00	5.15E-02	ADH6, AKR1C1, AKR1D1, DHRS2 (includes EG:10202), ADH4
NRF2-mediated Oxidative Stress Response	2.69E00	5.41E-02	HMOX1, FOS, JUN, GCLC, SQSTM1, GCLM, MAFF, TXNRD1, ACTA1, CBR1
VDR/RXR Activation	2.38E00	7.5E-02	SPP1, IGFBP3, IGFBP1, VDR, KLF4, SULT2A1
TGF-β Signaling	2.32E00	6.98E-02	FOS, JUN, SOS1, VDR, SERPINE1, ACVR1B
C21-Steroid Hormone Metabolism	2.28E00	4.35E-02	AKR1C1, AKR1D1, AKR1C3
Glycerophospholipid Metabolism	2.26E00	3.93E-02	TMEM87B, HMOX1, GPAM, PCYT1B, LCAT, ETNK1, AGPAT3
Glycerolipid Metabolism	2.22E00	4.14E-02	ADH6, GPAM, DHRS2 (includes EG:10202), AKR1B10, AGPAT3, ADH4
Glutamate Metabolism	2.21E00	5.13E-02	ABAT, GLS, GCLC, GCLM
IGF-1 Signaling	2.12E00	6.12E-02	FOS, JUN, SOS1, IGFBP3, IGFBP1, PRKAR1A
Baicalein treatment			
VDR/RXR Activation	5.06E00	1.25E-01	CYP24A1, GADD45A, MXD1, IGFBP3, CEBPB, IGFBP1, VDR, KLF4, NCOA3, SULT2A1
NRF2-mediated Oxidative Stress Response	3.32E00	6.49E-02	FOS, JUN, GSTA1, GCLC, HERPUD1, FOSL1, SQSTM1, JUNB, GCLM, DNAJB9, MAFF, ACTA1
Tyrosine Metabolism	2.81E00	3.23E-02	ADH6, DHRS2 (includes EG:10202), GOT1, SMOX, ADH4, MAOA
Methionine Metabolism	2.7E00	5.26E-02	DNMT3B, BHMT, MAT1A, CTH
LPS/IL-1 Mediated Inhibition of RXR Function	2.63E00	5.56E-02	ACSL3, JUN, ALDH1L2, GSTA1, SMOX, FABP3, IL1R1, FMO5, SULT2A1, CHST15, MAOA
Cysteine Metabolism	2.54E00	5.88E-02	CARS, GOT1, CTH, SULT2A1, CHST15
Glycolysis/Gluconeogenesis	2.48E00	4.93E-02	ADH6, ACSL3, HK2, DHRS2 (includes EG:10202), IL21R, RWDD2B, ADH4
IGF-1 Signaling	2.45E00	7.14E-02	FOS, JUN, IGFBP3, PDPK1, IGFBP1, CYR61, GRB10
Caveolar-mediated Endocytosis	2.28E00	7.32E-02	SRC, ALB, CD55, PTRF, ACTA1, EGFR
Tight Junction Signaling	2.08E00	5.49E-02	MYLK, FOS, F2RL2, CLDN11, JUN, ARHGEF2, CLDN2, CSTF3, ACTA1

IGF-1 signaling and VDR/RXR Activation, which implied the potential function of polyphenol on diabetic disease and other metabolic diseases.

Second, the top 10 networks were affected by treatment as shown in Table 2.5, which indicated that myricetin could most likely influence cardiovascular disease, metabolic disease and lipid metabolism, while baicalein could most likely influence cell death, amino acid metabolism, and small molecule biochemistry.

Third, top bio function results indicated that cancer is the most affected disease by both myricetin and baicalein treatments (Supplementary table 3, 4).

Table 2.5 Top 10 networks altered by polyphenolic compound treatment

Myricetin treatment			
ID	Top Functions	Molecules	Score
1	Cardiovascular Disease, Metabolic Disease, Lipid Metabolism	28	46
2	Connective Tissue Development and Function, Skeletal and Muscular System Development and Function, Tissue Morphology	22	33
3	Endocrine System Disorders, Hematological Disease, Metabolic Disease	22	32
4	Cardiovascular System Development and Function, Tissue Morphology, Amino Acid Metabolism	21	30
5	Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function	20	26
6	Cellular Movement, Dermatological Diseases and Conditions, Organismal Injury and Abnormalities	18	24
7	Cancer, Cell Death, Reproductive System Disease	17	23
8	Cancer, Cell Cycle, Cell Death	16	21
9	Cell Death, Cancer, Cell Cycle	16	21
10	Cancer, Hepatic System Disease, Liver Hyperplasia/Hyperproliferation	16	21
Baicalein treatment			
ID	Top Functions	Molecules	Score
1	Cell Death, Amino Acid Metabolism, Small Molecule Biochemistry	31	53
2	Cancer, Cell Death, Gastrointestinal Disease	26	41
3	Cellular Development, Embryonic Development, Tissue Development	26	41
4	Cellular Growth and Proliferation, Cell Cycle, Embryonic Development	20	28
5	Cancer, Cardiovascular System Development and Function, Cellular Movement	20	27
6	Cancer, Endocrine System Disorders, Cellular Growth and Proliferation	21	26
7	Cancer, Connective Tissue Disorders, Reproductive System Disease	18	24
8	Cancer, Cellular Movement, Endocrine System Disorders	18	24
9	Gene Expression, Cellular Development, Cellular Growth and Proliferation	18	24
10	Cardiovascular Disease, Developmental Disorder, Cancer	18	23

2.4.4 Confirmation by real time RT-PCR

To further confirm the microarray results, the expressions of some phase II genes especially in Nrf2-mediated pathway, such as GCLC/M, AKR1C1/2/3, HO1, TXNRD1, NQO1, GSR, DUSP1, SLC7A11 and SRXN1, were further detected by real-time PCR as shown in Figure 2.1. Most of these genes revealed a similar expression pattern between microarray and real-time PCR data. For example, after baicalein treatment, the expression level of GCLM increased 3.47-fold in real-time PCR, and 2.19-fold in DNA microarray. These data demonstrated that an Nrf2-mediated pathway was involved in polyphenolic compound-treated HepG2 cells.

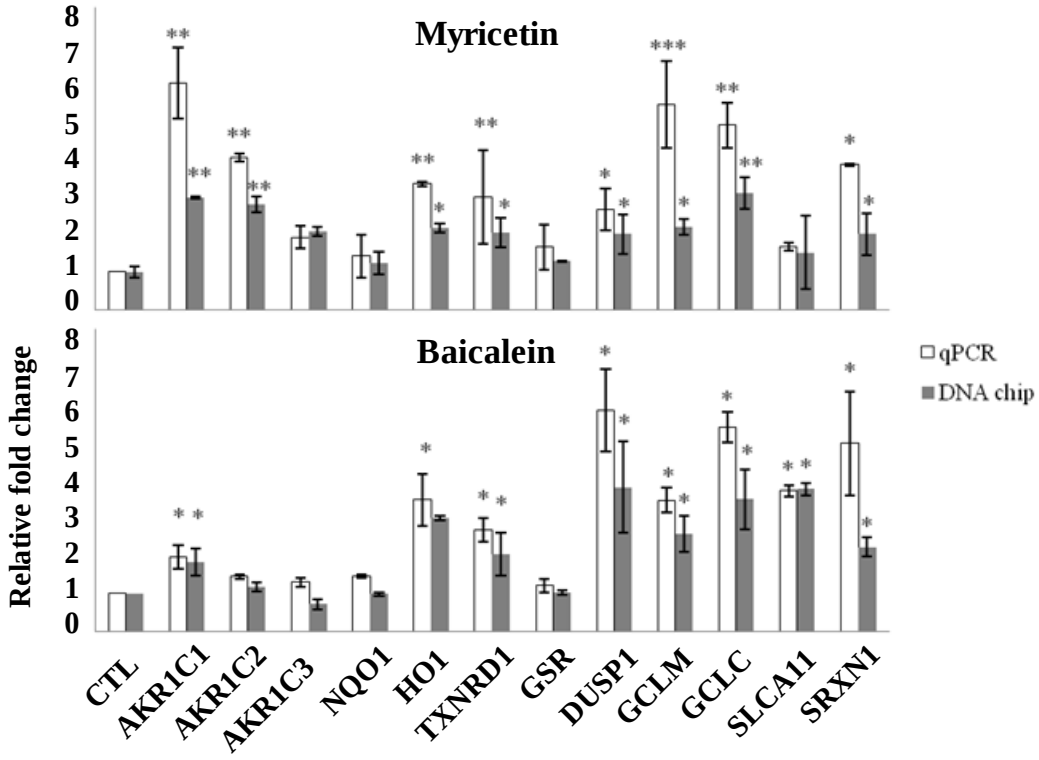

Figure 2.1 Comparison of gene expressions obtained by real time PCR with that obtained by DNA microarray. Real time PCR was performed with SYBR Green 2-Step qRT-PCR Kit as shown in materials and methods. The result was expressed as the relative expression level. Each value represents the mean ± SD of three separate experiments, * $p<0.05$ vs. control. CTL, control; AKR1C, aldo-keto reductase family 1, member C; NQO1, NAD(P)H:quinone oxidoreductase; HO-1, heme oxygenase-1; TXNRD1, thioredoxin reductase 1; GSR, glutathione reductase;

DUSP1, dual specificity protein phosphatase 1; GCLC, glutamate cysteine ligase, catalytic subunit; GCLM, glutamate cysteine ligase, modifier subunit; SLC7A11, solute carrier family 7, member 11; SRXN1, sulfiredoxin-1.

2.5 Discussion

Although a plenty of studies have showed that polyphenolic compounds have a number of pharmaceutical actions, the effects of them on hepatocytes, especially on the phase I, II metabolizing enzymes and transporters are still not clear. In this chapter, I profiled the gene expressions of polyphenolic compound-treated HepG2 cells by DNA microarray and analyzed the data by pathway analysis technologies.

Toxicological and pharmacological studies usually pay main attention on ADME enzymes, but the best target such as human hepatocyte is restricted to gain due to the ethical consideration, which leads to many other immortalized cell lines emerging, such as HepG2 and primary human hepatocytes. Both HepG2 and primary human hepatocytes are widely used in ADME, toxicological, and other basic research, but they still have their own insufficiencies. HepG2 cells are deficient in the expression of phase I drug-metabolizing enzymes such as P450 system [25, 26]. Human primary hepatocytes tend to dedifferentiate over time, eventually losing their drug-metabolizing capability, and are subject to various stimuli such as exposure to wide assortment of drugs, disease, diet, alcohol and so on [27]. Some reports used microarray to compare the gene expression differences between HepG2 and primary human hepatocytes treated by chemicals such as styrene, aflatoxin B1, and food promutagens [26, 28-30]; it seems that human hepatocytes are the preferred model for biotransformation in human liver, whereas HepG2 cells may be still useful to study regulation of drug metabolizing enzymes. In the present paper, I paid more attention on phase II metabolizing enzymes especially antioxidant proteins, so I chose HepG2 cells.

Among total 44K genes, as shown in Table 2.2, treatment with myricetin enhanced

1104 gene signals (2.51% of total genes) and reduced 2393 gene signals (5.44% of total) by ≥1.5-fold, while baicalein enhanced 1822 gene signals (4.14%) and reduced 1739 gene signals (3.95%). These results suggested that polyphenolic compounds could regulate gene expressions in HepG2 cells. Gene Ontology classification revealed that the drug metabolizing enzymes were significantly affected by polyphenol treatments, which play central roles on the metabolism, detoxification and elimination of xenobiotics and drugs including polyphenols. Thus, I listed out drug metabolizing enzymes based on the other publications [31, 32]. In total, the expression changes of 68 drug metabolizing enzymes were observed (Table 2.3). Especially, the gene expression of CYP1A1 (phase I enzymes), GCLC, GCLM (phase II and antioxidant enzymes) and SLC2A14 (transporters) are found enhanced in all the three polyphenolic compounds treatment. CYP family was well recognized as important metabolizing enzymes for toxic and carcinogenic xenobiotics. Flavonoids have been shown to modulate the CYP family, including the induction of specific CYP isozymes, the activation or inhibition of enzymes in this family [33]. In animal studies, inducers of CYP family usually decrease the carcinogenicity of chemical carcinogens, suggesting that induction of CYP family plays more important role in detoxification rather than formation of carcinogenic metabolites [34]. Our DNA microarray data showed polyphenolic compounds induced the expression of CYP1A1 gene which is supported by the previous report [35]. Previous study has suggested that flavonoid with ortho-hydroxyl substituent(s) on the aromatic ring(s) can induce the expressions of phase II and antioxidant enzymes like NQO1, HO1, GCLC and GCLM [36]. Polyphenolic compounds contain several ortho-hydroxyl substituents on its aromatic rings, which may raise the reactivity and facilitate addition of other substituent such as mercaptans, thereby raising its inducer potencies on expression of phase II and antioxidant enzyme genes GCLC and GCLM. Transporters like solute carriers group (SLC family) and ATP-binding cassette transporters (ABC family) have a substantial impact on systemic drug exposure and toxicity [37, 38], and some of the transporters also enhanced by polyphenolic compound treatment such as SLC2A14.

In order to know the regulation of these genes in signal network level, I performed

canonical pathway and network analysis by IPA software. The results showed that an Nrf2-mediated pathway was involved in the regulation of these gene expressions by myricetin and baicalein (Table 2.4). Top affected networks (Table 2.5) and bio functions further provided us the evidence of the biological effects of these polyphenolic compounds on human disease especially on cancer.

In summary, the DNA microarray data, for the first time, revealed gene expression profiles of myricetin and baicalein in HepG2 cells. Moreover, signaling pathway analysis demonstrated that Nrf2-mediated ARE activation is involved in polyphenolic compound-induced expressions of most hepatic drug metabolizing enzyme genes. These results provide a comprehensive data for understanding the hepatic metabolism, bioactive role of these polyphenolic compounds.

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Chapter 3

Molecular mechanism of Nrf2-ARE pathway activation by baicalein and myricetin based on microarray and network analysis

3.1 Abstract

The microarray data and IPA canonical pathway analysis in last chapter had revealed that the Nrf2-ARE pathway was involved in both baicalein-induced and myricetin-induced gene expressions. However, the underlying molecular mechanisms are still not clear. Due to the critical role of Nrf2-ARE pathway in chemopreventive effects of polyphenolic compounds, this chapter aimed to investigate the exact molecular mechanisms underlying the Nrf2-ARE pathway activation based on microarray with effective network analysis in HepG2 cells by baicalein and myricetin treatments.

The effect of baicalein on Nrf2 expression in HepG2 cells was investigated both at the transcriptional level and at the posttranscriptional level. At the transcriptional level, IPA network analysis and protein kinase signaling pathways data revealed that baicalein regulated the expression of Nrf2 mRNA by targeting the transcription factors and nuclear proteins via phosphorylating the protein kinases MEK, AKT and JNK signaling pathways. At the posttranscriptional level, molecular data revealed that baicalein activated Nrf2-ARE pathway by inhibiting Nrf2 ubiquitination and protein turnover via stimulating Keap1 modification and ubiquitination. Besides, baicalein-induced phosphorylation of protein kinases may lead to the phosphorylation of Nrf2, which also allowed Nrf2 escaping from Keap1 inhibition. All of these events finally increased Nrf2 nuclear accumulation, ARE binding activity and transcription activity to enhance ARE-mediated genes expressions. As well, myricetin was also found to exert its chemopreventive effect in the similar mechanism due to its highly

consistent molecular structure as baicalein.

Additionally, treatment with Nrf2 siRNA both attenuated the baicalein-induced and myricetin-induced ARE activity and gene expressions. Based on structure-activity analysis, we found that the basic flavan structure seems to be the key component of polyphenolic compounds to activate Nrf2.

The microarray based network analysis and molecular data provided comprehensive knowledge for understanding chemopreventive effects of baicalein and myricetin on Nrf2-ARE pathway activation and underlying molecular mechanisms.

3.2 Introduction

Accumulating evidences have strongly indicated the beneficial effects of consumption of polyphenol-rich fruits and vegetables in prevention against cancer, cardiovascular disease as well as other chronic diseases, which are proven to be associated with their antioxidant activity, at least partly [1]. To understand the antioxidant mechanisms of polyphenolic compounds at cellular and molecular level, we chose baicalein and myricetin as the subjects of this chapter because they are typical polyphenol and widely distributed in edible plants with many bioactivities such as antioxidation, antiinflammation, anticancer, antidiabetic effect and antimutagenicity [2-5].

The significantly changed expressions of drug metabolizing enzymes by both baicalein and myricetin treatment in chapter 2 suggested that they would be metabolized in liver. The bioavailability and bioactivity of quercetin, a similar compound to baicalein and myricetin, are reported to be dependent on modulation of the drug metabolizing enzymes of hepatocytes [6].

Nrf2 is a key mediator to regulate expressions of drug metabolizing enzyme genes since Nrf2 binds to the antioxidant-responsive element (ARE) with the consensus sequence 5′-TA/CANNA/GTGAC/TNNNGCA/G-3′ in promoter region of many drug

metabolizing enzyme genes [7, 8, 9]. Under unstimulated conditions, Nrf2 is sequestered in the cytoplasm, where it is associated with Keap1, an actin-binding protein [10]. Keap1 is critical regulator although there are many factors modulating the activation of Nrf2-ARE [11, 12]. Under stress stimulation, Nrf2 can be released from Keap1 to enter nuclear, bind to ARE, and induce phase II and antioxidant proteins expression, which is called Nrf2-ARE activation [13]. Besides, Nrf2 is a labile protein. Stabilizing Nrf2 is considered to be important to maintain the cellular defense system, which is likely dependent on the status of the Nrf2-Keap1 complex.

Based on the references in PubMed center during the last decade, the molecular mechanisms of Nrf2-ARE pathway activation can be summarized and classified into Keap1-dependent mechanisms and Keap1-independent mechanisms.

3.2.1 Modulation of Keap1-dependent mechanisms on Nrf2-ARE pathway

Keap1 comprises an N-terminal region (NTR), C-terminal region (CTR), a bric-a-brac, tramtrack and broad complex (BTB) domain, an intervening region (IVR) and a Kelch-repeat domain through which Keap1 binds to Nrf2. Keap1 is widely recognized as the central negative modulator of Nrf2 activation [14]. Several widely recognized models have been advanced to account for the repression of Nrf2 by Keap1 including 'Keap1 dissociation and Cul3-Rbx1 ubiquitination', 'Keap1 hinge and latch' and 'Keap1 ubiquitination' as shown in Figure 3.1.

The 'Keap1 dissociation and Cul3-Rbx1 ubiquitination' model originated from the phenomenon that the majority of ARE inducers are capable of modifying cysteines, suggesting that Keap1 cysteines are targeted by these compounds in signaling ARE induction [15]. Accumulated data showed that only 5 of 25 cysteines in mouse Keap1 are most reactive toward the electrophile dexamethasone 21-mesylate (Dex-Mes), that were identified as C257, C273, C288, C297, and C613 [16]. Of these five cysteines, only C273 and C288 are required for repression of Nrf2 nuclear accumulation in mouse-based ARE-reporter assays [17]. The importance of C273, C288 and C151 in

human Keap1 repression of ARE activation has been demonstrated by means of mutagenesis studies although the C151 is not important under normal conditions for Nrf2 repression [18]. Previous studies in yeast model noticed that different inducers of Yap1, a transcriptional master regulator of oxidative stress, depend on different cysteine residues to activate the signaling pathway [19-21]. Some studies proposed that different inducers of Nrf2 activation may react with Keap1 in different ways [22-24]. Subsequent studies further confirmed that Keap1 is a substrate adaptor for a Cul3-Rbx1 containing E3 ligase [25, 26].

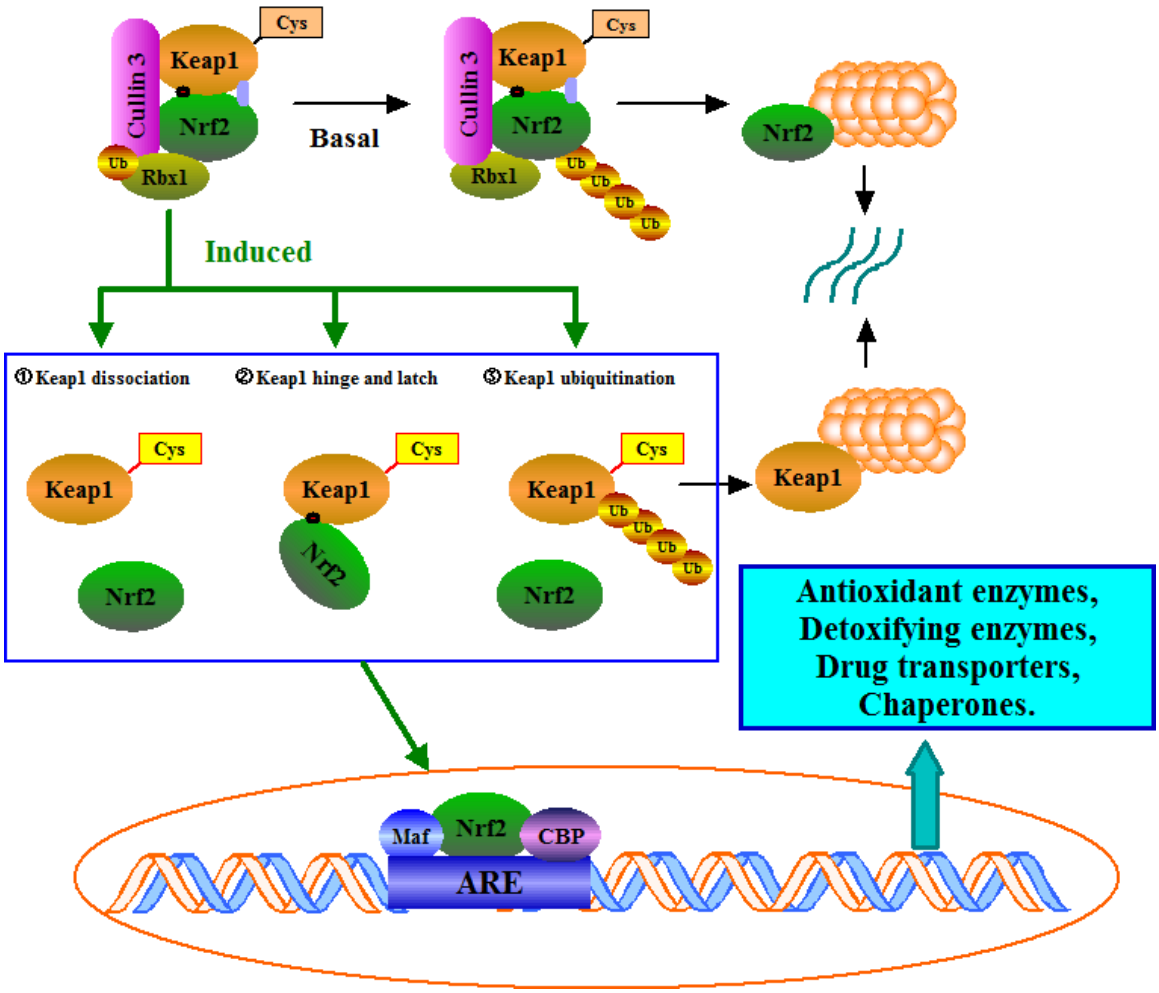


Figure 3.1 Modulation of Keap1 on Nrf2-ARE pathway. There are three widely accepted mechanisms on Keap1-dependent Nrf2-ARE pathway activation, which including (1) Keap1 dissociation: Keap1 cysteine modification may cause Nrf2 release from Keap1 in the cytoplasm; (2) Keap1 hinge and latch: Keap1 cysteine modification may cause a conformational change,

which likely disrupts the weak latch binding site to prevent ubiquitin conjugation onto Nrf2; (3) Keap1 ubiquitination: Keap1 cysteine modification may remove the ubiquitin binding from Nrf2 to itself. All of the above three pathways finally cause Nrf2 translocation to activate ARE.

Proposition of 'Keap1 hinge and latch' model was attributed to the studies of ETGE and DLG motifs of Nrf2. Either absence of ETGE or DLG motif, or both, Nrf2 may fail to bind to Keap1 [27, 28]. Moreover, modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2 [29]. Further studies proposed that these two binding sites within the Neh2 domain of Nrf2, a weak and a strong binding site, that each binds to one Kelch domain in Keap1, in a heterotrimer complex consisting of two Keap1 molecules and one Nrf2 molecule. It was speculated that binding of both the "hinge" and "latch" sites to the two Kelch domains of the Keap1 homodimer locks the seven lysine residues within the Neh2 domain in a precise orientation for ubiquitin conjugation. In response to activation signals, modification of cysteine residues in Keap1 may cause a conformational change, which likely disrupts the weak latch binding site, thus preventing ubiquitin conjugation onto Nrf2 [30, 31].

Another important model of 'Ubiquitination of Keap1' was noticed by researchers recently. Keap1 could be ubiquitinated by Cul3-dependent complex [32, 33]. Moreover, sequestosome-1 (SQSTM1, also called as p62), could activate Nrf2 through inactivation of Keap1 [34, 35]. Fumarate could modify cysteine residues within Keap1 by succination, and subsequently activate Nrf2 expression [36]. Nitric oxide could activate Nrf2 through S-nitrosylation of Keap1 [37]. Electrophilic metabolite tert-butylbenzoquinone (TBQ) could activate Nrf2 through covalent electrophilic modification of Keap1 [38], and stress-induced phosphorylation of Keap1 cause Nrf2 activation [39]. These recent investigations provide us a new view for understanding the role of Keap1 modification in Nrf2 activation.

3.2.2 Modulation of Keap1-independent factors on Nrf2-ARE pathway

Although Keap1 is primary factor for modulating Nrf2, there exist other factors may also play important roles in modulation of Nrf2-ARE pathway. These factors mainly include protein kinases and transcriptional factors as shown in Figure 3.2.

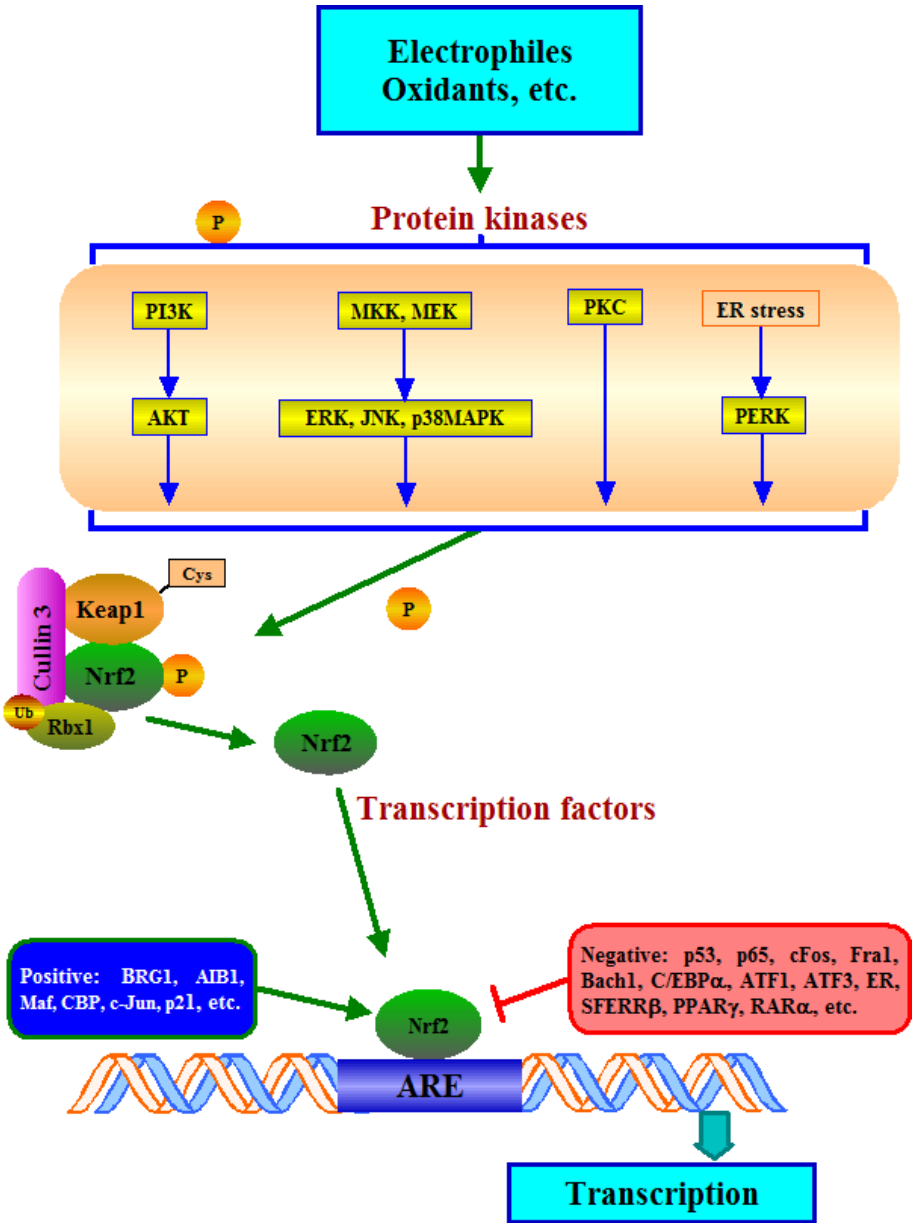


Figure 3.2 Modulation of Keap1-independent factors on Nrf2-ARE pathway. Keap1-independent factors include mainly protein kinases (such as PI3K/AKT, MAPKs, PKC) and other transcriptional factors (such as BRG1, AIB1, p53, p65).

3.2.2.1 Protein kinases

Since Nrf2 controls basal and inducible expression of ARE-driven genes, modification of Nrf2 stability, such as phosphorylation and ubiquitination, is important in modulation of Nrf2-ARE pathway. Under basal conditions, Nrf2 has a short half-life because it was ubiquitinated by Keap1-Cul3-Rbx1 ubiquitination system for degradation. The earlier studies had found that phosphorylation of Nrf2 could lead to an increase in its stability and subsequent transactivation activity [40]. It has been reported that protein kinase C [41-43], MAPKs [44, 45], GSK3 [46] and PI3K [47] could phosphorylate Nrf2 to affect Nrf2 activity in a Keap1-independent manner.

3.2.2.2 Transcriptional factors and other nuclear proteins

Although the exact mechanisms of nuclear proteins on modulation of Nrf2 activation are complicated, it is, at least, known that they contribute to the Keap1-independent adjustment. We classified these transcription factors and other nuclear proteins into several groups including NF-κB related inflammatory group, Caspase-3 related apoptosis group and other transcription factors group.

Small Maf proteins are widely accepted as the dimerization partners of Nrf2, which contain bZIP domain and adjacent extended homology region but without transactivation domain [48, 49]. Knockout of all small Maf proteins blocked the activation of Nrf2 and subsequent induction of antioxidant proteins [50, 51]. Besides, nuclear receptor coactivator 3 (AIB1) also serves as an essential coactivator for Nrf2 activation by physically interacting with Nrf2 to enhance its transcriptional activity [52]. In addition to small Maf proteins, many other nuclear proteins including c-Jun [53, 54], CBP [55, 56], BRG1 [57], and p21 [58], stimulate Nrf2 activation, but p53 [59] and p65 [60, 61] suppresses it. On the other hand, transcriptional factors, such as cFos [62], Fra1 [63], Bach1 [64], C/EBPα [65], ATF1 [66], ATF3 [67], ER (estrogen receptors) [68], SFERR (short form estrogen-related receptor) β [69], PPARγ [70] and

RAR (retinoic acid receptor) α [71], have been reported to negatively regulate Nrf2 transcription.

Besides, miRNAs have been reported to regulate the expression of Nrf2 at the posttranscriptional level [72, 73].

Accumulated data revealed that some phytochemical compounds could induce Nrf2-mediated ARE activation [74-76]. The effects and mechanisms of baicalein and myricetin on Nrf2-mediated ARE activation are poorly understood. Last chapter has revealed that both baicalein and myricetin could influence the gene expressions of hepatocytes and an Nrf2-mediated ARE activation was involved. This chapter further studied the molecular mechanism underlying and the results demonstrated that baicalein and myricetin both increased Nrf2 stabilization, Keap1 modification, Nrf2 nuclear accumulation and ARE binding activity to enhance ARE-mediated gene expressions. Furthermore, baicalein regulated the expression of Nrf2 mRNA by targeting several transcription factors and nuclear proteins via phosphorylating the protein kinases MEK, AKT and JNK signaling pathways.

3.3 Materials and methods

3.3.1 Materials, cell culture and cytotoxicity

Baicalein was purchased from Sigma (St. Louis, MO, USA) and myricetin purified by HPLC was obtained from Extrasynthese (Lyon Nord, Genay, France). HepG2 cells were obtained from the Cancer Cell Repository, Tohoku University, Japan, and cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The antibodies against Nrf2 (C-20), Keap1 (E-20), HO-1, α-tubulin (B-7), rabbit IgG and horseradish peroxidase-conjugated anti-goat secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). U0126, LY294002, horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling Technology (Beverly, MA, USA). SB202190 and RO318220 were

from Calbiochem (Nottingham, UK), and SP600125 was from Biomol Research Lab. (Plymouth Meeting, PA).

3.3.2 IPA network and JASPAR analysis

Network pathways were analyzed by Ingenuity Pathway Analysis (IPA) System (http://www.ingenuity.com) and manual curation based on references in Pubmed center. In order to predict transcription factors (TFs) that directly regulate NRF2 transcription, we queried the regulatory region of the NRF2 gene sequence against all transcription factor binding site (TFBS) information found in JASPAR [77], and some of the transcription factors were further proved by promoter analysis base on references.

3.3.3 RT-PCR

HepG2 (1×10^6) cells were precultured in 10-cm dishes for 24 h and then treated with various concentrations of baicalein in 0.1% DMSO, or with 0.1% DMSO alone as a control, for 9 h. Total RNA was extracted with an Isogen RNA Kit (Nippon Gene Co., Japan) as described in manufacturer's manual. The oligonucleotide primers forward, 5'-AGTGCAGTGGTGTGATCTCG-3', and reverse, 5'-GGTGGAGTCACGCCTGTAAT-3', were used to amplify human NQO1, the primers forward, 5'-AGACAAACATTCAAGCCGCT-3', and reverse, 5'-CCATCTCTTGTTTGCTGCAG-3', were used to amplify human Nrf2, the primers forward, 5'-CCTTCAGCTACACCCTGGAG-3', and reverse, 5'-AACATGGCCTTGAAGACAGG-3', were used to amplify human Keap1, and the primers forward, 5'-GACCCCTTCATTGACCTCAAC-3', and reverse, 5'-CATACCAGGAAATGAGCTTG-3', were used to amplify human GAPDH as a housekeeping gene. These primers were designed using the software PRIMER3 and sequence data from the NCBI database. RT-PCR was performed with Ready-to-Go RT-PCR beads (GE Healthcare, Little Chalfont, UK) as described previously [74].

The PCR products were separated on a 2% agarose gel and visualized under UV light after being stained with ethidium bromide. The relative densities of the PCR products were quantified, using Imager software (TAITEC Co., Saitama, Japan).

3.3.4 Transient transfection and luciferase reporter gene assay

The pGL2-hQR41 luciferase reporter plasmid containing ARE was described previously [75]. In brief, HepG2 cells were plated into each well of 12-well plates at the concentration of 1×10^5 and pre-cultured for 24 h in DMEM plus 10% FBS. The cells were then co-transfected with 0.1 μg of ARE promoter-encoding firefly luciferase plasmid and 0.1 μg of pGL4-TK-encoding Renilla luciferase plasmid (Promega, Madison, WI, USA) using Lipofect AMINE 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h incubation, the cells were treated by 20 μM of baicalein or myricetin in 0.1% DMSO, or 0.1% DMSO alone as a control, and further incubated for 24 h. The activities of firefly and renilla luciferase were measured in ARVO^{TM}SX multilabel counter (Perkin Elmer, Massachusetts, USA) with the Dual-Luciferase Reporter Assay System (Promega, Madison, USA). Luciferase activity values were normalized to transfection efficiency monitored by Renilla expression, and ARE transcription activity was expressed as fold induction relative to the control cells.

3.3.5 Cell fractionation and EMSA

Nuclear and cytosolic proteins were prepared according to the modified method as described previously [74]. In brief, cells were cultured on 100-mm dishes to 90% confluence and treated with 20 μM of baicalein or myricetin for 9 h. Cells were lysed with buffer A (10 mM Hepes-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) on ice for 20 min and then centrifuged at 14,000 g for 15 min at 4 °C. The supernatants were saved as the cytoplasmic fractions. The nuclear pellets were washed three times with buffer A and resuspended in buffer B (20 mM Hepes, 0.5 M KCl, 1 mM EDTA, 1

mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.9) for 30 min at 4 °C on a rotating wheel and then centrifuged at 14,000 g for 15 min at 4 °C. The cytosolic and nuclear fractions were used for immunoblot analysis and electrophoretic mobility-shift assay (EMSA). Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

In vitro protein-DNA interaction was examined using the Gelshift^{TM} Chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer. Briefly, ARE-specific EMSA DNA probes were synthesized, gel purified and 5'-biotin labeled (5'-TTTTATGCTGTGTCATGGTT-3'), unlabeled ARE-specific EMSA probes as competitor. Nuclear extract (4 μg) was combined with biotin-labeled DNA probes (20 fmol) in 20 μl binding buffer (50 ng/μl poly d (I-C), 2.5% glycerol, 5mM MgCl2, 0.05% NP-40) at room temperature for 20 min. The molar excess of unlabelled ARE-specific probe (4 pmol) was added to the binding reaction in competition experiments. Products of the binding reaction were run in 6% polyacrylamide gel with 0.5× TBE buffer. The binding reactions was transferred to a nylon membrane and immediately cross-linked for 10 min with the membrane face down on a transilluminator equipped with 312 nm bulbs. The biotin-labeled complexes were detected by chemiluminescence (TAITEC Co., Koshigaya-shi, Saitama, Japan).

3.3.6 Immunoprecipitation and Western blotting

HepG2 (3×10^6) cells were pre-cultured in 100-mm dishes for 24 h, and treated with baicalein or myricetin (20 μM) for additional 9 h. After that, cells were lysed with modified RIPA buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1% deoxycholate, 50 mM sodium fluoride, 50 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail (Nakarai Tesque, Kyoto, Japan). The lysates were homogenized in an

ultrasonicator for 10 s twice and incubated on ice for 30 min. The homogenates were centrifuged at 14,000 g for 15 min at 4℃. The supernatants were collected and protein concentration was determined by protein assay kit (Bio-Rad Laboratories). For immunoprecipitation, whole-cell lysates containing 0.5 mg of proteins were precleared with protein A-Sepharose beads (GE Healthcare) for 1 h and incubated with 1 μg of anti-Nrf2 or anti-Keap1 antibody for 4 h. Immunoprecipitated complexes were washed five times with RIPA buffer and then boiled in SDS sample buffer for 5 min. Either the immunoprecipitation products or the whole-cell lysates containing 20 μg of proteins were run on 8% SDS-PAGE and electrophoretically transferred to PVDF membrane (GE Healthcare). After blotting, the membrane was incubated with specific antibody overnight at 4℃ and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected using the ECL system and the relative amounts of proteins associated with specific antibody were quantified using Lumi Vision Imager soft ware (TAITEC Co., Koshigaya-shi, Saitama, Japan).

3.3.7 Transfection of small interfering RNA (siRNA)

Predesigned siRNA against human *Nrf2* (Catalog No. 115762) and control scrambled siRNA (Catalog No. 4611) were purchased from Ambion (Austin, TX, USA). HepG2 cells were plated at a density of 7×10^5 cells per 60-mm dish. Cells were transfected with 100 nM siRNA against Nrf2 or 50 nM scrambled duplex by using LipofectAMINE 2000 (Invitrogen). After 24 h incubation, fresh medium was added and the cells were cultured for another 48 h. The cells were then treated with 20 μM baicalein or myricetin for an additional 6 h and lysed for Western blotting.

3.3.8 Pull-down assay

Baicalein-Sepharose 4B beads were prepared as described previously [136]. In brief, baicalein (3 mg) was coupled to CNBr-activated Sepharose 4B beads (25 mg) in a coupling buffer overnight at 4 ℃ according to the manufacturer's instruction. The

mixture was washed in 5 volume of coupling buffer and then centrifuged at 1,000 rpm for 3 min at 4 ℃. The precipitate was resuspended in 5 volume of 0.1 M Tris–HCl buffer (pH 8.0) with 2 h rotation at room temperature. After washing three times with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, the mixture was further washed with 0.1 M Tris–HCl (pH 8.0) buffer containing 0.5 M NaCl. The cell lysates (500 μg for ex vivo assay) were incubated at 4 ℃ overnight with Sepharose 4B beads or Sepharose 4B baicalein-coupled beads (100 μl, 50% slurry) in a reaction buffer. The beads were then washed five times with a washing buffer. The proteins were applied to SDS-PAGE and then detected by immunoblotting.

3.3.9 Molecular modeling

Computer modeling of baicalein to MEK1 and AKT1 proteins (PDB codes: 1S9J and 3CQW) was performed using Molecular Operating Environment™ software (MOE, Version 2008.10, Chemical Computing Group Inc.). Hydrogen atoms were first added, and force field atomic charges were assigned. Docking of baicalein to protein kinases was done by using MOE-ASEDock 2005 software [134].

3.3.10 Statistical analysis

All the experimental data shown were repeated at least three times, unless otherwise indicated. Differences between treatments and the control were analyzed by the Student's *t*-test. A statistical probability of $p < 0.05$ was considered significant.

3.4 Results

3.4.1 Baicalein activates Nrf2-ARE pathway through Keap1-independent mechanisms

Several lines of data have revealed that PKC [41], MAPKs [44], and PI3K [47] could phosphorylate Nrf2 to enhance Nrf2 activity in a Keap1-independent manner. Thus, I first investigate whether these upstream protein kinases signaling are

important in baicalein-induced Nrf2-ARE pathway activation.

3.4.1.1 Upstream protein kinase signaling pathways are involved in baicalein-induced Nrf2 activation

HepG2 cells were treated with baicalein, inhibitors of PI3K, PKC, MEK, p38 and JNK alone or with combination of baicalein and these inhibitors. As shown in Figure 3.3, a significant reduction of both Nrf2 and HO-1 were observed in the cells cotreated with U0126 (MEK1/2 inhibitor), LY294002 (PI3K inhibitor) and SP600125 (JNK inhibitor), but not with SB202190 (p38 inhibitor) and R0318220 (PKC inhibitor). The results implicated that the activation of Nrf2-ARE pathway by baicalein may be at least partly due to the activation of MEK, PI3K and JNK signaling pathways.

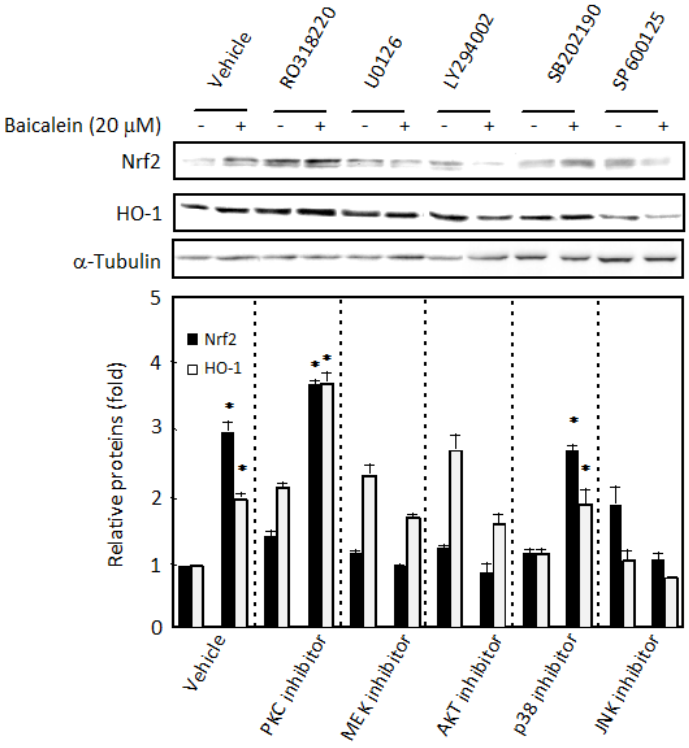


Figure 3.3 The effects of protein kinases inhibitors on Nrf2-ARE activation. HepG2 cells were pretreated with LY294002 (50 μM), RO318220 (10 μM), U0126 (10 μM), SB202190 (20 μM) and SP600125 (20 μM) for 1 h, and then treated with or without 20 μM baicalein for 9 h. Cell lysates were harvested, and Nrf2, HO-1, and α-tubulin were detected by Western blot analysis with their respective antibodies. Each value represents the mean ± SD of three separate

experiments. ∗ $p < 0.05$ vs control, respectively.

3.4.1.2 Baicalein stimulates phosphorylation of protein kinases MEK, JNK and PI3K signaling pathways

To further confirm whether baicalein stimulate phosphorylation of these protein kinases, we next examined the effect of baicalein on MEK, JNK and PI3K signaling

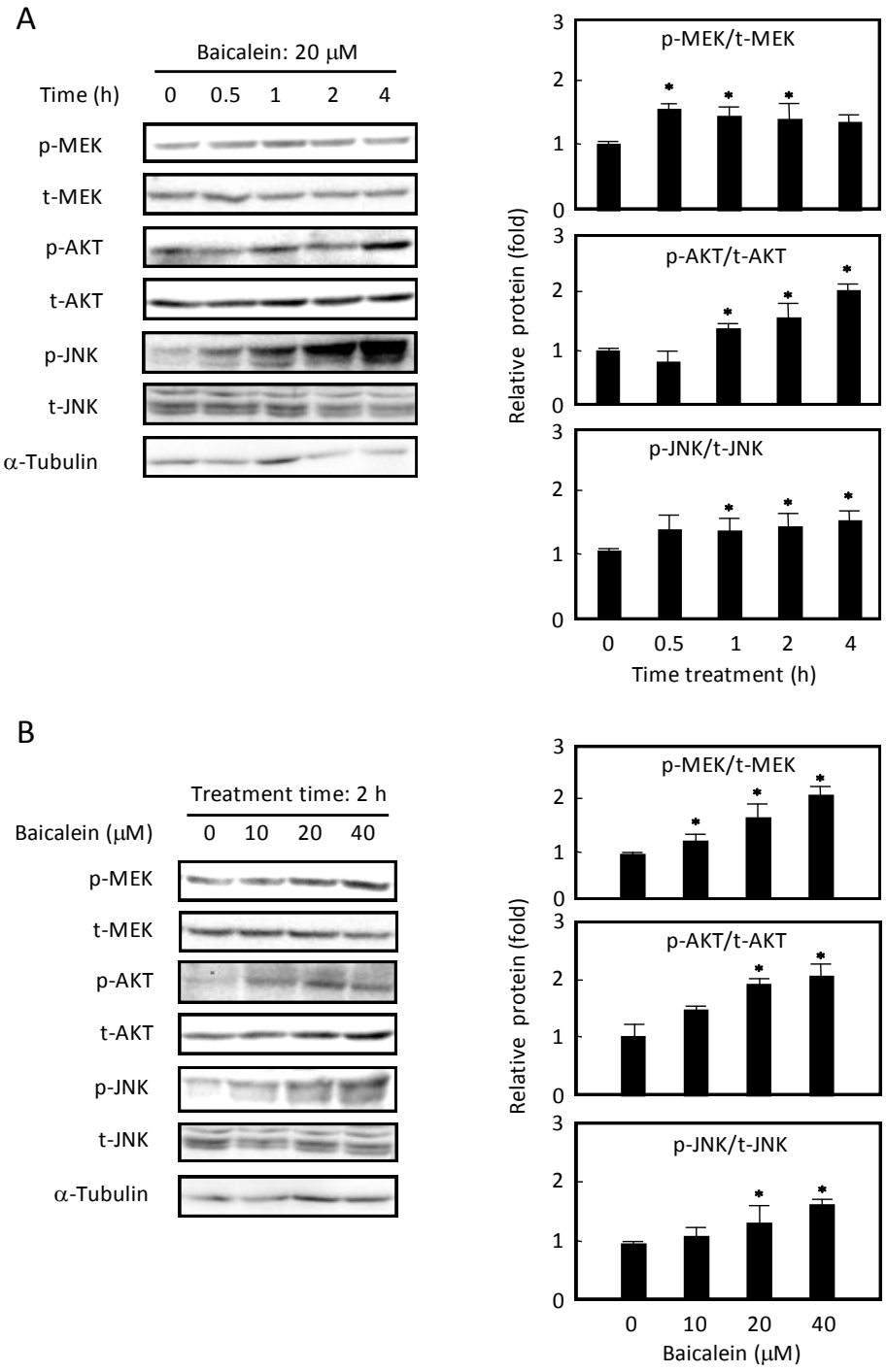


Figure 3.4 Baicalein increases phosphorylation of MEK, AKT and JNK upstream signaling of Nrf2-ARE pathway in HepG2 cells. Time and concentration response of baicalein on activation of MEK, AKT and JNK signaling pathway was determined. HepG2 cells were treated with baicalein at the indicated times (A) or concentrations (B), and then, the proteins were prepared for Western blotting with activation-specific antibodies. Each value represents the mean ± SD of three or four separate experiments. ∗ $p < 0.05$ vs control, respectively.

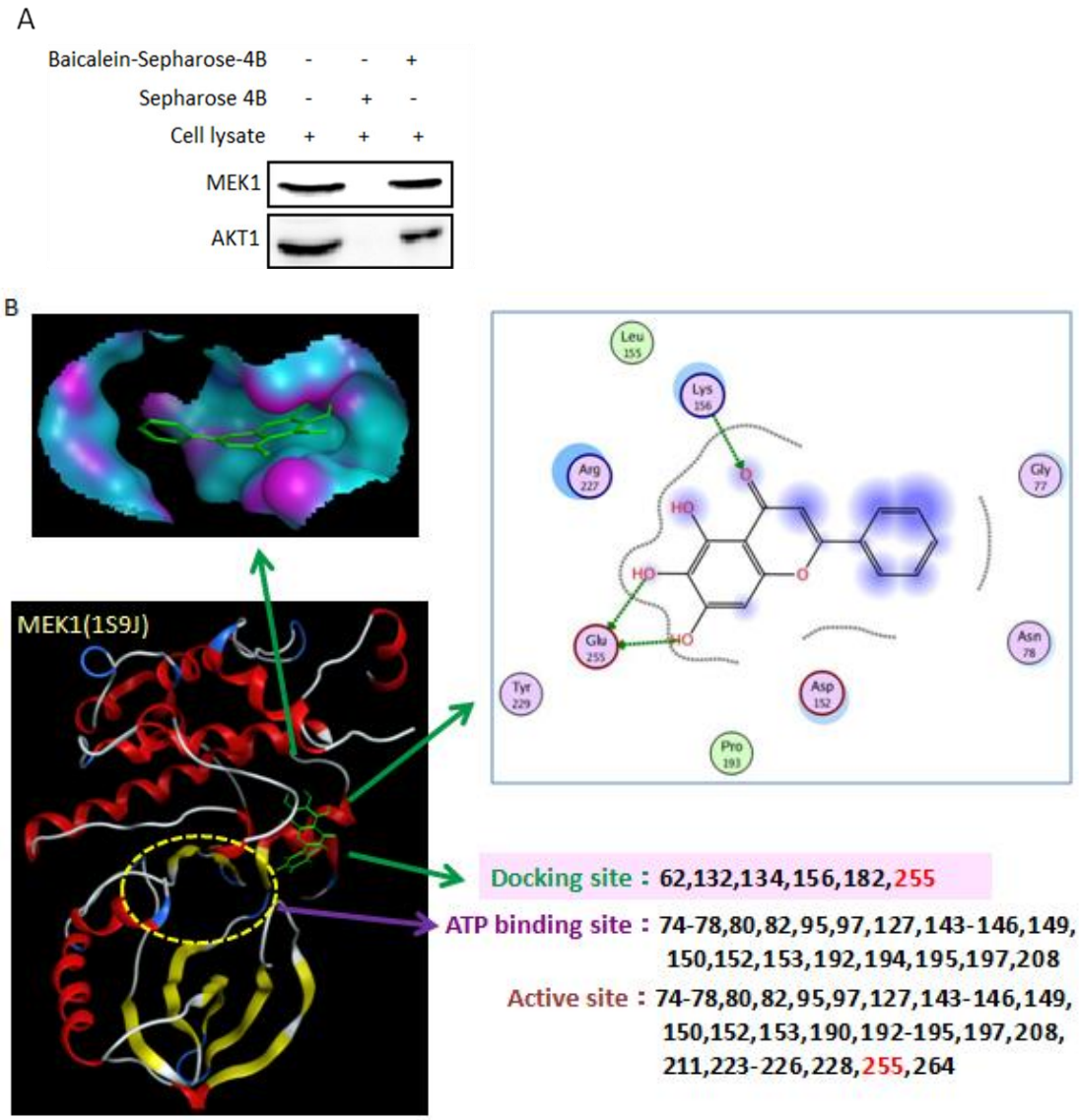
pathways in HepG2 cells by Western blot. As shown in Figure 3.4, baicalein increased the protein phosphorylation of MEK, AKT and JNK time dependently within 2 h of treatment (Figure 3.4A), and baicalein treatment for 2 h up-regulated the protein phosphorylation of MEK, AKT and JNK in a concentration-dependent manner (Figure 3.4B). Furthermore, we also tested the phosphorylation of Nrf2 at serine 40 residues by p-Nrf2 (Ser40) antibody which is reported to be the target of PKC pathway phosphorylation, and the result showed no significant change (data not shown here), indicating that PKC pathway had no significant effect on baicalein-induced Nrf2 activation. These results revealed that phosphorylation of MEK, AKT and JNK signaling pathways played important role in baicalein-induced Nrf2-ARE pathway activation.

3.4.1.3 Baicalein directly binds to MEK1 and AKT1

Myricetin, a compound with similar structure as baicalein, has been reported to directly bind with AKT1 and MEK1 to exert its function. *Ex vivo* and *in vitro* binding data showed that myricetin directly bound to the ATP-binding site of AKT1 and ATP-noncompetitive site of MEK1 [134, 135]. To further understand how baicalein stimulated phosphorylation of protein kinases, we hypothesis it is also caused by direct binding. Thus, we performed pull down assay and molecular modeling.

As shown in Figure 3.5A, MEK1 and AKT1 were detected in Sepharose 4B-baicalein-coupled beads (lane 3), but not in Sepharose 4B beads alone (lane 2) *ex vivo*. In order to further elucidate the properties of baicalein binding to MEK1 and

AKT1, we performed computational analysis based on the structure of these protein kinases and baicalein. As shown in Figure 3.5B, baicalein docked to the side of ATP-binding pocket of MEK1. Three hydrogen bonds were formed between the 4, 7 and 8 positions of baicalein and Lys156, Glu255 residues of MEK1, which configured the binding pocket (Figure 3.5B). For AKT1 binding, baicalein also docked side of its ATP-binding pocket (Figure 3.5C). Two hydrogen bonds were formed between the 7 and 8 positions of baicalein and Glu255 residue of AKT1, which configured the binding pocket (Figure 3.5C). These docking results further support our binding data *ex vivo* between baicalein and MEK1 and AKT1.



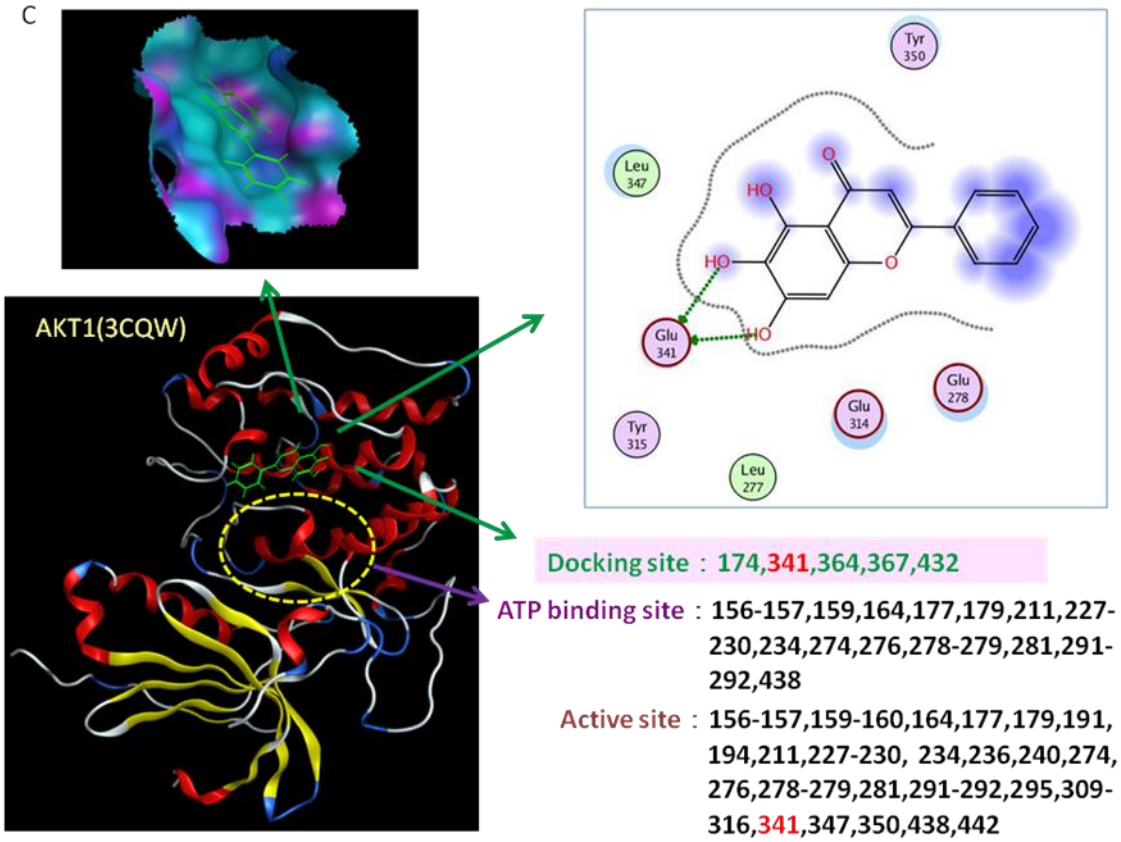


Figure 3.5 Baicalein directly binds to MEK1 and AKT1. (A) Ex vivo pull-down assay. Whole cell lysate (input control, lane 1), lysate precipitation with Sepharose 4B beads (negative control, lane 2) and Sepharose 4B-baicalein-coupled beads (lane 3) were applied to SDS-PAGE and then detected with MEK1 and AKT1 antibodies. (B) and (C) Docking model of baicalein with MEK1 and AKT1. Baicalein binds non-ATP-binding sites of both. Red: a-helix, yellow: b-sheet, blue: 3-turn, yellow-green: 4- or 5-turn and aqua: loop in left panel. Hydrogen bonds are indicated by blue (to backbone) and green (to sidechain) lines in right panel. Baicalein binding to the non-ATP-binding cleft represented as an electrostatic potential surface. Pink: hydrogen bonds, green: hydrophobic, blue: mild polar surfaces. Hydrogen bonds are indicated by yellow line.

3.4.1.4 Baicalein upregulates Nrf2 mRNA expression

To further know whether baicalein-induced Nrf2 protein expression is caused by transcriptional regulation of Nrf2 mRNA expression, we applied RT-PCR to detect the expressions of mRNA of Nrf2, Keap1 and NQO1. As shown in Figure 3.6A, baicalein increased the mRNA level of Nrf2 and NQO1 in a dose-dependent manner,

but Keap1 mRNA was not affected by the same treatment. The observation was further confirmed by using an inhibitor of transcription, actinomycin D. As shown in Figure 3.6B,

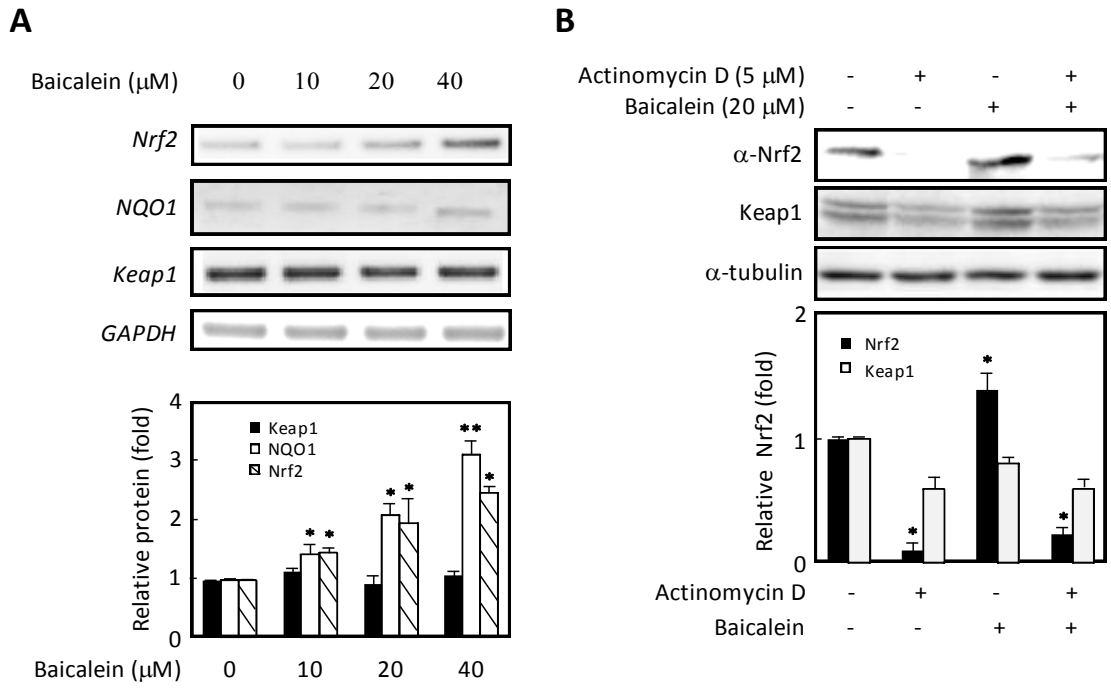


Figure 3.6 Baicalein upregulates Nrf2 expression at transcriptional level. (A) RT-PCR detection of m RNA expression of Nrf2, NQO1 and Keap1. (B) Actinomycin D's effect on Nrf2/Keap1 expression.

treatment with baicalein significantly increased the protein level of Nrf2, but not Keap1 (lane 3). Cotreatment with actinomycin D and baicalein significantly reduced baicalein-enhanced Nrf2 level (lane 4). These results indicate that baicalein upregulated mRNA of Nrf2 and NQO1, but not Keap1, at the transcriptional level.

3.4.1.5 Effect of baicalein on transcriptional regulation of Nrf2 mRNA

To understand how baicalein upregulated Nrf2 mRNA expression, we performed Nrf2-related network analysis based on IPA and JASPAR prediction. The regulation

of Nrf2 mRNA could be controlled by transcription factors (TFs) [57, 67], nuclear proteins [59, 60] and miRNAs [73, 73]. In order to predict TFs that directly regulate Nrf2 transcription, we queried the regulatory region of Nrf2 gene sequence against all TFBS information found in JASPAR [78, 79] and input the microarray data as shown in Table 3.1. In order to predict proteins that directly regulate Nrf2 transcription, we used IPA system to generate Nrf2-related network as shown in Table 3.2, mainly being classified into Keap1 system group, Protein kinases group, CBP-NF-κB group, Transcription factors group and others group, which showed the crosstalks between Nrf2-ARE pathway and other signaling pathways such as inflammatory pathway, apoptosis pathway, etc. No miRNA was found significantly upregulated by baicalein treatment.

Next, we listed out the TFs and proteins with significant change above 1.5-fold and Nrf2 mRNA itself. As shown in Figure 3.7, the expression of Nrf2 mRNA had increased 1.59-fold by baicalein treatment, which may be caused by induction of TFs and proteins. In TFs group, induced NF-κB, AHR and Nrf2 itself could bind the promoter region of Nrf2 mRNA [80-82], while Pax6 and ESR2 could enhance Nrf2 mRNA expression with other mechanism [83, 84]. In proteins group, the significantly induced expressions of MafF, c-Src, c-jun, Jun B, VEGF and SQSTM1 could enhance Nrf2 transcription, while c-Fos and FRA1 could inhibit that.

Several studies have reported that phosphorylation of protein kinases could induce TFs or nuclear proteins expression. For example, NF-κB can be induced by phosphorylation of AKT and TAK1 [85, 86], phosphorylation of ERK could upregulate Pax6 [87], and phosphorylation of JNK could induce c-Jun [88]. These phenomenons suggested that baicalein upregulated Nrf2 mRNA expression by targeting AKT/NF-κB, ERK/Pax6 and JNK/c-Jun pathways, at least.

Table 3.1 Transcription factors that can regulate expression of Nrf2

Name	Uniprot ID	Type	PMID	Microarray
EWSR1-FLI1	Q01844 Q01543			1.35 down (EWSR1) 1.21 down (FLI1)
FOXI1	Q8N6L8			1.12 down
MEF2A	Q7Z6C9	promoter of Nrf2	18222924	1.24 up
HNF1B	B4DKM3			1.24 up
NFIL3	Q16649			2.35 up
RREB1	F8WF59			1.45 down
RORA_1	P35398			2.35 up
ESR2	O60685	promoter of Nrf2	21167702	1.78 up
RELA	E9PRX2			1.28 up
TEAD1	E9PKB7			1.06 up
PBX1	Q53YC7			1.36 down
NR3C1	F5ATB8			1.26 up
TAL1::TCF3	P17542 Q9HCS4			1.33 up (TAL1) 1.66 down(TCF3)
IRF1	P10914			1.60 up
TLX1::NFIC	P31314 P08651			1.20 up (TLX1) 1.43 down(NFIC)
INSM1	Q01101			1.00 up
NR2F1	P10589			1.73 down
PPARG	E7EU07			1.16 up
Myf (MYOD1)	P15172			1.12 down
STAT1	E9PH66			1.69 down
FOXA1	G3V4B9			1.27 down
NFE2L2	E9PGJ7	promoter of Nrf2	11940647	1.59 up
E2F1	Q92768			1.29 down
ESR1	Q9NU51		14676828	1.16 up
FOXF2	Q5TGJ1			1.00 up
MAX	Q96CY8			1.27 up
MYC::MAX	P01106 P61244			1.89 up (MYC)
NF-kappaB	P19838	promoter of Nrf2	23077289	1.63 up
NHLH1	Q02575			1.31 up
Pax6	F1T0F8	promoter of MafK	29716	3.02 up
RORA_2	P35398			
SP1	Q8N907			1.33 down
AHR	P35869	promoter of Nrf2	15790560	1.50 up

Table 3.2 Nrf2-related network generated by IPA and manual curation

Group	Protein1	Protein2	Type	Effect	Details of the interaction	PMID	Microarray
Regulators of NRF2 transcription							**Protein 1**
Keap1 system	Keap1	NRF2	direct	inhibition	Neh2 domain of Nrf2 and the DGR domain of Keap1	9887101	1.01 down
	Keap1	IKKβ	direct	inhibition	the C-terminal Kelch domain of KEAP1 interacts with the IKKβ kinase domain	20600852	1.01 down
	Keap1	Cul3	direct	activation	BTB domain of KEAP1 binds to the N-terminal region of CUL3	15282312	1.01 down
	Keap1	ProTα	direct	undirected	carboxyl-terminal (Kelch repeats) of Keap1 binds ProT{alpha}	15657435	1.01 down
	ProTα	NRF2	indirect	activation		15657435	1.73 down
	ProTα	Keap1	direct	inhibition	carboxyl-terminal of Keap1 binds ProTα	15657435	1.73 down
	Cul3	Rbx1	direct	undirected		15572695	1.36 up
	CUL1	Rbx1	direct	activation		10230406	1.36 up
	Rbx1	IkBa	direct	inhibition	RBX1/Cul1 Ubiquitinates IκBα	10230406	1.64 down
	Rbx1	NRF2	direct	inhibition	ubiquitination of lysine residues of the N terminal Neh2 domain of NRF2	15572695	1.64 down
	COX-2	Keap1	indirect	inhibition		15917255	1.4 up
	HSP90	Keap1	direct	inhibition	NTR of Keap1 interacts with Hsp90 CLD regions	20864537	1.67 down
	SQSTM1	Keap1	direct	inhibition	KIR motif in p62 interacts with the Kelch-repeat domain of KEAP1	20452972	2.14 up
	CK2	Keap1	direct	inhibition	CK2 phosphorylates Keap1 at Thr55	20864537	1.10 down
	CAND1	Keap1	indirect	activation		16449638	1.12 down
	PGAM5	Keap1	direct	activation	DxESGE motif in PGAM5 binds to the NxETGE motif in Keap1	17046835	1.32 down
	PGAM5	NRF2	indirect	inhibition		18387606	1.32 down
Protein kinases	CK2	NRF2	direct	inhibition	CK2 phosphorylates Nrf2	17512459	1.10 down
	GSK3β	NRF2	direct	inhibition	phosphorylation at Ser residues	16551619	1.15 down
	p38α	NRF2	direct	inhibition	phosphorylates Nrf2	16951197	1.11 down
	p38β	NRF2	direct	inhibition	phosphorylates Nrf2	16951197	1.32 down
	p38γ	NRF2	direct	inhibition	phosphorylates Nrf2	16951197	1.13 down
	p38δ	NRF2	direct	inhibition	phosphorylates Nrf2	16951197	1.06 up
	PKCD	NRF2	direct	activation	PKC-delta phosphorylates Nrf2 S40	19920073	1.55 down
	PI3K	NRF2	indirect	activation	Phosphorylation	19272177	1.56 up
	JNK1	NRF2	direct	activation	phosphorylation of NRF2 by JNK1	16308312	1.22 up
	ERK2	NRF2	direct	activation	phosphorylation of NRF2 by ERK2	16308312	1.39 down
	Akt	NRF2	indirect	activation	Phosphorylation and Kinase docking	19931411	1.59 down
	ERK1	NRF2	indirect	activation	Phosphorylation and Kinase docking	19931411	1.36 down
	PKC	NRF2	direct	activation	Phosphorylation of Nrf2 at Ser-40	12198130	1.27 up

	PKAc	p65	direct	activation	phosphorylation of p65 at S276	9660950	1.34 up
CBP-NF-κ B	p65	NRF2	indirect	inhibition		18241676	1.28 up
	p65	NRF2	direct	activation	binding kappa B-site of Nrf2 promoter region	11940647	1.28 up
	p50	NRF2	direct	activation	binding kappa B-site of Nrf2 promoter region	11940647	1.34 up
	p65	HDAC3	direct	undirected		18241676	1.28 up
	p65	CBP	direct	inhibition	p65 binds CH1-KIX domain of CBP	18241676	1.28 up
	CBP	NRF2	direct	activation	Neh4 and Neh5 of NRF2 individually and cooperatively bind to TAD of CBP	11683914	1.29 up
	HDAC3	CBP	direct	inhibition	HDAC deacetylates CBP	18241676	1.11 down
	HDAC3	MafK	direct	inhibition	C-terminal Zip domain of MafK contains HDAC3 docking site	18241676	1.11 down
	HDAC1	NRF2	indirect	inhibition		18241676	1.15 down
	HDAC1	MafK	indirect	inhibition		18241676	1.15 down
	HDAC2	NRF2	indirect	inhibition		18241676	1.15 down
	HDAC3	NRF2	indirect	inhibition		18241676	1.11 down
	IKKβ	NRF2	indirect	inhibition	Phosphorylation and Kinase docking	18241676	1.27 down
	BTRC	IkBa	direct	inhibition	binds phosphorylated IkBa	10066435	1.23 down
	BTRC2	IkBa	direct	inhibition	binds phosphorylated IkBa	10066435	1.20 up
	BTRC	Skp-1	direct	undirected	F box near the N terminus of βTrCP interacts with Skp1	10531035	1.23 down
TFs	MafF	NRF2	direct	activation	ZIP-ZIP dimerization	12490281	3.63 up
	MafF	c-Jun	direct	activation	ZIP-ZIP dimerization	12490281	3.63 up
	MafG	NRF2	direct	activation	ZIP-ZIP dimerization	18585411	1.13 up
	MafG	BACH1	indirect	activation		21812759	1.13 up
	KAP1	NRF2	direct	activation	N-terminal region of KAP1 binds to Nrf2	21382013	1.55 down
	Skp-1	Cullin-1	direct	activation	N-terminal of CUL1 interacts with the N-terminal of Skp1	9663463	1.31 down
	BRG1	NRF2	direct	activation	Nrf2 recruits BRG1 to the distal E1 and E2 enhancers of the HO-1 promoter	16923960	1.75 down
	c-Jun	NRF2	direct	activation	heterodimer via leucine zipper region	9872330	3.20 up
	Jun-B	NRF2	direct	activation	heterodimer via leucine zipper region	9872330	2.28 up
	JunD	NRF2	direct	activation	heterodimer via leucine zipper region	9872330	1.19 up
	c-Fos	NRF2	direct	inhibition	binding with Nrf2	19671797	2.90 up
	FRA1	NRF2	direct	inhibition	binding with Nrf2	22393254	2.13up
	c-Src	NOX1	indirect	activation		15774483	2.61 up
	c-Src	NRF2	indirect	activation		18802114	2.61 up
	Bach1	NRF2	indirect	inhibition		19897490	1.90 up
Others	NOX1	NRF2	indirect	activation		20347035	1.1 up

	Casp-3	NRF2	direct	undirected	Cleavage NRF2 by casp-3 result in 2 product: 30 and 50 kDa	10510468	1.22 down
	CRIF1	NRF2	direct	inhibition	CRIF1 interacts with both N- and C-terminal regions of NRF2	20427290	1.41 down
	BRCA1	NRF2	indirect	activation	Binding on phosphorylated residues	15520196	1.09 up
	ERα	NRF2	indirect	inhibition	Binding to nuclear receptors	20623181	1.56 up
	MKK6	NRF2	indirect	inhibition	Phosphorylation and Kinase docking	16951197	1.55 down
	ENC1	NRF2	indirect	inhibition		19424503	1.15 down
	SIRT1	NRF2	indirect	inhibition		20623181	1.38 up
	YY1	CFTR	indirect	inhibition		20309604	1.21 up
	CUL1	NRF2	indirect	activation		17015834	1.36 up
	COX-2	NRF2	indirect	activation		15917255	1.41 up
	EPO	NRF2	indirect	activation		20229611	1.49 down
	KLF2	NRF2	indirect	activation		18467642	1.67 up
	MT-III	NRF2	indirect	activation		18554677	1.23 down
	VEGF	NRF2	indirect	activation		22033923	3.22 up
	TNF	NRF2	indirect	activation		18202225	1.00 down
	PMF-1	NRF2	direct	undirected	leucine-zipper region of Nrf-2 and a C-terminal coiled-coil region of PMF-1	11256947	1.40 down
	NRF2	YY1	direct	activation	Nrf2 binds to YY1 and promotes the YY1 nuclear localization	20309604	1.21 up
NRF2 transcriptional targets							**Protein 2**
	NRF2	VAMP-1	indirect	inhibition		16246346	1.23 up
	NRF2	MUC5AC	indirect	inhibition		20216230	1.37 up
	NRF2	CFTR	indirect	inhibition		20309604	1.18 up
	NRF2	MCP-1	indirect	inhibition		16246346	1.03 up
	NRF2	CBR3	indirect	activation		20806931	1.36 up
	NRF2	GCLM	indirect	activation		19808663	2.19 up
	NRF2	ABCC2	indirect	activation		18038766	1.00 up
	NRF2	IL-8	indirect	activation		16220540	6.95 up
	NRF2	IkBa	indirect	activation		16246346	1.22 up
	NRF2	MRP3	indirect	activation		19345732	1.43 up
	NRF2	VEGF	indirect	activation		20185790	3.22 up
	NRF2	GCLC	indirect	activation		16551616	3.50 up
	NRF2	MRP4	indirect	activation		20395535	1.30 up
	NRF2	ABCB11	indirect	activation		19821532	1.13 down
	NRF2	CYP2A6	indirect	activation		20887713	1.19 up
	NRF2	NQO1	indirect	activation		14985350	1.00 up
	NRF2	NQO2	indirect	activation		16545679	1.34 down
	NRF2	ATF3	indirect	activation		19864258	6.03 up
	NRF2	ATF4	indirect	activation		20185790	1.46 up

	NRF2	UGT1A1	indirect	activation		17259171	1.19 up
	NRF2	AKR1C2	indirect	activation		16478829	1.17 up
	NRF2	SQSTM1	indirect	activation		20452972	2.14 up
	NRF2	PREPL	indirect	activation		19575798	1.49 up
	NRF2	ABCG2	indirect	activation		20682644	1.54 down
	NRF2	Bach1	indirect	activation		21812759	1.90 up
	NRF2	C2ORF34	indirect	activation		19575798	1.52 down
	NRF2	GCS-1	indirect	activation		15890065	1.00 up
	NRF2	GI-GPx	indirect	activation		15923610	1.57 down
	NRF2	HO-1	indirect	activation		16123320	3.00 up
	NRF2	prx1	indirect	activation		17234762	1.15 down
	NRF2	SSAT	indirect	activation		11256947	1.24 up
	NRF2	Trx1	indirect	activation		12660821	1.15 up
	NRF2	TXAS	indirect	activation		11956185	1.17 up
	NRF2	JNK1	indirect	activation		16928811	1.22 up
	NRF2	CEBPB	indirect	activation		22138520	2.41 up

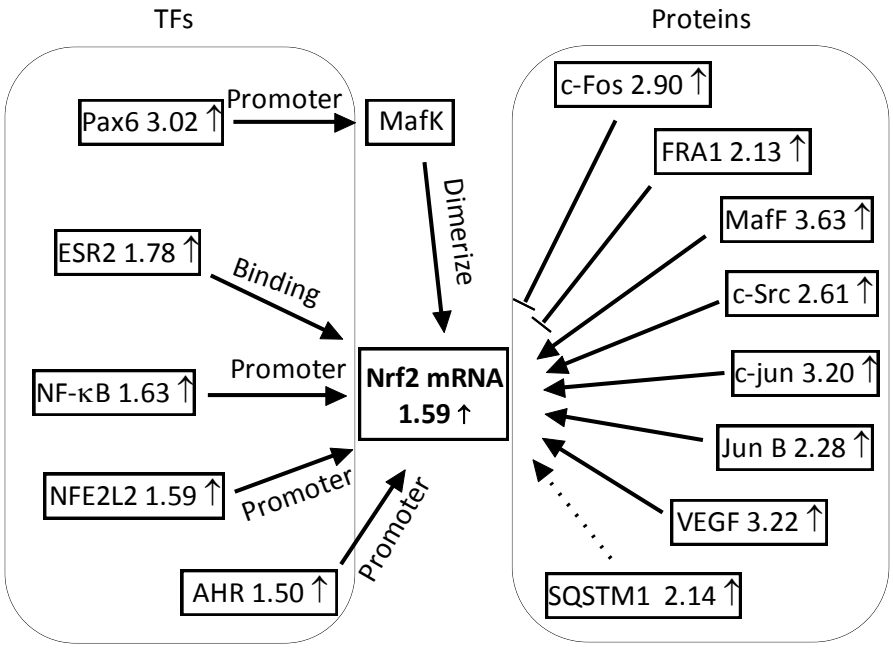


Figure 3.7 Nrf2-related network analysis of baicalein-induced Nrf2 mRNA expression by TFs and nuclear proteins. Fold change of TF with equal or larger than 1.5 is deemed to be significant, and fold change of protein with equal or larger than 2.0 is deemed to be significant.

These results based on microarray and Nrf2-related network analysis revealed that baicalein could regulate Nrf2-ARE pathway by Keap1-independent mechanisms.

3.4.2 Baicalein and myricetin activate Nrf2-ARE pathway through Keap1-dependent mechanisms

Accumulating data has shown that Nrf2-mediated antioxidant enzymes contain specific nucleotide sequences in their gene promoters, defined as ARE, with the consensus sequence 5′-TA/CANNA/GTGAC/TNNNGCA/G-3′ [89, 90]. ARE has been reported to contribute to the protection of cells against carcinogens and oxidative stress. Several molecules, such as nuclear factor-E2-related factor 2 (Nrf2), c-Jun, ATF2, and ATF4, have been proposed as potential modulators of ARE [91-95]. Of these, Nrf2, a member of the CNC family of bZIP proteins, is extensively proven to be a strong activator of ARE-mediated gene expression [89, 96]. Thus, we postulated that the mRNA expressions of these genes induced by baicalein and myricetin were due to Nrf2-mediated ARE transcriptional regulation. The consensus sequences of ARE are found in the promoter regions of genes of some hepatic drug metabolizing enzymes such as NQO1, AKR1C2, GCLM, GST, TXNRD1, SRXN1 and HO1 (Figure 3.8).

-426	5' TCACAG	TGA	CTCA	GC	AGAAT	NQO1
-5233	5' TCAGGG	TGA	CTCA	GC	TGCTTG	AKR1C2
-287	3' AGACAA	TGA	CTAA	GC	AGAAA	GCLM
-1048	5' TTTCTTAG	TGA	CTTA	GC	AGTATT	GST
-41	3' CTCGAA	TGA	CAAA	GC	AG	TXNRD1
-228	3' TTCACCC	TGA	GTCA	GC	GGCC	SRXN1
-291	3' CTGGGTG	TGA	TTTT	GC	TCCTTC	HO1
		TGA	nn nn	GC		ARE CONSENSUS

Figure 3.8 Promoter sequences assignment of selected human Phase II genes. All the promoter sequences are selected from human genes. Nucleotides at essential positions for ARE marked in bold in its consensus, and elliptical frame showed the same nucleotides of all the genes. The abbreviation follow standard IUPAC nomenclature (n = A, T, C or G). The promoter sequences begin with 5’ are shown as reverse complements, and the sequences begin with 3’ are shown as frontal complements.

3.4.2.1 Baicalein and myricetin stimulate Nrf2-mediated ARE activation

To evaluate whether they induces Nrf2-mediated ARE activity, we performed reporter gene assay by transfecting an ARE-luciferase reporter plasmid into HepG2 cells.

As shown in Figure 3.9A and 3.10A, baicalein and myricetin both induced ARE-driven activity in dose dependent manner. Next, we co-transfected Nrf2 expression plasmid with ARE-luciferase plasmid in HepG2 cells. Over-expression of Nrf2 stimulated ARE activation, and baicalein and myricetin enhanced the activation of ARE in a dose-dependent manner (Figure 3.9B and 3.10B). To further confirm the obtained results or previous results at protein level, we treated HepG2 cells with various concentrations of these polyphenolic compounds for 9 h and then detected the protein levels by Western blotting. As shown in Figure 3.9C and 3.10C, they both increased protein expressions of Nrf2, NQO1 and HO1 but decreased Keap1 expression significantly.

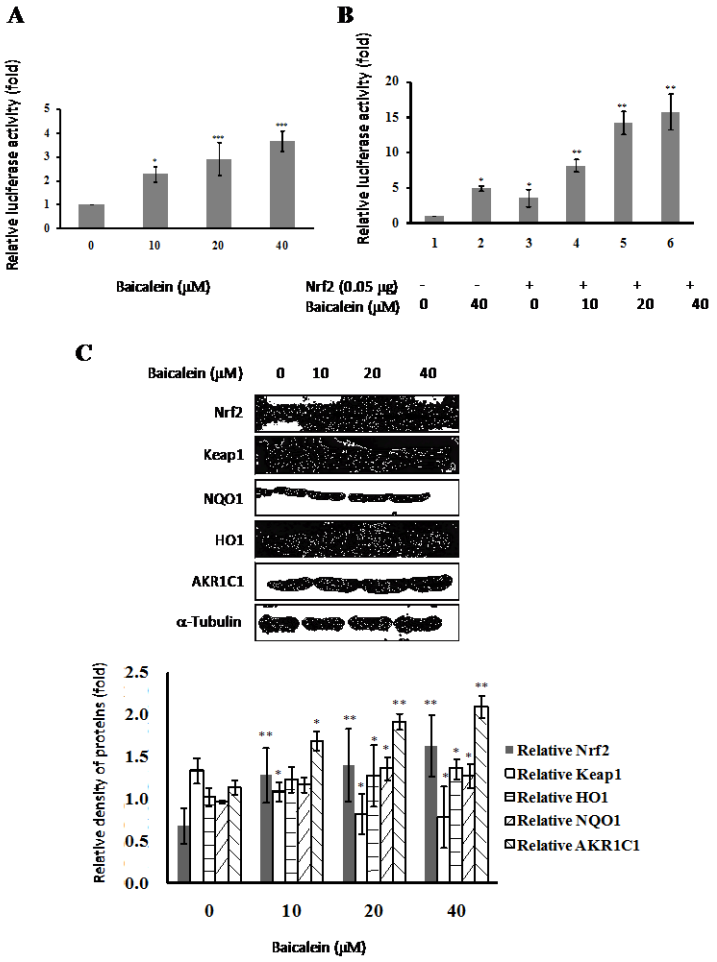


Figure 3.9 Baicalein stimulates Nrf2-mediated ARE transcription activity in HepG2 cells. (A) Effects of baicalein on the transcriptional activity of ARE in HepG2 cells. HepG2 cells were co-transfected with the pGL2-ARE *Firefly* and pGL4-TK-*Renilla* luciferase plasmids for normalization. After 5 h, cells were maintained in 10% serum medium for 20 h and then stimulated with 0-40 μM baicalein for an additional 24 h. Cells were lysed and analyzed for *Firefly* and *Renilla* luciferase activities. (B) Effects of baicalein on Nrf2-mediated ARE activity. HepG2 cells were co-transfected with 0.5 μg of pGL2-ARE-luciferase and 0.1 μg of the Nrf2 expression plasmid. Other steps are the same as shown in (A). (C) Baicalein induces the expressions of typical antioxidant proteins. HepG2 cells were treated with 0-40 μM baicalein for 9 h. Nrf2, Keap1, HO-1, AKR1C1, NQO1 and α-tubulin were detected by Western blot analysis with their respective antibodies. Each value represents the mean ± SD of three separate experiments, * $p < 0.05$; ** $p < 0.01$ vs control, respectively.

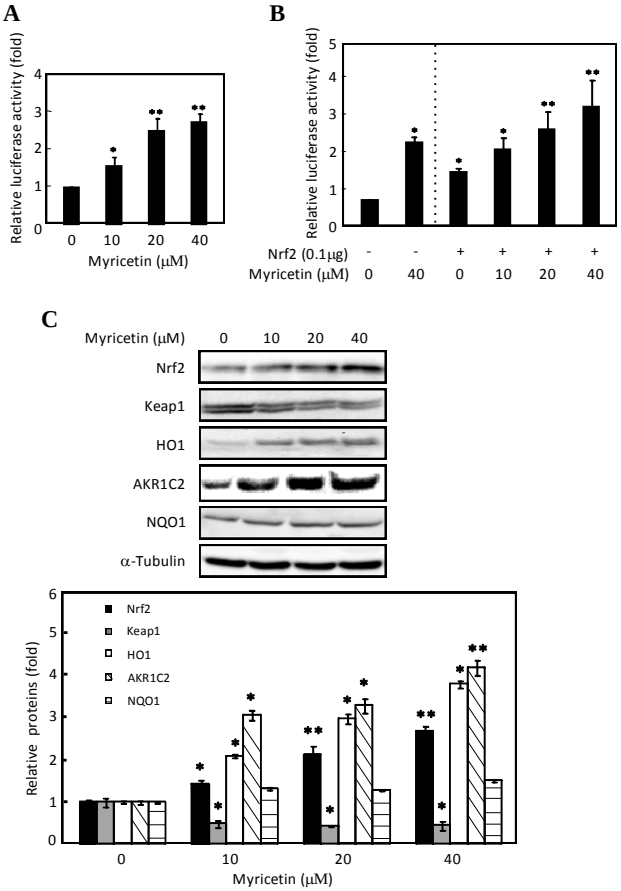


Figure 3.10 Myricetin stimulates Nrf2-mediated ARE transcription activity in HepG2 cells. Methods are the same as Figure 3.9.

These results indicated that baicalein and myricetin activated the Nrf2-ARE pathway by targeting Nrf2/Keap1 system.

3.4.2.2 Baicalein and myricetin promotes Nrf2 nuclear translocation and ARE binding ability

The nuclear accumulation and ARE binding ability of Nrf2 are essential primary actions during Nrf2-mediated ARE activation [97, 98]. To further clarify the effects of baicalein and myricetin on these actions, we first examined the localization of Nrf2 in the cells treated with or without baicalein or myricetin for 9 h by Western blotting.

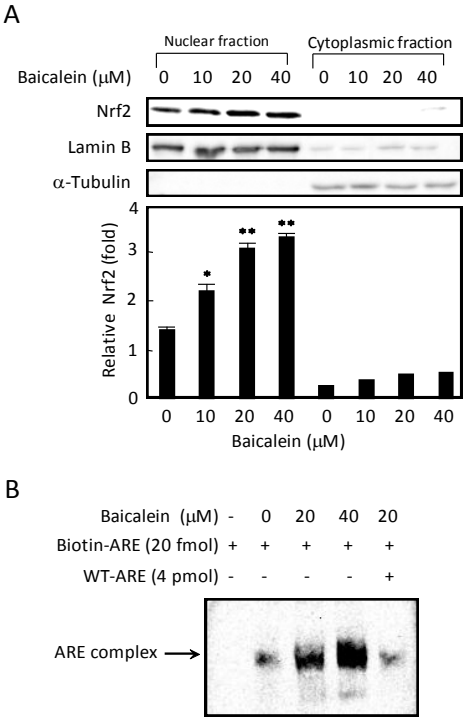


Figure 3.11 Baicalein promotes Nrf2 nuclear accumulation and ARE binding ability. (A) Baicalein increases the amount of endogenous nuclear Nrf2 in a dose-dependent manner. HepG2 cells were treated with the indicated concentrations of baicalein for 9 h. Cell fractionation were done as described in Materials and methods. The nuclear and cytosolic extracts containing 30 μg of proteins were prepared and subjected to Western blot analysis with indicated antibodies. (B) Baicalein enhances the ARE binding activity in a dose-dependent manner. Chemiluminescent EMSA were carried out as described under Materials and methods using biotin-labeled or not labeled doubled-stranded human ARE oligonucleotide and nuclear extracts from HepG2 cells treated with 0-40 μM baicalein for 9 h. Data represent means ±SD of three independent

experiments. * $p < 0.05$; ** $p < 0.01$ vs control, respectively.

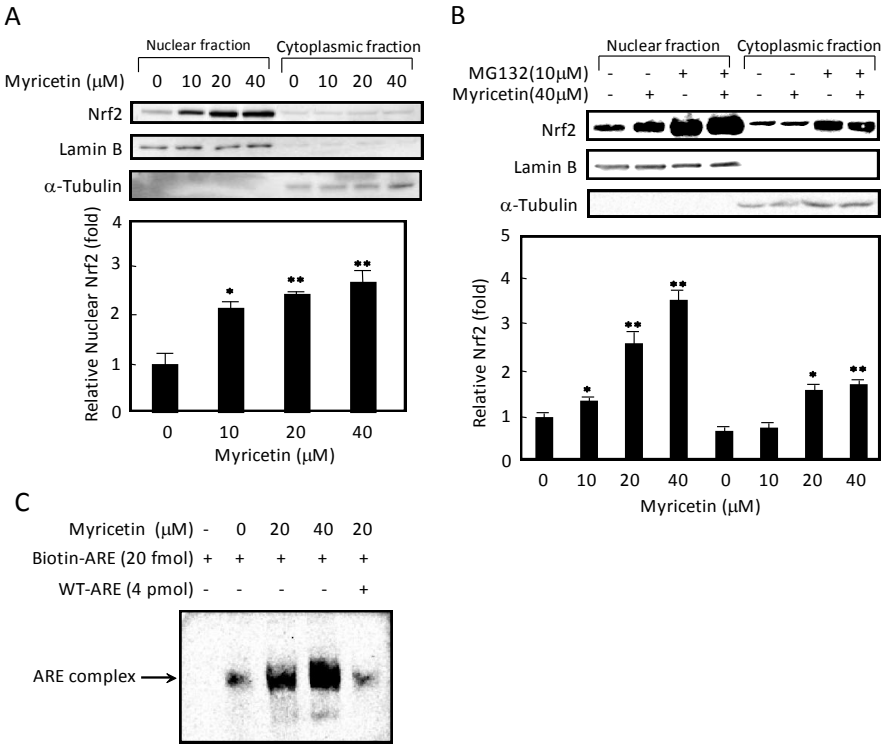


Figure 3.12 Myricetin promotes Nrf2 nuclear accumulation and ARE binding ability. (A) and (C) are the same as baicalein in Figure 3.10. (B) Myricetin increases Nrf2 in both cytoplasm and nucleus. HepG2 cells were pretreated with 10 μM MG132 for 1 h and then treated with or without 40 μM myricetin for 9 h.

As shown in Figure 3.11A and 3.12A, baicalein and myricetin both increased the amount of nuclear Nrf2 significantly in a dose-dependent manner, and little Nrf2 was detected in the cytosol of control cells. On the other hand, little Nrf2 was detected in the cytosol of control cells. The integrity of the cytosolic and nuclear fractions was confirmed by the analysis of the compartment-specific cytosolic α-tubulin and nuclear lamin B proteins. Furthermore, we pretreated the cells with 26S proteasome inhibitor MG132, and found that a clear Nrf2 band was also detected in the cytoplasm of MG132-treated cells, and myricetin further enhanced the Nrf2 level (Figure 3.12B). These data indicated that Nrf2 sequestered in the cytoplasm is rapidly degraded by ubiquitin-proteasome under homeostatic conditions.

To demonstrate whether Nrf2 accumulated in the nucleus by baicalein and myricetin actually binds to the ARE, we examined the ARE-binding complexes by chemiluminescent electrophoretic mobility shift assay (EMSA). The results revealed that baicalein and myricetin both enhanced ARE-binding complexes in a dose-dependent manner by detecting the dose of biotin-labeled human ARE oligonucleotides (Figure 3.11B and 3.12C, lane 1-4), while co-treated with 4 pmol of unlabeled human ARE oligonucleotides markedly blocked the formation of this DNA-protein complex (Figure 3.11B and 3.12C, lane 5). These results demonstrated that they both induced Nrf2-mediated ARE activation by enhancing the nuclear accumulation of Nrf2 and the binding of Nrf2 to the human ARE oligonucleotides.

3.4.2.3 Baicalein and myricetin inhibit ubiquitination of Nrf2 and modify Keap1

To further investigate the factors that affect Nrf2 nuclear accumulation, we examined the status of Nrf2 ubiquitination and Keap1 modification. After treatment with 26S proteasome-specific inhibitor MG132 or polyphenol, cellular Nrf2 or Keap1 was immunoprecipitated with its antibody, and ubiquitinated Nrf2 or Keap1 was then detected by ubiquitin antibody, respectively.

As shown in Figure 3.13A, the Nrf2 protein level was enhanced after treatment with baicalein or MG132 alone or in combination while Keap1 protein level was reduced in such treatment. Simultaneously, a significant reductive ubiquitination of Nrf2 (Figure 3.13B) and a significant inductive ubiquitination of Keap1 (Figure 3.13C) were observed in the cells cotreated with baicalein and MG132. These results indicate that the baicalein-enhanced Nrf2 is, partially at least, due to an inhibitory effect of baicalein on the ubiquitination of Nrf2 and an induction effect on the ubiquitination of Keap1. Recently, several studies revealed that some phytochemicals such as sulforaphane [99], quercetin [74] and 6-MSITC [75] induced formation of modified Keap1 protein, which is greater than 150 kDa. The formation of modified Keap1 protein caused a relative reduction to an approximately 70-kDa Keap1 band. Thus, we

next examined whether baicalein modifies Keap1. As shown in Figure 3.13D, a more than 150-kDa Keap1 protein was detected in the cells treated by 20 μM baicalein. Thus, baicalein-caused Keap1 reduction might be due to both the 26S proteasome-dependent degradation and the formation of modified Keap1 protein.

Furthermore, other study speculated that the up-regulation of Nrf2 protein by phytochemical may be due to the liberation of Nrf2 from Keap1 without dissociation

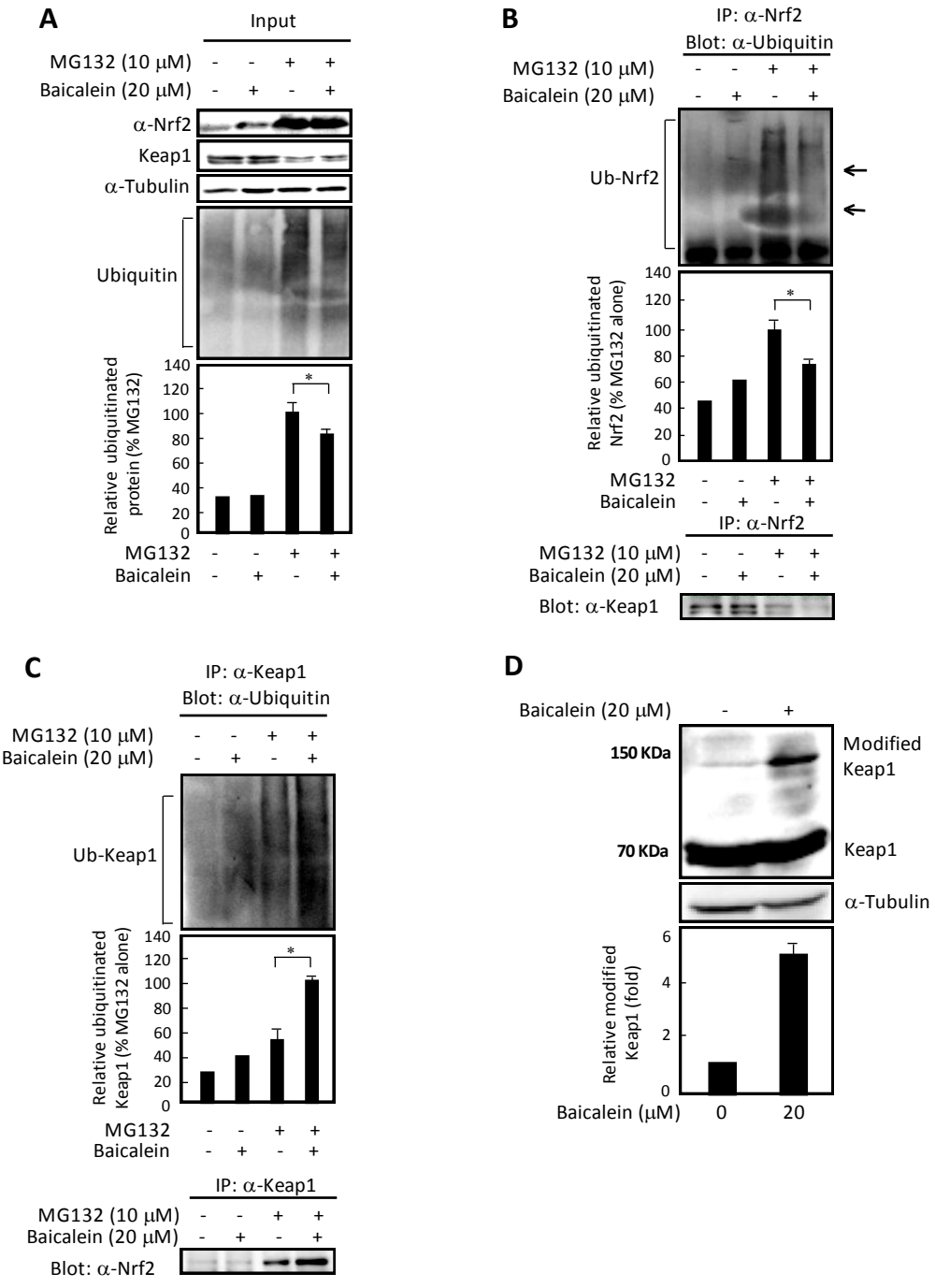


Figure 3.13 Baicalein inhibits Nrf2 ubiquitination and modifies Keap1. (A) Western blot analysis of endogenous Nrf2 and Keap1. HepG2 cells were pretreated with 10 μM MG132 for 1 h and then treated with or without 20 μM baicalein for 9 h. One part of the whole-cell lysates were analyzed by Western blot analysis with the indicated antibodies, the rest for immunoprecipitation. Equivalent amounts of proteins were immunoprecipitated with anti-Nrf2 antibody and visualized by Western blot analysis with ubiquitin antibody. Each arrow shows reduced ubiquitinated protein. * $p < 0.05$. (B) and (C) Effects of baicalein on ubiquitination of Nrf2 and Keap1. Equivalent amounts of proteins were immunoprecipitated with Keap1 antibody and visualized by Western blot analysis with ubiquitin antibody. (D) Modification of Keap1 by baicalein. HepG2 cells were treated with 20 μM baicalein for 9 h and analyzed by Western blot analysis with Keap1 antibody. Data represent means ±SD of three independent experiments. * $p < 0.05$ vs control.

[42,43]. To identify this possibility by baicalein treatment, we performed a coimmunoprecipitation experiment with anti-Nrf2 and anti-Keap1 antibodies. As shown in Figure 3.13B and C (bottom), treatment with baicalein did not cause dissociation of the Keap1-Nrf2 complex. These results suggest that the up-regulation of Nrf2 protein by baicalein was not due to the liberation of Nrf2 from Keap1.

Myricetin had the same effect as baicalein. As shown in Figure 3.14A, the Nrf2 protein level was enhanced after treatment with myricetin (lane 2) or MG132 alone (lane 3) or their combination (lane 4) while Keap1 protein level was reduced in such treatment. Simultaneously, a significant reduction of ubiquitination of Nrf2 (Figure 3.14A), but not Keap1 (Figure 3.14B), was observed in the cells cotreated with myricetin and MG132. These results indicate that the myricetin-enhanced Nrf2 is, partially at least, due to an inhibitory effect of myricetin on the ubiquitination of Nrf2 while myricetin-reduced Keap1 is not due to the inductive effect of myricetin on Keap1 ubiquitination. However, a more than 150-kDa Keap1 protein was also detected in the cells treated by 10-40μM myricetin (Figure 3.14C). Thus, myricetin-caused Keap1 reduction might be due to the formation of modified Keap1

protein, rather than 26S proteasome-dependent degradation.

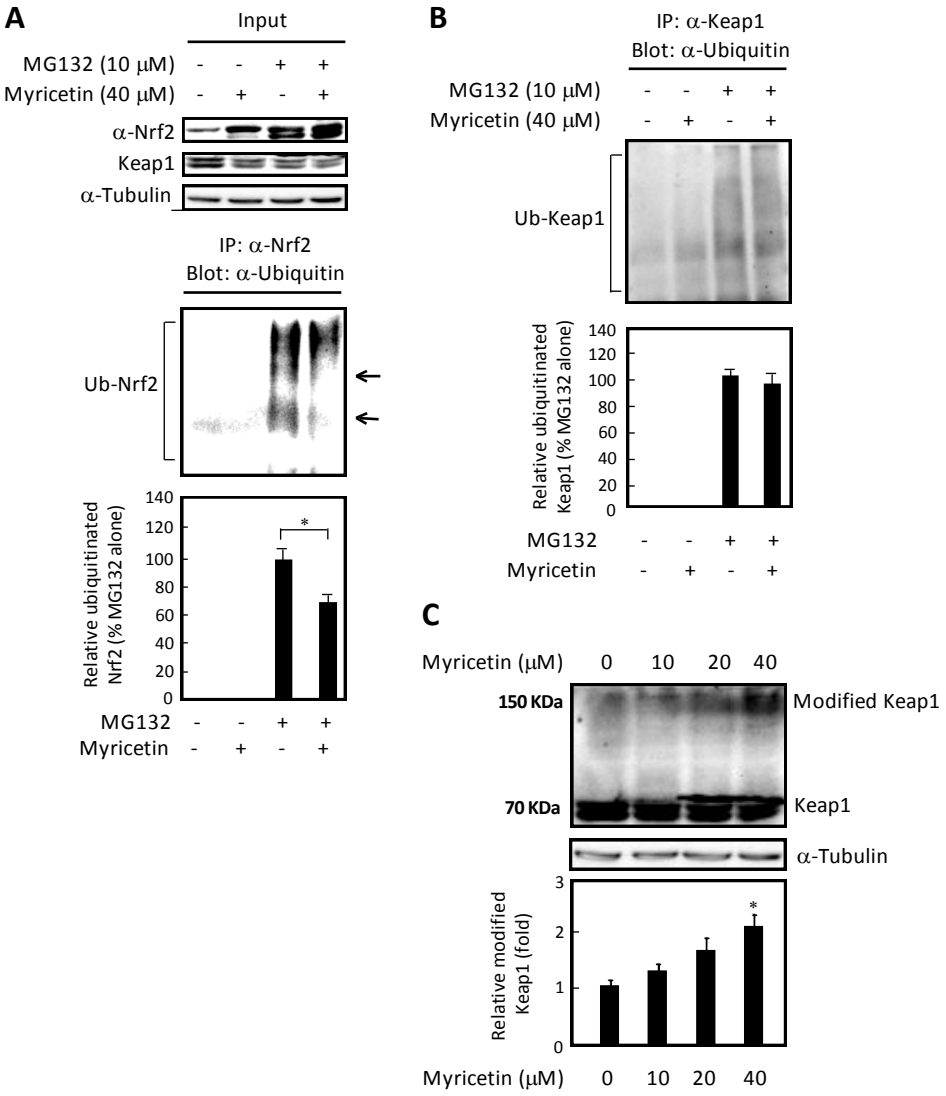


Figure 3.14 Myricetin inhibits Nrf2 ubiquitination and modifies Keap1. Methods are the same as Figure 3.13.

3.4.2.4 Baicalein and myricetin increase Nrf2 protein stability and reduce Nrf2 turnover

The significant increase of Nrf2 protein (Figure 3.13A and Figure 3.14A) by cotreatment with baicalein and MG132 suggests that baicalein and myricetin may

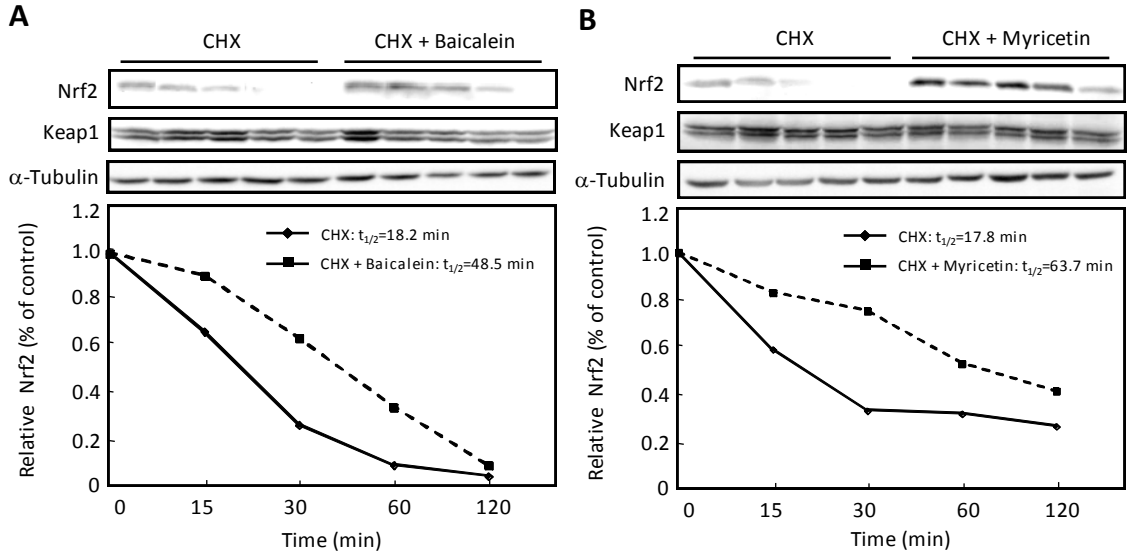


Figure 3.15 Baicalein (A) and myricetin (B) both stabilize Nrf2 protein. HepG2 cells were treated with 5 μg/ml CHX or pretreated with 20 μM baicalein or 40 μM myricetin for 2 h and then treated with CHX for the indicated times. Nrf2, Keap1, and α-tubulin were detected by Western blot analysis with their respective antibodies. Each value represents the mean ±SD of three or four separate experiments.

reduce proteasome turnover of Nrf2. We next examined the steady state of Keap1 and Nrf2 protein at different times after treatment with the protein synthesis inhibitor cycloheximide and 20 μM baicalein or 40 μM myricetin and then calculated the half-reduction time ($t_{1/2}$) from protein decay experiments. Treatment with baicalein extended the half-life ($t_{1/2}$) of Nrf2 protein from 18.2 to 48.5 min (Figure 3.15A) and myricetin extended that from 17.8 to 63.7 min, almost 4 times longer (Figure 3.15B), but the $t_{1/2}$ of Keap1 protein has no significant change in both. These results indicate that baicalein and myricetin increased Nrf2 protein also by inhibiting the turnover of Nrf2 at posttranscriptional levels.

3.4.2.5 siRNA of Nrf2 interrupts baicalein- and myricetin-induced ARE activation

To confirm whether the up-regulation of Nrf2 is essential for baicalein-induced

Nrf2-mediated ARE activity, we transfected siRNA molecules against Nrf2 into HepG2 cells. As shown in Figure 3.16A, siRNA of Nrf2 reduced the basal Nrf2 and HO-1 as well as baicalein-induced Nrf2 and HO-1 proteins, and scrambled siRNA showed no such effect, compared with control. To further determine whether the increased Nrf2 stimulates Nrf2-mediated ARE activation, ARE-luciferase reporter plasmid was co-transfected with or without siRNA against Nrf2. As shown in Figure 3.16B, treatment with siRNA of Nrf2 reduced the basal ARE activity as well as baicalein-induced ARE activity, and scrambled siRNA showed no effect on the activity compared with control. Moreover, myricetin treatment showed the same change as baicalein (Figure 3.17). These results demonstrate that an increase in Nrf2 is critical event for polyphenol-induced ARE activity.

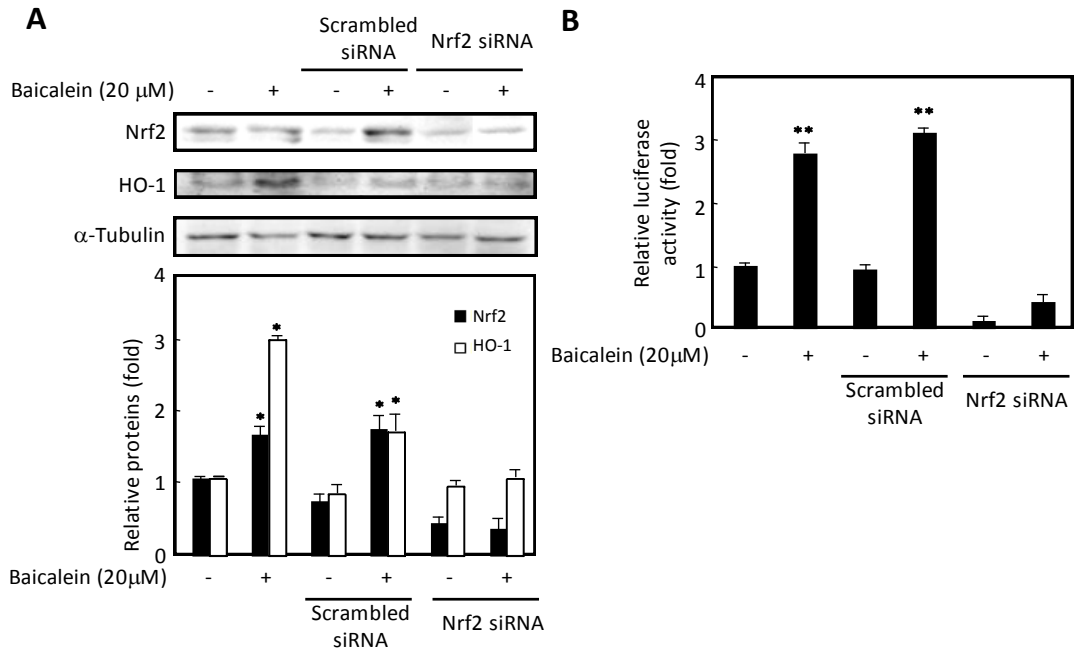


Figure 3.16 siRNA of Nrf2 interrupts baicalein-induced ARE activation. (A) HepG2 cells were transfected with 100 nM siRNA of Nrf2 or 50 nM scrambled duplex. After transfection, cells were harvested as described under Materials and methods. Nrf2, HO-1, and α-tubulin were detected by Western blot analysis with their respective antibodies. (B) HepG2 cells were cotransfected with pGL2-ARE-luciferase construct, pGL4-TK-Renilla, and 100 nM siRNA of Nrf2 or 50 nM

scrambled duplex. After 24 h, cells were placed in 5% serum medium for 24 h and then stimulated with 20 μM baicalein for an additional 24 h. Cells were lysed and analyzed for firefly and Renilla luciferase activities. Each value represents the mean ± SD of three or four separate experiments. * $p < 0.05$ vs control, respectively.

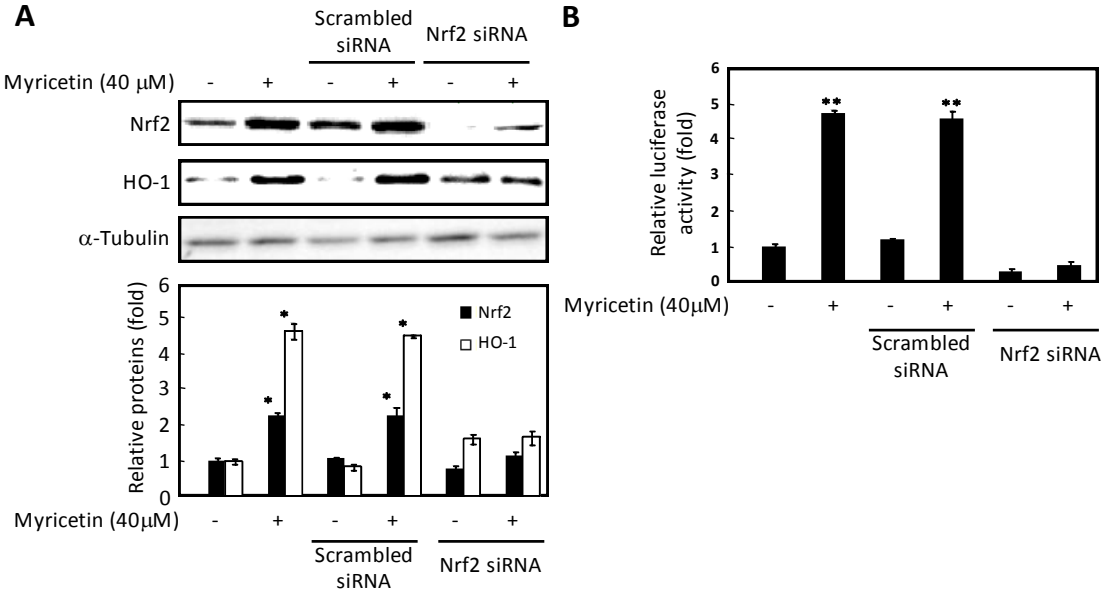


Figure 3.17 siRNA of Nrf2 interrupts myricetin-induced ARE activation. Methods are the same as shown in Figure 3.16.

3.5 Discussion

Although several lines of studies have showed that baicalein and myricetin have a number of biological activities [100-103], the effect of these polyphenolic compounds on Nrf2-mediated ARE pathway is still not clear. In the present chapter, we investigated molecular mechanism underlying the Nrf2-ARE pathway activation by baicalein and myricetin treatment.

The status of Nrf2-Keap1 determines Nrf2-mediated ARE activity [74, 75]. In the present study, we found that baicalein and myricetin both can affect status of Nrf2/Keap1 through multiple pathways. First, treatment with baicalein and myricetin reduced the steady-state level of Keap1 (Figure 3.9C and 3.10C), alternatively, a

modified Keap1 was observed in such treatment (Figure 3.13D and 3.14C). This result suggests that baicalein and myricetin might downregulate the steady-state level of Keap1 by modifying Keap1 protein to allow Nrf2 escaping from ubiquitination (Figure 3.13B and 3.14A). Similar actions were also observed in other antioxidant agents such as sulforaphane [99], quercetin [74] and 6-MTITC [75]. Second, the inhibition on Nrf2 ubiquitination was observed in the cotreatment with baicalien or myricetin and MG132 after immunoprecipitation with anti-Nrf2 antibody (Figure 3.13B and 3.14A), and the $t_{1/2}$ reduction time of Nrf2 protein was extended from 18.2 to 48.5 min by baicalein and from 17.8 to 63.7 min by myricetin in protein decay experiment (Figure 3.15). These data suggest that both of them increased the steady-state level of Nrf2 also by stabilizing Nrf2 protein through inhibition of Nrf2 ubiquitination and protein turnover at the posttranscriptional level. Third, pretreatment with actinomycin D suppressed both baicalein- and myricetin-induced Nrf2 expression, suggesting that the induced Nrf2 expression was at transcription levels. These actions caused by baicalein and myricetin finally resulted in a high ratio of Nrf2/Keap1, the surplus Nrf2, compared with Keap1, might bypass Keap1-Cul3 and accumulate in the nucleus to mediate ARE activation. Furthermore, we found that protein kinases including MEK, AKT and JNK were involved in baicalein-induced Nrf2-ARE pathway activation (Figure 3.3), and it was further confirmed by the increasing phosphorylation of these pathways by baicalein treatment in both time- and dose-dependent manners, implicating that baicalein activates Nrf2 pathway in a Keap1-independent mechanism (Figure 3.4). The RT-PCR data of increased expression of Nrf2 mRNA by baicalein treatment and Nrf2-related network analysis based on microarray further confirmed that baicalein upregulated Nrf2 mRNA in transcriptional level by many factors or pathways (Figure 3.6 and 3.7). Thus, activation of Nrf2-ARE pathway is at least partly due to the activation of MEK, AKT and JNK pathways by baicalein.

In vitro data have indicated that the antioxidant property of a flavonoid is

determined by its chemical structure, such as the number, positions and types of substitutions on the basic flavan nucleus, which influences radical scavenging and chelating activity [104]. Multiple hydroxyl groups seem to confer upon the flavonoid substantial antioxidant properties through their reducing capacities or other possible influences on intracellular redox status [105]. Especially, the di-OH substitution at 3' and 4' in ring B are particularly important and essential to the peroxyl radical absorbing activity of a flavonoid, which as indicated by the higher potency of luteolin (with two hydroxyls at 3' and 4' in ring B) than that of apigenin (with one hydroxyl at 4' in ring B) and chrysin (without any hydroxyl in ring B) [106, 107]. However, the above hydrogen-donating antioxidant activity is unlikely to be the sole explanation for Nrf2 activation in cell model. For example, a recent study indicated that chrysin, apigenin as well as luteolin could activate Nrf2 with the same concentration and treatment time in the order of chrysin > luteolin > apigenin [108]. Several lines of studies have revealed that different types of flavonoid have ability to induce Nrf2 activation, such as quercetin in flavonol [74], cyanidin in anthocyanidin [109], genistein in isoflavone [110], chrysin in flavone [108] and epicatechin in flavanol [111]. Based on these reports with our data, the basic flavan structure seems to be the key component of flavonoid to activate Nrf2, and flavonoids with basic flavan structure might exert their chemopreventive effect through Nrf2 pathway, at least partly.

Baicalein- and Myricetin-induced gene expression changes have been classified into 10 canonical pathways (Table 2.4) by canonical pathway analyses with IPA software. Nrf2-mediated pathway was listed up at the 2nd and the 3rd by baicalein and myricetin treatment, respectively, suggesting that Nrf2-mediated ARE pathway was linked to their antioxidant property [55, 56, 112-115]. Besides, involvement of VDR/RXR pathway in both baicalein- and myricetin-induced gene expressions may partially support their anticancer property of baicalein [63, 64, 102, 103 and 116] since VDR/RXR plays a crucial role in the regulation/metabolism of calcium and

phosphorus involving in immune function, tumor suppression, growth regulation and parathyroid hormone secretion [117]. Involvement of metabolism of xenobiotics by cytochrome P450 in myricetin-induced gene expressions suggests that myricetin acted as a xenobiotic to cause expressions of drug metabolizing enzyme genes. Other canonical pathways including bile acid biosynthesis, c21-steroid hormone metabolism, glycerophospholipid metabolism, glycerolipid metabolism and IGF-1 signaling were also involved in myricetin-induced gene expressions, suggesting that myricetin may play important role in lipid metabolism and insulin resistance, which in keeping with the antidiabetic function of myricetin such as inhibition of hyperglycemia and glucose uptake [118-120].

It has been reported that polyphenolic compounds act on a variety of signal transduction pathways related to cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis and metastasis [121]. Moreover, Nrf2-ARE signaling pathway has been reported to contribute to chemoprevention against many human chronic diseases by modulating expressions of genes including detoxifying enzymes, drug transporters, and cellular redox regulators [122-126], and many polyphenolic compounds are proven to enhance ARE activation [74, 76, 126, 127]. Baicalein and myricetin are typical polyphenolic compounds, thus, are considered to modulate Nrf2-ARE signaling pathway to exert their chemopreventive effects although there is limited data regarding this. Our data from DNA microarray and signaling pathway analyses will be useful for future studies on chemopreventive effect of these polyphenolic compounds and underlying molecular mechanisms.

The absorption and metabolism of flavonoids play critical roles in maintenance of their bioactivities. Dietary flavonoid glycosides can be transformed to aglycones in gastrointestinal tract, and only the aglycones of flavonoids can be absorbed in intestinal with the form of passing through the gut wall and entering into the plasma [128]. Thus, we directly added the aglycone of baicalein and myricetin into culture cells to mimic the *in vivo* situation of flavonoids. A recent study, that myricetin

glycosides (myricetin-3-*O*-galactoside and myricetin-3-*O*-rhamnoside) were added into human chronic myelogenous leukemia cells, was carried to investigate the expression patterns of oxidative stress genes, using DNA microarray with only 21 probes [129]. Although the gene expressions of some antioxidant proteins such as *TXNRD1, GPX1, TXN and SOD1* were increased, the concentrations (220 μg/ml for myricetin-3-*O*-galactoside and 150 μg/ml for myricetin-3-*O*-rhamnoside) were higher than that in our experiment [129]. A previous study found that treatment with 20 μM of myricetin for 72 h had no significant effect on HepG2 cell growth [130]. I had also confirmed that treatment with 40 μM of myricetin and 20 μM of baicalein for 48 h showed no cytotoxicity in HepG2 cells, but with significant induction effect of several antioxidant proteins and detoxifying enzymes. That is why we chose the dose of 20 μM in the present study.

It is also worth of noting that two bands were observed for Nrf2 and Keap1 (such as Figure 3.14) in our Western blot results although the predicted molecular mass are ~66 and ~69 kDa, respectively. Although the exact reason still remained unknown, one previous study had identified the two bands of Nrf2 as phosphorylated forms with the aid of protein kinase CK2 [131]. Another recent paper provided solid evidence that the biologically relevant molecular weight of Nrf2 is ~95-110 kDa, not the predicted ~66 kDa based on its 2-kilobase open reading frame [132]. Moreover, our previous studies also found two bands of Keap1 [74-76], this may be caused by binding to other proteins that can induce higher-order oligomeric forms of Keap1 [133].

It is the first time to use microarray-based Nrf2-related network to analyze the Nrf2 pathway activation caused by baicalein on genome wide. As shown in Table 3.1, among all the 35 transcription factors are reported to transcriptional regulate Nrf2 mRNA, baicalein increased expressions of 5 of them. In other word, baicalein stimulated 5 positive feedback loops of TFs on Nrf2 expression. Several studies have reported that phosphorylation of protein kinases could induce TFs or nuclear proteins

expressions. For example, NF-κB can be induced by phosphorylation of TAK1 and AKT [137, 138], phosphorylation of ERK could upregulates Pax6 [139], and phosphorylation of JNK could induce c-Jun [140], all of which could upregulate the Nrf2 expression in positive feedback. These phenomenons suggested that baicalein upregulated Nrf2 mRNA expression by targeting AKT/NF-κB, ERK/Pax6 and JNK/c-Jun pathways, at least. As shown in Table 3.2, all the proteins reported to posttranscriptional regulate Nrf2 expression were classified into Keap1 system group, Protein kinases group, CBP-NF-κB group, Transcription factors group and others group, which not only showed the crosstalks between Nrf2-ARE pathway and other signaling pathways such as inflammatory pathway [141] and apoptosis pathway [142], but also revealed that baicalein stimulated 6 positive and 2 negative feedback loops of proteins on Nrf2 expression.

Protein kinases play crucial roles in the regulation of multiple cell signaling pathways and cellular functions. Deregulation of protein kinase function has been implicated in carcinogenesis. The inhibition of protein kinases has emerged as an important target for cancer chemoprevention and therapy [143]. Accumulated data revealed that flavonoids can bind directly to some protein kinases and act as protein kinase inhibitors for cancer chemoprevention [135, 144 and 145]. However, a series of other studies on phytochemicals-induced Nrf2 activation indicated that flavonoids act as protein kinase activators [146-148]. And pull down assay and molecular modeling data in the present study further confirmed that baicalein directly binds to MEK1 and AKT1 and acts as protein activator in HepG2 cells. These contradictory results may be caused by the different cell status, which flavonoids act as protein kinase inhibitors in cells stimulated by LPS or TPA, but act as protein kinase activators in cells without stimulation. Thus, we hypothesize that the Site Occupying Theory: flavonoids directly bind to protein kinases, occupying a small number of their active sites and protect them, which gently stimulate their phosphorylation in normal cells; after stimulation by LPS or TPA, excess ROS occupies only the rest active sites

and stimulate dramatically the phosphorylation of protein kinases. The more sites occupied by flavonoids (hormesis dose), the more inhibition activity showed in stimulated cells. Excessive dose of flavonoids can act as LPS or TPA in normal cells.

In conclusion, DNA microarray allowed us to obtain gene expression profiling and pathway networks in HepG2 cell. Molecular data demonstrated baicalein and myricetin both inhibited Nrf2 ubiquitination and protein turnover, and stimulated Keap1 modification. All of these events finally increased nuclear Nrf2 accumulation and ARE binding activity to enhance Nrf2-ARE-mediated gene expressions. Furthermore, RT-PCR data and Nrf2-related network analysis revealed that baicalein could upregulate expression of Nrf2 mRNA in transcriptional level by targeting transcription factors and nuclear proteins via phosphorylating protein kinases. It is the first time to use network analysis based on microarray data in interactome wide to study the molecular mechanism both at the transcriptional and posttranscriptional levels of Nrf2-ARE pathway activation caused by baicalein. This chapter provides a comprehensive data for understanding the bioactivities and the molecular mechanisms of baicalein and myricetin in hepatic metabolism and chemoprevention.

3.6 References

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Chapter 4

Discussion and Conclusion

4.1 Discussion

In the present study, gene expression profiling was performed by DNA microarray to evaluate the chemopreventive function and underlying genes targeted by polyphenolic compounds myricetin and baicalein in HepG2 cells. Then, Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) system were used to analyze the huge microarray data by classifying significantly changed genes, generating canonical pathways and networks. Finally, further investigation was performed to study the molecular mechanisms of the chemopreventive effects of baicalein and myricetin by regulating Nrf2/Keap1-mediated ARE system. These data will help us understanding the effects of these polyphenolic compounds on the genome wide.

4.1.1 Microarray-based network analysis of Nrf2-ARE activation

It is the first time to analyze the effect of polyphenol on the Nrf2-related network basing on microarray data. As summarized in chapter 3, Nrf2-related network is mainly including Nrf2 targets and regulome of Nrf2, in which, there exist 34 TFs and 115 proteins feedback loops (Table 3.1 and 3.2). As shown in Figure 4.1, the present study revealed that baicalein upregulated Nrf2 expression both at the transcriptional level and the posttranscriptional level. In the total promising 34 TFs, baicalein significantly activated 5 positive feedback loops which upregulated Nrf2 mRNA at the transcriptional level by binding its promoter region. In the total promising 115 proteins, baicalein significantly activated 6 positive feedback loops (MafF, c-Src, c-Jun, Jun B, VEGF and SQSTM1) and 2 negative feedback loops (c-Fos and FRA1). All of the above significant changed feedback loops played important roles on regulation of Nrf2 mRNA at the posttranscriptional level. These results could help us fully understand the effect of baicalein on Nrf2 interactome, regulome and feedback

regulatory loops at the same time, and provide an efficient method for the medical research on Nrf2.

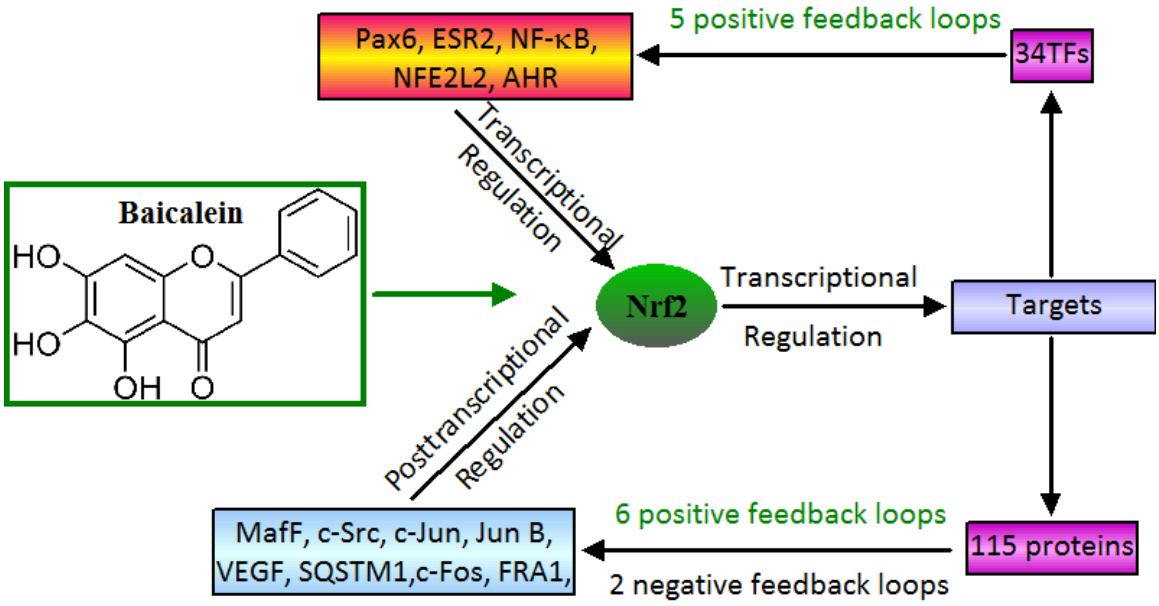

Figure 4.1 Nrf2-related network analysis of baicalein-induced Nrf2 expression based on microarray. Among thousands of Nrf2 targets, 115 proteins and 34TFs are reported to be able regulating Nrf2 transcription in feedback. In the present study, baicalein treatment only stimulated 5 positive feedback loops in TFs, and 6 possitive feedback loops and 2 negative feedback loops in proteins.

Thus, DNA microarray combined with efficient professional analysis software is a very potent and promising method to analyze the multi-function of polyphenols which could speed up the application of polyphenols in drug or functional food, and it could provide constructive guide for the future studies.

4.1.2 Gene expression profiling, pathway network analysis and underlying molecular mechanism of Nrf2 activation by baicalein and myricetin

DNA microarray data revealed the molecular basis of chemopreventive effects of myricetin and baicalein (chapter 2). Among the total 44K gene probes, gene ontology results showed that myricetin and baicalein treatment both affected expressions of many drug metabolizing enzymes in HepG2 cells, which were further confirmed by IPA canonical pathway analysis that polyphenolic compounds treatment not only

affected Nrf2-ARE pathway, but also affected many other metabolic pathways such as metabolisms of xenobiotics, tyrosine, methionine and cysteine. Besides, network and bio-function analysis revealed that these polyphenolic compounds played important roles in many cell functions and human diseases such as cancer (Figure 4.5), cardiovascular disease (Figure 4.4), metabolic disease (Figure 4.7 and 4.8), cell death, amino acid metabolism, infection mechanism and antimicrobial response. Thus, these results provided a comprehensive data for understanding the gene expression, hepatic metabolism and bioactive role of myricetin and baicalein, and supplied us promising route map for the future research related the pathology of many human chronic diseases.

The molecular mechanisms underlying the activation of Nrf2-ARE pathway by baicalein treatment were investigated both at the transcriptional level and at the posttranscriptional level of Nrf2 expression in HepG2 cells. As shown in Figure 4.2, at the transcriptional level, Nrf2-related network analysis and upstream protein kinases signaling pathways data revealed that baicalein regulated the expression of Nrf2 mRNA by targeting transcription factors (such as NF-κB and AHR) and nuclear proteins (such as c-Src and c-Jun) via phosphorylating the upstream protein kinases MEK, AKT and JNK signaling pathways. At the posttranscriptional level, molecular data revealed that baicalein activated Nrf2-ARE pathway by inhibiting Nrf2 ubiquitination and protein turnover via stimulating Keap1 modification and ubiquitination. Besides, baicalein-induced phosphorylation of protein kinases may lead to the phosphorylation of Nrf2, which also allowed Nrf2 escaping from Keap1 inhibition. All of these events finally increased nuclear Nrf2 accumulation, ARE binding activity and transcription activity to enhance ARE-mediated genes expressions. These results revealed that baicalein activates Nrf2-ARE pathway not only by Keap1-dependent mechanisms, but also by Keap1-independent mechanisms. Moreover, myricetin was found to exert its chemopreventive effect in the same mechanism due to its similar structure as baicalein. Additionally, treatment with Nrf2 siRNA attenuated both the baicalein-induced and myricetin-induced ARE activity and gene expressions.

Structure-activity relationship was analyzed between baicalein and myricetin, and the result indicated that the basic flavan structure seems to be the key component of polyphenolic compounds to activate Nrf2 based on structure-activity analysis (Figure 4.3).

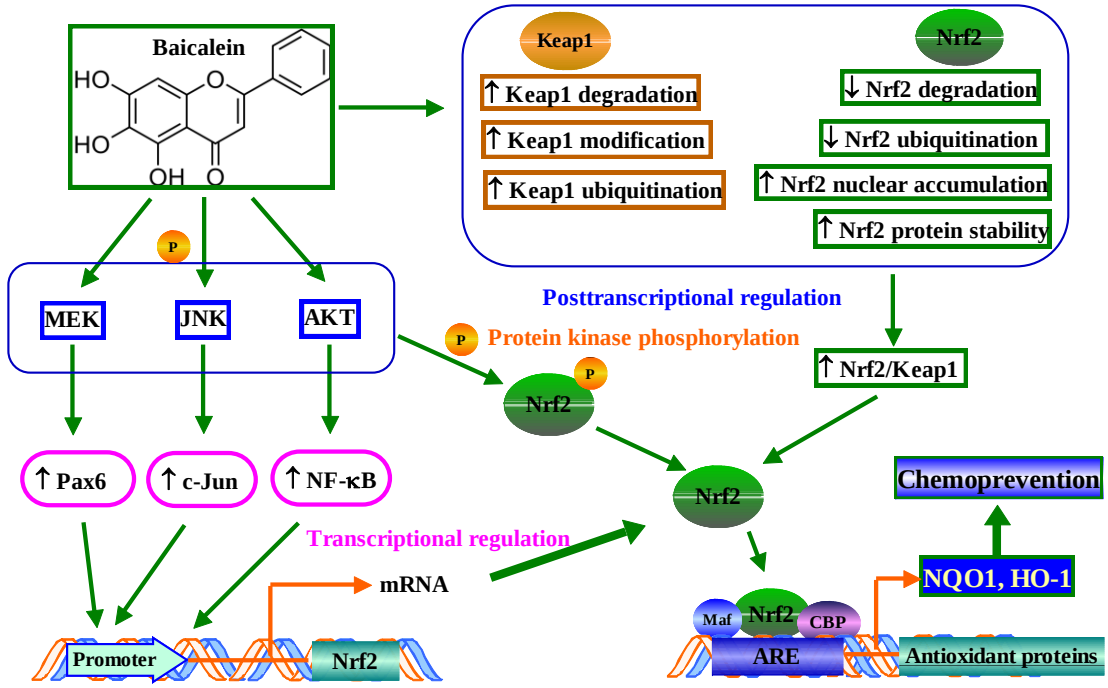

Figure 4.2 Schematic molecular models for cytoprotective role of baicalein targeting Nrf2-ARE pathway. Baicalein activates Nrf2-ARE pathway both at transcriptional and at posttranscriptional levels, by both Keap1-dependent and Keap1-independent mechanisms.

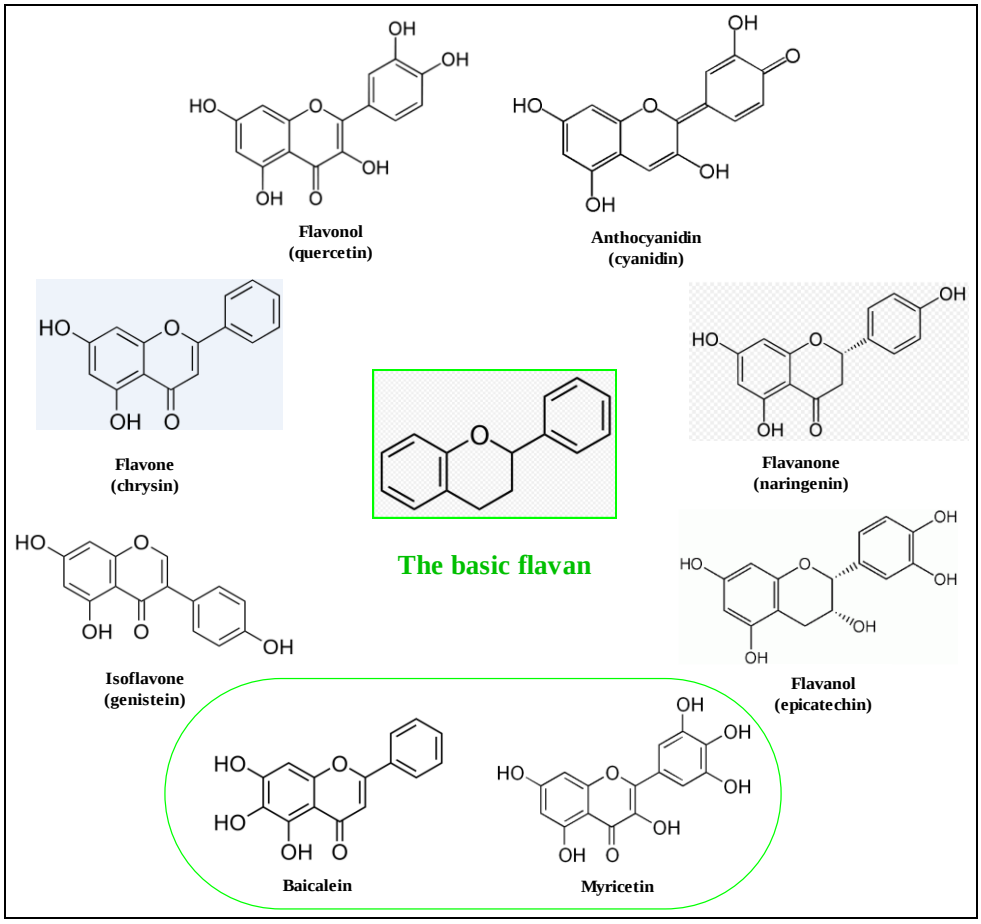

Figure 4.3 Structure-activity relationships of polyphenols. The basic flavan structure seems to be the key component of polyphenolic compounds to activate Nrf2. In the present study, baicalein and myricetin activates Nrf2, the other polyphenols such as quercetin, cyanidin, chrysin, naringenin, genistein and epicatechin, are also reported to activate Nrf2-ARE pathway in the Discussion of Chapter 3.

4.1.3 Phytochemicals activate Nrf2-ARE pathway by different mechanisms

Accumulating studies revealed that besides polyphenols, many other phytochemicals can activate Nrf2-ARE pathway to exert their multi-function in human health. To expand the vision of this field, I have summarized series of phytochemicals which can activate Nrf2-ARE pathway effectively during the last decade since it has been discovered. As shown in Table 1.1, these dietary phytochemicals have been classified into 5 groups: fruits & vegetables, spices, teas & coffee, herb medicines and marine products.

Table 1.1 Summary of phytochemicals activating Nrf2-ARE pathway.

Classification		Mechanism	Dose	Model	Reference
Fruits and vegetables					
Quercetin	Apple, tea, onion	Modified Keap1 Nrf2 stability	0-40 μM	HepG2 cells	[1]
		p38 MAPK and ERK	100-200 μM	Human hepatocytes	[2]
Sulforaphane	Cruciferous vegetables	Modification of Keap1	0-200 μM	Human HEK293 cells	[3]
		↓p38 MAPK isoforms	20 μM	HepG2 cells	[4]
		ERK and PI3K	20 μM	Caco-2 cells	[5]
PEITC	Cruciferous vegetables	ERK and JNK	5 μM	PC-3 cells	[6]
I3C and DIM	Cruciferous vegetables	JNK	6.25 μM	HepG2-C8	[7]
3H-1, 2-dithiole -3-thione	Cruciferous vegetables	Stability of Nrf2	50 μM	PC12 Cells	[8]
Diallyl sulfide	Garlic and onion	ERK and p38 MAPK	1 mM	HepG2 cells	[9]
Diallyl trisulfide	Garlic and onion	Calcium signaling	100 μM	HepG2 cells	[10]
Carnosic acid	Rosemary	p38 MAPK	1-20 μM	Rat Clone 9 liver cells	[11]
		S-alkylation of Keap1	10 μM	PC12h and COS7 cell	[12]
Hydroxytyrosol	Olive	PI3K/Akt,MEK1/2-ERK1/2	50 μM	VE cells	[13]
		JNK	0-200 μM	HRPE cells	[14]
Resveratrol	Red grape	Modified Nrf2, Keap1	10 μM	A549 cells	[15]
		ERK and PI3K	15 μM	PC12 cells	[16]
Lycopene	Tomato	ERK and p38 MAPK	0-10 μM	HepG2 cells	[17]
Luteolin	Celery, green pepper	ERK1/2, HO1, ARE binding	0-20 μM	PC12 cells	[18]
Kaempferol	Tea and broccoli	JNK, HO-1, GCLC	0-10 μM	HEI-OC1 cells	[19]
CE	Soybean	Modification of Keap1	10 μM	RAW264.7	[20]
3',4'-Dideme-thylnobiletin	Citrus peels	PI3K/Akt	0-20 μM	PC12 cells	[21]
Procyanidin B2	Cocoa, red wine	ERKs and p38-MAPK	10 μM	Human colonic cells	[22]
Anthocyanins	Purple sweet potato	Akt and ERK1/2	10-200 μg/ml	HepG2	[23]
Glyceollins	Soybean	PI3K/Akt Keap1 modification		Hepa1c1c7 and BPRc1 cells	[24]
Fisetin	Strawberries	PKC-σ and p38 MAPK	0-25 μM	HUVE cells	[25]
Pterostilbene	Blueberries and grapes	Nrf2,HO-1	5 mg/kg BW	Male BALB/c mice	[26]
Sesamin and episesamin	Sesame seeds	p38 MAPK	0-10 μM	Rat PC12 cells	[27]
Chlorophyllin	Spinach, green leafy	PI3K/Akt	50 μM	HUVE cells	[28]
Hesperidin	Citrus fruits	ERK1/2	0-80 μM	Human hepatic L02 cells	[29]
Ferulic acid	Apple, coffee	PI3K and ERK	0-5 μM	HUVE cells	[30]
Xanthohumol	hops	Modification of Keap1	4 μM	Murine Hepa1c1c7 cells	[31]

Cinnamalde-hyde	Cinnamomum cassia Presl	Nrf2,HO-1	50-100 μM	Endothelial cells	[32]
Chalcone	Plant phenols	Nrf2,HO-1	10-25 μM	Endothelial cells	[33]
Herbs					
Kaurenoic acid	Aralia continentalis	Nrf2, phase 2	0-10 μM	RAW 264.7 macrophages	[34]
Iso-cubebenol	Schisandra chinensis	PI3k/Akt and ERK	20 μM	THP-1 cells	[35]
Oleanolic acid	Pokeweed, garlic	Akt and ERK	10-50 μM	PRVSM cells	[36]
Eupatilin	Artemisia	ERK	0-150 μM	FISM cells	[37]
Genipin	Gardenia jasminoides	PI3K-JNK1/2	0-100 μM	RAW 264.7 macrophages	[38]
Oridonin	Rabdosia rubescens	↓Nrf2 ubiquitination, Keap1 ubiquitination	0-8.4 μM	MDA-MB-231 cells	[39]
Isoorientin	Sasa borealis	PI3K/Akt		HepG2 cells	[40]
Butin	Vernonia	PI3K/Akt	10 μg/ml	V79-4 cells	[41]
Guggulsterone	Commiphora mukul	PI3K/Akt		HME cells	[42]
Alantolactone	Inula helenium	PI3K and JNK	0-10 μM	Hepa1c1c7 cells	[43]
Phytoestrogen puerarin	Inula helenium	PI3K/Akt	0-100 μM	Hepa1c1c7 cells	[44]
ACE	Aralia continentalis	ERK1/2 and p38 MAPK	0-200 μg/ml	Hepa1c1c7 cell line	[45]
GBE	Ginkgo biloba	p38 MAPK	100 μg/ml	Human aortic endothelial cells	[46]
CAD	Salicornia herbacea	PI3K/Akt	1-20 μM	Hepa1c1c7 cells	[47]
Fraxetin	Fraxinus	Nrf2,HO-1	30-100 μM	Vascular smooth muscle cells	[48]
Rottlerin	Mallotus	ERK and p38 MAPK	1-10 μM	HT29 cells	[49]
Falcarindiol	Apiaceae	Modification of Keap1	50-100 μM	HEK293 cells	[50]
Ketopinoresinol	Adlay	PI3K/AKT, phos. of Nrf2	3.12-50 μM	HSC3-ARE9 cells	[51]
Berberine	Rhizoma coptidis	PI3K/AKT, phos. of Nrf2	1-10 μM	Rat brain astrocyte cell line	[52]
Acteoside	Scrophulariaceae	ERK and PI3K/Akt	30 μM	PC12 cells	[53]
Z-ligustilide	Rhizoma Chuanxiong	PI3K/Akt	0-50 μM	PC12 cells	[54]
DSE	Salvia miltiorrhiza	PI3K/Akt and MEK1	0-50 μg/ml	RAW 264.7 macrophages	[55]
Tanshinone IIA	Salvia miltiorrhiza	ERK and PKB	0-5 μM	Human smooth muscle cells	[56]
Celastrol	Tripterygium	ERK and p38 MAPK	0-1 μg/ml	HaCaT cells	[57]
Schisandrin B	Schisandra chinensis	ERK		AML12 cells	[58]
Mollugin	Rubia cordifolia L.	p38 MAPK	20 μM	HT22 and BV2 cells	[59]
Sappanchalcone	Caesalpinia sappan L.	JNK	40 μM	HDP and HPDL cells	[60]

Piceatannol	Euphorbia lagascae	Akt, modification of Keap1	30 μM	MCF10A cells	[61]
RGE	Red ginseng	MEK1/2, ERK1/2, PI3K/Akt	0.5 mg/ml	PC12 cells	[62]
NDGA	Creosote bush	PI3K, JNK, p38, Nrf2 stability	15 μM	Mouse embryo fibroblasts	[63]
Teas and coffee					
EGCG	Tea	p38 MAPK and Akt	20 μM	B lymphoblasts	[64]
		ERK and PI3K/Akt	50 μM	Bovine aortic endothelial cells	[65]
Epicatechin	Cocoa and tea	ERK and PI3K/Akt	30 mg/kg BW	Ischemic damaged mice	[66]
Kahweol	Coffee	Akt and p38MAPK	0-10 μM	SH-SY5Y cells	[67]
Polymeric TP	Black tea	PKC and PI3K	0-2 μM	HepG2	[68]
MRP	Coffee	Nrf2	1-4 mg/mL	Macrophages	[69]
NMP	Coffee	Nrf2, phase 2	10-100 μg/ml	Human colon carcinoma cells	[70]
Spices					
6MITC	Wasabi	Keap1 modification Nrf2 stability	0-10 μM	HepG2 cells	[71]
Capsaicin	Red peper	PI3K/Akt	200 μM	HepG2 cells	[72]
Curcumin	Turmeric	PKC-σ and p38 MAPK	15 μM	Human monocytes	[73]
		p38 MAPK	10-30 μM	NRK-52E cells, LLC-PKi cells	[74]
		p38 MAPK and PI3K/Akt	15 μM	VSMC cells	[75]
LA	Lindera strychnifolia	ERK	0-40 μM	Mouse HT22 cells	[76]
[6]-gingerol	Ginger	Nrf2, phase 2	10 μM	SH-SY5Y cells	[77]
Zerumbone	Zingiber zerumbet	Nrf2, phase 2	0-25 μM	RL34 cells	[78]
Piperine	Black pepper	JNK and p38 MAPK	50 μM	HEI-OC1 cells	[79]
Marine products					
Eckol	Kelps and rockweeds	ERk and PI3K/Akt	0-10 μM	V79-4 cells	[80]
SGP-8	Marine sponge	Nrf2	5 μM	HT22 cells	[81]
Fucoxanthin	Brown sea algae	ERK and p38	5-20 μM	Mouse hepatic BNL CL.2 cells	[82]
Oligosaccharide	Seaweed	Nrf2	0.05% (w/v)	PC12 cells	[83]

Note: mechanism column means all induced by phytochemical treatment except specially indicated.

The molecular mechanisms of Nrf2 activation caused by these phytochemicals have also been summarized as shown in Table 1.1. Phytochmeicals seems to activate Nrf2 pathway by targeting MAPKs, PI3K/AKT, PKC signaling pathways and modifying Keap1.

4.1.3.1 Targeting MAPKs pathway

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens, osmotic stress, heat shock and proinflammatory cytokines) and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival or apoptosis [84].

MAP kinases are activated with the protein kinase cascades that consist of at least three types of enzymes including MAP kinase kinase kinase (MKKK, MEKK or MAP3K), MAP kinase kinase (MKK, MEK, or MAP2K) and MAP kinase. Because both tyrosine and threonine phosphorylations are required to activate the MAP kinases, phosphatases that remove phosphate from either site will inactivate them. The MAP kinase signaling cascades convey information to effectors, coordinate incoming information from other signaling pathways, amplify signals, and allow for a variety of response patterns. They respond to different stimuli by phosphorylating cytoplasmic components and nuclear transcription factors depending on the cellular context [85-87].

Activation of MAPKs is required for phosphorylation of Nrf2 which subsequently causes Nrf2-ARE pathway activation [88-91]. ERKs, JNKs, and p38 MAPK finally contributes to the phosphorylation of Nrf2 with subsequent activation of Nrf2-ARE pathway.

As shown in Table 1.1, ERK could be phosphorylated by fruits and vegetables constituents such as quercetin from apple and onion, sulforaphane/PEITC from broccoli, diallyl sulfide from garlic, hydroxytyrosol from olive, resveratrol from grape, lycopene from tomato, luteolin from celery, procyanidin B2 from cocoa, anthocyanins from purple sweet potato, hesperidin from citrus fruit, ferulic acid from artichoke; herbs constituents such as α-iso-cubebenol and schisandrin B from *Schisandra chinensis*, oleanolic acid from *American pokeweed*, eupatilin from *Artemisia*, *Aralia continentalis* extract, rottlerin from *Mallotus philippinensis*, acteoside from *Scrophulariaceae*, tanshinone IIA from *Salvia miltiorrhiza*, celastrol from Tripterygium wilfordii, red ginseng extract; teas and spices constituents such as EGCG from tea, epicatechin from cocoa, lindenenyl acetate from *Lindera*

strychnifolia and marine products such as eckol from kelps, fucoxanthin from *Brown sea algae*. They all enhanced Nrf2 activation via ERKs-dependent phosphorylation of Nrf2.

p38 MAPK is also activated by some fruits and vegetables constituents such as quercetin, sulforaphane, diallyl sulfide, carnosic acid, lycopene, procyanidin B2, fisetin, sesamin/episesamin; herbs constituents such as *Aralia continentalis* extract, ginkgo biloba extract, rottlerin, celastrol, mollugin; teas and coffee constituents such as EGCG, kahweol; spices such as curcumin, piperine and marine product fucoxanthin. These compounds stimulate Nrf2 activation via p38 MAPKs-dependent phosphorylation of Nrf2.

JNK activation has been reported by PEITC, hydroxytyrosol, kaempferol, indole-3-carbinol/ 3, 3'-diindolylmethane, genipin and sappanchalcone. They enhanced Nrf2 activation via JNKs-dependent phosphorylation of Nrf2.

4.1.3.2 Targeting PI3K/Akt pathway

Phosphatidylinositol 3-kinases (PI3-kinases or PI3Ks) are a family of enzymes involved in cell growth, proliferation, differentiation, motility, survival and intracellular trafficking, which in turn are involved in cancer and other chronic diseases. Many of these functions relate to the ability of class I PI3-kinases to activate protein kinase B (PKB, aka Akt) in the PI3K/Akt/mTOR pathway [93]. Several studies have demonstrated that PI3K was involved in Nrf2-ARE pathway activation [91, 92].

A series of studies have showed that dietary phytochemicals can activate Nrf2-ARE pathway through PI3K (Table 1.1). These compounds include fruits and vegetables constituents such as sulforaphane from broccoli, hydroxytyrosol from olive, resveratrol from grape, chlorophyllin from spinach, 3',4'-didemethylnobiletin from citrus peels, glyceollins from soybean, ferulic acid from apple; herbs constituents such as α-iso-cubebenol from *Schisandra chinensis*, genipin from *Gardenia jasminoides*, isoorientin from *Sasa borealis*, butin from *Vernonia anthelmintica*, guggulsterone from *Commiphora mukul*, alantolactone and phytoestrogen puerarin from *Inula*

helenium, chlorogenic acid derivative from *Salicornia herbacea*, 4-ketopinoresinol from adlay, berberine from *Rhizoma coptidis*, acteoside from *Scrophulariaceae*, z-ligustilide from *Rhizoma Chuanxiong*, salvia miltiorrhiza (Danshen) extract, red ginseng extract, piceatannol from *Euphorbia lagascae*; teas and spices constituents such as EGCG, epicatechin, polymeric black tea polyphenols, capsaicin, curcumin and marine product like eckol from kelps or rockweeds. They all modulate Nrf2 activation via PI3K-Akt-dependent phosphorylation of Nrf2.

4.1.3.3 Targeting protein kinase C pathway

Protein kinase C also known as PKC, is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes in turn are activated by signals such as increases in the concentration of diacylglycerol (DAG) or calcium ions (Ca^{2+}).

Recent studies have revealed that PKC activation is also involved in Nrf2-ARE pathway activation [94, 95]. As shown in Table 1.1, fisetin from strawberry, polymeric polyphenols from black tea, and curcumin from turmeric could activate Nrf2-ARE pathway through PKC-dependent phosphorylation of Nrf2.

4.1.3.4 Targeting Keap1 modification

Cysteine modification of Keap1 is critical event on antioxidant defenses in response to oxidative stress [96-98]. Some dietary phytochemicals could modify Keap1cysteines to activate Nrf2-ARE pathway (Table 1.1). These compounds include fruits and vegetables constituents such as quercetin, sulforaphane, carnosic acid, resveratrol, catechol estrogens, glyceollins; herbs constituents such as oridonin, falcarindiol, piceatannol, xanthohumol; and spices constituent 6MITC.

It is interesting that many phytochemicals exert their Nrf2/ARE activation pathway by several mechanisms, such as quercetin, sulforaphane, and resveratrol.

4.1.4 Human clinical evidence on induction of Nrf2-ARE pathway dependent phase II enzymes by dietary phytochemicals

A number of cells and animals studies had provided further supportive evidence of phytochemicals' role on induction of Nrf2-ARE pathway dependent phase II antioxidant proteins and detoxifying enzymes (Table 1.1). However, to fully understand the potential impact of phytochemicals on human health and wellness, the results of cells and animal studies need to be translated to human clinical evidence. Consumption of phytochemical-containing foods results in systemic exposure to phytochemicals. Accordingly, a handful of human intervention studies have attempted to elucidate the *in vivo* effects of high phytochemical content food consumption on ARE-mediated enzyme induction in a limited number of human organisms (Table 1.2).

The sulforaphane, isothiocyanates (ITC) and glucosinolate hydrolysis products from broccoli, sprouts and other cruciferous vegetables are the best confirmed effective phytochemicals to protect the body from cancer by induction of Nrf2-ARE pathway phase II enzymes. The first study reported that human subjects ingested with 10 and 20% Brussels sprouts diets caused a 1.4- and 2.3-fold induction of quinone oxidoreductase (QR) in the pancreas, a 1.5- and 2.5-fold induction in liver and a 3.1- and 3.6-fold induction in colonic epithelium, respectively [99]. After that, sixteen subjects were recruited into a randomized, 3-phase crossover dietary trial of standard broccoli, high glucosinolate broccoli, and water. Global changes in gene expression that occurred 6 h after consuming broccoli soups or water were quantified in gastric mucosal tissue, using Affymetrix whole genome microarrays (n=4), and in selected genes by real-time RT-PCR in the other individuals. Consumption of high glucosinolate broccoli resulted in up-regulation of several xenobiotic metabolizing genes, including thioredoxin reductase (TRX), aldo-keto reductases (AKRs), Solute carrier family 7, member 11 (SLC7A11) and glutamate cysteine ligase modifier subunit (GCLM) [100]. In a subsequent pilot study, eight healthy women undergoing reduction mammoplasty were given a single dose of a broccoli sprout preparation containing 200 μmol of sulforaphane. Following oral dosing, sulforaphane

metabolites were readily measurable in human breast tissue enriched for epithelial cells. The results showed expression levels of NQO1 and HO-1 transcripts were highly correlated, indicating similar mechanisms of induction. These findings provide a strong rationale for evaluating the protective effects of a broccoli sprout preparation in clinical trials of women at risk for breast cancer [101]. Another dose-escalation safety study in healthy human subjects revealed no adverse reactions when doses as high as 340 nmol of sulforaphane in the form of broccoli sprout extracts were applied topically to the center of a 1-cm-diameter circle drawn on the volar forearm. A subsequent efficacy study showed that despite the interindividual differences in basal levels, the enzyme activity of NQO1 in homogenates of 3-mm full thickness skin punch biopsies increased in a dose-dependent manner, with maximum increases of 1.5- and 4.5-fold after application of 150 nmol doses, once or three times (at 24 h-intervals), respectively, thus providing direct evidence for induction of the phase 2 response in humans [102]. Furthermore, a recent study confirmed that oral sulforaphane safely and effectively induced mucosal phase II enzyme expression in the upper airway of human subjects [103].

Besides, other phytochemicals also showed their induction effect of phase II enzymes in human clinical trials. Recently, the effect of curcumin has been investigated in the activities of drug-metabolizing enzymes such as CYP1A2, CYP2A6, N-acetyltransferase (NAT2), and xanthine oxidase (XO) in 16 healthy male Chinese volunteers, using caffeine as a probe drug. After 14 days, in the curcumin-treated (1,000 mg/day) group, CYP1A2 activity was decreased by 28.6%, while CYP2A6 activity was increased by 48.9% [104]. In another study, fifteen patients with advanced colorectal cancer refractory to standard chemotherapies consumed capsules compatible for 4 months. Total GST activity and M1G levels in leukocytes differed substantially between patients with reasonable reproducibility for each patient across the 4-week study period with curcumin 3.6 g daily, as borne out by average coefficients of variation within each patient of 15% and 31% for GST and M1G, respectively [105]. Moreover, bioflavonoid curcumin and quercetin therapy improved early graft function in cadaveric renal transplantation possibly through

HO-1 induction because of urinary HO-1 was higher in bioflavonoid groups [106]. Even St John's wort high in quercetin could induce CYP3A4 and CYP2E1 expression in twelve healthy volunteers between the ages of 60 and 76 years (mean age 67 years) randomly assigned to receive each botanical supplement for 28 days followed by a 30-day washout period [107]. Besides, resveratrol intervention in a recent study was found to induce CYP1A2, GST-pi level and UGT1A1 activity in individuals [108].

Table 1.2 Summary of human clinical evidences for induction of ARE-mediated enzymes by phytochemicals

Phytochemical type	Enzyme	Tissue	Effective dose	Reference
Brussels sprouts diets	NQO1	Pancreas, liver, colonic epithelium	10-20%/day, 6 days	99
High glucosinolate broccoli	SLC7A11, TRX, AKRs, GCLM	Gastric mucosal tissue	150 ml soup/day, 21 days	100
Broccoli sprouts	NQO1, HO-1	Breast tissue	200 μmol	101
Sulforaphane	NQO1, HO-1	Skin	150 nmol	102
Sulforaphane	NQO1, HO-1, GSTs	Upper airway	64 μmol/day, 3 days	103
Curcumin	CYP2A6	Blood and urine	1000 mg/day, 14 days	104
Curcumin	GST, M1G	Blood leukocytes	3.6 g/day, 4 weeks	105
Curcumin, quercetin	HO-1	Transplanted renal	480 mg curcumin and 20 mg quercetin/month, 2 years	106
Even St John's wort	CYP3A4	Blood	3 × 300 mg/day, 28 days	107
Resveratrol	CYP1A2, GST-pi, UGT1A1	Blood	1 g resveratrol/day, 4 weeks	108

4.1.5 Phytochemicals targeted Nrf2-ARE pathway in regulation of chronic diseases

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) modulated by exogenous factors, such as ultraviolet lights, ionizing radiation, chemotherapeutics, inflammatory cytokines and environmental toxins, or by endogenous factors including mitochondria, peroxisomes, lipoxygenases, NADPH oxidase, cytochrome P450, SOD and glutathione [109-112], have been proposed as main second messengers in the activation of several signaling pathways leading to mitogenesis or apoptosis [113]. Although ROS or RNS is constantly generated for essential biologic functions, excess

generation or an imbalance between oxidants and antioxidants can produce a common pathophysiological condition in the form of perturbations in redox circuity, known as oxidative stress [114, 115]. Accumulated data have revealed that excess oxidative stress is closely related to many kinds of chronic diseases in both humans and animals, such as cardiovascular diseases, cancer, neurodegenerative diseases, diabetes, obesity, ageing and other chronic inflammatory diseases [112, 114, 116-120].

Organisms are continuously threatened and intermittently exposed to oxidative damage caused by environmental factors. To counterbalance this, organisms and cells have created a variety of adaptation mechanisms to maintain their genomic stability. One of the most versatile mechanisms of adaptation is Nrf2-ARE antioxidant pathway [121]. Oxidative stress may trigger the transactivation of a battery of cytoprotective genes such as antioxidant proteins, drug-metabolizing enzymes, drug-efflux pumps, heat shock proteins, 26S proteasomes, growth factors, growth factor receptors and various transcription factors, which may play important roles in DNA repair, genomic surveillance and cell growth [122]. Recent studies both *in vitro* and *in vivo* including cell models, animal models and even human models imply that dietary phytochemicals could provide an inexpensive, adequate safe, readily applicable and easily accessible approach to activate Nrf2-ARE pathway and exert its role on chronic diseases control and management. Strengthening of cellular defense mechanism or restoration of stress-response signaling by administrating dietary phytochemicals provides an important strategy for chronic diseases chemoprevention [123].

4.1.5.1 Cardiovascular diseases

Cardiovascular diseases (CVD) are a class of diseases that involve the heart or blood vessels (arteries and veins) including atherosclerosis, coronary heart disease, cardiomyopathy, ischaemic heart disease, heart failure, hypertensive heart disease, inflammatory heart disease, valvular heart disease and myocardial infarction [124-128]. Cardiovascular diseases remain the biggest cause of deaths worldwide, more than 17 million people died from cardiovascular diseases in 2008 [129].

The main cause of the majority of cardiovascular diseases comes from

complications of atherosclerosis, and oxidized low-density lipoprotein (Ox-LDL) formation under the stimulation of reactive oxygen species which come from several different sources contributes the pathology of atherosclerosis [146]. This is likely to occur at the sites of endothelial damage which are caused by Ox-LDL itself as well as physical or chemical forces and infection. Endothelial cells, smooth muscle cells (SMCs), and macrophages are the sources of oxidants for the oxidative modification of phospholipids [132]. As shown in Figure 4.4, Ox-LDL can damage endothelial cells and induce the expression of adhesion molecules such as P-selectin, intracellular/vascular cell adhesion molecule-1 (I/VCAM-1) and proinflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage colony stimulating factor (mCSF). These processes lead to the tethering, activation, and attachment of monocytes and T-lymphocytes to the endothelial cells. Endothelial cells, leukocytes, and SMCs then secrete growth factors, chemoattractants and other proinflammatory cytokines that act on the migration of monocytes and leukocytes into the subendothelial space [130, 131]. Monocytes ingest lipoproteins and morph into macrophages. Macrophages generate reactive oxygen species (ROS), which convert Ox-LDL into highly oxidized LDL, which is, in turn, taken up by macrophages themselves to form foam cells. Foam cells combine with leukocytes to become the fatty streak, and as the process continues foam cells secrete growth factors that induce SMC migration into the intima. SMC proliferation, coupled with the continuous influx and propagation of monocytes and macrophages, converts fatty streaks to more advanced lesions and ultimately to a fibrous plaque that will protrude into the arterial lumen. Later, calcification can occur and fibrosis continues, yielding a fibrous cap that surrounds a lipid-rich core. This formation may also contain dead or dying SMCs. In acute coronary syndromes (e.g., myocardial infarction), when fibrous plaques rupture, the formation and release of thrombi may ultimately occlude vessels [132-134]. The pathology that oxidative stress causes cardiovascular injury is further confirmed by animal and human studies [135].

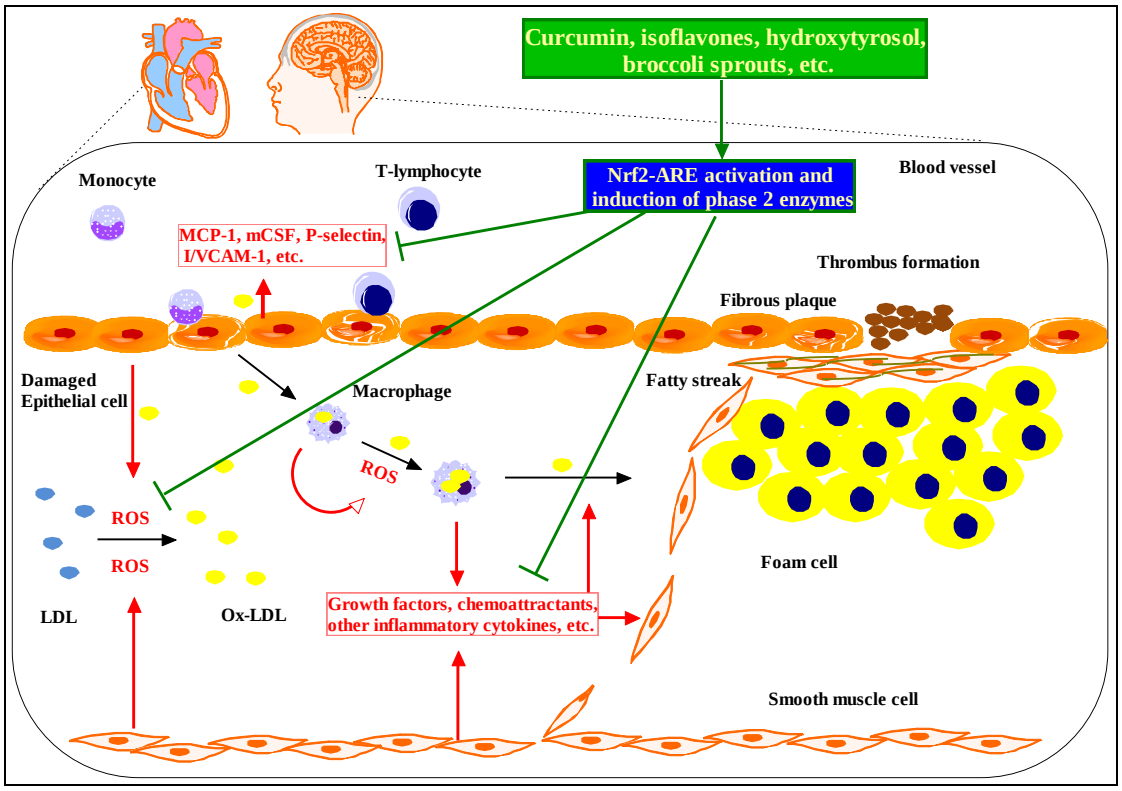

Figure 4.4 Pathology of cardiovascular disease, ROS and dietary phytochemicals. ROS produced by endothelial cells, SMCs, and macrophages oxidizes LDL to Ox-LDL in the subendothelial space. At the sites of endothelial damage, the produced ROS and the simultaneous secreting cell adhesion molecule (I/VCAM-1), proinflammatory cytokines (MCP-1) and growth factors, together initiating events that culminate in the formation of a fibrous plaque. Rupture of fibrous plaque leads to thrombus formation and occlusion of the vessel. Phytochemicals could prevent cardiovascular disease by eliminating ROS, inhibiting the production of proinflammatory cytokines, cell adhesion molecules and growth factors.

Howard first proposed, based on epidemiological studies, that phytochemicals such as plant sterols, flavonoids, and plant sulfur compounds distributed in vegetable and fruit could prevent coronary heart disease [137]. Following investigations further revealed that phytochemicals in fruits and vegetables, including phytoestrogens in soy, hydroxytyrosol in olives, resveratrol in nuts or red wine, lycopene in tomatoes, organosulfur compounds in garlic or onions, isothiocyanates in cruciferous vegetables, monoterpenes in citrus fruits, polyphenols in teas or wines, could be independently or jointly responsible for the apparent reduction in CVD risk [136, 138, 139]. However,

the mechanism underlying the prevention activity caused by phytochemicals remained unclear. Recent studies in animal model and cultured vascular cells have established that isoflavones increase the activity and expression of eNOS, and upregulate expression of detoxifying and antioxidant enzymes genes [140, 141]. Nrf2 has been suggested to be a valuable therapeutic target for cardiovascular disease [142] and it strongly inhibits the initial stage of atherosclerotic progression in the form of leading to repression of adhesion molecules such as MCP-1 and VCAM-1 [231, 232]. For example, hydroxytyrosol from olive could protect against oxidative injury in vascular endothelial cells via induction of Nrf2-mediated HO-1 [143]. Ginkgo biloba extract could exert its anti-atherogenesis and vascular protective effects by inducing vascular HO-1 expression that is regulated by p38-Nrf2 pathway [144]. A relatively short dietary treatment with broccoli sprouts could strongly protect the heart against oxidative stress and cell death caused by ischemia-reperfusion via activating Nrf2 pathway in rats [145].

In human clinical trials, curcumin administration reduced the serum levels of cholesterol and lipid peroxides in 10 healthy human volunteers receiving 500 mg of curcumin daily for 7 days [227]. Furthermore, another interventional study with randomized, double-blind, controlled trial found that the administration of low-dose curcumin also showed a trend of reduction in total cholesterol level and LDL cholesterol level in patients with acute coronary syndrome [228]. Long-term (2–12 months) supplementation with genistein or soy isoflavones showed benefits to cardiovascular including reducing arterial stiffness, lowering blood pressure and improving vascular function [229, 230].

4.1.5.2 Cancer

Cancer is the second leading cause of death, after cardiovascular diseases, in occidental countries [147]. Every year, around 10 million people worldwide are diagnosed with cancer, and approximately 6.2 million die of this disease [147, 148]. Only 5–10% of all cancer cases can be attributed to genetic defects, whereas the remaining 90–95% cancers have their roots in the environmental factors, among

which, almost 30-35% are linked to diet [149]. It is nearly impossible to prove what caused a cancer in any individual, because most cancers have multiple possible causes, but it is clear that the ratio in a number of cancers is low in the population having diets rich in vegetables, fruits and whole grains while it is high in the population having diets rich in processed or red meats [150].

Cancer development in humans is a multistep with long-term process, in which cancer cells acquired several biological capabilities such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion & metastasis, reprogramming of energy metabolism, evading immune destruction, genome instability & mutation and tumor-promoting inflammation [151]. Due to cancer pathogenesis is traceable back to DNA mutations that impact cell growth and metastasis, oxidative stress is one of main causes for carcinogenesis.

Oxidative stress promotes damage to the cell structure including proteins, lipids, membranes and DNA, thus, plays a key role in the development of cancer [152] (Figure 4.5). Mitochondrial electron-transport chain, proinflammatory cytokines and other oxidizing agents are the prime pathways that generate excess ROS in vivo, leading to several types of DNA damage, including depurination and depyrimidination, single and double-stranded DNA breaks, base and sugar modifications and DNA-protein crosslinks [153, 154]. Permanent modification of genetic material resulting from the oxidative damage is one of the vital steps involved in mutagenesis that leads to carcinogenesis. Stimulation of DNA damage can cause transcription disorder, replication errors and genomic instability. All of these happenings are associated with carcinogenesis [155]. Excess oxidative stress has been considered to promote cancer [156, 157].

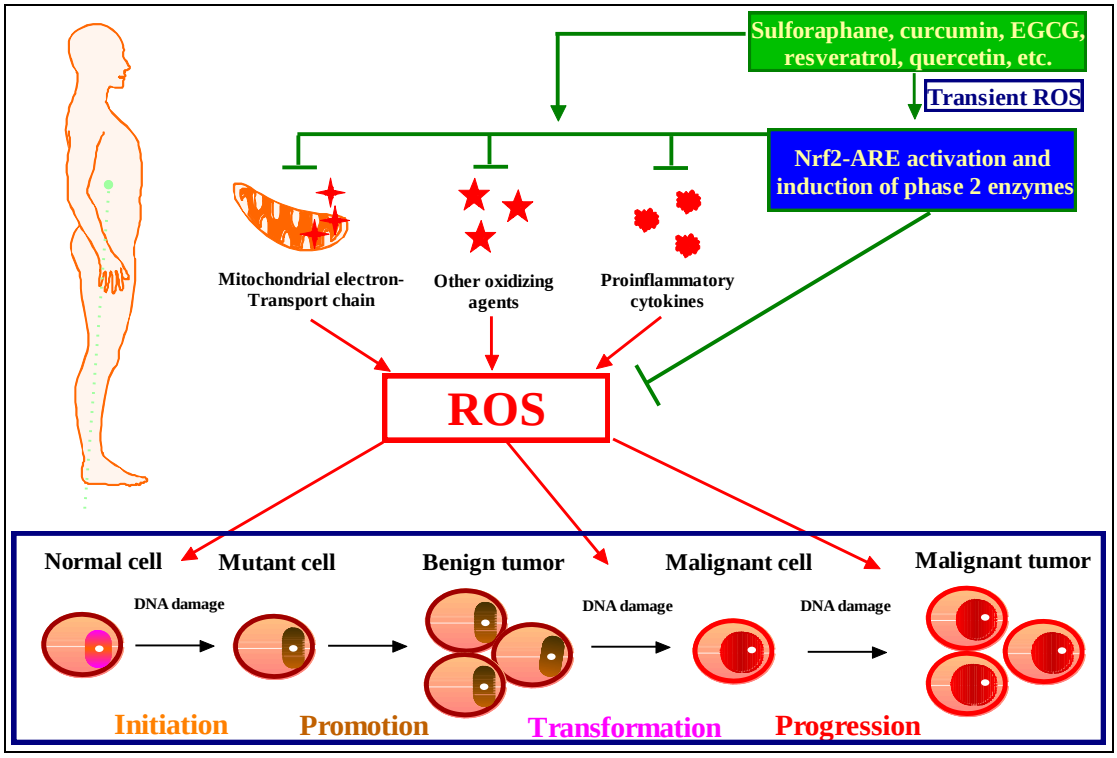

Figure 4.5 Carcinogenesis, ROS and dietary phytochemicals. ROS produced by mitochondrial electron-transport chain, proinflammatory cytokines and other oxidizing agents is the main cause for carcinogenesis. Phytochemicals prevent carcinogenesis either by directly eliminating ROS or by the induction of Nrf2-mediated phase 2 enzymes. In addition, anti-inflammatory property of phytochemicals to Nrf2-ARE pathway activation also plays an important role in carcinogenesis.

The first study that found phytochemicals may play positive role in cancer prevention was carried out half a century ago, in which researchers administrated small quantities of phytochemicals decreased the incidence of cancer in rats [147]. From then on, many phytochemicals have been proved gradually to possess the chemoprevention activity by mediating Nrf2-ARE pathway. These phytochemicals can be classified into "blocking agents" who impede the initiation stage and "suppressing agents" which arrest or reverse the promotion and progression of cancer [158-160]. The well investigated phytochemicals with obvious cancer prevention ability include sulforaphane from broccoli and wasabi, curcumin from turmeric root, EGCG from green tea, resveratrol from grape, and caffeic acid phenethyl ester from coffee. Additionally, quercetin, myricetin, garlic oranosulfur compounds, lycopene, purpurogallin, avicins widely distributed in fruits and vegetables are also found to

have cancer chemoprevention activity [161]. These phytochemicals exert their chemoprenventive activity through the induction of Nrf2-dependent adaptive responses including phase II detoxifying enzymes, antioxidant proteins and transporters that protect cells from carcinogens or other stimulations [163].

In human clinical trials, administration of glucosinolate-rich broccoli sprout reduced aflatoxin-caused DNA adducts through induction of GST activity [233, 234] Curcumin works as chemosensitizer and radiosensitizer for tumors therapy through activation of Nrf2-mdiated expression of antioxidant enzymes in preclinical trials [235]. Another recent study using microarray to analyze gene expression and regulation pathways in tissue of men with prostate cancer in a randomized clinical trial found that lycopene supplementation could activate Nrf2 pathway [236].

It is noticed that Nrf2 plays dual roles in normal cells and cancer cells. In normal cells, Nrf2-mediated induction of phase II antioxidant proteins and detoxifying enzymes is beneficial to cancer chemoprevention while in cancer cells, Nrf2-mediated induction of phase II detoxifying enzymes have reported to increase drug resistance and promote proliferation of cancer cells [162]. Thus, it needs to clarify the effects and mechanisms of dietary phytochemicals in cancer cells although the role of dietary phytochemicals in cancer prevention is clear.

4.1.5.3 Neurodegenerative Diseases

Neurodegenerative diseases are causes with the progressive loss of structure or function of neurons including death of neurons due to the expression of certain gene alleles, toxicant administration and aging [164]. Many neurodegenerative diseases including Parkinson's Disease (PD), Alzheimer's Disease (AD), Huntington's Disease (HD) and Amyotrophic Lateral Sclerosis (ALS) occur as a result of neurodegenerative processes. The commonalities among neurodegenerative diseases include protein aggregation, proteasomal or autophagic dysfunction, inflammation, neuronal apoptosis, oxidative stress, mitochondrial dysfunction and interactions between neurons and glia [164-172]. Among these pathologies, the causal nature of mitochondrial dysfunction and oxidative stress in neurodegeneration is widely

considered, and accumulated evidence suggests that free radicals are extremely important in causing neuronal death [164, 173].

Consecutive and long stimulation from excess oxidative stress caused by ROS or RNS could induce damage in neurons. The central nervous system (CNS) is particularly sensitive to oxidative stress, owing to a high oxygen consumption and exposure under excess polyunsaturated fatty acids, making it particularly vulnerable to lipid peroxidation. Oxidative damage to key intracellular targets such as DNA or proteins by free radicals has been shown to be a major cause of the neuronal cell damage related to degenerative diseases [172, 174]. In brief, excess oxidative stress ROS and RNS lead to aggregation and accumulation of bad proteins like α-synuclein protein of Lewy bodies, amyloid precursor protein (APP) and amyloid β peptides which are major neuropathological alterations in neurodegenerative diseases. These proteins released from neurons lead to the activation of transcription factor NF-κB and AP-1 in microgials and astrocytes, and sequentially induce ROS, iNOS, COX-2, NAPDH oxidase, proinflammatory cytokines and inflammatory mediators, which in turn to damage neurons and finally cause neurodegenerative diseases [175] (Figure 4.6).

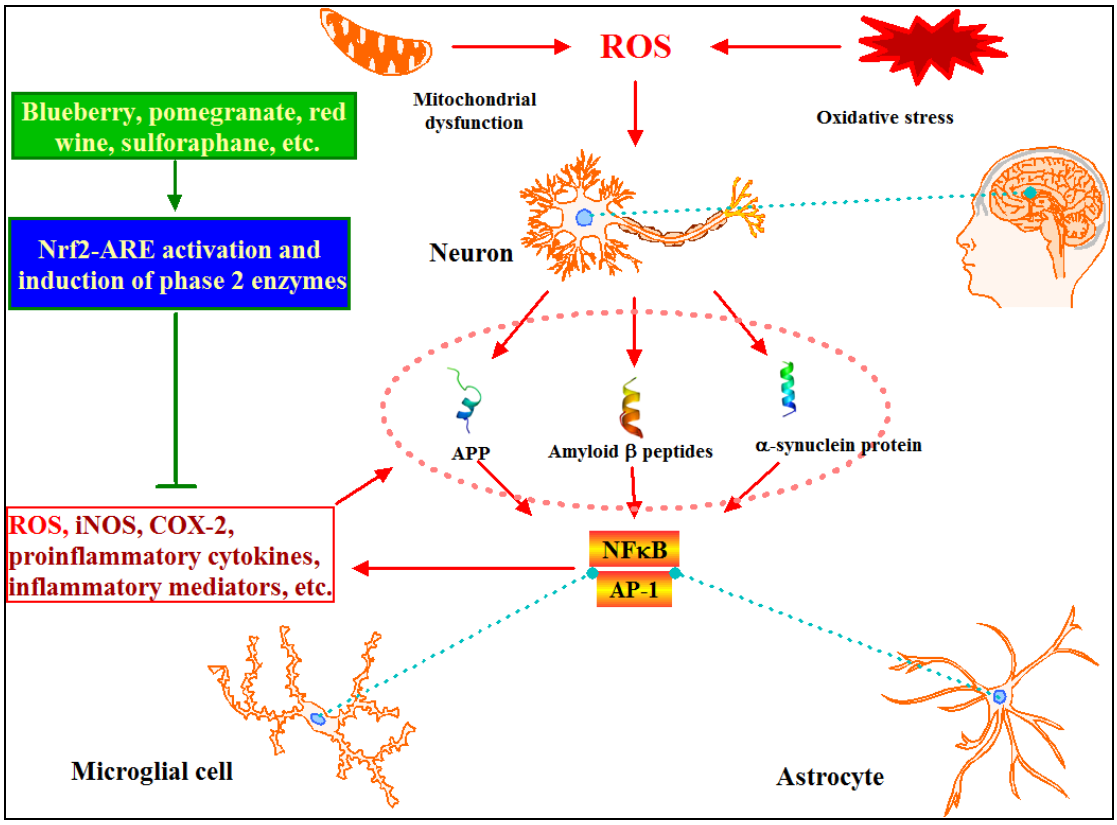

Figure 4.6 Neurodegenerative disease, ROS and dietary phytochemicals. ROS produced by mitochondrial dysfunction, and oxidative stress leads to aggregation and accumulation of bad proteins like α-synuclein protein, amyloid precursor protein (APP) and amyloid β peptides, which activated the transcription factor NF-κB and AP-1 in microgials and astrocytes, leading to the induction of ROS, iNOS, COX-2, NAPDH oxidase, proinflammatory cytokines and inflammatory mediators, which in turn to damage neurons and finally cause neurodegenerative diseases. Phytochemicals could prevent neurodegenerative diseases by eliminating ROS, blocking bad protein aggregation and accumulation and inhibiting proinflammatory cytokines.

Targeting the prevention of oxidative stress and mitochondrial dysfunction shows priority on chemoprevention of neurodegenerative diseases that can be achieved through stimulation of endogenous cellular mechanisms. Nrf2-ARE antioxidant pathway is considered to play central role in prevention of neurodegenerative diseases due to their induction of gene expression of downstream phase II detoxifying enzymes and antioxidant proteins. A number of studies in animal models revealed that the activation of Nrf2-ARE pathway is beneficial for prevention of various diseases of the

central nervous system [238]. In the human AD brain, the amount of Nrf2 is reduced in the hippocampus, and histochemical analyses confirmed that Nrf2-mediated transcription is not induced in AD patients [239]. Further study on a plenty of AD patients have found that common variants of the Nrf2 gene may affect disease progression, potentially altering clinically recognized disease onset [240].

Phytochemicals have been reported to activate Nrf2-ARE pathway to induce the expressions of genes that encode antioxidant enzymes, protein chaperones, phase II enzymes, neurotrophic factors, and other cytoprotective proteins [176], which then eliminate excess oxidative stress to prevention against neurodegenerative diseases. Data from epidemiological studies of human populations suggest that phytochemicals in fruits and vegetables can protect the nervous system against disease. For example, people who consume higher than average amounts of vegetables and fruits had low risk for Alzheimer's disease [177]. Numerous studies of cell culture and animal models have demonstrated that dietary supplementation with specific fruits or vegetables, or their extracts or specific chemical components could activate Nrf2-ARE pathway. For example, dietary supplementation with blueberries protected dopaminergic neurons against dysfunction and degeneration in a rat model of Parkinson's disease [178], and improved learning and memory without affecting amyloid pathology in a mouse model of Alzheimer's disease [179]. Drinking pomegranate juice reduced the amount of amyloid and improved behavioral deficits in a mouse model of Alzheimer's disease [180]. Moderate consumption of red wine reduced amyloid pathology in a mouse model of Alzheimer's disease [181]. Sulforaphane showed its neuroprotective effect in animal model of neurodegenerative condition [182]. Curcumin, resveratrol and green tea catechins revealed a positive relationship between consumption of these compounds and the prevention of AD [183], companying reduction of the formation of neurotoxic β-amyloid fibrils [184-186].

Although no human clinical trial could verify the neuroprotection of phytochemicals now, an interesting study revealed that PD patient-derived cells had alterations in cellular responses of Nrf2 signaling to sulforaphane. Thus, the PD

patient-derived cell models would be useful for pre-clinical testing of pharmaceuticals and phytochemicals that target Nrf2 for Neurodegenerative therapy [237].

4.1.5.4 Diabetes

Diabetes is one of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because cells do not respond to the insulin. This high blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) [187]. Diabetic patients have an increased risk to develop many complications such as diabetic retinopathy with progression of the disease leading to blindness and end-stage renal failure [191], cardiovascular disease (CVD) with leading atherosclerosis [188, 192], diabetic nephropathy with leading scarring changes in the kidney tissue, loss of small or progressively larger amounts of protein in the urine, and eventually chronic kidney disease requiring dialysis [193]. During hyperglycaemia, reactive oxygen species and mitochondrial dysfunction caused excess oxidative stress that plays a causal role in the development and progression of the above diabetic complications [189, 190]. In brief, as shown in Figure 4.7, ROS provoked by hyperglycemia and free fatty acids leads to activation of several signaling pathways including NF-κB, p38 MAPK, and JNK, which cause chronic inflammation and production of series of cytokines through suppressing secretion of insulin and promoting cell dysfunction [193].

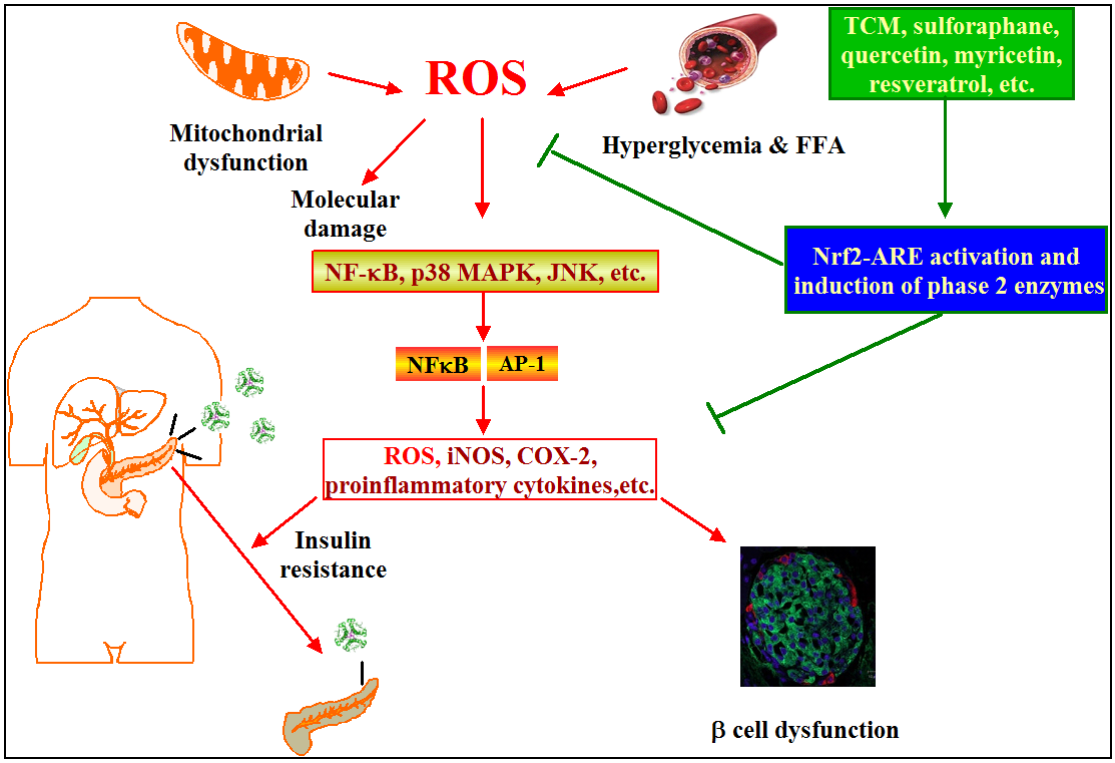

Figure 4.7 Diabetes, ROS and dietary phytochemicals. ROS produced by mitochondrial dysfunction, hyperglycemia and free fatty acids leads to activation of several inflammatory signaling pathways such as NF-κB, p38 MAPK, and JNK. The chronic inflammation activates inflammatory transcription factors including NF-κB and AP-1, finally leads to production of ROS, iNOS, COX-2 and series of proinflammatory cytokines through suppressing secretion of insulin and promoting cell dysfunction. Phytochemicals could prevent diabetes by inhibiting inflammatory factors production and eliminating ROS generated by mitochondrial dysfunction and hyperglycemia.

A series of studies have showed the priority of phytochemical in diabetic disease prevention. For example, traditional Chinese medicines (TCM) are usually served as adjuvants to improve diabetic syndromes in combination of routine antidiabetic drugs [197]. A soya phytochemical extract, namely as an oestrogenic agent, acts as an inhibitor of intestinal glucose-uptake and a preventive agent for glucose-induced lipid peroxidation [200]. The leaf extract of *Annona squamosa* showed antidiabetic activity in rat model [201]. Malmea depressa administration can improve glycemic control through blocking hepatic glucose production [198]. A medicinal plant from Thailand

also showed hypoglycemic activity in normoglycemic and alloxan-induced diabetic mice [199]. However, the underlying mechanism of antidiabetic phytochemicals remained unclear.

Recent investigations using experimental diabetic Nrf2-knockout mice based on administration of streptozotocin (STZ) clearly demonstrated a protective role of Nrf2 pathway in diabetic nephropathy [241-243]. Especially one of these studies also documented that the glomeruli of patients with diabetic nephropathy were under oxidative stress and had elevated Nrf2 levels [242], which suggesting that dietary or pharmacological activation of Nrf2 could be used as a strategy to prevent or retard the progression of this debilitating complication of diabetes in humans.

Phytochemicals show their promising potency on diabetes prevention through the activation of Nrf2-ARE pathway. For example, sulforaphane can suppress ROS-induced hyper glycemia and metabolic dysfunction in human microvascular endothelial cells by activating Nrf2 pathway [194]. Quercetin and myricetin could reduce the risk of diabetic cataract formation via affecting multiple pathways including Nrf2 pathway [195, 196]. Cinnamic aldehyde significantly attenuated common metabolic disorder symptoms associated with diabetes in Nrf2 (+/+) but not in Nrf2 (-/-) mice [202]. Resveratrol was demonstrated to have the ability to protect diabetic kidney by attenuating markers of hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling [203].

4.1.5.5 Obesity

Obesity is a medical condition in which excess body fat has accumulated to the extent and is reaching worldwide unprecedented prevalence in persons of all ages [204, 205]. Obesity causes a series of health problems like metabolic syndrome [206, 207] and likelihood of various diseases especially including heart disease, type 2 diabetes, obstructive sleep apnea, certain types of cancer, and osteoarthritis [208]. Oxidative stress shows a strong relation to obesity (Figure 4.8). In brief, hyperglycemia and excess oxidative stress stimulate adipocyte generation. Mature adipocyte could induce more oxidative stress and secret a series of proinflammatory

cytokines such as IL6, PAI-1, MCP-1, and TNF-α, companying inhibition of adiponectin and leptin production to damage tissues and cause chronic inflammation. All of these events finally lead to metabolic syndromes including diabetes, insulin resistance, atherosclerosis and hypertension. Besides, obesity per se may induce systemic oxidative stress in accumulated fat that, in turn, causes dysregulation of adipocytokines and further development of metabolic syndrome [209, 224 and 225].

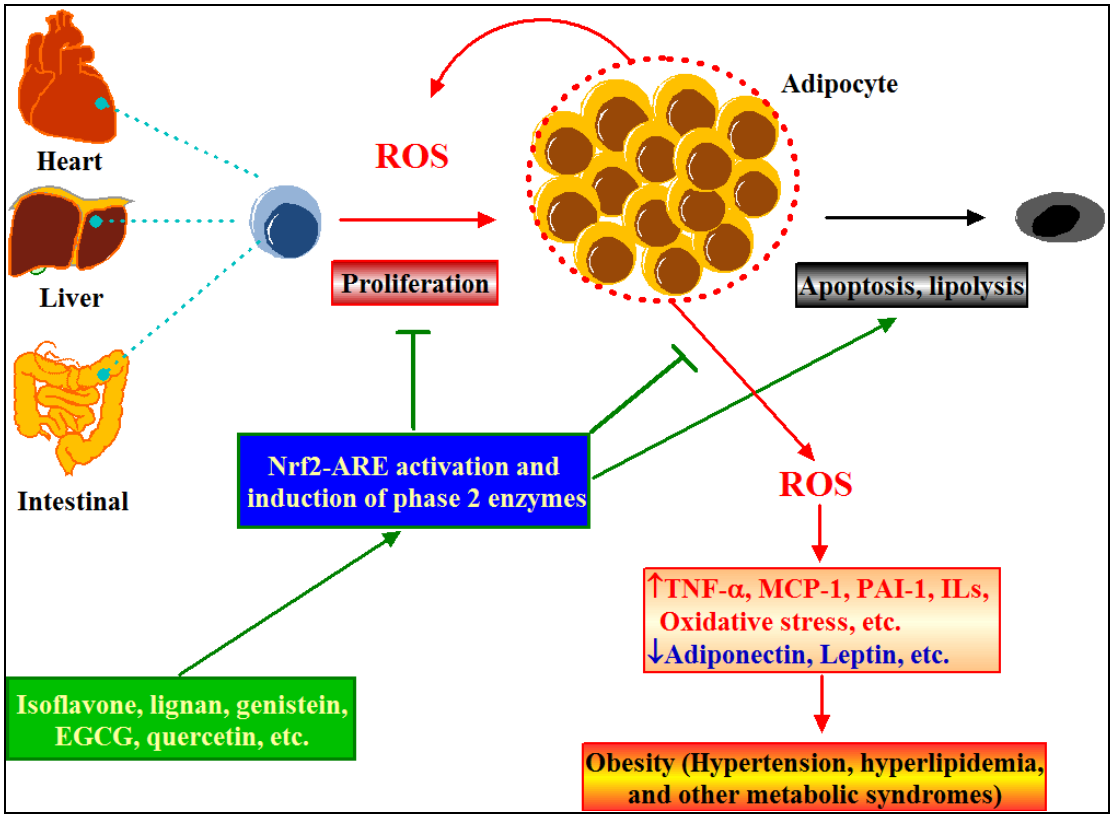

Figure 4.8 Obesity, ROS and phytochemicals. Hyperglycemia and excess oxidative stress cause adipocyte generation which could induce more oxidative stress in turn and damage tissues as well as activate chronic inflammation with secreting series of proinflammatory cytokines (IL6, PAI-1, MCP-1, TNFα, etc.) and inhibit production of adiponectin and leptin, finally lead to metabolic syndromes. Phytochemicals could prevent obesity by inhibiting adipocyte formation and proinflammatory factors secretion, inducing adipocyte apoptosis or lipolysis and eliminating ROS or other oxidative stress.

The obesogenic environment of highly palatable foods with hidden fats and sugars

can promote metabolic syndrome and obesity, whereas fruit and vegetables with phytochemicals can counteract metabolic syndrome and obesity [210]. For example, isoflavones and lignans have been proved to exert anti-obesity activity in nutritional intervention studies in both animals and humans [211]. Genistein, conjugated linoleic acid (CLA), docosahexaenoic acid, epigallocatechin gallate, quercetin, resveratrol and ajoene affect adipocytes during various stages of the adipocyte life cycle, resulting in either inhibition of adipogenesis or induction of adipocyte apoptosis which decreasing lipid accumulation and inducing lipolysis [212]. Moreover, phytochemicals can target obesity-induced oxidative stress through activation of Nrf2-ARE pathway [213, 214]. Oxidative stress in accumulated fat appears as an earlier instigator of obesity-associated metabolic syndrome, thus, it is an important target for therapies.

4.1.5.6 Other chronic diseases (chronic inflammatory diseases, COPD, ageing and longevity)

Phytochemicals showed their potency in prevention on many other chronic diseases through activation of Nrf2-ARE pathway.

Inflammation is a complex set of interactions among soluble factors and cells, and can arise in any tissue in response to traumatic, infectious, post-ischaemic, toxic or autoimmune injury [215]. Thus, inflammation is at the root of several degenerative disorders, such as autoimmune diseases, rheumatoid arthritis, asthma, emphysema, gastritis, colitis, osteoarthritis, chronic obstructive pulmonary disease, ageing, atherosclerosis, cancer [216]. As shown in Figure 4.9, inflammation occurs as part of the immune reaction and produces ROS to inactivate the foreign molecules and fight against the invading pathogens. A cascade of cytokine- and chemokine-mediated inflammatory reactions initiate and maintain a host response, involving activation and attraction of immune and non-immune cells. Leukocytes (neutrophils, monocytes and eosinophils), macrophages, lymphocytes, and plasma cells in venous system infiltrate into the disrupted and damaged tissue to recover from infection and to heal [215]. However, cytokines and chemokines persisting at inflammatory sites cause chronic oxidative stress that can mediate subsequent tissue injuries. Oxidative stress activates

the redox-sensitive transcription factors such as NF-κB and AP-1, resulting in the production of pro-inflammatory cytokines and chemokines. Excess oxidative stress is also involved in the pathophysiologies of many inflammation-associated disorders. Therefore, the induction of antioxidant proteins and detoxifying enzymes through activation of Nrf2-ARE pathway is essential for body's protection against inflammatory tissue injuries [217, 218].

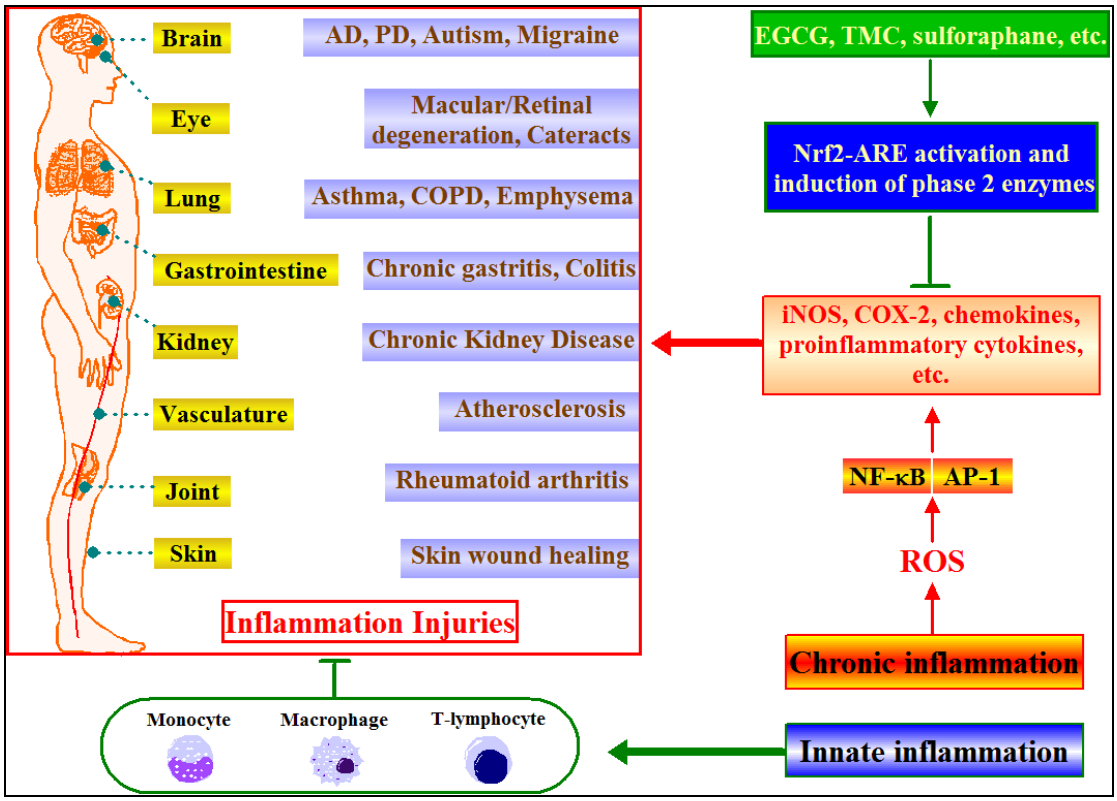

Figure 4.9 Other chronic diseases, ROS and phytochemicals. Innate inflammation occurs aiming to fight against the invading pathogens and to inactivate the foreign molecules, which protect human organs through activation immune system including production of monocytes, macrophages, and lymphocytes. However, chronic inflammation causes excess oxidative stress and induces proinflammatory cytokines and chemokines persisting at inflammatory sites by activation of NF-κB and AP-1, which could lead to subsequent tissue injuries. Phytochemicals could prevent chronic diseases by inhibiting proinflammatory factors during chronic inflammation.

Recent studies confirmed that phytochemicals could prevent chronic inflammatory related disorders by activation of Nrf2-ARE pathway. For example, EGCG has been proved to prevent lupus nephritis in mice by enhancing the Nrf2 signaling pathway, decreasing renal NLRP3 inflammasome activation, and increasing systemic Treg cell activity [219]. EGCG also enhanced Nrf2-mediated expression of Phase II enzymes with subsequent restraint inflammation during bleomycin-induced pulmonary fibrosis [221]. Curcumin mediates its anti-inflammatory effects through induction of phase II enzymes that was confirmed by pharmacodynamics and pharmacokinetics in pulmonary disease in both animals and humans [222]. Traditional Chinese medicine Fuzi from lateral roots of *Aconitum carmichaeli Debx* could play beneficial effect on rheumatoid arthritis patients by regulating the Nrf2 pathway [220]. Sulforaphane in broccoli sprouts could enhance protection of gastric mucosa against oxidative stress in vitro and exerted anti-inflammatory effects on gastric mucosa during *H. pylori* infection in mice and human through activation of Nrf2 pathway. The potential use of these phytochemical antioxidants in the treatment of chronic obstructive pulmonary disease (COPD) has been supported by *in vitro* data, animal models or human preclinical studies [223, 226].

Phytochemicals also showed their potential effects on calorie restriction and longevity. Nrf2 is recently identified as a mediator of caloric restriction [246, 247], and as an effector of longevity signals, providing new therapeutic perspectives [248, 249]. Resveratrol has been found to extend lifespan in several organism models [250], but further investigations are required to determine whether Nrf2 is required for the lifespan extension or other salutary effects caused by resveratrol.

It is clear that since the discovery of Nrf2-ARE pathway, enormous advances have been made over the past decade in understanding the mechanisms of action of phytochemicals. Obviously, phytochemicals, especially polyphenolic compounds, can play a central role on prevention of series of oxidative stress-related chronic diseases by regulating Nrf2-ARE pathway. However, much of the data has been undertaken in cell models or animal models, which not only because of the nearsightedness of company researchers just paid main attention on limited biomarkers, but also because

of the neglect and deficiency on study of crosstalks between Nrf2-ARE pathway and other disease related signaling transduction pathways. Polyphenolic compounds definitely contribute to human health, so there is now an urgent need to translate cell or animal based knowledge into the human.

4.2 Conclusion

The present study performed gene expression and IPA analysis to screen out the most potent functions of polyphenolic compounds which including chemoprevention, and further characterized the molecular mechanism of baicalein and myricetin on chemopreventive Nrf2-ARE pathway activation.

The conclusions include that

1. DNA microarray data of HepG2 cells treated by baicalein and myricetin revealed that the expressions of drug metabolizing enzymes were significantly regulated by these polyphenolic compounds. IPA pathway network analysis further revealed that an Nrf2 -mediated ARE pathway is involved in both baicalein- and myricetin-induced gene expressions.

2. Baicalein and/or myricetin activate Nrf2-ARE pathway by up-regulating expression of Nrf2 mRNA via phosphorylation of protein kinases at transcriptional level and by increasing Nrf2/Keap1 overplus at posttranscriptional level. Structure-activity relationship analysis revealed that the basic flavan structure seems to be the key component of polyphenolic compounds to activate Nrf2.

3. DNA microarray based Nrf2-related network analysis is a promising and potent method for clarifying chemopreventive function of polyphenolic compounds.

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Appendix

Supplementary table 1 The number of genes that regulated by purpurogallin treatment

Fold change	Purpurogallin treatment	
	Numbers	Regulation
≥ 4	6	up
	145	down
2 ≤ ~ < 4	260	up
	1379	down
1.5 ≤ ~ < 2	3401	up
	3253	down
Total	3667	up
	4777	down

Supplementary table 2 Gene profiling comparison between polyphenolic compounds by Gene Ontology ID

Catalytic activity (GO: 0003824)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
ACSL3	acyl-CoA synthetase long-chain family member 3	2.613	up	1.164	down	1.259	down	AL525798
ACSL3	acyl-CoA synthetase long-chain family member 3	2.198	up	1.226	down	1.525	down	NM_004457
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	2.006	up	1.217	up	1.068	down	NM_006636
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	2.464	up	2.463	up	1.605	up	NM_004566
PRSS8	protease, serine, 8	2.658	up	1.483	down	2.462	down	NM_002773
NNT	nicotinamide nucleotide transhydrogenase	1.286	down	1.681	down	2.233	down	U40490
NNT	nicotinamide nucleotide transhydrogenase	1.184	down	1.720	down	2.194	down	NM_012343
HK2	hexokinase 2	2.320	up	1.382	up	1.050	up	AI761561
UPP1	uridine phosphorylase 1	2.569	up	1.336	up	1.700	down	NM_003364
AGL	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (glycogen debranching enzyme, glycogen storage disease	1.787	down	2.420	down	5.394	down	NM_000645
RTCD1	RNA terminal phosphate cyclase domain 1	1.044	up	2.186	down	3.306	down	NM_003729
LCAT	lecithin-cholesterol acyltransferase	1.691	down	2.231	down	1.429	down	NM_000229
DPYD	dihydropyrimidine dehydrogenase	1.353	down	1.438	down	2.100	down	NM_000110
NMT2	N-myristoyltransferase 2	1.622	down	2.120	down	3.518	down	AW293531
NMT2	N-myristoyltransferase 2	1.365	down	1.691	down	3.046	down	NM_004808
PSPH	phosphoserine phosphatase	1.489	up	1.791	down	2.329	down	NM_003832
MAN2A1	mannosidase, alpha, class 2A, member 1	1.128	down	1.337	down	2.255	down	NM_002372
PSPH	phosphoserine phosphatase	1.523	up	1.612	down	2.241	down	NM_004577
ATP7A	ATPase, Cu++ transporting, alpha polypeptide (Menkes syndrome)	1.360	up	1.637	down	3.411	down	NM_000052
PRSS2	protease, serine, 2 (trypsin 2)	1.539	up	1.395	up	2.136	up	NM_002770
CTH	cystathionase (cystathionine gamma-lyase)	4.282	up	1.066	down	1.791	down	NM_001902
PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	1.195	down	1.446	down	2.609	down	NM_005084
PGGT1B	protein geranylgeranyltransferase type I, beta subunit	1.236	up	1.044	down	2.033	down	NM_005023
AGXT	alanine-glyoxylate aminotransferase	1.842	down	1.553	down	2.083	down	NM_016236
EYA4	eyes absent homolog 4 (Drosophila)	2.016	up	1.037	down	1.166	down	NM_004100
ADH6	alcohol dehydrogenase 6 (class V)	2.364	down	2.508	down	3.285	down	NM_000672
HPR	haptoglobin-related protein	3.169	down	2.081	down	2.382	down	NM_020995
GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	2.239	up	1.006	up	1.072	down	BC000498
CBR1	carbonyl reductase 1	1.261	up	2.122	up	1.531	up	BC002511
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1.298	down	1.663	down	3.943	down	M95541
AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.526	down	2.030	down	1.938	down	AI796120
AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.332	down	2.792	down	3.297	down	AA888589
AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.160	down	1.860	down	2.129	down	AF047020
PPAT	phosphoribosyl pyrophosphate amidotransferase	1.013	down	1.446	down	2.656	down	AI457120
PPAT	phosphoribosyl pyrophosphate amidotransferase	1.010	down	1.272	down	2.987	down	U00238
ABAT	4-aminobutyrate aminotransferase	1.602	down	2.313	down	1.837	down	AF237813
ABAT	4-aminobutyrate aminotransferase	1.472	down	2.182	down	1.510	down	AF237813
HSDL2	hydroxysteroid dehydrogenase like 2	1.533	down	1.888	down	2.211	down	BC004331
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	1.255	down	1.342	down	2.336	down	U51120
ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	1.182	down	1.409	down	2.310	down	M55905
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	1.366	down	1.458	down	2.599	down	AF189723
GPR56	G protein-coupled receptor 56	1.055	up	1.952	down	2.432	down	AL554008
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	1.168	down	1.239	down	2.521	down	AK001684
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.349	up	2.141	down	5.681	down	NM_000819
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.576	up	1.777	down	2.952	down	BE966876
CBS	cystathionine-beta-synthase	1.487	up	1.747	down	2.787	down	BE613178
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1.017	up	1.621	down	6.612	down	AW576457
ATP8A1	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	1.324	down	1.446	down	2.291	down	AI769688
SCAMP4	secretory carrier membrane protein 4	1.515	down	1.919	down	2.093	down	AI207792
PGAP1	post-GPI attachment to proteins 1	2.104	down	1.273	down	1.005	up	AV705244
RABGGTB	Rab geranylgeranyltransferase, beta subunit	1.188	up	1.366	down	2.235	down	AA129753
DHRS2	dehydrogenase/reductase (SDR family) member 2	2.073	down	2.630	down	2.223	down	AK000345
ADH6	alcohol dehydrogenase 6 (class V)	2.125	down	2.462	down	2.645	down	H71135
PDE3B	phosphodiesterase 3B, cGMP-inhibited	1.326	down	1.694	down	2.169	down	NM_000753
AASS	aminoadipate-semialdehyde synthase	2.245	down	2.284	down	2.389	down	AK023446
HSDL2	Hydroxysteroid dehydrogenase like 2	1.682	down	1.744	down	2.166	down	AK023959
LOC653188 /// SMA4	glucuronidase, beta pseudogene /// similar to Beta-glucuronidase-like protein SMA3	1.535	down	1.535	down	3.003	down	X83300
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1.133	down	1.433	down	5.800	down	L14561
CTH	cystathionase (cystathionine gamma-lyase)	3.378	up	1.203	down	1.874	down	AL354872
MDM2	Mdm2 p53 binding protein homolog (mouse)	1.455	up	1.236	up	3.592	up	AJ276888
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.445	up	1.840	down	7.813	down	AF008655
MOCS2	molybdenum cofactor synthesis 2	2.066	down	1.498	down	1.580	down	NM_004531
UBA5	ubiquitin-like modifier activating enzyme 5	1.014	up	1.696	down	2.878	down	NM_024818
C12orf5	chromosome 12 open reading frame 5	1.011	up	1.190	up	2.115	up	NM_020375
RP11-35N6.1	plasticity related gene 3	3.263	down	3.098	down	2.273	down	NM_017753
THNSL1	threonine synthase-like 1 (S. cerevisiae)	1.093	up	1.845	down	2.667	down	NM_024838

PGAP1	post-GPI attachment to proteins 1	1.304	down	1.565	up	2.825	up	NM_024989
GPHN	gephyrin	2.266	up	1.117	down	1.049	down	NM_020806
UEVLD	UEV and lactate/malate dehydrogenase domains	1.056	down	1.444	down	2.401	down	NM_018314
AGXT2L1	alanine-glyoxylate aminotransferase 2-like 1	1.739	down	2.133	down	1.703	down	NM_031279
ELP3	elongation protein 3 homolog (S. cerevisiae)	1.133	down	1.394	down	2.389	down	NM_018091
CSAD	cysteine sulfinic acid decarboxylase	1.648	down	1.762	down	3.190	down	NM_015989
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	1.201	up	1.186	down	3.723	down	BG035985
SCLY	selenocysteine lyase	1.443	down	1.266	down	2.816	down	AA911739
UBA5	ubiquitin-like modifier activating enzyme 5	1.146	up	1.408	down	2.468	down	AW516242
UBA5	ubiquitin-like modifier activating enzyme 5	1.108	up	1.653	down	2.180	down	BE549973
SRR	serine racemase	1.386	up	1.400	down	2.242	down	AF169974
ARSD	arylsulfatase D	1.236	down	1.704	down	2.220	down	BC003660
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	2.098	down	2.104	down	2.139	down	M15943
PDE11A	phosphodiesterase 11A	3.303	down	4.267	down	3.894	down	AB038041
STEAP2	six transmembrane epithelial antigen of the prostate 2	1.149	up	1.351	down	2.176	down	BF680588
FAM80B	family with sequence similarity 80, member B	1.062	down	1.732	up	2.056	up	AI743612
MAN2A1	mannosidase, alpha, class 2A, member 1	1.085	down	1.592	down	2.777	down	AV700323
ATP11C	ATPase, class VI, type 11C	1.305	down	1.876	down	4.430	down	BF475862
ELP3	elongation protein 3 homolog (S. cerevisiae)	1.033	down	1.702	down	3.510	down	AI949204
SPTLC3	serine palmitoyltransferase, long chain base subunit 3	2.531	down	2.047	down	1.523	down	AA005105
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	3.652	up	2.441	up	2.313	up	AL038787
HINT3	histidine triad nucleotide binding protein 3	2.252	up	1.463	up	1.501	up	AW731710
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.088	down	2.307	up	1.543	up	BE566894
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.505	down	3.297	up	1.513	up	BE566894
NRD1	nardilysin (N-arginine dibasic convertase)	1.394	down	2.043	down	2.012	down	AA448346
ALDH1L2	Aldehyde dehydrogenase 1 family, member L2	2.310	up	1.274	down	1.940	down	AI654224
LOC136242	similar to RIKEN cDNA 1700016G05	1.361	up	1.282	up	2.128	up	AI805861
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	2.752	down	3.001	down	5.745	down	AV651117
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.108	up	2.116	up	1.424	up	AL135787
ARSB	arylsulfatase B	1.172	down	1.004	down	2.377	down	AW168942
MASP1	mannan-binding lectin serine peptidase 1 (C4/C2 activating component of Ra-reactive factor)	2.002	up	1.262	up	1.221	down	AI274095
PCYT1B	phosphate cytidylyltransferase 1, choline, beta	2.011	down	2.056	down	1.294	down	AI857508
SCAMP4	secretory carrier membrane protein 4	1.351	down	1.844	down	2.210	down	BF207216
MAN2A1	mannosidase, alpha, class 2A, member 1	1.232	down	1.810	down	3.223	down	AA029155
MDH1	Malate dehydrogenase 1, NAD (soluble)	1.914	down	1.302	down	2.103	down	AW952547
KLB	klotho beta	4.010	down	3.293	down	2.046	down	AI677905
ERN1	endoplasmic reticulum to nucleus signaling 1	2.390	up	1.216	up	1.486	down	AV704183
PDE11A	phosphodiesterase 11A	1.670	down	1.962	down	2.407	down	AI919276
ARSK	arylsulfatase family, member K	1.214	down	2.478	down	4.286	down	AI243677
PGAP1	post-GPI attachment to proteins 1	2.049	down	1.531	down	1.082	down	T90703
ATP11C	ATPase, class VI, type 11C	1.129	down	2.059	down	2.138	down	AI371849
KLB	klotho beta	3.121	down	2.385	down	1.739	down	AI668605
PAH	phenylalanine hydroxylase	2.290	down	2.014	down	1.927	down	H47984
DIP2A	DIP2 disco-interacting protein 2 homolog A (Drosophila)	1.009	up	1.492	down	2.475	down	NM_015151
AK7	adenylate kinase 7	1.214	up	1.687	up	2.138	up	NM_152327
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	1.094	down	1.388	down	2.673	down	BC012536
HMGCLL1	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase-like 1	1.455	up	1.577	up	2.045	up	BC024194
ALDH1L2	aldehyde dehydrogenase 1 family, member L2	2.271	up	1.152	up	1.244	down	AI378916
RDH13	Transcribed locus /// Retinol dehydrogenase 13 (all-trans/9-	1.069	down	1.464	down	2.701	down	AL833150

Oxidoreductase activity (GO: 0016491)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
TXNRD1	thioredoxin reductase 1	1.923	up	2.042	up	2.089	up	NM_003330
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	2.006	up	1.217	up	1.068	down	NM_006636
ALDH3A2	aldehyde dehydrogenase 3 family, member A2	1.472	down	1.193	down	2.103	down	NM_000382
ACADM	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	1.162	down	1.650	down	2.070	down	NM_000016
ZMYM2	zinc finger, MYM-type 2	1.219	up	1.254	down	3.230	down	NM_003453
NNT	nicotinamide nucleotide transhydrogenase	1.286	down	1.681	down	2.233	down	U40490
NNT	nicotinamide nucleotide transhydrogenase	1.184	down	1.720	down	2.194	down	NM_012343
HMOX1	heme oxygenase (decycling) 1	1.163	up	3.001	up	1.157	down	NM_002133
GCLM	glutamate-cysteine ligase, modifier subunit	1.661	up	2.593	up	2.294	up	NM_002061
SUOX	sulfite oxidase	1.827	down	1.902	down	2.099	down	AA129776
AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	1.025	down	2.960	up	1.929	up	NM_001353
MAOA	monoamine oxidase A	2.013	down	1.440	down	2.112	down	NM_000240
DPYD	dihydropyrimidine dehydrogenase	1.353	down	1.438	down	2.100	down	NM_000110
ACADSB	acyl-Coenzyme A dehydrogenase, short/branched chain	1.527	down	1.438	down	2.124	down	NM_001609
ACOX2	acyl-Coenzyme A oxidase 2, branched chain	1.153	down	2.084	down	2.659	down	NM_003500
ETFDH	electron-transferring-flavoprotein dehydrogenase	1.084	up	2.168	down	2.031	down	NM_004453
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	5.999	up	2.807	up	5.714	up	NM_000499
FMO5	flavin containing monooxygenase 5	1.830	down	2.396	down	2.943	down	NM_001461
CYP4F11	cytochrome P450, family 4, subfamily F, polypeptide 11	1.188	down	2.818	up	1.034	up	NM_021187
DIO1	deiodinase, iodothyronine, type I	2.588	down	1.950	down	3.221	down	NM_000792
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	2.420	up	1.521	up	1.353	down	NM_000782
AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	1.250	up	2.295	up	1.584	up	NM_020299
AKR1D1	aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)	1.504	down	3.146	down	2.041	down	NM_005989
ADH6	alcohol dehydrogenase 6 (class V)	2.364	down	2.508	down	3.285	down	NM_000672
AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	1.390	down	2.073	up	1.485	up	AB018580
CBR1	carbonyl reductase 1	1.261	up	2.122	up	1.531	up	BC002511
AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.526	down	2.030	down	1.938	down	AI796120
AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.332	down	2.792	down	3.297	down	AA888589

AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.160	down	1.860	down	2.129	down	AF047020
HSDL2	hydroxysteroid dehydrogenase like 2	1.533	down	1.888	down	2.211	down	BC004331
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	1.022	down	2.604	up	1.746	up	U05598
SARDH	sarcosine dehydrogenase	1.643	down	2.455	down	1.608	down	AF162428
ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	1.182	down	1.409	down	2.310	down	M55905
SMOX	spermine oxidase	3.011	up	2.134	up	1.679	up	BC000669
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	1.076	down	2.787	up	1.736	up	M33376
JMJD1A	jumonji domain containing 1A	2.358	up	1.598	up	1.085	up	AA524505
DHRS2	dehydrogenase/reductase (SDR family) member 2	2.073	down	2.630	down	2.223	down	AK000345
ADH6	alcohol dehydrogenase 6 (class V)	2.125	down	2.462	down	2.645	down	H71135
AASS	aminoadipate-semialdehyde synthase	2.245	down	2.284	down	2.389	down	AK023446
FMO5	flavin containing monooxygenase 5	2.016	down	1.791	down	1.903	down	AK022172
HSDL2	Hydroxysteroid dehydrogenase like 2	1.682	down	1.744	down	2.166	down	AK023959
LOX	lysyl oxidase	2.098	up	1.495	up	1.491	up	L16895
AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	1.120	up	2.929	up	2.027	up	S68290
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9	1.109	up	1.156	down	2.048	up	M15331
SRD5A3	steroid 5 alpha-reductase 3	1.312	down	1.683	down	2.600	down	NM_024592
CYP20A1	cytochrome P450, family 20, subfamily A, polypeptide 1	1.139	down	1.200	down	2.300	down	NM_020674
CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1	1.872	up	1.035	up	2.236	down	NM_016593
FOXRED2	FAD-dependent oxidoreductase domain containing 2	2.074	down	1.878	down	1.462	down	NM_024955
UEVLD	UEV and lactate/malate dehydrogenase domains	1.056	down	1.444	down	2.401	down	NM_018314
ALDH6A1	aldehyde dehydrogenase 6 family, member A1	1.518	down	1.689	down	2.054	down	AW612403
ALOXE3	arachidonate lipoxygenase 3	1.309	up	1.208	up	2.111	up	AW003512
ETFDH	electron-transferring-flavoprotein dehydrogenase	1.152	up	2.096	down	2.648	down	S69232
EGLN1	egl nine homolog 1 (C. elegans)	2.026	up	1.484	up	1.037	up	AL117352
PBRM1	polybromo 1	1.376	down	1.965	down	2.409	down	AF197569
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	2.098	down	2.104	down	2.139	down	M15943
SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	2.226	up	1.998	up	1.603	up	AL121758
MSRB3	methionine sulfoxide reductase B3	1.434	down	1.230	down	2.120	down	AL048386
STEAP2	six transmembrane epithelial antigen of the prostate 2	1.149	up	1.351	down	2.176	down	BF680588
ZMYM2	zinc finger, MYM-type 2	1.115	up	1.371	down	2.748	down	AW340955
AOF1	amine oxidase (flavin containing) domain 1	1.187	down	2.323	down	3.585	down	BE348688
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.088	down	2.307	up	1.543	up	BE566894
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.505	down	3.297	up	1.513	up	BE566894
ALDH1L2	Aldehyde dehydrogenase 1 family, member L2	2.310	up	1.274	down	1.940	down	AI654224
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	2.752	down	3.001	down	5.745	down	AV651117
FOXRED2	FAD-dependent oxidoreductase domain containing 2	2.205	down	1.510	down	1.415	down	AK026975
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.108	up	2.116	up	1.424	up	AL135787
EPS15L1	epidermal growth factor receptor pathway substrate 15-like 1	2.216	down	1.379	down	1.655	down	AK023744
FOXRED2	FAD-dependent oxidoreductase domain containing 2	2.221	down	1.475	down	1.401	down	AL022313
MDH1	Malate dehydrogenase 1, NAD (soluble)	1.914	down	1.302	down	2.103	down	AW952547
GCLM	glutamate-cysteine ligase, modifier subunit	2.188	up	2.589	up	2.076	up	AI753488
JMJD2C	Jumonji domain containing 2C	3.164	up	1.313	up	1.058	up	AA766126
PAH	phenylalanine hydroxylase	2.290	down	2.014	down	1.927	down	H47984
AOF1	amine oxidase (flavin containing) domain 1	1.426	down	3.786	down	4.327	down	NM_153042
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	1.094	down	1.388	down	2.673	down	BC012536
SMOX	spermine oxidase	3.039	up	2.196	up	1.745	up	AY033891
ALDH1L2	aldehyde dehydrogenase 1 family, member L2	2.271	up	1.152	up	1.244	down	AI378916
RDH13	Transcribed locus /// Retinol dehydrogenase 13 (all-trans/9-	1.069	down	1.464	down	2.701	down	AL833150
CHDH	choline dehydrogenase	1.602	down	3.645	down	5.446	down	AA609488
AKR1C1	Aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	1.186	up	4.874	up	2.480	up	BC014579

Antioxidant activity (GO: 0016209)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
ALB	albumin	2.956	down	2.014	down	2.380	down	AF116645
ALB	albumin	2.011	down	1.804	down	2.785	down	M12523
SELS	selenoprotein S	2.280	up	1.551	up	1.129	up	AF328864
SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	2.226	up	1.998	up	1.603	up	AL121758

Ligase activity (GO: 0016874)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
		1.139	up	1.128	down	2.033	down	AFFX-BioB-3
WARS	tryptophanyl-tRNA synthetase	2.133	up	1.075	down	1.184	down	NM_004184
SARS	seryl-tRNA synthetase	2.248	up	1.046	up	1.002	down	NM_006513
ACSL3	acyl-CoA synthetase long-chain family member 3	2.613	up	1.164	down	1.259	down	AL525798
ACSL3	acyl-CoA synthetase long-chain family member 3	2.198	up	1.226	down	1.525	down	NM_004457
UBE3C	ubiquitin protein ligase E3C	1.012	down	1.538	down	2.447	down	NM_014671
MYCBP2	MYC binding protein 2	1.031	down	1.331	down	2.283	down	NM_015057
CARS	cysteinyl-tRNA synthetase	2.116	up	1.083	down	1.432	down	NM_001751
GCLC	glutamate-cysteine ligase, catalytic subunit	3.006	up	3.364	up	2.334	up	BF676980
GCLC	glutamate-cysteine ligase, catalytic subunit	3.093	up	3.503	up	2.490	up	NM_001498
RTCD1	RNA terminal phosphate cyclase domain 1	1.044	up	2.186	down	3.306	down	NM_003729
GCLM	glutamate-cysteine ligase, modifier subunit	1.661	up	2.593	up	2.294	up	NM_002061
ASNS	asparagine synthetase	3.194	up	1.253	up	1.525	down	NM_001673
MDM2	Mdm2 p53 binding protein homolog (mouse)	1.321	up	1.067	up	2.207	up	NM_002392
DZIP3	DAZ interacting protein 3, zinc finger	1.365	down	1.992	down	2.641	down	NM_014648
HERC4	hect domain and RLD 4	1.288	up	1.767	down	3.600	down	NM_015601
UBR5	ubiquitin protein ligase E3 component n-recognin 5	1.369	down	1.719	down	2.612	down	U69567
UBR5	ubiquitin protein ligase E3 component n-recognin 5	1.007	up	1.712	down	2.553	down	BF515424

CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	1.243	up	1.227	down	2.195	down	U26710
UBE3A	ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome)	1.252	up	1.224	down	2.873	down	U84404
UBE3A	ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome)	1.127	up	1.360	down	2.965	down	AF116702
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.349	up	2.141	down	5.681	down	NM_000819
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.576	up	1.777	down	2.952	down	BE966876
NEDD4L	neural precursor cell expressed, developmentally down-regulated 4-like	1.255	down	2.551	down	2.874	down	AB007899
CARS	cysteinyl-tRNA synthetase	2.210	up	1.079	up	1.081	down	AI769685
UBE3A	ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome)	1.216	up	1.336	down	3.346	down	AA527499
UBE3A	ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome)	1.289	up	1.223	down	2.793	down	AA160522
MARS	methionyl-tRNA synthetase	1.247	up	1.518	down	2.009	down	AA621558
MDM2	Mdm2 p53 binding protein homolog (mouse)	1.455	up	1.236	up	3.592	up	AJ276888
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.445	up	1.840	down	7.813	down	AF008655
HERC1	hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	1.021	down	1.518	down	2.163	down	NM_003922
QRSL1	glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1	1.360	up	2.633	down	2.374	down	AL136679
QRSL1	glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1	1.209	up	1.636	down	2.776	down	NM_018292
FARSB	phenylalanyl-tRNA synthetase, beta subunit	1.261	down	1.901	down	2.912	down	D84430
RNF19A	ring finger protein 19A	2.088	up	1.203	up	1.556	up	AB029316
KIAA1333	KIAA1333	1.311	down	2.066	down	3.542	down	AA887053
BIRC6	baculoviral IAP repeat-containing 6 (apollon)	1.165	down	1.599	down	2.778	down	AI017106
ZNRF1	zinc and ring finger 1	1.713	down	1.798	down	2.889	down	AI199541
HERC4	hect domain and RLD 4	1.128	up	1.920	down	4.270	down	AI819938
HERC4	hect domain and RLD 4	1.392	up	1.433	down	2.759	down	AB046813
TARSL2	threonyl-tRNA synthetase-like 2	1.068	down	1.275	down	2.315	down	AA442856
UBE2CBP	ubiquitin-conjugating enzyme E2C binding protein	1.270	down	1.367	down	2.085	down	AV692609
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	1.513	up	1.473	down	2.013	down	AV701750
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	1.136	up	1.304	down	2.447	down	AU157259
UBE2G2	ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast)	1.174	up	1.850	up	2.153	up	AL355686
BIRC6	baculoviral IAP repeat-containing 6 (apollon)	1.190	down	1.530	down	2.614	down	AK023788
UBR3	ubiquitin protein ligase E3 component n-recognin 3	1.161	down	1.078	down	2.201	down	BF577193
RNF125	ring finger protein 125	1.248	up	1.199	up	2.617	down	AI969697
GCLM	glutamate-cysteine ligase, modifier subunit	2.188	up	2.589	up	2.076	up	AI753488
HECTD1	HECT domain containing 1	1.043	down	1.309	down	2.132	down	AW207734
SH3RF2	SH3 domain containing ring finger 2	1.115	down	2.178	down	1.823	down	AW082633
UBE3C	ubiquitin protein ligase E3C	1.138	down	1.066	down	2.357	down	BC014029

Transferase activity (GO: 0016740)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
POLR2B	polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	1.137	down	1.188	down	2.222	down	NM_000938
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	2.734	up	1.041	up	1.414	down	AW157070
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	2.931	up	1.450	up	1.045	up	NM_005228
RIOK3	RIO kinase 3 (yeast)	1.213	up	1.946	down	2.741	down	AW006290
RIOK3	RIO kinase 3 (yeast)	1.513	up	2.094	down	3.054	down	AA725102
RIOK3	RIO kinase 3 (yeast)	1.149	up	2.315	down	3.454	down	NM_003831
NNMT	nicotinamide N-methyltransferase	3.624	up	1.082	up	1.229	down	NM_006169
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	2.464	up	2.463	up	1.605	up	NM_004566
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	1.701	down	1.558	down	2.120	down	AI356412
POLR2A	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	2.108	down	1.414	down	1.458	down	NM_000937
HK2	hexokinase 2	2.320	up	1.382	up	1.050	up	AI761561
GALNAC4S-6ST	B cell RAG associated protein	2.113	up	1.718	up	2.346	up	NM_014863
UPP1	uridine phosphorylase 1	2.569	up	1.336	up	1.700	down	NM_003364
HISPPD1	histidine acid phosphatase domain containing 1	1.162	down	2.194	down	3.871	down	NM_015216
EPHA2	EPH receptor A2	2.223	up	1.331	up	1.641	up	NM_004431
AGL	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (glycogen debranching enzyme, glycogen storage disease	1.787	down	2.420	down	5.394	down	NM_000645
BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	1.410	down	1.943	down	3.438	down	NM_001211
VRK1	vaccinia related kinase 1	1.013	down	2.396	down	3.805	down	NM_003384
GSTA1	glutathione S-transferase A1	2.079	down	1.233	up	1.005	up	NM_000846
ADK	adenosine kinase	1.325	down	1.334	down	2.266	down	NM_001123
KIAA0999	KIAA0999 protein	1.047	down	1.403	down	2.054	down	AA044154
FGFR3	fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	1.879	down	2.980	down	3.165	down	M58051
LCAT	lecithin-cholesterol acyltransferase	1.691	down	2.231	down	1.429	down	NM_000229
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1	1.575	down	1.578	down	2.069	down	NM_005424
CDC7	cell division cycle 7 homolog (S. cerevisiae)	1.468	down	1.454	down	2.150	down	NM_003503
PDPK1	3-phosphoinositide dependent protein kinase-1	2.170	up	1.127	down	1.106	down	NM_002613
TTK	TTK protein kinase	1.262	down	1.575	down	4.364	down	NM_003318
POLA1	polymerase (DNA directed), alpha 1	1.366	down	1.980	down	2.607	down	NM_016937
PLK3	polo-like kinase 3 (Drosophila)	2.079	up	1.148	up	1.390	up	NM_004073
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	1.279	up	1.035	down	2.603	up	NM_016817
NMT2	N-myristoyltransferase 2	1.622	down	2.120	down	3.518	down	AW293531

NMT2	N-myristoyltransferase 2	1.365	down	1.691	down	3.046	down	NM_004808
MAP3K8	mitogen-activated protein kinase kinase kinase 8	1.088	down	2.056	down	2.389	down	NM_005204
DDR2	discoidin domain receptor tyrosine kinase 2	2.797	up	1.470	down	1.419	down	NM_006182
PIGB	phosphatidylinositol glycan anchor biosynthesis, class B	1.547	down	1.879	down	3.543	down	NM_004855
CDS1	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	1.197	up	1.042	up	2.495	down	NM_001263
MAT1A	methionine adenosyltransferase I, alpha	2.170	down	1.046	down	1.155	down	NM_000429
JAK2	Janus kinase 2 (a protein tyrosine kinase)	1.100	down	1.961	down	2.308	down	NM_004972
JAK2	Janus kinase 2 (a protein tyrosine kinase)	1.052	down	1.177	down	2.221	down	AF001362
POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)	2.137	down	1.399	down	3.033	down	NM_002692
BHMT	betaine-homocysteine methyltransferase	2.470	down	1.144	down	1.410	down	NM_001713
PGGT1B	protein geranylgeranyltransferase type I, beta subunit	1.236	up	1.044	down	2.033	down	NM_005023
SULT2A1	sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	4.024	down	1.925	down	1.404	down	NM_003167
SULT2A1	sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	4.037	down	2.348	down	1.951	down	U08024
PDK1	pyruvate dehydrogenase kinase, isozyme 1	2.616	up	2.308	up	1.200	up	NM_002610
AGXT	alanine-glyoxylate aminotransferase	1.842	down	1.553	down	2.083	down	NM_016236
CDC2L5	cell division cycle 2-like 5 (cholinesterase-related cell division controller)	1.061	up	1.306	down	2.189	down	AJ297710
CDC2L5	cell division cycle 2-like 5 (cholinesterase-related cell division controller)	1.087	up	1.345	down	2.353	down	NM_003718
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	1.147	up	1.400	down	2.095	down	NM_003605
NCOA3	nuclear receptor coactivator 3	2.010	down	1.558	down	2.522	down	NM_006534
METTL7A	methyltransferase like 7A	2.456	down	2.277	down	1.227	down	NM_014033
TGM5	transglutaminase 5	1.108	up	1.527	up	2.197	up	NM_004245
SNF1LK	SNF1-like kinase	2.307	up	1.141	down	1.498	down	NM_030751
ACVR1B	activin A receptor, type IB	1.526	down	2.153	down	2.162	down	NM_020327
ATM	ataxia telangiectasia mutated	1.255	up	1.962	down	2.284	down	NM_000051
PRKDC	protein kinase, DNA-activated, catalytic polypeptide	1.217	down	1.651	down	2.001	down	U47077
GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	2.239	up	1.006	up	1.072	down	BC000498
NCOA3	nuclear receptor coactivator 3	1.682	down	1.441	down	2.025	down	AI438999
NCOA3	nuclear receptor coactivator 3	2.033	down	1.466	down	1.975	down	AI761748
NCOA1	nuclear receptor coactivator 1	1.294	down	1.367	down	2.010	down	BF576458
PIM1	pim-1 oncogene	2.034	up	1.060	down	1.155	up	M24779
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	1.010	down	1.683	down	2.539	down	AU153366
PPAT	phosphoribosyl pyrophosphate amidotransferase	1.013	down	1.446	down	2.656	down	AI457120
PPAT	phosphoribosyl pyrophosphate amidotransferase	1.010	down	1.272	down	2.987	down	U00238
ABAT	4-aminobutyrate aminotransferase	1.602	down	2.313	down	1.837	down	AF237813
ABAT	4-aminobutyrate aminotransferase	1.472	down	2.182	down	1.510	down	AF237813
TST	thiosulfate sulfurtransferase (rhodanese)	2.293	up	1.172	up	1.194	down	D87292
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	1.255	down	1.342	down	2.336	down	U51120
BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	1.343	down	2.096	down	6.372	down	AF043294
ATR	ataxia telangiectasia and Rad3 related	1.145	up	2.270	down	4.734	down	U49844
ATR	ataxia telangiectasia and Rad3 related	1.023	down	2.761	down	5.496	down	U49844
BMPR2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	1.010	down	1.347	down	2.066	down	U20165
SMG1	PI-3-kinase-related kinase SMG-1	1.069	up	1.689	down	2.856	down	U32581
HIPK3	homeodomain interacting protein kinase 3	1.175	up	1.083	up	2.126	down	AF305239
NAT8	N-acetyltransferase 8	2.594	down	1.699	down	1.711	down	AB013094
ST3GAL6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	1.170	down	1.467	down	3.209	down	AB022918
GYG2	glycogenin 2	1.895	down	2.074	down	1.922	down	U94364
NCOA3	nuclear receptor coactivator 3	2.467	down	1.977	down	3.126	down	U80737
SULT1C2	sulfotransferase family, cytosolic, 1C, member 2	1.679	down	1.421	down	2.058	down	AF186255
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.349	up	2.141	down	5.681	down	NM_000819
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.576	up	1.777	down	2.952	down	BE966876
ATM	ataxia telangiectasia mutated	1.043	up	1.911	down	2.756	down	U82828
GNPTAB	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	1.863	down	1.424	down	2.004	down	AK001821
ZDHHC17	zinc finger, DHHC-type containing 17	1.049	up	1.390	down	2.296	down	AI621223
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	1.108	up	1.481	down	2.462	down	AV682436
NEK1	NIMA (never in mitosis gene a)-related kinase 1	1.141	up	1.673	down	2.523	down	AV700007
DIMT1L	DIM1 dimethyladenosine transferase 1-like (S. cerevisiae)	1.423	up	2.191	down	6.168	down	W87688
MBOAT5	Membrane bound O-acyltransferase domain containing 5	2.206	up	1.252	up	1.527	up	AA773554
RABGGTB	Rab geranylgeranyltransferase, beta subunit	1.188	up	1.366	down	2.235	down	AA129753
MINK1	misshapen-like kinase 1 (zebrafish)	1.677	down	2.187	down	1.486	down	AI859060
ADH6	alcohol dehydrogenase 6 (class V)	2.125	down	2.462	down	2.645	down	H71135
CLK1	CDC-like kinase 1	1.163	up	1.680	down	2.922	down	AI251890
XYLB	xylulokinase homolog (H. influenzae)	1.126	down	1.467	down	2.459	down	AA777793
BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	1.307	down	1.614	down	2.964	down	AL137654
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	1.445	up	1.840	down	7.813	down	AF008655
CDC42BPB	CDC42 binding protein kinase beta (DMPK-like)	1.549	down	1.930	down	3.170	down	NM_006035
CMAS	cytidine monophosphate N-acetylneuraminic acid synthetase	1.132	down	2.268	down	6.334	down	NM_018686
SUV420H1	suppressor of variegation 4-20 homolog 1 (Drosophila)	1.000	down	2.298	down	2.273	down	NM_017635
UGCGL2	UDP-glucose ceramide glucosyltransferase-like 2	1.111	up	1.594	down	2.137	down	NM_020121
TRMT11	tRNA methyltransferase 11 homolog (S. cerevisiae)	1.106	down	1.279	down	3.249	down	NM_021820
C9orf95	chromosome 9 open reading frame 95	1.011	up	1.613	down	2.044	down	NM_017881
ZDHHC13	zinc finger, DHHC-type containing 13	1.251	down	2.581	down	3.432	down	NM_019028

CASD1	CAS1 domain containing 1	1.251	down	1.666	down	3.243	down	NM_022900
MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	2.151	down	1.141	down	1.541	down	NM_012214
BHMT2	betaine-homocysteine methyltransferase 2	2.095	down	1.135	down	1.329	down	NM_017614
C8orf44 /// SGK3	serum/glucocorticoid regulated kinase family, member 3 /// chromosome 8 open reading frame 44	1.288	down	1.365	down	2.383	down	NM_013257
PIGZ	phosphatidylinositol glycan anchor biosynthesis, class Z	3.391	up	1.536	up	1.018	down	NM_025163
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	2.834	down	1.426	down	1.783	down	NM_006892
TAOK3	TAO kinase 3	1.317	down	1.589	down	2.083	down	NM_016281
CSNK1G3	casein kinase 1, gamma 3	1.419	up	1.045	up	2.153	down	NM_004384
AGXT2L1	alanine-glyoxylate aminotransferase 2-like 1	1.739	down	2.133	down	1.703	down	NM_031279
ELP3	elongation protein 3 homolog (S. cerevisiae)	1.133	down	1.394	down	2.389	down	NM_018091
TPK1	thiamin pyrophosphokinase 1	2.070	down	1.507	down	1.287	down	NM_022445
FLJ20628	hypothetical protein FLJ20628	1.184	down	1.886	down	3.345	down	NM_017910
SOAT1	sterol O-acyltransferase (acyl-Coenzyme A: cholesterol acyltransferase) 1	1.508	down	1.459	down	2.269	down	L21934
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	1.201	up	1.186	down	3.723	down	BG035985
C3orf64	chromosome 3 open reading frame 64	1.664	down	1.421	down	2.348	down	AK023140
DPH5	DPH5 homolog (S. cerevisiae)	1.116	up	1.389	down	2.062	down	AI291720
SCLY	selenocysteine lyase	1.443	down	1.266	down	2.816	down	AA911739
GALNT7	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GalNAc-T7)	1.595	down	1.437	down	2.454	down	BF699855
REV1	REV1 homolog (S. cerevisiae)	1.006	up	1.496	down	2.250	down	N51427
ZDHHC2	zinc finger, DHHC-type containing 2	1.244	down	1.487	down	2.566	down	AK001608
AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	1.502	down	2.167	down	2.895	down	AI337300
AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	1.316	down	2.092	down	1.810	down	BC004219
POLK	polymerase (DNA directed) kappa	1.050	up	1.104	down	2.165	down	AF194973
TRPM7	transient receptor potential cation channel, subfamily M, member 7	1.162	down	1.448	down	3.046	down	AF346629
POLR1B	polymerase (RNA) I polypeptide B, 128kDa	1.069	up	1.066	down	2.326	down	BC004882
CHST9	carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9	2.830	down	3.440	down	1.648	down	AF239821
CHST9	carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9	2.816	down	3.455	down	1.452	down	AF332473
ETNK1	ethanolamine kinase 1	1.164	down	2.258	up	2.657	up	BC006111
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	2.640	up	3.480	up	1.424	up	BC006236
MYLK	myosin light chain kinase	2.165	down	2.109	down	1.953	down	AA526844
SMG1	PI-3-kinase-related kinase SMG-1	1.245	up	1.029	down	2.464	down	AK025794
SETD7	SET domain containing (lysine methyltransferase) 7	2.101	up	1.128	up	1.148	up	AK024846
BMPR2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	1.054	down	1.884	down	2.472	down	AI457436
ETNK1	ethanolamine kinase 1	1.020	down	1.221	down	2.055	down	AV692425
GPAM	glycerol-3-phosphate acyltransferase, mitochondrial	1.283	down	2.292	down	3.052	down	AV699379
GPAM	glycerol-3-phosphate acyltransferase, mitochondrial	1.032	down	1.928	down	4.011	down	AB046780
DDR2	discoidin domain receptor tyrosine kinase 2	2.797	up	1.996	down	2.069	down	AI799915
ALG2	asparagine-linked glycosylation 2 homolog (S. cerevisiae, alpha-1,3-mannosyltransferase)	2.091	up	1.529	up	1.261	up	BE967331
DGAT2	diacylglycerol O-acyltransferase homolog 2 (mouse)	2.275	down	1.916	down	1.969	down	AW469523
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	1.025	up	1.609	down	2.575	down	AI401379
CHST11	Carbohydrate (chondroitin 4) sulfotransferase 11	1.045	up	1.373	up	2.157	up	AI123348
PDK1	pyruvate dehydrogenase kinase, isozyme 1	2.151	up	1.713	up	1.110	down	AU146532
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	1.359	up	1.599	down	2.185	down	AV706396
ELP3	elongation protein 3 homolog (S. cerevisiae)	1.033	down	1.702	down	3.510	down	AI949204
B3GALTL	beta 1,3-galactosyltransferase-like	2.062	down	1.684	down	1.864	down	N51325
PDIK1L	PDLIM1 interacting kinase 1 like	1.140	down	1.183	down	2.043	down	AI806633
DDR2	discoidin domain receptor tyrosine kinase 2	2.950	up	2.066	down	2.135	down	W73819
SPTLC3	serine palmitoyltransferase, long chain base subunit 3	2.531	down	2.047	down	1.523	down	AA005105
CSNK1G3	casein kinase 1, gamma 3	1.622	up	1.015	up	2.429	down	AI073822
SEPSECS	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase	2.246	up	1.587	down	2.717	down	AI806592
ACVR2A	activin A receptor, type IIA	1.211	up	1.023	down	2.202	down	AI149508
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	3.652	up	2.441	up	2.313	up	AL038787
CLK4	CDC-like kinase 4	1.211	up	1.454	down	3.010	down	AW975057
NTRK3	neurotrophic tyrosine kinase, receptor, type 3	1.127	up	1.145	up	2.081	up	AI140305
PCM1	pericentriolar material 1	1.069	down	1.574	down	3.809	down	BE672700
PARP11	poly (ADP-ribose) polymerase family, member 11	1.101	up	1.573	down	2.059	down	AV747166
PGM2L1	phosphoglucomutase 2-like 1	1.105	down	1.321	down	3.986	down	AV724329
PGM2L1	phosphoglucomutase 2-like 1	1.506	down	1.736	down	4.059	down	AA736452
EXT1	exostoses (multiple) 1	1.012	up	1.766	down	3.978	down	AW189467
CHPT1	choline phosphotransferase 1	1.434	down	1.536	down	2.112	down	BF940025
PRKCB1	Protein kinase C, beta 1	1.000	down	1.308	up	2.034	up	AA724722
SGK2	Serum/glucocorticoid regulated kinase 2	1.980	down	1.229	down	2.344	down	AI631895
CROT	carnitine O-octanoyltransferase	2.101	down	1.247	up	1.261	up	BE674103
SEPSECS	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase	1.884	up	1.238	down	2.305	down	NM_016955
BMPR2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	1.119	down	1.339	down	3.233	down	AL046696
PCYT1B	phosphate cytidylyltransferase 1, choline, beta	2.011	down	2.056	down	1.294	down	AI857508
POLR1B	polymerase (RNA) I polypeptide B, 128kDa	1.094	up	1.166	down	3.131	down	AK025574
MAP3K2	mitogen-activated protein kinase kinase kinase 2	1.304	up	1.121	up	2.204	down	BG504375
SETDB2	SET domain, bifurcated 2	1.206	down	1.682	down	2.442	down	W65369
DDR2	Discoidin domain receptor tyrosine kinase 2	2.229	up	1.105	up	1.028	up	AA545764
ERN1	endoplasmic reticulum to nucleus signaling 1	2.390	up	1.216	up	1.486	down	AV704183
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	1.009	up	1.433	down	2.187	down	AU154663
PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit	1.268	up	1.656	down	2.247	down	AI928203
BMPR2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	1.182	down	1.543	down	2.462	down	BF247383
TNIK	TRAF2 and NCK interacting kinase	1.017	up	1.522	up	2.035	up	BF431017
PTK2	PTK2 protein tyrosine kinase 2	1.474	down	1.017	down	2.180	down	AA912743
PIK3C2A	Phosphoinositide-3-kinase, class 2, alpha polypeptide	1.094	up	1.924	up	2.655	up	AA579047
PIGB	phosphatidylinositol glycan anchor biosynthesis, class B	1.682	down	1.667	down	2.712	down	AA808203
MKNK1	MAP kinase interacting serine/threonine kinase 1	1.231	up	1.335	down	2.180	down	AW796364

SEPSECS	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase	1.808	up	1.599	down	3.008	down	BC023539
METTL6	methyltransferase like 6	1.345	up	1.578	down	2.581	down	NM_152396
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	1.123	up	1.480	down	2.396	down	NM_002645
AK7	adenylate kinase 7	1.214	up	1.687	up	2.138	up	NM_152327
POLR2B	polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	1.129	down	1.240	down	3.683	down	BE614461
PDSS2	prenyl (decaprenyl) diphosphate synthase, subunit 2	1.239	down	2.067	down	2.586	down	BC029491
CSNK1A1	Casein kinase 1, alpha 1	1.272	down	1.305	down	2.143	down	BQ025347
PAPOLG	Poly(A) polymerase gamma	1.045	up	1.215	up	2.001	up	R73588
LMTK3	lemur tyrosine kinase 3	2.188	down	2.158	down	1.476	down	BE868592
STK38	Serine/threonine kinase 38	1.449	down	1.229	down	2.255	down	BU617137
POLH	Polymerase (DNA directed), eta	2.006	down	1.107	down	1.310	up	AW665155
MAP3K13	Mitogen-activated protein kinase kinase kinase 13	2.589	down	2.328	down	2.270	down	BC026249
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	2.614	up	1.330	up	1.408	up	AF272982
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	2.339	up	1.309	up	1.441	up	AF272982
POLE4	Polymerase (DNA-directed), epsilon 4 (p12 subunit)	1.697	down	1.057	down	2.119	down	AY034104
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.833	up	1.780	up	2.861	up	AF277897
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.752	up	1.624	up	2.126	up	AF277897

Transcription activator activity (GO: 0016563 or 0003710)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
EGR1	early growth response 1	3.654	up	2.272	up	1.343	up	AV733950
EGR1	early growth response 1	3.159	up	1.750	up	1.093	up	NM_001964
MED13	mediator complex subunit 13	1.103	up	1.368	down	2.637	down	AI984051
HLTF	helicase-like transcription factor	1.280	down	1.466	down	3.218	down	AI760760
FOSL1	FOS-like antigen 1	2.132	up	1.707	up	1.557	up	BG251266
CEP290	centrosomal protein 290kDa	1.232	down	1.269	down	2.653	down	NM_014684
HNRNPD	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)	1.394	down	2.103	down	2.411	down	D55674
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	1.010	down	1.683	down	2.539	down	AU153366
ARID1A	AT rich interactive domain 1A (SWI-like)	1.614	down	2.065	down	1.716	down	AF231056
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	2.406	up	1.169	down	1.347	down	AL564683
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	1.369	down	1.443	down	2.249	down	AI652586
MED17	mediator complex subunit 17	1.128	up	1.101	down	2.014	down	AF105421
CEP290	centrosomal protein 290kDa	1.055	down	1.400	down	2.768	down	AF317887
KLF4	Kruppel-like factor 4 (gut)	3.223	up	2.387	up	1.299	up	BF514079
MED17	mediator complex subunit 17	1.305	up	1.055	down	2.213	down	AK001674
MED13	mediator complex subunit 13	1.398	up	2.072	up	2.226	up	AF151055
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	1.222	down	2.360	down	1.720	down	AA489681
EGR1	Early growth response 1	4.439	up	2.796	up	1.399	up	AI459194
KLF6	Homo sapiens, clone IMAGE:4096273, mRNA /// Kruppel-like factor 6	2.320	up	1.417	up	1.949	up	BU683415
PPARA	peroxisome proliferator-activated receptor alpha	1.933	down	1.436	down	2.386	down	AF086231

Transcription regulator activity (GO: 0030528)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
BHLHB2	basic helix-loop-helix domain containing, class B, 2	4.873	up	2.442	up	1.574	up	BG326045
BHLHB2	basic helix-loop-helix domain containing, class B, 2	10.183	up	4.456	up	2.401	up	NM_003670
ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1.018	up	1.662	down	2.254	down	NM_002166
ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1.117	up	1.483	down	2.567	down	D13891
MXI1	MAX interactor 1	2.243	up	1.549	up	1.282	up	NM_005962
PROX1	prospero homeobox 1	2.231	down	1.743	down	1.697	down	NM_002763
NCOA3	nuclear receptor coactivator 3	2.010	down	1.558	down	2.522	down	NM_006534
NCOA3	nuclear receptor coactivator 3	1.682	down	1.441	down	2.025	down	AI438999
NCOA3	nuclear receptor coactivator 3	2.033	down	1.466	down	1.975	down	AI761748
NCOA1	nuclear receptor coactivator 1	1.294	down	1.367	down	2.010	down	BF576458
TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	1.301	down	1.398	down	2.287	down	AA768906
MSC	musculin (activated B-cell factor-1)	2.022	up	1.319	up	1.184	up	AF060154
TAF5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa	1.117	down	1.547	down	3.311	down	AW138827
CNOT3	CCR4-NOT transcription complex, subunit 3	1.116	down	1.547	down	2.069	down	AF180474
NCOA3	nuclear receptor coactivator 3	2.467	down	1.977	down	3.126	down	U80737
ID2 /// ID2B	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein /// inhibitor of DNA binding 2B, dominant negative helix-loop-helix protein	1.201	up	1.207	down	2.401	down	AI819238
MYCL1	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	2.663	up	1.106	up	1.117	up	M19720
TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	1.662	down	1.855	down	2.281	down	X52078
HEY1	hairy/enhancer-of-split related with YRPW motif 1	2.249	up	1.777	up	1.103	down	NM_012258
HAND2	heart and neural crest derivatives expressed 2	1.181	up	1.283	up	2.060	up	NM_021973
ARNTL2	aryl hydrocarbon receptor nuclear translocator-like 2	1.361	down	1.340	down	2.075	down	NM_020183
MED17	mediator complex subunit 17	1.128	up	1.101	down	2.014	down	AF105421
PDCD6	Aryl-hydrocarbon receptor repressor	1.192	down	2.037	down	1.858	down	AI907083
HEY1	hairy/enhancer-of-split related with YRPW motif 1	2.292	up	1.833	up	1.244	down	R61374

LOC100131851 /// LOC100134497 /// LOC401002 /// LOC646674 /// SSBP3	single stranded DNA binding protein 3 /// similar to single stranded DNA binding protein 3 /// hypothetical LOC646674 /// hypothetical protein LOC100131851 /// hypothetical protein LOC100134497	1.962	down	2.110	down	2.354	down	AA102468
MED17	mediator complex subunit 17	1.305	up	1.055	down	2.213	down	AK001674
ARNTL2	aryl hydrocarbon receptor nuclear translocator-like 2	1.026	up	2.026	down	3.371	down	AF256215
SSBP3	single stranded DNA binding protein 3	2.062	down	2.387	down	1.687	down	BC003605
MXD1	MAX dimerization protein 1	2.766	up	1.080	down	1.192	down	AI188653
KIAA2018	KIAA2018	1.382	down	1.455	down	2.138	down	AI651814
KIAA2018	KIAA2018	2.067	down	2.071	down	2.541	down	AI962192
MXD1	MAX dimerization protein 1	2.475	up	1.240	up	1.680	up	AW071793
ZNF711	zinc finger protein 711	1.503	up	1.351	down	2.366	down	AU157017
ZFX	zinc finger protein, X-linked	1.141	down	2.179	down	2.395	down	AI745209
CNOT3	CCR4-NOT transcription complex, subunit 3	1.694	down	2.096	down	2.328	down	AW449353
TWIST2	twist homolog 2 (Drosophila)	1.534	down	1.611	down	2.534	down	AI086614
TCFL5	transcription factor-like 5 (basic helix-loop-helix)	1.714	down	1.531	down	2.002	down	N49233
RBAK	RB-associated KRAB zinc finger	1.409	up	1.097	up	2.239	down	BE618393
LOC100134475 /// LOC388720	ribosomal protein S27a /// ubiquitin B /// ubiquitin C /// similar to ubiquitin /// similar to rCG23287 /// hypothetical LOC100134475	1.028	up	1.419	down	2.725	down	AU152194
TAF5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa	1.346	down	1.859	down	4.546	down	NM_139052

Transcription factor activity (GO: 0003700 or 0000130)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
BHLHB2	basic helix-loop-helix domain containing, class B, 2	4.873	up	2.442	up	1.574	up	BG326045
BHLHB2	basic helix-loop-helix domain containing, class B, 2	10.183	up	4.456	up	2.401	up	NM_003670
ZFP36L2	zinc finger protein 36, C3H type-like 2	1.488	down	1.495	down	2.178	down	AI356398
ZFP36L2	zinc finger protein 36, C3H type-like 2	1.380	down	1.445	down	2.256	down	NM_006887
JUN	jun oncogene	3.152	up	2.251	up	2.502	up	BG491844
JUN	jun oncogene	3.198	up	1.877	up	1.943	up	NM_002228
JUNB	jun B proto-oncogene	2.284	up	1.261	up	1.330	down	NM_002229
EGR1	early growth response 1	3.654	up	2.272	up	1.343	up	AV733950
EGR1	early growth response 1	3.159	up	1.750	up	1.093	up	NM_001964
HDAC2	histone deacetylase 2	1.053	down	1.571	down	2.069	down	NM_001527
KLF10	Kruppel-like factor 10	3.211	up	1.303	up	1.142	down	NM_005655
ATF3	activating transcription factor 3	6.027	up	1.255	up	2.360	up	NM_001674
SYNE2	spectrin repeat containing, nuclear envelope 2	1.414	down	2.269	down	3.578	down	NM_015180
RB1	retinoblastoma 1 (including osteosarcoma)	1.074	down	1.620	down	3.527	down	NM_000321
NFIL3	nuclear factor, interleukin 3 regulated	2.349	up	1.049	down	1.320	down	NM_005384
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	1.180	down	1.508	down	2.030	down	NM_004364
CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	2.054	up	1.142	down	1.598	down	NM_001806
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	2.184	down	1.848	down	1.751	down	NM_000376
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	2.121	down	2.082	down	1.518	down	AA772285
SOLH	small optic lobes homolog (Drosophila)	1.413	down	1.482	down	2.233	down	AI796687
FOSL1	FOS-like antigen 1	2.132	up	1.707	up	1.557	up	BG251266
NR1D1 /// THRA	thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian) /// nuclear receptor subfamily 1, group D, member 1	1.069	up	1.746	down	3.079	down	NM_021724
NR2C1	nuclear receptor subfamily 2, group C, member 1	1.211	up	1.681	down	3.634	down	NM_003297
ATF5	activating transcription factor 5	1.450	down	1.859	down	2.456	down	BC005174
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	2.642	up	1.487	up	1.605	up	NM_012323
PAX6	paired box 6	2.375	up	1.282	up	1.157	down	NM_000280
PHTF1	putative homeodomain transcription factor 1	1.558	down	1.649	down	2.318	down	NM_006608
NR1H4	nuclear receptor subfamily 1, group H, member 4	1.383	up	1.559	down	2.188	down	NM_005123
SOX5	SRY (sex determining region Y)-box 5	3.953	down	1.946	down	1.701	down	NM_006940
ONECUT2	one cut homeobox 2	1.142	up	1.303	up	2.021	up	NM_004852
SNF1LK	SNF1-like kinase	2.307	up	1.141	down	1.498	down	NM_030751
RCAN1	regulator of calcineurin 1	2.003	up	1.221	down	1.434	up	NM_004414
TSC22D3	TSC22 domain family, member 3	4.384	up	1.040	down	1.041	up	AL110191
ATRX	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	1.102	down	2.100	down	3.690	down	AI650257
ATRX	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	1.098	down	1.783	down	3.366	down	U09820
ATRX	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	1.208	up	1.529	down	3.053	down	U72937
NR2F2	nuclear receptor subfamily 2, group F, member 2	2.113	down	1.754	down	1.661	down	AL037401
NR2F2	nuclear receptor subfamily 2, group F, member 2	2.161	down	2.041	down	1.518	down	M64497
TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	1.301	down	1.398	down	2.287	down	AA768906
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	2.901	up	2.046	up	1.273	up	BC004490
KLF5	Kruppel-like factor 5 (intestinal)	4.039	up	2.058	up	1.908	up	AF132818
KLF5	Kruppel-like factor 5 (intestinal)	2.876	up	1.834	up	2.452	up	AB030824
NR2F6	nuclear receptor subfamily 2, group F, member 6	1.425	down	1.679	down	2.657	down	BF000629
DDIT3	DNA-damage-inducible transcript 3	3.538	up	1.159	down	1.131	down	BC003637
MSC	musculin (activated B-cell factor-1)	2.022	up	1.319	up	1.184	up	AF060154
TAF5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa	1.117	down	1.547	down	3.311	down	AW138827
C2orf3	chromosome 2 open reading frame 3	2.006	down	1.162	down	1.101	down	BC000853
PHTF1	putative homeodomain transcription factor 1	1.632	down	2.427	down	4.811	down	BC002447
L3MBTL	l(3)mbt-like (Drosophila)	1.233	down	1.909	down	2.349	down	U89358
RORA	RAR-related orphan receptor A	2.355	up	1.999	up	1.412	up	U04897
RORA	RAR-related orphan receptor A	2.017	up	2.069	up	1.888	up	L14611
PBX2	pre-B-cell leukemia homeobox 2	1.654	down	1.285	down	2.018	down	BC003111

RB1	retinoblastoma 1 (including osteosarcoma)	1.053	up	1.565	up	2.023	up	M19701
MTA1	metastasis associated 1	1.319	down	1.978	down	3.173	down	BC006177
ZFP36L1	zinc finger protein 36, C3H type-like 1	1.074	down	1.931	down	3.046	down	BE620915
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	2.406	up	1.169	down	1.347	down	AL564683
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	1.746	down	2.096	down	1.623	down	AI684141
NFIB	nuclear factor I/B	1.660	down	1.893	down	2.028	down	AI186739
JUN	Jun oncogene	3.077	up	2.277	up	1.847	up	BE327172
MYCL1	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	2.663	up	1.106	up	1.117	up	M19720
NR2F2	nuclear receptor subfamily 2, group F, member 2	2.004	down	1.971	down	2.611	down	AL554245
RCAN1	regulator of calcineurin 1	2.268	up	1.350	up	1.372	up	AL049369
TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	1.662	down	1.855	down	2.281	down	X52078
PHTF1	putative homeodomain transcription factor 1	1.441	down	1.298	down	2.065	down	AA927671
C2orf3	chromosome 2 open reading frame 3	1.053	down	1.145	down	2.026	down	AC005034
SFXN3	sideroflexin 3	1.907	down	1.804	down	2.164	down	M95929
MDM2	Mdm2 p53 binding protein homolog (mouse)	1.455	up	1.236	up	3.592	up	AJ276888
SLC2A4RG	SLC2A4 regulator	1.868	down	2.117	down	2.405	down	NM_020062
HEY1	hairy/enhancer-of-split related with YRPW motif 1	2.249	up	1.777	up	1.103	down	NM_012258
SOX18	SRY (sex determining region Y)-box 18	1.453	down	1.416	down	4.719	down	NM_018419
ZSCAN16	zinc finger and SCAN domain containing 16	1.110	up	2.029	down	2.204	down	NM_025231
ALS2CR8	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 8	1.230	up	1.992	down	2.188	down	NM_024744
HAND2	heart and neural crest derivatives expressed 2	1.181	up	1.283	up	2.060	up	NM_021973
ELF5	E74-like factor 5 (ets domain transcription factor)	3.180	down	1.404	down	1.466	down	AF115403
ARNTL2	aryl hydrocarbon receptor nuclear translocator-like 2	1.361	down	1.340	down	2.075	down	NM_020183
KLF4	Kruppel-like factor 4 (gut)	3.223	up	2.387	up	1.299	up	BF514079
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	3.631	up	2.126	up	1.804	up	AL021977
HEY1	hairy/enhancer-of-split related with YRPW motif 1	2.292	up	1.833	up	1.244	down	R61374
SLC2A4RG	SLC2A4 regulator	1.898	down	2.123	down	2.503	down	BE898559
ZHX1	zinc fingers and homeoboxes 1	1.237	down	1.529	down	2.005	down	AF195766
ARNTL2	aryl hydrocarbon receptor nuclear translocator-like 2	1.026	up	2.026	down	3.371	down	AF256215
KLF3	Kruppel-like factor 3 (basic)	1.349	up	2.566	down	1.883	down	AA130132
FOSL2	FOS-like antigen 2	2.369	up	1.123	down	1.117	up	AI670862
CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	2.485	up	1.025	up	1.203	down	BE622659
MXD1	MAX dimerization protein 1	2.766	up	1.080	down	1.192	down	AI188653
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	1.222	down	2.360	down	1.720	down	AA489681
FOXP4	forkhead box P4	1.248	down	2.109	down	1.990	down	AI673539
NFXL1	nuclear transcription factor, X-box binding-like 1	2.497	up	1.202	up	1.101	down	AI743731
EGR1	Early growth response 1	4.439	up	2.796	up	1.399	up	AI459194
THRB	thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)	1.424	down	2.592	down	2.517	down	BG494007
MXD1	MAX dimerization protein 1	2.475	up	1.240	up	1.680	up	AW071793
NR2F2	nuclear receptor subfamily 2, group F, member 2	1.446	down	1.650	down	2.295	down	AI420144
ZNF367	zinc finger protein 367	2.053	down	1.542	down	1.874	down	N62196
IRX3	iroquois homeobox 3	2.134	down	1.449	down	1.261	down	AI681917
THRB	thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)	1.081	down	2.242	down	2.434	down	BF431989
IKZF4	IKAROS family zinc finger 4 (Eos)	1.646	down	1.704	down	2.933	down	BF115531
TCFL5	transcription factor-like 5 (basic helix-loop-helix)	1.714	down	1.531	down	2.002	down	N49233
PAX6	paired box 6	3.018	up	1.230	down	1.496	down	AW088232
ZNF83	zinc finger protein 83	2.268	down	1.174	up	1.528	down	AI831874
FOXA1	Forkhead box A1	1.051	up	1.653	down	2.592	down	AI693336
CEP110	centrosomal protein 110kDa	1.176	up	1.518	down	3.861	down	AA642477
DLX6	distal-less homeobox 6	1.177	down	1.201	down	2.034	down	AA040332
ZNF452	zinc finger protein 452	2.140	up	1.234	up	1.030	down	AA480069
TAF5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa	1.346	down	1.859	down	4.546	down	NM_139052
NR1H4	nuclear receptor subfamily 1, group H, member 4	1.530	up	1.842	down	2.382	down	AF478446
ATF3	activating transcription factor 3	2.557	up	1.068	up	1.364	up	AB066566
ZKSCAN1	zinc finger with KRAB and SCAN domains 1	1.163	up	2.113	down	1.761	down	BG761185
PPARA	peroxisome proliferator-activated receptor alpha	1.933	down	1.436	down	2.386	down	AF086231

Transcription cofactor activity (GO: 0003712)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
BTG1	B-cell translocation gene 1, anti-proliferative	2.930	up	1.047	up	1.192	up	AL535380
BTG1	B-cell translocation gene 1, anti-proliferative	2.309	up	1.018	up	1.100	down	NM_001731
MED13	mediator complex subunit 13	1.103	up	1.368	down	2.637	down	AI984051
NMI	N-myc (and STAT) interactor	1.827	down	1.326	down	2.992	down	NM_004688
MED17	mediator complex subunit 17	1.128	up	1.101	down	2.014	down	AF105421
MED17	mediator complex subunit 17	1.305	up	1.055	down	2.213	down	AK001674
MED13	mediator complex subunit 13	1.398	up	2.072	up	2.226	up	AF151055
MXD1	MAX dimerization protein 1	2.766	up	1.080	down	1.192	down	AI188653
MXD1	MAX dimerization protein 1	2.475	up	1.240	up	1.680	up	AW071793

Immune response (GO: 0006955)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
SQSTM1	sequestosome 1	1.692	up	2.079	up	1.024	up	NM_003900
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	2.220	up	1.166	up	1.316	down	NM_000574
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	2.020	down	1.201	down	1.339	up	BC002666
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	2.280	down	1.145	down	1.166	up	NM_002053
IKBKAP	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	2.042	down	1.709	down	1.646	down	AF153419
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	2.460	down	1.714	down	1.728	down	U57059

TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	2.450	down	1.722	down	1.252	down	NM_003810
IL8	interleukin 8	6.950	up	2.145	up	3.413	up	NM_000584
IL1R1	interleukin 1 receptor, type I	3.140	down	1.786	down	2.827	down	NM_000877
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	1.122	down	2.041	down	1.035	down	U53470
NFIL3	nuclear factor, interleukin 3 regulated	2.349	up	1.049	down	1.320	down	NM_005384
CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	2.054	up	1.142	down	1.598	down	NM_001806
FAS	Fas (TNF receptor superfamily, member 6)	1.446	down	1.173	up	2.096	up	AA164751
FAS	Fas (TNF receptor superfamily, member 6)	1.474	down	1.071	up	2.062	up	NM_000043
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	1.133	down	1.582	down	2.306	down	BE856546
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	1.279	up	1.035	down	2.603	up	NM_016817
IL1B	interleukin 1, beta	1.344	up	1.289	up	2.118	up	NM_000576
LIF	leukemia inhibitory factor (cholinergic differentiation factor)	2.641	up	1.123	up	1.352	up	NM_002309
CCL20	chemokine (C-C motif) ligand 20	3.979	up	1.591	up	3.208	up	NM_004591
CD28	CD28 molecule	1.141	down	1.957	down	2.025	down	NM_006139
LY96	lymphocyte antigen 96	1.324	up	1.077	up	2.278	down	NM_015364
IL7	interleukin 7	1.684	down	1.497	down	2.323	down	NM_000880
CD209	CD209 molecule	1.138	up	1.306	up	2.016	up	AF290886
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	2.483	up	1.364	up	1.671	up	NM_001561
SNF1LK	SNF1-like kinase	2.307	up	1.141	down	1.498	down	NM_030751
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	1.243	up	1.227	down	2.195	down	U26710
IL1RL1	interleukin 1 receptor-like 1	1.325	up	1.576	up	2.156	up	AB012701
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	1.389	down	1.674	down	2.208	down	AB015706
TNFSF11	tumor necrosis factor (ligand) superfamily, member 11	1.030	up	1.392	up	2.015	up	AB037599
IL8	interleukin 8	2.970	up	1.498	up	2.025	up	AF043337
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	2.406	up	1.169	down	1.347	down	AL564683
SQSTM1	sequestosome 1	2.141	up	2.449	up	1.098	up	N30649
ERAP1	endoplasmic reticulum aminopeptidase 1	1.235	up	1.554	down	2.142	down	BE551138
FAS	Fas (TNF receptor superfamily, member 6)	1.377	down	1.021	down	2.047	up	Z70519
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.127	up	1.512	down	2.896	down	NM_014314
IFIH1	interferon induced with helicase C domain 1	1.470	down	1.664	down	2.942	down	NM_022168
C5AR1	complement component 5a receptor 1	5.501	up	1.149	up	1.297	down	NM_001736
HAMP	hepcidin antimicrobial peptide	1.212	down	2.513	down	4.906	down	NM_021175
FAIM3	Fas apoptotic inhibitory molecule 3	1.034	down	1.105	up	2.007	up	AF057557
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.310	down	2.293	down	3.371	down	AK023661
CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	2.485	up	1.025	up	1.203	down	BE622659
ORAI1	ORAI calcium release-activated calcium modulator 1	1.372	down	1.632	down	2.019	down	AL530596
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	1.513	up	1.473	down	2.013	down	AV701750
TICAM2 /// TMED7	transmembrane emp24 protein transport domain containing 7 /// toll-like receptor adaptor molecule 2	3.087	up	1.789	up	1.417	up	AI423165
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	3.080	down	1.506	down	1.269	up	AW014593
IL1F8	interleukin 1 family, member 8 (eta)	1.548	up	1.266	up	2.374	up	NM_014438
MASP1	mannan-binding lectin serine peptidase 1 (C4/C2 activating component of Ra-reactive factor)	2.002	up	1.262	up	1.221	down	AI274095
RNF125	ring finger protein 125	1.248	up	1.199	up	2.617	down	AI969697
TICAM2 /// TMED7	transmembrane emp24 protein transport domain containing 7 /// toll-like receptor adaptor molecule 2	2.133	up	1.627	up	1.209	up	AI400110
CLEC7A	C-type lectin domain family 7, member A	1.291	up	1.191	up	2.187	up	BC013385
IL23A	Enhancer of polycomb homolog 1 (Drosophila)	1.100	up	1.287	up	2.344	up	AJ296370

Metabolic process (GO: 0008152)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
ACSL3	acyl-CoA synthetase long-chain family member 3	2.613	up	1.164	down	1.259	down	AL525798
ACSL3	acyl-CoA synthetase long-chain family member 3	2.198	up	1.226	down	1.525	down	NM_004457
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	2.006	up	1.217	up	1.068	down	NM_006636
ALDH3A2	aldehyde dehydrogenase 3 family, member A2	1.472	down	1.193	down	2.103	down	NM_000382
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	2.464	up	2.463	up	1.605	up	NM_004566
ACADM	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	1.162	down	1.650	down	2.070	down	NM_000016
NNT	nicotinamide nucleotide transhydrogenase	1.286	down	1.681	down	2.233	down	U40490
NNT	nicotinamide nucleotide transhydrogenase	1.184	down	1.720	down	2.194	down	NM_012343
AGL	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (glycogen debranching enzyme, glycogen storage disease	1.787	down	2.420	down	5.394	down	NM_000645
GSTA1	glutathione S-transferase A1	2.079	down	1.233	up	1.005	up	NM_000846
DPYD	dihydropyrimidine dehydrogenase	1.353	down	1.438	down	2.100	down	NM_000110
ASNS	asparagine synthetase	3.194	up	1.253	up	1.525	down	NM_001673
PSPH	phosphoserine phosphatase	1.489	up	1.791	down	2.329	down	NM_003832
MAN2A1	mannosidase, alpha, class 2A, member 1	1.128	down	1.337	down	2.255	down	NM_002372
PSPH	phosphoserine phosphatase	1.523	up	1.612	down	2.241	down	NM_004577
ATP7A	ATPase, Cu++ transporting, alpha polypeptide (Menkes syndrome)	1.360	up	1.637	down	3.411	down	NM_000052
ACADSB	acyl-Coenzyme A dehydrogenase, short/branched chain	1.527	down	1.438	down	2.124	down	NM_001609
ACOX2	acyl-Coenzyme A oxidase 2, branched chain	1.153	down	2.084	down	2.659	down	NM_003500
AGXT	alanine-glyoxylate aminotransferase	1.842	down	1.553	down	2.083	down	NM_016236
EYA4	eyes absent homolog 4 (Drosophila)	2.016	up	1.037	down	1.166	down	NM_004100
ADH6	alcohol dehydrogenase 6 (class V)	2.364	down	2.508	down	3.285	down	NM_000672
SH3PXD2A	SH3 and PX domains 2A	1.314	down	1.656	down	2.815	down	NM_014631
METTL7A	methyltransferase like 7A	2.456	down	2.277	down	1.227	down	NM_014033
CBR1	carbonyl reductase 1	1.261	up	2.122	up	1.531	up	BC002511
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1.298	down	1.663	down	3.943	down	M95541
AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.526	down	2.030	down	1.938	down	AI796120
AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.332	down	2.792	down	3.297	down	AA888589
AMACR /// C1QTNF3	alpha-methylacyl-CoA racemase /// C1q and tumor necrosis factor related protein 3	2.160	down	1.860	down	2.129	down	AF047020
PPAT	phosphoribosyl pyrophosphate amidotransferase	1.013	down	1.446	down	2.656	down	AI457120
PPAT	phosphoribosyl pyrophosphate amidotransferase	1.010	down	1.272	down	2.987	down	U00238

HSDL2	hydroxysteroid dehydrogenase like 2	1.533	down	1.888	down	2.211	down	BC004331
ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	1.182	down	1.409	down	2.310	down	M55905
NAT8	N-acetyltransferase 8	2.594	down	1.699	down	1.711	down	AB013094
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	1.366	down	1.458	down	2.599	down	AF189723
GPR56	G protein-coupled receptor 56	1.055	up	1.952	down	2.432	down	AL554008
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	1.168	down	1.239	down	2.521	down	AK001684
CBS	cystathionine-beta-synthase	1.487	up	1.747	down	2.787	down	BE613178
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1.017	up	1.621	down	6.612	down	AW576457
ATP8A1	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	1.324	down	1.446	down	2.291	down	AI769688
DHRS2	dehydrogenase/reductase (SDR family) member 2	2.073	down	2.630	down	2.223	down	AK000345
ADH6	alcohol dehydrogenase 6 (class V)	2.125	down	2.462	down	2.645	down	H71135
CAPN3	calpain 3, (p94)	1.624	down	1.232	down	2.947	down	AF127764
IREB2	iron-responsive element binding protein 2	1.122	down	1.117	down	2.647	down	AI204981
AASS	aminoadipate-semialdehyde synthase	2.245	down	2.284	down	2.389	down	AK023446
HSDL2	Hydroxysteroid dehydrogenase like 2	1.682	down	1.744	down	2.166	down	AK023959
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1.133	down	1.433	down	5.800	down	L14561
MDM2	Mdm2 p53 binding protein homolog (mouse)	1.455	up	1.236	up	3.592	up	AJ276888
UBA5	ubiquitin-like modifier activating enzyme 5	1.014	up	1.696	down	2.878	down	NM_024818
ACTR6	ARP6 actin-related protein 6 homolog (yeast)	1.089	up	1.776	down	5.466	down	NM_022496
C12orf5	chromosome 12 open reading frame 5	1.011	up	1.190	up	2.115	up	NM_020375
THNSL1	threonine synthase-like 1 (S. cerevisiae)	1.093	up	1.845	down	2.667	down	NM_024838
UEVLD	UEV and lactate/malate dehydrogenase domains	1.056	down	1.444	down	2.401	down	NM_018314
ELP3	elongation protein 3 homolog (S. cerevisiae)	1.133	down	1.394	down	2.389	down	NM_018091
ALDH6A1	aldehyde dehydrogenase 6 family, member A1	1.518	down	1.689	down	2.054	down	AW612403
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	1.201	up	1.186	down	3.723	down	BG035985
DPH5	DPH5 homolog (S. cerevisiae)	1.116	up	1.389	down	2.062	down	AI291720
SCLY	selenocysteine lyase	1.443	down	1.266	down	2.816	down	AA911739
UBA5	ubiquitin-like modifier activating enzyme 5	1.146	up	1.408	down	2.468	down	AW516242
UBA5	ubiquitin-like modifier activating enzyme 5	1.108	up	1.653	down	2.180	down	BE549973
SRR	serine racemase	1.386	up	1.400	down	2.242	down	AF169974
AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	1.502	down	2.167	down	2.895	down	AI337300
AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	1.316	down	2.092	down	1.810	down	BC004219
PNPLA8	patatin-like phospholipase domain containing 8	1.417	up	1.575	down	2.943	down	BG025248
PNPLA8	patatin-like phospholipase domain containing 8	1.729	up	1.896	down	9.544	down	AF217519
ARSD	arylsulfatase D	1.236	down	1.704	down	2.220	down	BC003660
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	2.098	down	2.104	down	2.139	down	M15943
PNPLA8	patatin-like phospholipase domain containing 8	1.436	up	2.117	down	6.806	down	AB041261
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	2.640	up	3.480	up	1.424	up	BC006236
GPAM	glycerol-3-phosphate acyltransferase, mitochondrial	1.283	down	2.292	down	3.052	down	AV699379
GPAM	glycerol-3-phosphate acyltransferase, mitochondrial	1.032	down	1.928	down	4.011	down	AB046780
STEAP2	six transmembrane epithelial antigen of the prostate 2	1.149	up	1.351	down	2.176	down	BF680588
IREB2	iron-responsive element binding protein 2	1.037	up	1.232	down	3.254	down	BF438417
MAN2A1	mannosidase, alpha, class 2A, member 1	1.085	down	1.592	down	2.777	down	AV700323
ATP11C	ATPase, class VI, type 11C	1.305	down	1.876	down	4.430	down	BF475862
AOF1	amine oxidase (flavin containing) domain 1	1.187	down	2.323	down	3.585	down	BE348688
ELP3	elongation protein 3 homolog (S. cerevisiae)	1.033	down	1.702	down	3.510	down	AI949204
SPTLC3	serine palmitoyltransferase, long chain base subunit 3	2.531	down	2.047	down	1.523	down	AA005105
TMEM68	transmembrane protein 68	1.266	down	1.701	up	2.037	up	AI671172
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	3.652	up	2.441	up	2.313	up	AL038787
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.088	down	2.307	up	1.543	up	BE566894
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.505	down	3.297	up	1.513	up	BE566894
PHOSPHO2	phosphatase, orphan 2	1.123	up	1.641	down	2.305	down	AA769615
ALDH1L2	Aldehyde dehydrogenase 1 family, member L2	2.310	up	1.274	down	1.940	down	AI654224
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	2.752	down	3.001	down	5.745	down	AV651117
LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.108	up	2.116	up	1.424	up	AL135787
ARSB	arylsulfatase B	1.172	down	1.004	down	2.377	down	AW168942
KIAA1161	KIAA1161	2.278	down	1.814	down	1.845	down	AB032987
MAN2A1	mannosidase, alpha, class 2A, member 1	1.232	down	1.810	down	3.223	down	AA029155
MDH1	Malate dehydrogenase 1, NAD (soluble)	1.914	down	1.302	down	2.103	down	AW952547
ARSK	arylsulfatase family, member K	1.214	down	2.478	down	4.286	down	AI243677
ATP11C	ATPase, class VI, type 11C	1.129	down	2.059	down	2.138	down	AI371849
PAH	phenylalanine hydroxylase	2.290	down	2.014	down	1.927	down	H47984
DIP2A	DIP2 disco-interacting protein 2 homolog A (Drosophila)	1.009	up	1.492	down	2.475	down	NM_015151
PITPNM2	phosphatidylinositol transfer protein, membrane-associated 2	1.002	up	1.157	down	2.397	down	AL133612
AOF1	amine oxidase (flavin containing) domain 1	1.426	down	3.786	down	4.327	down	NM_153042
AK7	adenylate kinase 7	1.214	up	1.687	up	2.138	up	NM_152327
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	1.094	down	1.388	down	2.673	down	BC012536
HMGCLL1	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase-like 1	1.455	up	1.577	up	2.045	up	BC024194
IREB2	iron-responsive element binding protein 2	1.020	up	2.028	up	2.697	up	BC017880
ALDH1L2	aldehyde dehydrogenase 1 family, member L2	2.271	up	1.152	up	1.244	down	AI378916
RDH13	Transcribed locus /// Retinol dehydrogenase 13 (all-trans/9-	1.069	down	1.464	down	2.701	down	AL833150

Cellular metabolic process (GO: 0044237)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	1.359	up	1.599	down	2.185	down	AV706396

Apoptosis (GO: 0006915 or 0008632)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
BAD	BCL2-antagonist of cell death	1.153	down	1.267	down	2.033	down	U66879
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	4.316	up	1.798	up	1.020	down	BF594446
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	2.469	up	1.553	up	1.173	up	AI275690
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	2.629	up	1.515	up	1.059	up	NM_021960
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	1.231	down	1.397	down	2.292	down	NM_022037
SQSTM1	sequestosome 1	1.692	up	2.079	up	1.024	up	NM_003900
IER3	immediate early response 3	2.460	up	1.787	up	1.678	up	NM_003897
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	2.056	up	1.814	up	1.270	up	U15174
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	2.016	up	1.680	up	1.106	up	NM_004052

PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	3.905	up	1.177	up	1.390	up	NM_014330
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	2.460	down	1.714	down	1.728	down	U57059
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	2.450	down	1.722	down	1.252	down	NM_003810
DDIT4	DNA-damage-inducible transcript 4	4.248	up	1.447	down	2.657	down	NM_019058
BAG5	BCL2-associated athanogene 5	1.052	down	1.332	down	2.029	down	AA457021
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	1.122	down	2.041	down	1.035	down	U53470
GADD45A	growth arrest and DNA-damage-inducible, alpha	2.637	up	1.374	up	2.036	up	NM_001924
BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	1.410	down	1.943	down	3.438	down	NM_001211
SHB	Src homology 2 domain containing adaptor protein B	2.664	up	1.465	up	1.483	up	AL138752
SHB	Src homology 2 domain containing adaptor protein B	2.316	up	1.493	up	1.279	up	NM_003028
FAS	Fas (TNF receptor superfamily, member 6)	1.446	down	1.173	up	2.096	up	AA164751
FAS	Fas (TNF receptor superfamily, member 6)	1.474	down	1.071	up	2.062	up	NM_000043
LOC652755 /// NAIP	NLR family, apoptosis inhibitory protein /// similar to Baculoviral IAP repeat-containing protein 1 (Neuronal apoptosis inhibitory protein)	1.062	down	1.760	down	2.653	down	NM_004536
IL1B	interleukin 1, beta	1.344	up	1.289	up	2.118	up	NM_000576
HIP1	huntingtin interacting protein 1	1.056	down	2.391	down	2.131	down	NM_005338
HIP1	huntingtin interacting protein 1	1.521	down	2.625	down	2.007	down	U79734
JAK2	Janus kinase 2 (a protein tyrosine kinase)	1.100	down	1.961	down	2.308	down	NM_004972
JAK2	Janus kinase 2 (a protein tyrosine kinase)	1.052	down	1.177	down	2.221	down	AF001362
FASTKD2	FAST kinase domains 2	1.079	down	1.558	down	2.445	down	NM_014929
GADD45B	growth arrest and DNA-damage-inducible, beta	4.218	up	1.197	up	1.296	up	NM_015675
GML	glycosylphosphatidylinositol anchored molecule like protein	1.142	up	1.504	up	2.627	up	NM_002066
PTPRH	protein tyrosine phosphatase, receptor type, H	2.082	up	1.163	down	1.082	down	NM_002842
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	2.272	up	1.273	up	1.976	up	BC001281
GADD45B	growth arrest and DNA-damage-inducible, beta	3.110	up	1.274	up	1.353	up	AF087853
GADD45B	growth arrest and DNA-damage-inducible, beta	3.028	up	1.262	up	1.168	up	AF078077
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	1.255	down	1.342	down	2.336	down	U51120
HIPK3	homeodomain interacting protein kinase 3	1.175	up	1.083	up	2.126	down	AF305239
P2RX1	purinergic receptor P2X, ligand-gated ion channel, 1	2.459	up	1.248	up	1.381	up	U45448
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	2.340	up	1.197	up	1.775	up	AF153687
BIRC3	baculoviral IAP repeat-containing 3	4.176	up	1.834	down	1.215	up	U37546
RTEL1 /// TNFRSF6B	tumor necrosis factor receptor superfamily, member 6b, decoy /// regulator of telomere elongation helicase 1	1.383	down	1.168	down	2.023	down	BC000673
SQSTM1	sequestosome 1	2.141	up	2.449	up	1.098	up	N30649
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	3.073	up	1.776	up	1.102	up	BF981280
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	2.450	up	1.558	up	1.089	up	H71805
FAS	Fas (TNF receptor superfamily, member 6)	1.377	down	1.021	down	2.047	up	Z70519
PHLDA1	pleckstrin homology-like domain, family A, member 1	2.521	up	1.747	up	1.066	up	AA576961
PHLDA1	pleckstrin homology-like domain, family A, member 1	3.528	up	1.722	up	1.058	down	AI795908
PHLDA1	pleckstrin homology-like domain, family A, member 1	2.983	up	1.225	up	1.015	down	NM_007350
PHLDA1	pleckstrin homology-like domain, family A, member 1	3.207	up	1.953	up	1.120	up	NM_007350
TRIB3	tribbles homolog 3 (Drosophila)	4.387	up	1.360	up	1.307	down	NM_021158
RHOT1	ras homolog gene family, member T1	1.106	down	1.679	down	2.252	down	NM_018307
TNFRSF12	tumor necrosis factor receptor superfamily, member 12A	2.217	up	1.708	up	1.225	up	NM_016639
C8orf4	chromosome 8 open reading frame 4	2.776	up	1.120	down	1.144	up	NM_020130
EAF2	ELL associated factor 2	1.293	up	1.375	down	2.669	down	NM_018456
FAIM	Fas apoptotic inhibitory molecule	1.771	down	1.240	down	2.008	down	NM_018147
CIDEB	cell death-inducing DFFA-like effector b	2.193	down	1.511	down	1.876	down	NM_014430
FAM130A1	family with sequence similarity 130, member A1	2.111	up	1.026	down	1.047	down	NM_030809
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	3.600	up	2.612	up	1.207	up	AL132665
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	3.066	up	2.085	up	1.591	up	AF060922
RHOT1	ras homolog gene family, member T1	1.030	up	1.530	down	2.158	down	BF688108
PDCD6	Aryl-hydrocarbon receptor repressor	1.192	down	2.037	down	1.858	down	AI907083
PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	3.341	up	1.056	up	1.267	up	U83981
BIRC6	baculoviral IAP repeat-containing 6 (apollon)	1.165	down	1.599	down	2.778	down	AI017106
PHLDA1	pleckstrin homology-like domain, family A, member 1	2.510	up	1.067	down	1.014	down	AK026181
ADAMTSL4	ADAMTS-like 4	1.133	down	1.934	down	2.175	down	AF217974
HIP1	Huntingtin interacting protein 1	1.442	down	2.116	down	2.064	down	AU145049
UNC5B	unc-5 homolog B (C. elegans)	5.380	up	1.158	down	1.134	down	AK022859
DIDO1	death inducer-obliterator 1	1.459	down	4.271	down	2.535	down	AW664953
BIRC6	baculoviral IAP repeat-containing 6 (apollon)	1.190	down	1.530	down	2.614	down	AK023788
ERN1	endoplasmic reticulum to nucleus signaling 1	2.390	up	1.216	up	1.486	down	AV704183
CCAR1	Cell division cycle and apoptosis regulator 1	1.877	down	2.940	down	2.270	down	W73136
RTKN	Rhotekin	1.282	down	1.883	down	2.533	down	AA403118
SHB	Src homology 2 domain containing adaptor protein B	2.545	up	1.127	up	1.056	up	AI589280
TRIB3	tribbles homolog 3 (Drosophila)	4.397	up	1.119	up	1.383	down	AF250311
SHB	Src homology 2 domain containing adaptor protein B	3.006	up	1.931	up	1.557	up	BU685917

Cellular metabolic process (GO: 0044237)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	1.359	up	1.599	down	2.185	down	AV706396

Stress response (GO: 0006950)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
HSPA1A /// HSPA1B	heat shock 70kDa protein 1A /// heat shock 70kDa protein 1B	2.533	down	3.345	up	1.457	up	NM_005345
HSPA1A /// HSPA1B	heat shock 70kDa protein 1A /// heat shock 70kDa protein 1B	1.764	down	3.243	up	1.409	up	NM_005345
DUSP1	dual specificity phosphatase 1	3.816	up	2.000	up	2.086	up	NM_004417
SQSTM1	sequestosome 1	1.692	up	2.079	up	1.024	up	NM_003900
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	2.734	up	1.041	up	1.414	down	AW157070
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	2.931	up	1.450	up	1.045	up	NM_005228

PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	3.905	up	1.177	up	1.390	up	NM_014330
HSPA1A /// HSPA1B	heat shock 70kDa protein 1A /// heat shock 70kDa protein 1B	1.660	down	2.976	up	1.466	up	NM_005346
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	2.642	up	1.487	up	1.605	up	NM_012323
HSPA4L	heat shock 70kDa protein 4-like	1.757	down	3.009	down	6.049	down	NM_014278
EPO	erythropoietin	1.493	down	1.116	down	2.922	down	NM_000799
GADD45B	growth arrest and DNA-damage-inducible, beta	4.218	up	1.197	up	1.296	up	NM_015675
GADD45B	growth arrest and DNA-damage-inducible, beta	3.110	up	1.274	up	1.353	up	AF087853
GADD45B	growth arrest and DNA-damage-inducible, beta	3.028	up	1.262	up	1.168	up	AF078077
SMG1	PI-3-kinase-related kinase SMG-1	1.069	up	1.689	down	2.856	down	U32581
SQSTM1	sequestosome 1	2.141	up	2.449	up	1.098	up	N30649
MINK1	misshapen-like kinase 1 (zebrafish)	1.677	down	2.187	down	1.486	down	AI859060
SNN	stannin	2.285	down	1.000	down	1.368	down	AF070673
TRIB3	tribbles homolog 3 (Drosophila)	4.387	up	1.360	up	1.307	down	NM_021158
OXR1	oxidation resistance 1	1.089	down	1.625	down	2.157	down	NM_018002
HSPBAP1	HSPB (heat shock 27kDa) associated protein 1	1.379	up	1.351	down	2.543	down	NM_024610
C8orf44 /// SGK3	serum/glucocorticoid regulated kinase family, member 3 /// chromosome 8 open reading frame 44	1.288	down	1.365	down	2.383	down	NM_013257
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	3.631	up	2.126	up	1.804	up	AL021977
PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	3.341	up	1.056	up	1.267	up	U83981
OXR1	oxidation resistance 1	1.105	down	1.690	down	2.847	down	AL541048
OXR1	oxidation resistance 1	1.122	up	1.411	down	2.054	down	AF309387
SMG1	PI-3-kinase-related kinase SMG-1	1.245	up	1.029	down	2.464	down	AK025794
CIRBP	cold inducible RNA binding protein	1.021	up	1.691	down	2.157	down	AL565767
HSPB6	heat shock protein, alpha-crystallin-related, B6	1.086	up	1.616	up	2.174	up	AA563621
AHSA2	AHA1, activator of heat shock 90kDa protein ATPase homolog 2 (yeast)	1.643	down	1.474	down	2.005	down	AI986239
EIF1	Eukaryotic translation initiation factor 1	2.115	up	1.146	up	1.214	up	BE964053
KIN	KIN, antigenic determinant of recA protein homolog (mouse)	1.077	down	1.500	down	2.198	down	AA768850
TNIK	TRAF2 and NCK interacting kinase	1.017	up	1.522	up	2.035	up	BF431017
TRIB3	tribbles homolog 3 (Drosophila)	4.397	up	1.119	up	1.383	down	AF250311
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.833	up	1.780	up	2.861	up	AF277897
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.752	up	1.624	up	2.126	up	AF277897

Autophagy (GO: 0006914)

Gene Symbol	Gene Title	Fold[Bai]	Regulation [Bai]	Fold[Myr]	Regulation [Myr]	Fold[PG]	Regulation [PG]	Public ID
MAP1LC3B	microtubule-associated protein 1 light chain 3 beta	2.390	up	1.064	up	1.040	up	AF183417
ATG4C /// CTR9	Ctr9, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae) /// ATG4 autophagy related 4 homolog C (S. cerevisiae)	1.172	down	1.853	down	2.151	down	BF197009

Summary of Analysis

Analysis Name: Bain HepG2 - 2009-02-24 02:09
Analysis Creation Date: 2009-02-24
IPA version: Pre 8.0
Content version: Not available.

Analysis settings

View

Reference set: Human Genome U133 Plus 2.0 Array

Relationship to include: Direct and Indirect

Includes Endogenous Chemicals

Optional Analyses: My Pathways My List

Filter Summary:

Consider all molecules and/or relationships

Top Networks

ID	Associated Network Functions	Score
1	Cell Death, Amino Acid Metabolism, Small Molecule Biochemistry	53
2	Cancer, Cell Death, Gastrointestinal Disease	41
3	Cellular Development, Embryonic Development, Tissue Development	41
4	Cellular Growth and Proliferation, Cell Cycle, Embryonic Development	28
5	Cancer, Cardiovascular System Development and Function, Cellular Movement	27

Top Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Cancer	4.43E-13 - 2.65E-03	167
Cardiovascular Disease	1.13E-09 - 1.19E-03	58
Inflammatory Disease	3.29E-08 - 1.14E-03	59
Reproductive System Disease	3.32E-08 - 2.27E-03	98
Neurological Disease	1.67E-07 - 9.00E-04	18

Molecular and Cellular Functions

Name	p-value	# Molecules
Cellular Growth and Proliferation	3.60E-16 - 2.24E-03	132
Cell Death	7.08E-14 - 2.71E-03	125
Cell Cycle	1.38E-10 - 2.65E-03	65
Cellular Development	5.99E-09 - 2.57E-03	86
Cellular Movement	3.59E-08 - 2.33E-03	76

Physiological System Development and Function

Name	p-value	# Molecules
Cardiovascular System Development and Function	1.05E-06 - 2.65E-03	38
Connective Tissue Development and Function	3.05E-06 - 2.19E-03	52
Hematological System Development and Function	6.62E-06 - 2.44E-03	52
Organismal Survival	1.21E-05 - 2.24E-03	52
Organismal Development	1.43E-05 - 2.66E-03	55

Top Canonical Pathways

Name	p-value	Ratio
VDR/RXR Activation	8.77E-06	10/80 (0.125)
NRF2-mediated Oxidative Stress Response	4.75E-04	12/185 (0.065)
Tyrosine Metabolism	1.55E-03	6/186 (0.032)
Methionine Metabolism	1.99E-03	4/76 (0.053)
LPS/IL-1 Mediated Inhibition of RXR Function	2.37E-03	11/198 (0.056)

Top Molecules

Fold Change up-regulated

Molecules	Exp. Value	Exp. Chart
SERPINE1*	↑15.330	
BHLHB2*	↑10.183	
IGFBP1	↑9.460	
IL11	↑8.384	
IL8*	↑6.950	
INHBE	↑6.515	
ATF3*	↑6.027	
CYP1A1	↑5.999	
C5AR1	↑5.501	
UNC5B	↑5.380	

Fold Change down-regulated

Molecules	Exp. Value	Exp. Chart
AQP11	↓-4.826	
PLXNC1*	↓-4.565	
SULT2A1*	↓-4.037	

KLB*	-4.010
CD36*	-3.967
SOX5 (includes EG:6660)	-3.953
NTN4	-3.860
DKK1	-3.639
SEMA6B	-3.476
KLHL13	-3.461

Top My Lists

Name	p-value	Ratio

Top Pathways

Name	p-value	Ratio

Top Tox Lists

Name	p-value	Ratio
VDR/RXR Activation	8.77E-06	10/77 (0.13)
Oxidative Stress Response Mediated by Nrf2	2.96E-04	13/205 (0.063)
Oxidative Stress	1.41E-03	6/57 (0.105)
LPS/IL-1 Mediated Inhibition of RXR Function	2.37E-03	11/187 (0.059)
CAR/RXR Activation	3.57E-03	4/29 (0.138)

Top Tox Functions

Cardiotoxicity

Name	p-value	# Molecules
Cardiac Necrosis/Cell Death	3.72E-05 - 2.93E-01	11
Cardiac Hypertrophy	3.79E-03 - 7.56E-02	14
Cardiac Arteriopathy	7.37E-03 - 7.37E-03	6
Cardiac Output	3.96E-02 - 3.96E-02	2
Cardiac Damage	4.52E-02 - 8.84E-02	1

Hepatotoxicity

Name	p-value	# Molecules
Liver Necrosis/Cell Death	1.75E-03 - 1.88E-01	10
Liver Steatohepatitis	3.04E-03 - 3.33E-02	3
Liver Proliferation	7.13E-03 - 6.70E-02	5
Liver Dysfunction	2.29E-02 - 2.29E-02	1
Liver Hyperplasia/Hyperproliferation	2.29E-02 - 8.93E-02	2

Nephrotoxicity

Name	p-value	# Molecules
Renal Hypertrophy	1.33E-02 - 1.33E-02	2
Renal Nephritis	1.69E-02 - 2.41E-01	5
Kidney Failure	1.78E-02 - 8.93E-02	6
Renal Degeneration	2.29E-02 - 8.84E-02	2
Renal Dilation	2.29E-02 - 2.29E-02	1

Summary of Analysis

Analysis Name: Myin HepG2 - 2009-02-25 12:54
Analysis Creation Date: 2009-02-25
IPA version: Pre 8.0
Content version: Not available.

Analysis settings

View

Reference set: Human Genome U133 Plus 2.0 Array

Relationship to include: Direct and Indirect

Includes Endogenous Chemicals

Optional Analyses: My Pathways My List

Filter Summary:

Consider all molecules and/or relationships

Top Networks

ID	Associated Network Functions	Score
1	Cardiovascular Disease, Metabolic Disease, Lipid Metabolism	46
2	Connective Tissue Development and Function, Skeletal and Muscular System Development and Function, Tissue Morphology	33
3	Endocrine System Disorders, Hematological Disease, Metabolic Disease	32
4	Cardiovascular System Development and Function, Tissue Morphology, Amino Acid Metabolism	30
5	Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function	26

Top Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Cancer	4.74E-09 - 2.00E-02	124
Gastrointestinal Disease	5.65E-07 - 1.98E-02	54
Genetic Disorder	4.15E-06 - 2.00E-02	122
Metabolic Disease	1.13E-05 - 1.53E-02	38
Reproductive System Disease	7.15E-05 - 2.00E-02	70

Molecular and Cellular Functions

Name	p-value	# Molecules
Cellular Growth and Proliferation	1.23E-05 - 1.98E-02	79
Cell Morphology	9.73E-05 - 2.00E-02	48
Carbohydrate Metabolism	1.22E-04 - 1.92E-02	25
Cellular Assembly and Organization	1.46E-04 - 1.82E-02	32
Cell Cycle	1.89E-04 - 2.00E-02	39

Physiological System Development and Function

Name	p-value	# Molecules
Organismal Development	2.78E-05 - 2.00E-02	40
Connective Tissue Development and Function	9.83E-05 - 2.00E-02	29
Skeletal and Muscular System Development and Function	9.83E-05 - 2.00E-02	26
Embryonic Development	1.35E-04 - 2.00E-02	25
Hematological System Development and Function	3.07E-04 - 2.00E-02	31

Top Canonical Pathways

Name	p-value	Ratio
Metabolism of Xenobiotics by Cytochrome P450	2.79E-04	8/209 (0.038)
Bile Acid Biosynthesis	9.16E-04	5/97 (0.052)
NRF2-mediated Oxidative Stress Response	2.03E-03	10/185 (0.054)
VDR/RXR Activation	4.18E-03	6/80 (0.075)
TGF-β Signaling	4.75E-03	6/86 (0.07)

Top Molecules

Fold Change up-regulated

Molecules	Exp. Value	Exp. Chart
F2RL2	↑7.653	
SERPINE1*	↑5.997	
IGFBP1	↑5.064	
AKR1C1*	↑4.874	
TMCC1	↑4.638	
BHLHB2*	↑4.456	
NCF2	↑4.267	
FST*	↑4.220	
NDRG1	↑3.767	
EID3	↑3.744	

Fold Change down-regulated

Molecules	Exp. Value	Exp. Chart
BBS7	↓-5.413	
SLC2A2	↓-4.894	

ARHGAP26	-4.578
DMXL1	-4.375
DIDO1	-4.271
PDE11A	-4.267
RHOBTB3*	-4.203
AOF1*	-3.786
CCDC18*	-3.718
SORBS2*	-3.705

Top My Lists

Name	p-value	Ratio

Top Pathways

Name	p-value	Ratio

Top Tox Lists

Name	p-value	Ratio
Xenobiotic Metabolism	2.57E-04	8/123 (0.065)
Oxidative Stress Response Mediated by Nrf2	1.14E-03	11/205 (0.054)
TGF-β Signaling	3.92E-03	6/77 (0.078)
VDR/RXR Activation	4.18E-03	6/77 (0.078)
Fatty Acid Metabolism	4.21E-03	7/129 (0.054)

Top Tox Functions

Cardiotoxicity

Name	p-value	# Molecules
Cardiac Damage	4.40E-03 - 1.32E-01	3
Cardiac Fibrosis	1.47E-02 - 2.62E-01	4
Pulmonary Hypertension	1.47E-02 - 1.47E-02	3
Cardiac Dilation	3.97E-02 - 1.01E-01	4
Cardiac Hemorrhaging	3.97E-02 - 1.00E00	2

Hepatotoxicity

Name	p-value	# Molecules
Liver Hepatitis	3.68E-03 - 4.09E-01	4
Liver Cholestasis	4.18E-03 - 1.32E-01	6
Liver Hyperplasia/Hyperproliferation	2.00E-02 - 2.00E-02	1
Liver Necrosis/Cell Death	2.00E-02 - 2.91E-01	4
Liver Regeneration	2.70E-02 - 2.70E-02	2

Nephrotoxicity

Name	p-value	# Molecules
Renal Proliferation	1.67E-03 - 1.67E-03	4
Renal Damage	2.34E-03 - 3.33E-01	2
Kidney Failure	1.15E-02 - 1.83E-01	4
Renal Nephritis	1.31E-02 - 2.00E-01	4
Renal Degeneration	2.00E-02 - 2.00E-02	1

Summary of Analysis

Analysis Name: PG HepG2 - 2009-02-24 02:41
Analysis Creation Date: 2009-02-24
IPA version: Pre 8.0
Content version: Not available.

Analysis settings

View

Reference set: Human Genome U133 Plus 2.0 Array

Relationship to include: Direct and Indirect

Includes Endogenous Chemicals

Optional Analyses: My Pathways My List

Filter Summary:

Consider all molecules and/or relationships

Top Networks

ID	Associated Network Functions	Score
1	Infection Mechanism, Antimicrobial Response, Cell-To-Cell Signaling and Interaction	34
2	Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair	34
3	Molecular Transport, Small Molecule Biochemistry, Cancer	33
4	Protein Degradation, Protein Synthesis, Cell Morphology	32
5	Small Molecule Biochemistry, Cell Cycle, Cellular Assembly and Organization	31

Top Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Cancer	1.94E-09 - 1.22E-02	329
Reproductive System Disease	1.68E-06 - 1.21E-02	192
Hematological Disease	2.07E-05 - 1.20E-02	111
Organismal Injury and Abnormalities	1.89E-04 - 1.06E-02	15
Skeletal and Muscular Disorders	2.14E-04 - 1.22E-02	16

Molecular and Cellular Functions

Name	p-value	# Molecules
Cell Death	2.96E-06 - 1.08E-02	208
Cell Cycle	3.15E-06 - 1.22E-02	117
Gene Expression	4.57E-06 - 1.21E-02	94
Post-Translational Modification	4.65E-06 - 4.92E-03	88
DNA Replication, Recombination, and Repair	7.91E-06 - 1.03E-02	94

Physiological System Development and Function

Name	p-value	# Molecules
Organismal Survival	5.51E-06 - 2.86E-03	101
Organismal Development	1.17E-04 - 1.06E-02	84
Hematological System Development and Function	2.54E-04 - 9.52E-03	42
Hematopoiesis	2.54E-04 - 6.83E-03	24
Reproductive System Development and Function	7.25E-04 - 9.52E-03	27

Top Canonical Pathways

Name	p-value	Ratio
Role of BRCA1 in DNA Damage Response	8.32E-05	11/52 (0.212)
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.01E-03	8/43 (0.186)
LPS/IL-1 Mediated Inhibition of RXR Function	1.12E-03	21/198 (0.106)
IGF-1 Signaling	1.21E-03	13/98 (0.133)
PPARα/RXRα Activation	1.26E-03	19/185 (0.103)

Top Molecules

Fold Change up-regulated

Molecules	Exp. Value	Exp. Chart
CYP1A1	↑5.714	
TMCC1	↑4.678	
EDN1*	↑4.532	
SERPINE1*	↑4.231	
FST*	↑3.892	
IGFBP1	↑3.796	
MDM2*	↑3.592	
IL8*	↑3.413	
INSIG2	↑3.238	
CCL20	↑3.208	

Fold Change down-regulated

Molecules	Exp. Value	Exp. Chart
DMXL1	↓-13.135	
KRIT1*	↓-12.239	
BBS7*	↓-10.613	

CNOT6L*	-10.255
PNPLA8*	-9.544
DLGAP5	-9.230
TBC1D8B*	-9.034
CD36*	-9.016
DIMT1L*	-8.922
C1ORF25*	-8.910

Top My Lists

Name	p-value	Ratio

Top Pathways

Name	p-value	Ratio

Top Tox Lists

Name	p-value	Ratio
PPARα/RXR Activation	1.08E-03	19/166 (0.114)
LPS/IL-1 Mediated Inhibition of RXR Function	1.12E-03	21/187 (0.112)
G2/M Transition of the Cell Cycle	2.32E-03	7/34 (0.206)
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα	2.75E-03	13/95 (0.137)
Hepatic Cholestasis	3.48E-03	16/135 (0.119)

Top Tox Functions

Cardiotoxicity

Name	p-value	# Molecules
Cardiac Dilation	1.79E-02 - 6.12E-01	7
Cardiac Necrosis/Cell Death	2.93E-02 - 4.13E-01	11
Cardiac Fibrosis	3.51E-02 - 5.89E-01	8
Cardiac Hypertrophy	4.82E-02 - 4.13E-01	19
Cardiac Degeneration	5.75E-02 - 1.12E-01	2

Hepatotoxicity

Name	p-value	# Molecules
Hepatocellular Carcinoma	1.12E-02 - 1.12E-02	13
Liver Necrosis/Cell Death	1.22E-02 - 6.35E-01	10
Liver Hyperplasia/Hyperproliferation	1.68E-02 - 5.75E-02	4
Liver Hematopoiesis	1.83E-02 - 1.83E-02	2
Liver Cholestasis	2.98E-02 - 2.99E-01	9

Nephrotoxicity

Name	p-value	# Molecules
Renal Proliferation	8.89E-03 - 2.99E-01	6
Kidney Failure	1.21E-02 - 4.47E-01	8
Nephrosis	4.82E-02 - 2.56E-01	5
Renal Degeneration	5.75E-02 - 5.75E-02	1
Renal Dilation	5.75E-02 - 5.75E-02	1