IDENTIFICATION OF NOVEL GROWTH SIGNALLING PATHWAY IN SHEEP SKELETAL MUSCLE BY COMPARATIVE MICROARRAY ANALYSIS

Research Project

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ABSTRACT

The feed conversion efficiency can be improved through increasing the ratio of lean to fat deposition in animal. The benefits of this involve decrease production costs, increase produce quality, reducing grazing pressure and decreasing nitrogenous excretion into the environment. Since beta agonists and growth hormone result in muscle fibre hypertrophy and changes in muscle fibre type composition, also promote a rise in muscle mass and abatement in fat, therefore there are good methods to increase the feed conversion efficiency. In order to improve feed conversion efficiency, the mechanisms of reaction need to be study.

For this project, the aim is to identify growth signaling pathways in skeletal muscle. Since an appropriate sheep-specific microarray was unavailable, a human affymetric arrary and genomic DNA hybridization were used to investigate effects of BA and GH on the sheep muscle transcriptome. Microarray data was analyzed using Ingenuity Pathway Analysis software and GeneSpring. The microarray analysis results were conformed by real-time quantitative RT-PCR.

As the results of this project, there were some target genes and signaling pathways had been identified, these included AKT1 pathway and PSAT1 pathway which associated with protein synthesis; and citrate cycle pathway, oxidative phosphorylation pathway and glycolysis pathway which correlated with energy metabolism. These pathways were predominantly associated with beta-agonists response in comparison there were only relatively minor effects of growth hormone.

CONTENTS

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ABBREVIATIONS

4EBP1	eIF4E Binding Protein 1,
ADP	adenosine diphosphate
AKT1	two of them respectively included v-akt murine thymoma viral oncogene homolog 1
ANOVA	one way analysis of variance
ATP	Adenosine triphosphate
BA	beta agonists
camp	Cyclic adenosine monophosphate,
CK	control group
CLTA	These genes were clathrin, light chain
CREB	cAMP response element binding protein.
CSA	cross sectional area
D120 age	12o days of age
dsDNA	double-stranded DNA
eIf2B	Eukaryotic translation initiation factor 2B,
eIf4E	Eukaryotic Translation Initiation Factor 4E,
Epac	Exchange protein activated by cAMP,
ERK	Extracellular Signal Regulated Kinase,
ESTs	expressed sequence tags
FCE	feed conversion efficiency
FOXO	forkhead box, sub-group O,
FOXP1	forkhead box P1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH	growth hormone
GHRs	growth hormone receptors
GO analysis	Gene ontology analysis
GS	Glycogen Synthase,
GSK3	Glycogen Synthase Kinase-3
HDAC5	Histone deacetylase 5,
ICDH	The isocitrate dehydrogenase
IGF-1	insulin-like growth factor 1
IGFBPs	insulin-like growth factor binding proteins
IGFRs	insulin-like growth factor receptors
IKB	Ingenuity's Knowledge Base
IPA	Ingenuity Pathway Analysis software
IRS-1	Insulin Receptor Substrate-1,
KEGG	Kyoto Encyclopedia of Genes and Genomes
KPCR	kinetic polymerase chain reaction

LD	*longissimus dorsi*
MAFbx	Muscle Atrophy F-box,
MEK	Mitogen-Activated Protein Kinase/ERK Kinase,
MHC	myosin heavy chain
mRNA	messenger RNA
MTOR	Mammalian Target of Rapamycin,
MURF1	Muscle-Specific RING Finger 1,
p70s6K	70 kDa Ribosomal Protein S6 Kinase 1,
PCA	Principal components analysis
Pi	phosphate
PI3K	phosphoinositol 3-kinase
PI3K	Phosphoinositide 3-Kinase,
PIP2	Phosphatidylinositol 4,5-Bisphosphate,
PIP3	Phosphatidylinositol 3,4,5-Trisphosphate,
PKA	Protein Kinase A, Rap 1 - repressor activator protein,
PSAT1	phosphoserine aminotransferase 1
PSAT1	Phospho Serine Amino Transferase 1
qPCR/qrt-PCR/	
Q-PCR	quantitative real time polymerase chain reaction
RHBDL1	rhomboid, veinlet-like 1
RIP140	co-repressor receptor-interacting protein 140
SIK1	salt-inducible kinase 1
β-ARs	beta-adrenergic receptors

1. INTRDUCTION

1.1 Background

In today's world, the control of lean and fat tissue deposition in the meat of livestock is a very popular and welcomed subject for producers, consumers and environmentalists. If the ratio of lean to fat deposition in animal is improved, the feed conversion efficiency will be improved at the same time. The cost of feed determines the quantity of profit, so raising feed conversion efficiency (FCE) is very important for producers. Besides this, in many countries of South East Asia, with the increased economy and population, the meat demand and feed supply of livestock are also increased. This make a heavy pressure effecting the whole world. If the FCE is improved, more meat can be supplied to people currently with limited resources. Last but not least, FCE is important because increase FCE is good for reducing environmental pressure and decreased nitrogenous excretion into atmosphere (Coffey, 1996; Sillence, 2004). A lot of lamb producers also pay close attention to the feed conversion efficiency, even if their production relies on grazing to a great extent. For these lamb producers, increasing feed conversion efficiency is a good method to improve meat production and at the same time reduce the pasture pressure on the affluent, but fragile natural landscapes (Hunter et al., 1993). However, finding a method to complete these aims that is affordable, acceptable and also reliable, to the vast majority person has demonstrated to be one great challenge for the researchers and producers.

Since the European Union forbade hormonal growth promoters about fifteen years ago, some countries such like the United States and Australia got new license to produce domestic animal production. The new license indicates that beta adrenergic agonist, bovine growth hormone, porcine growth hormone and equine growth hormone can be used into livestock production (Sillence, 2004).

The producers consider how to improve their produces, while the researchers study how to develop the novel technologies. Opportunities to develop beta adrenergic agonist consist of reducing the effects of meat toughness, decreasing bad influences on treated livestock, and lengthening the time of action. It is noteworthy that the jointed use of the bate-agonist with growth hormone, which can up-regulates beta

adrenoceptors, can bring an significant improvement in feed efficiency and carcass composition (Sillence, 2004). In endocrinology, another outstanding improvement is that the hormones, which are secreted by fat and muscle cells, adjust energy metabolism, body composition and feed intake. By researching double-muscled or genetically obese livestock, adiponectin, myostatin and leptin have been found. Although transgenic liverstock's production is still effected by the low level control of gene expression, and also challenges to the acceptance of the consumer, these results can be exploited potentially though genetic management.

Because the beta agonists (BA) and growth hormone (GH) can promote a rise in muscle mass and abatement in fat, therefore there are good methods to increase the feed conversion efficiency (Enright, 1989; Buttery and Dawson, 1990). The beta agonists and growth hormone have different ways to reach this goal. GH principally indirectly affects is though insulin-like growth factor 1 (IGF-1) production's stimulation (Scarth, 2006). However, as Lynch and Ryall explained (2008) that beta agonists is trend to directly effect via beta-adrenergic receptors (β-ARs). Lefaucheur and Gerrard pointed out (2000) that the efficacy of BA and GH in changing muscle mass trended to depends on the composition of fibre type and/or the transitions of muscle fibre type.

1.2Skeletal muscle function and metabolism

As skeletal muscle makes movement, it is an important part of the body and play a principal role in metabolism (Lawrence and Fowler, 2002). The interaction of myosin and actin, dominated by the binding of calcium ions to troponin-C (a kind of protein has relation with actin filaments) is the reason of the muscle's contraction (Frayn, 2003). Adenosine triphosphate (ATP) can supply energy for muscle contraction, and it can be hydrolyzed to inorganic phosphate (Pi) and adenosine diphosphate (ADP). Through aerobic or anaerobic metabolism, the different substrates can be used to create adenosine triphosphate (Frayn, 2003). Besides this, skeletal muscle action as the amino acids' storage can be used for gluconeogenesis and the synthesis of de novo protein and has a main role in glucose regulation, it store as glycogen (Lawrence and Fowler, 2002; Frayn, 2003). The contraction of muscle and substrate utilization are displayed in Figure 1.1.

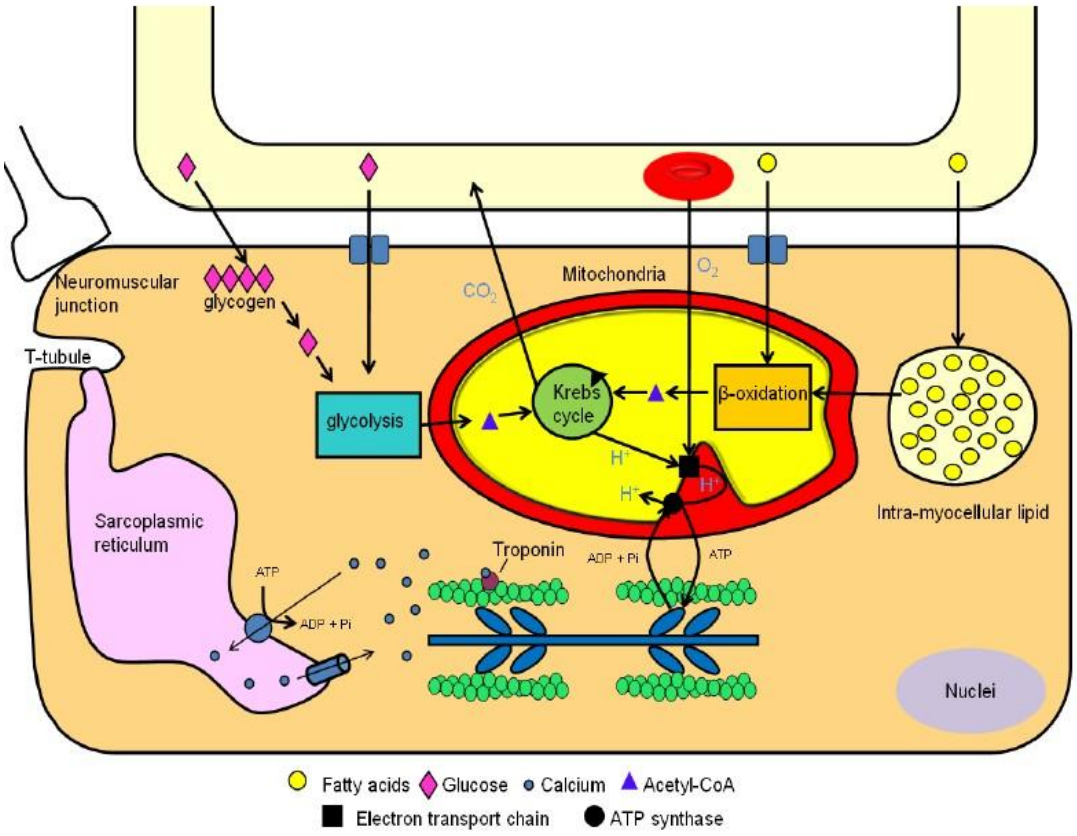

Figure 1.1 Skeletal muscle contraction and substrate utilization pathways (Flück and Hoppeler, 2003)

An action at the neuromuscular junction leads to depolarisation of the T-tubules, which in turn activates calcium ions release from sarcoplamic reticulum into the cytoplasm. Calcium ions binds to troponin, this permitting the interaction of actin with myosin, and subsequent shortening of sarcomere. This excitation contraction process depends on adenosine triphosphate. The creatine and arginine ATP storages provides the initial adenosine triphosphate. Then glycolysis supplies adenosine triphosphate efficiently. Whereas the lactate production effects the performance of muscle and the glycolysis is inefficient with that. Hence for prolonged muscle contraction ATP is generated by carbohydrate metabolites and the fatty acid's mitochondrial oxidation. The intracellular storages and blood can provide substrates. After the cessation of an action, the potential calcium ions are pumped into sarcoplasmic reticulum.

1.3Growth promoting agents and muscle fibre type

1.3.1Growth promoting agents

Recently the control of lean and fat tissue deposition in the meat of livestock is a very welcomed topic for many people. An increase of the lean to fat tissue deposition can lead to an improvement of the meat production of every animal, this always correlates with the improvements of feed conversion efficiency (FCE). Methods for adjusting lean and fat tissue deposition in the meat include using growth promoting agents, such as beta agonists (BA) and growth hormone (GH).

Beta-agonists can promote the meat growth of livestock and increase the proportion of protein (Lynch and Ryall 2008). The beta-agonists' anabolic influence is highly selective for skeletal muscle compare with other growth promoters (Sillence,2004). If the protein of intake is restricted, the effect of it should reduce. However, even the underfed animals show an effect, because beta-agonists can redistribute protein from other tissues to skeletal muscle (Sillence 2000). Therefore, beta-agonists are also called repartitioning agents.

Growth hormone can decrease body fat of livestock, while increasing muscle mass (Sillence,2004). The growth hormone receptors (GHRs) of target organs have a very important role in the whole growth process. The functions of GH have been demonstrated in two ways. First， GH effects target organs, like liver, binding to GHRs and producing insulin-like growth factors (IGFs). Then IGFs go into the blood and bind to insulin-like growth factor binding proteins (IGFBPs). They arrive to target organ and act on insulin-like growth factor receptors (IGFRs), thereby regulating growth and development. Second, GH can directly affect target organs, like muscle, to produce IGF through paracrine or autocrine to adjust the growth and development (Scarth, 2006).

These two growth agents can increase fat and muscle mass, however their effects have different action mechanisms (Figure 1.2). Beta-agonists exert this effect primarily directly via β-ARs, while growth hormone expresses this effect through stimulating liver to produce IGF-1. Then IGF-1 can bind to aim tissues (Bell et al., 1998). It is worth to note that signaling pathways of them appear a overlap part, which is phosphoinositol 3-kinase (PI3K) pathway.

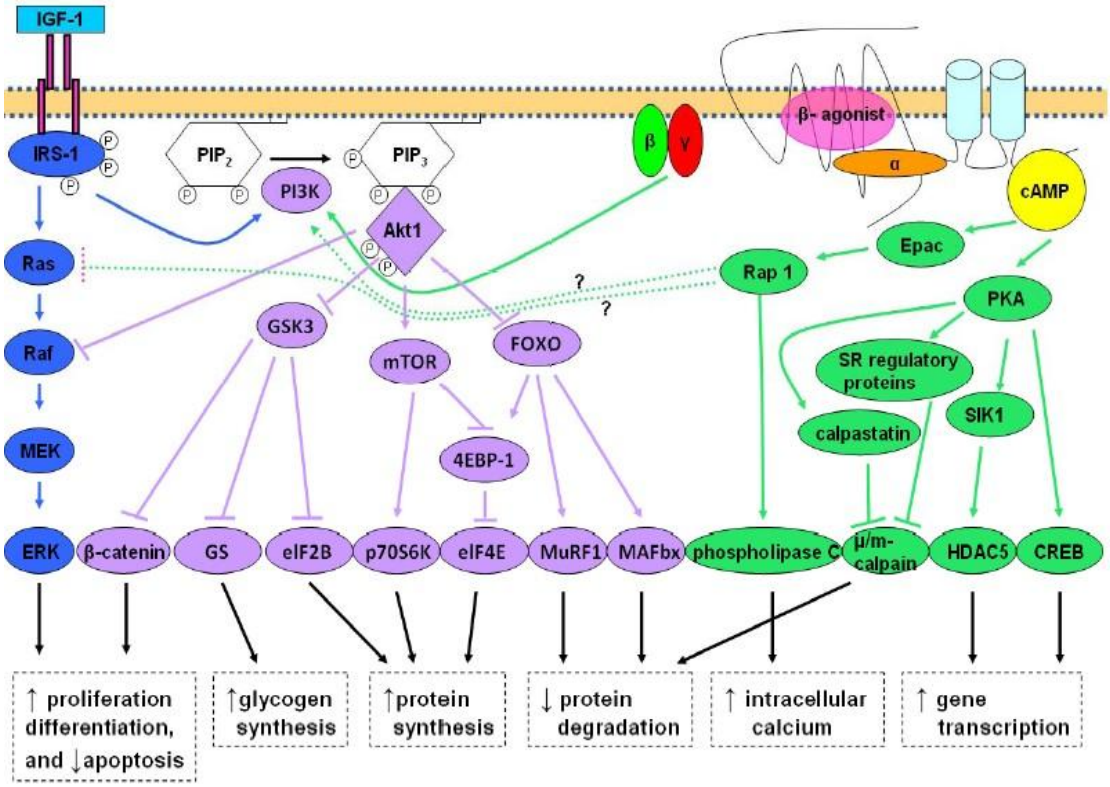

Figure 1.2: Growth hormone and Beta-agonists signalling pathways. (Glass 2003; Chang 2007 and Lynch and Ryall 2008)

This figure shows the signalling pathways correlated with beta-agonists and growth hormone administration. The effects form Growth hormone are primarily exerted by IGF-1, then IGF-1 acts on tyrosine kinase receptors of muscle. The effects form Beta-agonists primarily via acting on β2-AR's direct.

4EBP1 - eIF4E Binding Protein 1, cAMP-Cyclic adenosine monophosphate, CREB - cAMP response element binding protein. eIf2B - Eukaryotic translation initiation factor 2B, eIf4E – Eukaryotic Translation Initiation Factor 4E, Epac - Exchange protein activated by cAMP, ERK - Extracellular-Signal-Regulated Kinase, FOXO - forkhead box, sub-group O, GS - Glycogen Synthase, GSK3 - Glycogen Synthase Kinase-3HDAC5 - Histone deacetylase 5, IRS-1 - Insulin Receptor Substrate-1,MAFbx - Muscle Atrophy F-box, MEK - Mitogen-Activated Protein Kinase/ERK Kinase, mTOR - Mammalian Target of Rapamycin, MURF1 -Muscle-Specific RING Finger 1, p70s6K - 70 kDa Ribosomal Protein S6 Kinase 1, PI3K - Phosphoinositide 3-Kinase, PIP2 -Phosphatidylinositol 4,5-Bisphosphate, PIP3 - Phosphatidylinositol 3,4,5-Trisphosphate, PKA - Protein Kinase A, Rap 1 - repressor activator protein, SIK1 - salt-inducible kinase 1,

1.3.1.1 Growth hormone

The anterior pituitary is responsible for secreting growth hormone into blood, then stimulates and produces IGF-1 in liver (mainly). Growth hormone also has a direct influence on some target tissues, for example, adipose tissue is stimulated direct by growth hormone to produce IGF-1 (Scarth, 2006). Then IGF-1 acts on many cell types in the body's individual tissues. Figure 1.2 displays the pathways relate to skeletal

muscle cells. IGF-1 acts on tyrosine kinase receptors, this leads to IRS-1's recruitment, then the signals are sent to mitogen activated protein kinase and phosphoinositide 3-Kinase (PI3K) pathways (Glass, 2003). This makes the muscles trend to hypertrophic and/or hyperplastic growth and this process depends on the muscle development stage.

1.3.1.2 Beta-agonists

Murdoch et al (2006) pointed out that adrenergic receptors adjusted the influences of physiological agonists. The catecholamines, including noradrenaline, dopamine and adrenaline, are released in times of stress and under other metabolic challenges. Adrenaline and noradrenaline stimulate glycogenolysis in liver and muscle, lipolysis in adipose cell and gluconeogenesis in liver (Gerrard and Grant, 2006), hence releasing the energy reserves of body, correlated with the demand for a raise in energy consume (Murdoch et al., 2006). In skeletal muscle, β2-AR stimulation also changes the protein turnover's rate, leads protein deposition (such as, growth) (NRC, 1994). Figure1.2 Shows the signalling pathways of cells educing the β2-AR mediated influences on skeletal muscle. The β2-AR's stimulation leads to a conformational variation in the correlated G protein, guanosine diphosphate is devolved from the α subunit and guanosine triphosphate binds. This leads to the α subunit dissociating from the β/γ subunits. The α subunit then stimulates adenylyl cyclase to produce cAMP, which mediates downstream effects, then the adenylyl cyclase is stimulated by α subunit and bring cAMP, which can adjusts the effects of downstream, the most well characterized being PKA pathway. The PI3K pathway can be activated by remaining β or γ subunit (Lynch and Ryall, 2008).

1.3.2Muscle fibre type

The muscle is according as shortening velocity to class as fast and slow-twitch muscles (Scott *et al.*, 2001). Based on the metabolic and contractile properties of muscles, muscle fibres also can been classified (Figure 1.3). The contractile muscle fibre type according to the distinct myosin heavy chain isoforms' expression and muscle fibre types are always named based on the myosin heavy chain (MHC) isoforms expressed in them (Lefaucheur and Gerrard, 2000). Thereby fast fibres can express the IIA or IIX or IIB MHC isoforms, while slow fibres just can express type I myosin heavy chain isoform. Fibre types are also relevant to the fibre's metabolic capacity. The

metabolic capacity of fibre is an entia across muscle fibre types, and with all of the fibre types having an entia of glycolytic and oxidative metabolism, whereas every fibre type owns a distinct capacity for every kind of metabolism (Gerrard and Grant, 2006).

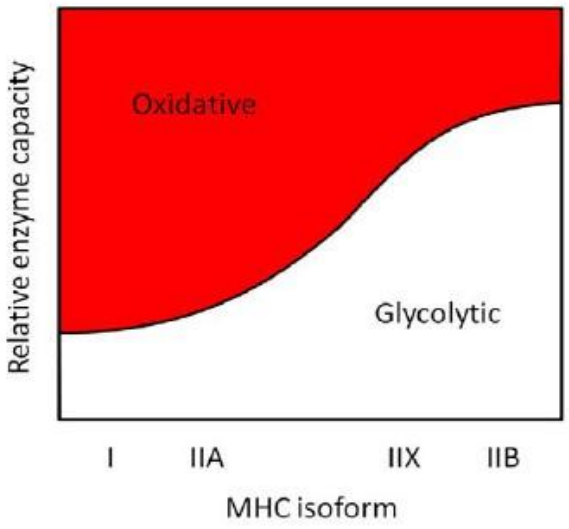

Figure 1.3: Skeletal muscle fibre types' characterization based on metabolic and contractile features (Gerrard and Grant, 2006)

In this figure, red means slow-twitch muscles and white means fast-twitch muscles.

1.3.3Growth promoting agents and the relationship with muscle fibre type

The responses of muscle hypertrophy to growth promoting agents may depend on the muscle fibre types or include the changes of fibre type composition. Growth hormone trends to rise all of fibres' cross sectional area (CSA), however no influence on muscle fibre type composition (Lefaucheur and Gerrard, 2000).Whereas there was a human study indicated that a rise in myosin heavy chain IIX in response to growth hormone, hence growth hormone may also change fibre type (Lange *et al.* 2002). BA trends to increase the fast fibres' CSA (Kim *et al.* 1987; Sainz *et al.* 1993).

A recent experiment indicated that the growth promoters, particularly BA, induced muscle growth and this appeared to be a switch to a muscle type that was a fast type with an apparent reduction on the oxidative metabolic capacity (produced by Krystal Hemmings).

1.4Technology

1.4.1Microarry analysis

1.4.1.1What is microarry

The microarray is a kind of miniature analytical installation that is used to the genomic exploration. It has the highest precision and speed in the biology' history. These small glass chips which include tens of thousands of genes can be used to check the fluorescent samples. The messenger RNA (mRNA) of cells, tissues, organs and the other biological materials labels in the fluorescent samples (Figure 1.4). The fluorescent samples' molecules react with homologous sequences in the chip, this leads every spot to shine with an intensity, which proportional to the expressed gene's activity. The large capacity of this small device can analyze the whole human genome in one experiment. Since the gene expression is strongly related to function, microarrys can prove much information on disease, drug action, hormone action, diet, mental illness, aging and a lot of other clinical problems. Beside that, micrarrays can find the gene sequences' changes, pave a new way for genetic screening, genetic diagnostics and genetic testing. Protein and tissue microarrys are abbreviated traditional biochemical and histological assays, quickening the analysis of protein-protein interactions, enzymes and tumor specimens. Because the microarrays can study the genomes of viruses, bacteria, worms, plants, fruit flies, primates, cows, mice, rats and chickens, it is called the biochemistry's Noah's Ark (Mark Schena 2002).

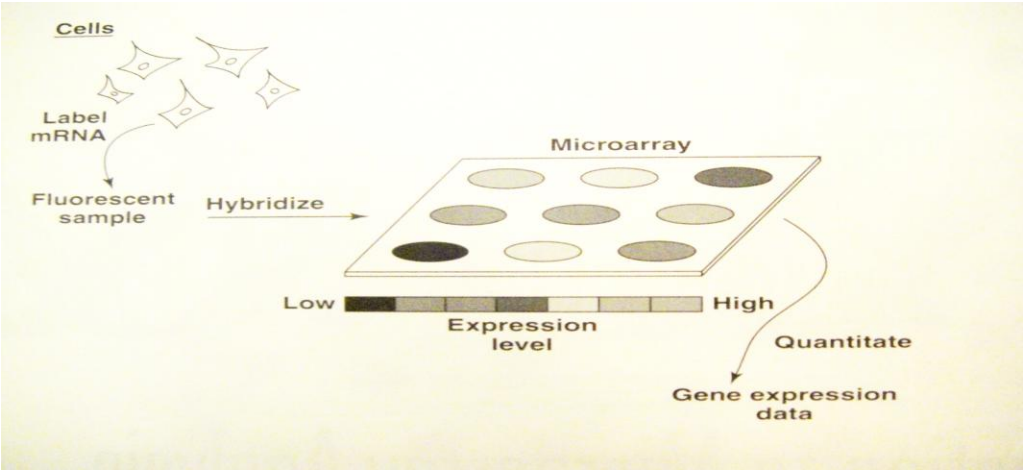

Figure 1.4 The process of microarrys analysis (Mark Schena, 2002)

In the process of microarrys study, mRNA is isolated form sample cells, then labeled by fluorescent tags, and then hybridized to the chip, which includes the gene sequence. The fluorescent samples' molecules react with homologous sequences in the chip, this leads every spot to shine with an intensity, which proportional to the expressed gene's activity. These intensities are coded into a

color palette, which shows as horizontal bar in this figure. From every location of microarrys, the quantitative data of gene expression can be got by determining the intensity of fluorescence.

1.4.1.2Microarrays types and applications

The microarrays have many types, that include DNA microarrays (like oligonucleotide microarrays, SNP microarrays and cDNA microarrays), MMChips (for surveillance of microRNA populations), tissue microarrays, protein microarrays, chemical compound microarrays, cellular microarrays (or transfection microarrays), carbohydrate arrays (or glycoarrays) and antibody microarrays (http://en.wikipedia.org/wiki/Microarray). In this project, the DNA microarrays (Affymetrix-GeneChip) had been used. The applications of the DNA microarrays showed in Table 1.1 (http://en.wikipedia.org/wiki/DNA_microarray).

Table 1.1 The applications of the DNA microarrays

Application technology	Synopsis
Gene expression profiling	In a gene expression profiling or mRNA experiment, the thousands of genes' expression levels are monitored together to research the influences of certain diseases, treatments and developmental process on gene expression. For instance, people can use microarray-based gene expression profiling to study the genes whose expression is changed in response to other organisms or pathogens through comparing gene expression in uninfected to that in infected tissues or cells (Adomas. et al, 2008).
Comparative genomic hybridization	Estimating genome content in closely related organisms or in different cells (Pollack et. al. 1999; Moran et.al.2004).
Gene ID	Small microarrays to detect organisms' IDs in feed or food, for example, GMO (Kulesh et al 1987), pathogens for disease detection or mycoplasms in cell, mainly combining microarray technology and PCR.
Chromatin immunoprecipitation on Chip	DNA sequences bind to a special protein that can be isolated by immunoprecipitating the protein, these fragments hybridizes to a microarray permitting the determination of protein binding site occupancy throughout the genome. For instance, the protein to immunoprecipitate are RNA Polymerase II to research transcription landscape or histone modifications (H3K9me3, H3K27me3, H3K4me2, etc.), trithorax-group protein (Ash1) and Polycomb-group protein (PRC1:YY1, PRC2:Suz12) to research the epigenetic landscape.
Dam ID	Genomic regions bound by an interesting protein can be separated and used to hybridize a microarray to define binding site occupancy. Dam ID is different form ChIP, it

	does not need antibodies, however it uses adenine methylation close to the protein's binding sites to amplify those regions, introduced through expressing minute quantity of interesting protein combined to the DNA adenine methyltransferase of bacterium.
SNP detection	Detection single nucleotide polymorphism in alleles within populations (Hacia et al 1999). Microarrays' several applications use SNP detection, involving forensic analysis, genotyping, determining drug-candidates, surveying predisposition to disease, assessing body mutations in cancers or germline mutations in individuals, analyzing genetic linkage or evaluating loss of heterozygosity.
Alternative splicing detection	The design of an exon junction array uses probes particular to the predicted exons' potential splice sites. It is of intermediate coverage, or density, to a genomic tiling array (with thousands or hundreds of probes each gene) and a classical gene expression array (with 1 to 3 probes each gene). It can be used to test alternative splice forms' expression. Exon arrays have a distinct design, using probes designed to test every individual exon for predicted or known genes, and also can be utilized for exploring splicing isoforms.
Fusion genes microarray	A Fusion gene microarray can explore fusion transcripts, for example, from cancer specimens. The principle is building on alternative splicing microarrays. Oligo design method makes chimeric transcript's combined measurements junctions with individual fusion partners' exon-wise measurements.
Tiling array	Genome tiling arrays is composed by overlaying probes designed to densely show a genomic interesting region, sometimes as big as one whole human chromosome. The aim is to explore empirically expression of alternatively splice forms or transcripts which may unknown.

1.4.1.3Cross-species microarray

The gene expression's modulation represents the basic mechanism of biology process underlining pathological derailments and physiological developments. The application of microarrays to explore all of the transcriptional profiles is a widely used and valuable tool for studying biological systems, containing the disease responses and developmental processes, and it also can provide significant new ideas into the biological process's molecular mechanisms (Brown and Botstein 1999; Lockhart and Winzeler 2000). Although microarray improves greatly the genetic analysis in biological systems, however because this powerful technology has been primarily

restricted to a few model animals, plants and human. To make high-density DNA microarrays have to spend a lot of money and time. This method includes ranking the gene transcripts on a big scale from different tissues, classing the expressed sequence tags (ESTs) to produce special transcripts, annotating these transcripts, probe design, and synthesizing these probes on glass chips. Therefore, people would like to find a new method to avoid this large undertaking and use existing microarray to do cross-species transplant.

Recently, some researchers pointed out that all of the mammalian own a common ancestor before more or less 65 to 100 million years (Lewin, 1999). The protein functions' preservation plus not long time span of the geology result that a high quality of nucleotide sequence has been saved in mammalian species. The sequences of human and 2820 orthologous rodent have been compared by Makalowski and Boguski in 1998, the result shows that these two far related mammals have a large resemblance in their gene transcripts. They pointed out that, between mouse and human, average identity is 85.9 percent in protein-coding nucleotide sequences, in 3'-untranslated region is 71.0 percent and in 5'-untranslated region is 69.7 percent.

In 2004, Ji. et al. used cross-species hybridization with human high-density oligonucleotide arrays (Affymetrix GeneChip) to study far related mammals species: pig, dog and cattle. Based on the Affymetrix GeneChip's special feature that each gene is embodied by more than one probes, they hypothesized the sequence conservation of mammals is sufficient strong to bring enough signals from some probes for gene expression analysis. They proved that although all of the hybridization signals were low in the cross-species hybridization experiment, a spot probes of the majority of genes still brought signals. The finally result was same as same-species hybridization. It showed that it was possible to use cross-species hybridization to study other mammals for which species-specific microarrays are not available, but that not all probes worked. Recently, the researchers agree widely that the cross-species method can be used to research the sample do not has accomplished genome arrays (Chismar et al., 2002; Higgins et al., 2003; Spiewak and Gerin, 2004; Khaitovich et al., 2004; Hammond et al., 2005, 2006; Bar-Or et al., 2007; Broadley et al., 2008). Since current available sheep microarrays are more targeted to study genes involved in immune response, not suitable for studies on muscle growth and metabolism. And also the ovine does not have

accomplished genome arrays available. Hence, for this project the sheep RNA had been hybridized by human microarry.

1.4.2Software analysis

1.4.2.1GeneSpring

GeneSpring is a kind of powerful software that can provide accessible statistical tools and professional analysis of genomic structural variation and expression data. The core features of transcriptomics applications of GeneSpring (as described in Agilent GeneSpring GX user manual 2009) include: First, Gene-level expression analysis on all the microarray platforms, including Affymetrix, Illumina and Agilent. Second, using intact TargetScan information to analyze microRNA and identify gene targets. Third, can be applied to analyze Real-time PCR data. Fourth, NCBI Gene Expression Omnibus Importer tool for expression datasets.
(http://www.chem.agilent.com/en-US/products/software/lifesciencesinformatics/genespringgx/Pages/default.aspx).

For this project, the main functions of GeneSpring: profile plots analysis, statistical analysis, principal components analysis, gene ontology analysis, filtered on volcano plot, venn diagram analysis and pathway analysis had been used.

1.4.2.2Ingenuity Pathway Analysis software

The target genes which identified by GeneSpring had been analyzed by Ingenuity Pathway Analysis software (IPA version: 8.6-3003 Build: 93815;2010 Ingenuity Systems, Inc) to identify specific signaling pathways.

IPA is a kind of powerful and web-based software that can provide bioinformaticians and biologists to identify a visualization of an underlying mechanism, specific biological signaling pathways and functions most correlative to genes of interest or experimental of researchers (Ganter and Corner, 2007).

The IPA dynamically combines relevant biological functions and networks focused on the experimental interesting genes. Scientists can create new pathways for

specific biomarkers, targets, biological functions or disease areas. IPA gives researchers a deeper and more complete understanding of cellular and molecular mechanisms, which ultimately influences future experimental direction and improves experimental process (Alessia et. al. 2010; and www.ingenuity.com).

IPA uses a manually management and robust database, Ingenuity's Knowledge Base (IKB). It includes up-to-date information on more than 20,000 mammalian proteins and genes, over one hundred canonical pathways and 1.4 million biological interactions. The Ingenuity's Knowledge Base is structured into an ontology consisting of more than 60 thousands processes and objects, onto which inputted protein or gene identifiers are mapped to. IPA has many kinds of main functions that can help researchers to deeply analyze and understand data. First function, the IPA Core Analysis can provide biological processes and pathways that are most significant in the analyzed results. Second, the IPA Function Analysis associates biological diseases and functions to dataset. Third, the IPA Canonical Pathway Analysis can be used to identify the most relevant signaling and metabolic pathways among those present in up-to-date dataset. Fourth, the IPA Tox Analysis can analyze the particular proteins or genes are observably associated with important function genes based on present biological processes, toxicity lists and main toxicological responses. The finally score can be found in the sumarry lists (Alessia et.al. 2010). In the analysis of this project, the IPA Core Analysis had been utilized.

1.4.3Quantitative real time polymerase chain reaction

The another name of real-time polymerase chain reaction is kinetic polymerase chain reaction (KPCR) or quantitative real time polymerase chain reaction (qPCR/qrt-PCR/Q-PCR). It based on the traditional PCR, is a laboratory technique of molecular biology, can be used to amplify and synchronously quantify an aim DNA molecule. It can explore and quantify (relative amount when normalized to import DNA or as absolute copies number) the particular sequences in the target DNA molecule. The key feature of real-time polymerase chain reaction is amplified DNA is explored as a reaction progress called real time. It is a new method compared to traditional PCR. In real-time PCR, two common approaches for exploring products are:

(1) sequence-specific DNA probes is composed by oligonucleotides. A fluorescent sign labels with them, it only allows exploration which after the probe has been hybridized with its corresponding DNA sequence.
(2) another one is non-specific fluorescent dyes, it intercalates with any double-stranded DNA.

Normally, reverse transcription is integrated with real-time polymerase chain reaction to quantify Non-coding RNA and messenger RNA in tissues or cells.

The abbreviations of real-time PCR can be Q-PCR, RTQ-PCR or qPCR (VanGuilder et al, 2008), RRT-PCR (Spackman and Suarez 2008), qRT-PCR (Udvardi et al.2008) or RT-rt PCR (Gertsch et.al.2002) often means real-time reverse-transcription PCR. RT-PCR normally means reverse-transcription PCR but not real-time PCR, however not all of the authors insist on this convention (Logan et. al. 2009).

1.4.3.1Real-time PCR with double-stranded DNA-binding dye

The DNA-binding dye combines to all of the double-stranded DNA in PCR, causing the dye's fluorescence. During PCR, a rise in DNA product, results in a rise in the intensity of fluorescence and is surveyed at every cycle, therefore permitting to quantify the DNA concentrations. Whereas double-stranded DNA (dsDNA) dyes (like SYBR Green) can bind to all of the double-stranded DNA PCR products, involving non-specific PCR products (for example, primer dimer). This may prevent or interfere with the target sequence's accurate quantification.

The process shows below:

1. Usual reaction preparation added fluorescent double-stranded DNA dye.
2. A Real-time PCR instrument (like Light Cycle 480) is responsible for running this reaction. The fluorescence levels are surveyed by a detector after every cycle. Only when the dye bind to dsDNA (like PCR product), the fluorescence has been shown. With reference to standard dilution, the double-stranded DNA concentration can be mensurated.

As explained above, comparing the determined RNA/DNA sample to the standard dilution will just provide a ratio or fraction of the RNA/DNA sample relevant to the standard, only permitting correlative comparisons between experimental condition or different tissues. In order to make sure the accuracy of quantification, it needs to normalize the target gene's expression to a steadily expressed gene. This can amend possible discrepancy in RNA quality or quantity across the samples of experiment (http://en.wikipedia.org/wiki/Real_time_PCR).

In this project, the SYBR Green had been used to do Real Time PCR, in next part the principle of them will be explained.

1.4.3.2SYBR Green principle

SYBR Green is a kind of fluorescent dye that only can bind to double-stranded DNA. Fluorescence sent out proportionally to double-stranded DNA's amount. The input cDNA or DNA of PCR reaction is minimal, hence just double stranded DNA has enough quantity to be explored in PCR product. The read-out is showed by the number of PCR cycles (like cycle threshold, Ct for short) need to achieve the fluorescence's given level. During the original PCR cycles, fluorescence sent out by SYBR Green bound to PCR products, normally it is too weak to note. In the PCR's exponential phase, the fluorescence signal doubles at every cycle. A exact fluorescence signal doubling at every cycle is a main indicator of an appropriate assay. The fluorescent intensity usually trends to plateau means the PCR has attained saturation after more or less 32 cycles. Since cycle threshold relates to the original quantity of target of sample, the correlative concentration of one target with respect to another is showed in the cycle number's differences ($\Delta C_t = C_t^{sample} - C_t^{reference}$) need to reach the same fluorescence's level. There are many commercially SYBR Green chemistry kits can be used, like Roche's DNA master SYBR® Green, Qiagen's QuantiTech SYBR® Green PCR master mix, Biorad's IQ™SYBR® Green supermix, MJ Research's DyNAsin™ II DNA polymerase with SYBR® Green and so on (Frederique, 2006).

It is worth to note that once your experiment has been optimized by one chemistry, if you want to change chemistry kits, the re-optimization is necessary. Although the master mixes may be more expensive than individual reagents, in order to minimize the variability, the master mixes are recommended, or through using

homemade SYBR Green master mixes to decrease the cost (Karsai et al., 2002).

1.5 Relationship with the prior experiment

Growth promoter administration results in muscle fibre hypertrophy and changes in muscle fibre type composition, responses that are important in terms of human ageing and disease (such as sarcopenia and type 2 diabetes), as well as animal growth efficiency.

As can be seen form the description above the produce of animals can be changed by the of utilization of growth promoters. Fundamentally the object is to change lean tissue production which will enable the more efficient production of the meat. This is important because of the limitations in the availability of food supplies for the world population. Also illustrated are the potential methodologies which will allow the dissection of this mechanisms by which these growth promoter agents work. Through the use of microarray it may be possible to identify these mechanisms which will allow manipulation of the growth process to enable more efficient production systems.

On going research within the deviation of nutritional sciences has been examining the effects of growth promoter on sheep muscle growth..

Specifically, a recent experiment has examined the effects on muscle physiology and target gene expression in response to short term (6 day) treatment of sheep (120 days of age) with either beta-agonist (BA) and growth hormone(GH).

The main finding of this study were (produced by Krystal Hemmings):

1. Although just a short-term treatment with growth promoters, there has a trend for the weight of muscle to rise in response to beta-agonist in the 120 days of age lambs, however no influences were found with growth hormone.

2. BA treatment significantly affected the metabolic activity in the *longissimus dorsi* (LD). The isocitrate dehydrogenase (ICDH) activity has been decreased, which

suggests a relational decrease in oxidative capacity, pointing metabolic transition towards a more glycolytic fibre type. This decrease also correlate with relevant varieties in signaling, involving an rise in the mRNA expression of the co-repressor receptor-interacting protein 140 (RIP140) and a decrease in the co-activator PGC-1β.

3. Although both growth promoters can lead a rise in muscle mass though different mechanisms, only BA has effects on fibre type composition of muscle. In other words, BA has strong effect on Myosin heavy chain mRNA expression in longissimus dorsi.

In summary, the main finding of this study has been that the growth promoters, particularly BA, induced muscle growth and this appeared to be a switch to a muscle type that was a fast type with an apparent reduction on the oxidative metabolic capacity.

As current available sheep microarrays are more targeted to study genes involved in immune response and not suitable for studies on muscle growth and metabolism. A human Affymetric arrary and genomic DNA hybridization were used to investigate effects of beta-agonist and growth hormone on the sheep muscle transcriptome (in collaboration with Sean May).

This microarrays dataset is available but in order to identify muscle growth signaling pathways specific or common to beta-agonist and growth hormone, the GeneSpring GX11 and Ingenuity Pathway Analysis software had been used to fully analyze the data. Identification of the microarray results was performed using real-time quantitative RT-PCR.

1.6Hypothesis and Objectives

The aim of the project was to further analyze and explain the results from Krystal Hemmings, which would enable a better understanding increase the animal growth efficiency.

Hypothesis: Growth promoter administration to sheep will lead to the effects on muscle gene expression which when detected by microarray analysis will indicate the specific pathways and target genes which mediate this response.

Therefore the objectives of this project were

1. To analyze the data produced from a microarray examination of the effects of growth promoters on sheep skeletal muscle. This will include:
    a. Identify up and down regulated genes and commonality and differences between the two growth promoters.
    b. Examination of potential interactions between genes whose expression had been altered to try to identify signaling pathways that are effected by the agents
    c. The identification gene expression alterations which are associated with specific pathways which maybe involved in mediating the response top the growth agents
2. On selected genes associated with specific pathways that respond to the agents confirm changes in gene expression identified by microarray using quantitative RT-PCR

2 MATERIALS AND METHODS

2.1Animal trial

Male lambs were kept with their mothers until 53 ± 4 days of age then they were weaned and penned individually until 12o days of age (D120 age). Creep feed started about 28 days before weaning and lambs were given free access from weaning onwards. Before the three treatments, lambs were penned with ad-libitum access to creep feed for 7 days.

These D120 age lambs were separated into three treatment groups. The difference of each group's mean body weights was not significant. First group, control group (n=11) were fed ad-libitum. Second group, growth hormone group (n=10) were fed ad-libitum and injected prolonged release bovine growth hormone (POSILAC®, Monsanto) on the first day. The dose was 3.75mg/kg BW. Third group, beta-agonists group (n=10) were fed ad-libitum and 10 ppm cimaterol (supplied by Pfizer Ltd). All of these treatments started at 120 ± 4 days of age and sustained six days.

At slaughter, the samples from LD (from left side of sheep carcass at the 10th rib) were snap-frozen in liquid nitrogen then stored at 80 degrees below zero centigrade. All of the frozen LD muscles were dissected from the right side of the sheep carcass, crushed and mixed for next experiment (Supplied by Zoe Daniel).

2.2 Cross-species microarray

As current available sheep microarrays are more targeted to study genes involved in immune response and not suitable for studies on muscle growth and metabolism, and the aim of this project is to identify the growth signaling pathways in sheep skeletal muscle, therefore the human GeneChip array (Affymetrix-GeneChip Human Genome U133 Plus 2.0) was selected to hybridize with Sheep genomic DNA .

The RNA samples from sheep muscle were analyzed by NASC Xspecies technique (Hammond et al. 2005). The GeneChip operating system (GCOS; Affymetrix), GeneSpring GX 11 (Agilent Technologies) and NASC Xspecies filter were used to analyze this cross-species microarray data, as described by Daniel et al (2008).

2.3 Bioinformatics

2.3.1 Software analysis

2.3.1.1 GeneSpring GX 11

In this project, GeneSpring GX 11 (Agilent Technologies) was used to analyze the changes of gene expressions after three treatments (beta agonists, growth hormone and normal), how well does the sheep skeletal muscle on human microarray works and try to identify sheep muscle growth signaling pathways specific or common to beta agonists (BA) group and growth hormone (GH) group. The steps of GeneSpring GX 11.0 analysis showed below.

1) Loaded initial data and created experiments in GeneSpring GX 11.0. By using RMA normalization algorithm to load the RNA files into GeneSpring GX 11.0 (Irizarry et al.,2003). This RNA files were analyzed by the .CDF files emerged from the genomic DNA hybridization, as described by Neil S. et al (2009). The probes with hybridization signal less than 450 in the genomic DNA hybridization had been removed.

2) Profile plots analysis. Profile plots is an important visualizations that can cleanly display the change trend of gene expression against the fold change and different colors. In this project, the profile plots function was used to show the different expression and fold change of every gene in beta agonist treatment group, control group and growth hormone treatment group.

3) Statistical analysis. Used one way analysis of variance (ANOVA) to test the initial data. Chose the genes which meet P value less than 0.05 (named “After ANOVA gene group”) to analyze in next step.

4) Principal components analysis (PCA). It was used to view the separation of the sample groups of beta agonist (BA) treatment, control and growth hormone (GH) treatment. The quality of data was checked by this step.

5) Gene ontology analysis (GO analysis). This module was used to explore gene

ontology and display the percent of the genes which have similar functions. The genes (from "After ANOVA gene group") which met p value less than 0.05 had been explored.

6) Filtered on volcano plot. Volcano plot can represent a visual summary of fold change value and p value. In this case, the corrected p-value cutoff was 0.05; the fold change cutoff was 1.5 (Fold change = Condition 1/Condition 2), T Test unpaired, asymptotic p-value computation, no multiple testing correction. The up and down regulated genes were found by this step.

By comparing the gene expression of the treatment groups (BA and GH) with control group, four gene groups had been built. The first group named "Up-regulated by BA group", the genes of this group up-regulated by beta-agonist (BA) relative to the control group. The second group named "Down-regulated by BA group", the genes of this group down-regulated by beta-agonist (BA) relative to the control group. The third group named "Up-regulated by GH group", the genes of this group up-regulated by growth hormone (GH) relative to the control group. The fourth group named "Down-regulated by GH group", the genes of this group down-regulated by growth hormone (GH) relative to the control group.

By merging "Up-regulated by BA group" and "Down-regulated by BA group", a new group had been made, named "Up or down-regulated by BA group". By merging "Up-regulated by GH group" and "Down-regulated by GH group", another new group had been made, named "Up or down-regulated by GH group".

7) Venn diagram analysis. Venn diagram was used to capture the common and different genes between two of the gene groups which from "Filter on volcano plot" step. Used venn diagram function to compare "Up or down-regulated by BA group" and "Up or down-regulated by GH group", the common genes of them had been found and it will showed in Figure 3.6. This common gene group named "common group"

8) Pathway analysis. The four gene groups which from "Filter on volcano plot" step were mapped into the GeneSpring pathway database. This software integrated the information regarding dependencies of the genes and the dynamics in a new pathway.

GeneSpring had two different options of this pathway analysis step, Simple Analysis and Advanced Analysis. In this project, the Direct Interactions function of Simple Analysis had been used to find the direct relations between the target genes or proteins.

9) Mapped into Kyoto Encyclopedia of Genes and Genomes (KEGG) database. In "pathway analysis" step, some new pathways had been found. In order to complete these pathway results and research the biology process of them, the GeneSpring GX 11.0 was used to map data in KEGG pathway database. This step of GeneSpring can supply a clearly visualization pathway that includes functional genes and biology process and it is useful for understanding and analyzing biology process and determine future experimental direction.

2.3.1.2 Ingenuity Pathways Analysis

IPA has eight kinds of analysis types, such as Core Analysis, IPA Metabolomics analysis, IPA Tox Analysis and IPA Biomarker Analysis. In this project, Core Analysis had been used. It allows users to interpret small or large datasets in the context of pathways, molecular networks and biological processes. The first step of IPA analysis was uploading data. "Up or down-regulated by BA group", "Up or down-regulated by GH group", and "common group" had been mapped in Ingenuity Pathway Analysis software (IPA version: 8.6-3003 Build: 93815;2010 Ingenuity Systems, Inc). Artificially changed different gene ID (Probe set ID, UniGene ID, GenBank and GI Number), the "mapped NO./unmapped NO." of three gene groups were 369/76 ("Up or down-regulated by BA group"), 237/49 ("Up or down-regulated by GH group") and 68/16 ("common group"). All of the unmapped genes had been checked, a vast majority of them referred to transcripts of unassigned function or were suspected to be ALU-repeat sequences, which were most unlikely to map anyway. This artificially method can map much more genes than the "Ingenuity pathway analysis connector" function of Genespring and give researchers more opportunities to identify the pathways they haven't thought of.

The corn pathway functions of IPA, Networks Explorer and Canonical pathways, had been used in this project. The introductions of them showed below.

Networks Explorer. It is a useful function for visualizing molecular relationships. Genes, chemicals, and proteins are indicated as various shapes. In this project,"Up or down-regulated by GH group"and"common group"respectively produced 25 (Table 3.7) and 9 networks (Table 3.8). The analysis result of"Up or down-regulated by BA group"was composed of 25 networks (showed in Table 3.6), two of them respectively included v-akt murine thymoma viral oncogene homolog 1 (AKT1) and phosphoserine aminotransferase 1 (PSAT1). They were the core genes of the pathway result from GeneSpring.

The Canonical Pathways Analysis. This step can dynamic and deeply display cell and metabolic signaling pathways which have been up-to-date and managed by Ph.D. level researchers. The renewed information and networks come from review articles, specific journal articles, textbooks and KEGG database. In this project, some important pathway results associated with oxidative metabolism and maybe involved in mediating the response of the growth agents had been found by the canonical pathway analysis.

The flow chart and important gene groups of all software analysis process were showed by Figure 2.1

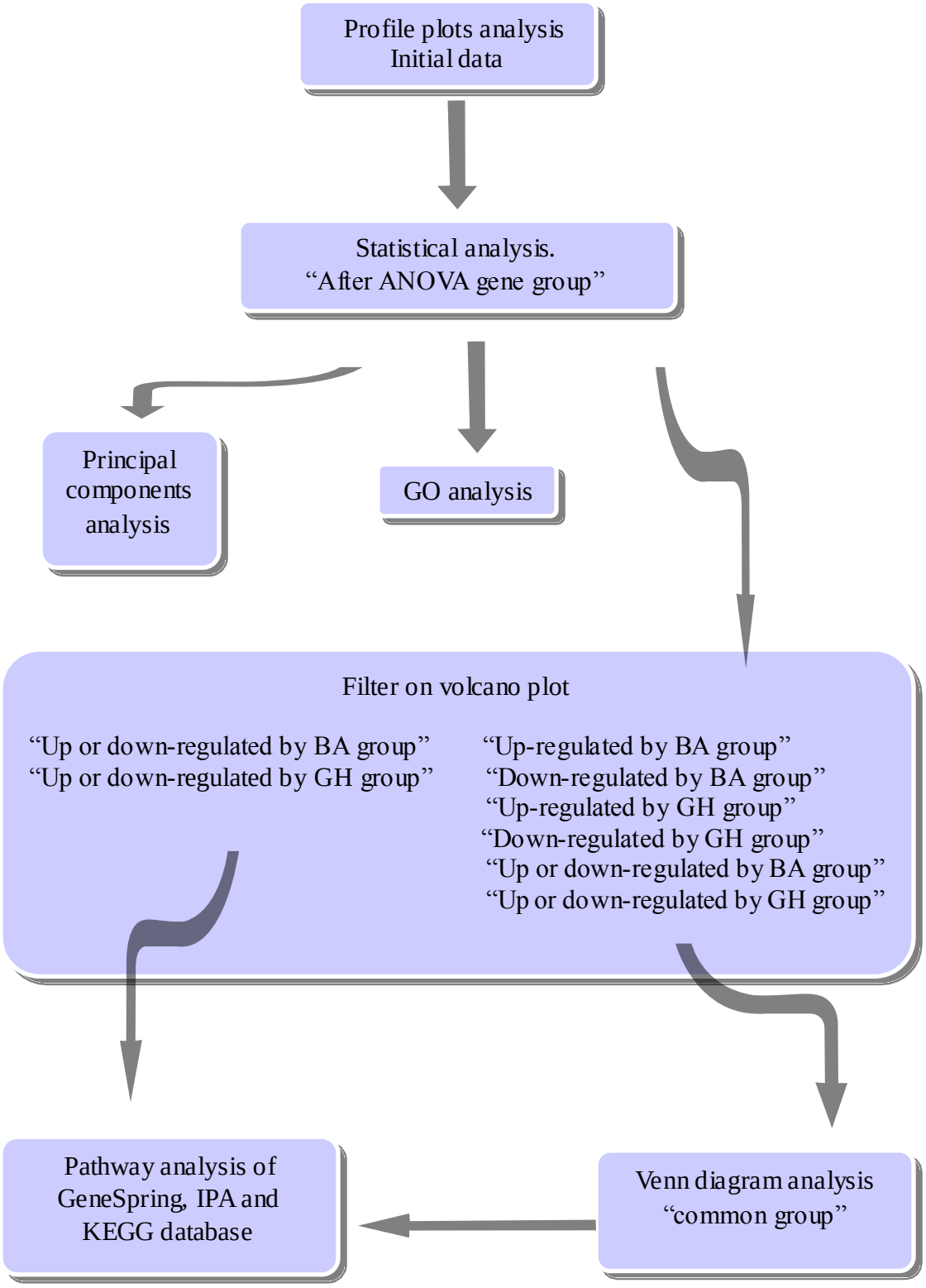

Figure 2.1 The summary chart of software analysis

After loaded initial data in GeneSpring, used profile plots analysis to look at the expression and fold change of all gene. Then statistical analysis (one way ANOVA test) had been used to find the significant data (p value less than 0.05) and these data made up of “After ANOVA gene group”. Then used PCA method to check the quality of samples. In the next step, GO analysis had been used to class

the genes in “After ANOVA gene group” according to biology function. Then “After ANOVA gene group” was filtered on volcano plot and six important gene groups had been built. There were “Up-regulated by BA group”, “Down-regulated by BA group”, “Up-regulated by GH group”, “Down-regulated by GH group”, “Up or down-regulated by BA group” and “Up or down-regulated by GH group”. All of the six gene groups had been analyzed by Venn diagram function. The common genes of “Up or down-regulated by BA group” and “Up or down-regulated by GH group” built the “common group”. Finally, “Up or down-regulated by BA group”, “Up or down-regulated by GH group” and “common group” had been mapped in pathway analysis function of GeneSpring, IPA and KEGG database to explore pathways.

2.3.2 Sequence search

In this experiment, ovine DNA was hybridized to the human Affymetrix microarray in order to analyze sheep muscle gene expression. Therefore this project needs researcher to search ovine ESTs sequence instead of using the human's sequence from GeneSpring directly for primer design. The searched genes from AKT1 pathway (Figure 3.9), PSAT1 pathway (Figure 3.15) and citrate cycle pathway (Figure 3.17). The flow chart of searching process showed below (Figure 2.2).

Target genes were searched in NCBI database (http://www.ncbi.nlm.nih.gov/).

Status 1 Had ovine cDNA sequence.

▼

Aligned different ovine mRNA sequences found by different people (Clustal W2)

▼

Used the same part to design primer.

Status 2. No ovine cDNA sequences but had bovine cDNA sequences.

▼

Aligned different bovine mRNA sequences found by different people (Clustal W2)

▼

Blasted the similar bovine CDS sequence in sheep ESTs database (www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9940). Chose the result with highest score. (If the CDS number is not showed in NCBI, used human's CDS to define the bovine's)

▼

Used Clustal W2 (www.ebi.ac.uk/Tools/clustalw2/index.html) and EMBOSS Pairwise Alignment Algorithms (www.ebi.ac.uk/Tools/emboss/align/index.html) to do alignment and checked the similarity between bovine mRNA and ovine EST sequences.

▼

The ovine EST sequence had high similarity score (more than 75 or 18.0 percent) can be used to design primer.

Status 3. No ovine and bovine cDNA sequences but had human cDNA sequences.

▼

Aligned different human mRNA sequences found by different people (Clustal W2)

▼

Blasted the similar human CDS sequence in sheep ESTs database. Chose the result with highest score.
(www.ncbi.nlm.nih.gov /genome/seq/BlastGen/BlastGen.cgi?Taxid =9940).

▼

Used ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html) and EMBOSS Pairwise Alignment Algorithms(www.ebi.ac.uk/Tools/emboss/align/index.html) to do alignment and checked the similarity between human mRNA and ovine EST sequences.

▼

The ovine ESTs sequence had high similarity score (more than 75 or 18.0 percent) can be used to design primer.

Figure 2.2 The flow chart of searching process

2.4 Primer design

After sequences searching, ovine EST sequences had been load into Cow BLAT Search online software (http://genome.ucsc.edu/cgi-bin/hgBlat) to find the exon boundaries for primer design. Designed primers used Primer Express 3.0 (Applied Biosystems) to suit real time PCR parameters. These parameters were shows below (Table 2.1) and the summary table of finally designed primers will show in Appendix 1.

Table 2.1 Parameters of primer design

Parameter	Value
Primer Temperature	
Min Primer Temperature	58
Max Primer Temperature	60
Max Difference in Temperature of Two Primers	2
Primer GC Content	
Mix Primer %GC Content	30
Max Primer %GC Content	80
Max Primer 3' GC's	2
Primer 3'End Length	5
Primer 3'GC Clamp Residues	0
Primer Length	
Min Primer Length	9
Max Primer Length	40
Optimal Primer Length	20
Primer Composition	
Max Primer G Residues	3
Max Num Ambig Residues in Primer	0
Primer Secondary Structure	
Max Primer Consec Base Pair	4
Max Primer Total Base Pair	8
Primer Site Uniquencess	
Max % Match in Primer	75
Max % Consec Match in Primer	9
Max % 3' Consec Match in Primer	7
Amplicon	

Min Amplified Region Temperature	0
Max Amplified Region Temperature	85
Min Amplified Region Length	50
Max Amplified Region Length	150

2.5 Quantitative real-time PCR

Total RNA was isolated from all samples using Trizol as per manufacturers instructions including glycogen removal (Invitrogen,Paisley,UK) and an additional DNase step (Promega) and phenol/ chloroform/ isoamylalcohol clean up. The isolated total RNA was resuspended in RNase-free water and frozen at –80 ℃ until required. Yield and purity were determined using the Nanodrop DN-1000 (Thermo Scientific). (Supplied by Zoe Daniel)

First Strand cDNA synthesis. Every reaction tube (thin walled 0.2ml PCR tube) added 5 ul total RNA stock (0.1ug/ul; 10pg-5ug total RNA), 1ul Random Primers (50-250ug random primers) and 9 ul water. Then put them in PCR machine, incubated 70 degrees centigrade for 5 minutes and then put on ice for 1 minute. After that added 5ul MMLV Reverse Transcriptase buffer (x5), 1.25 l Nucleotides (10mM each), 0.5 l RNase inhibitor, 1 l MMLV Reverse Transcriptase and 2.25 l RNase free water in each tube. The final volume was 25 uL. Mixed and spun down, then incubated at room temperature for 10 minutes and returned to the PCR machine for 42 degrees centigrade for 60 minutes and held at 4 degrees centigrade. This process as described by the manufacturer (Promega, Southampton, UK).

Oligreen for determining cDNA quantity. Made 1x TE buffer by diluting 20 x TE buffer 1:20 in RNAse free water, then made working reagent of Oligreen cDNA reagent by diluting 1:200 in 1 x TE buffer. After this added 5uL working reagent and 5uL cDNA into one well of 384 well plate (Poche cat). Incubate plate in dark for 5 minutes and ran on Roche Light Cycler 480. The data from Light Cycler 480 was used to make standard curve to check the quantity of cDNA.

Checked primer pairs(Sigma Alorich) on serial dilution of LD cDNA. Real-time PCR reactions using the primers described in Appendix 1. Ran samples and standard curve for 3 selected genes and cyclophillin. These genes were clathrin, light chain (Lca)

(CLTA), forkhead box P1 (FOXP1) and rhomboid, veinlet-like 1 (RHBDL1). The real time PCR process was described below.

Every well of 384 well plates (Poche cat) carried 7.5 uL 1x SYBR Green PCR Master Mix (Roche), 0.45 uL forward primer (0.3 uM), 0.45 uL reverse primer (0.3 uM), 1.6 uL water (molecular grade), and 5 uL cDNA (1 in 4 or 1in 8 dilution). The final volume was 15 uL. Mixed and spun down by centrifuge for 5 minutes, and then read on Light Cycler 480 PCR machine (Roche): 95℃ for 5 minutes, then 95℃ For 10 seconds, and then 45 cycles of 95℃ for 1 second and 60℃ for 20 seconds. The fluorescence was detected in real time and threshold CP values were calculated using LC480 software. Used melt curve analysis to make sure only giving one produce and that the standard curve was linear with an efficiency of less than 2. The data had been test by one way ANOVA test (Genstat 12th).

3 RESULTS

3.1 Checking the quality of microarray data

The result of this part mainly proved that the microarray data after one way analysis of variance test had good quality.

The initial data had been loaded in GeneSpring, all of the raw data was cleanly displayed in profile plots (Figure 3.1). This figure showed the changes of expression of all the initial gene against the fold change and three different colors. After this step, used ANOVA to test the initial data, genes which meet P value less than 0.05 had been chose to make up of "After ANOVA gene group". The Figure 3.2 showed the expression's changes of genes which meet P value less than 0.05. By comparing Figure 3.1 and Figure 3.2, it is easy to see that the number of genes in Figure 3.2 is less than in Figure 3.1. This trended to prove that the ANOVA test worked.

In the next step, "After ANOVA gene group" had been analyzed by Principle Component Analysis. In the result figure (Figure 3.3), the different treatment groups separated from each other and the replicates of each group clustered together in one side of three-dimensional photo. This demonstrated that the three sample groups had significant differences and the microarray data has good quality.

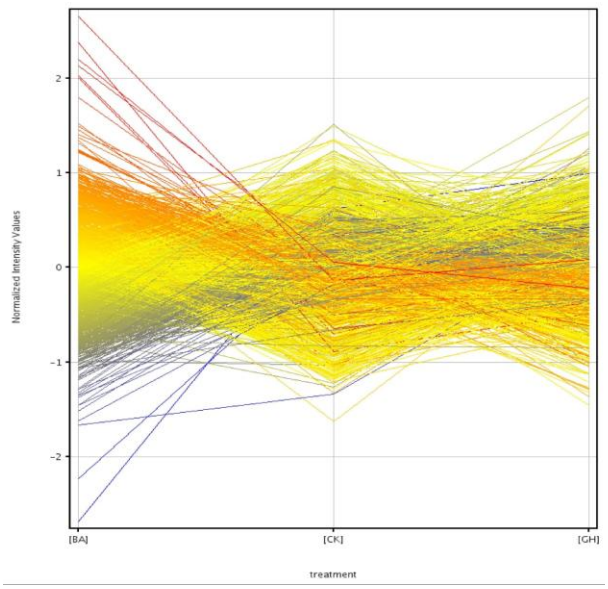

Figure 3.1 Profile plots of initial data

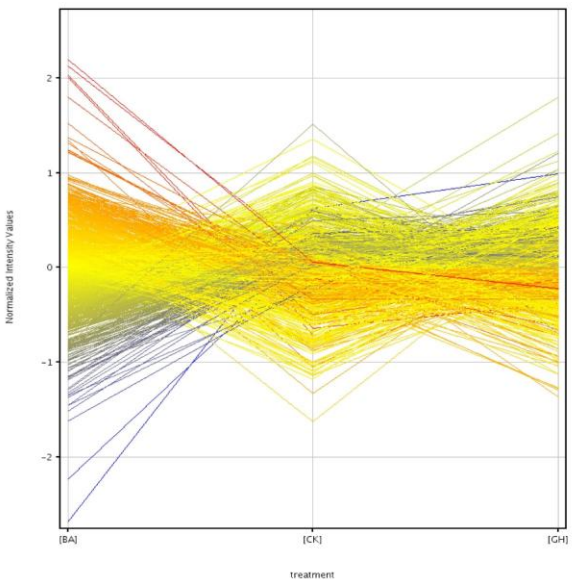

Figure 3.2 Profile plots of the data after one way ANOVA test (p value less than 0.05).

In Figure 3.1 and Figure 3.2, X axis shows different treatments: beta agonist (BA), control (CK) and growth hormone (GH). Y axis displays the expression intensity value of genes. Red lines indicate the genes up-regulated by beta-agonist (BA) relative to the control group (CK), and the genes down-regulated by beta-agonist (BA) relative to the control group (CK) were colored by blue. Yellow lines means the genes have similar expression relative to the control group (CK).

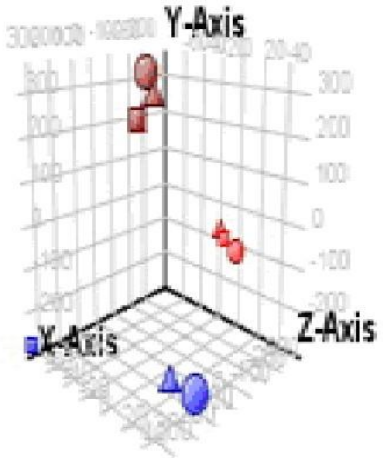

Figure 3.3 Principle Component Analysis (PCA) of the data after one way ANOVA test.

Red color shows the triplicate samples that were treated by beta agonist, and the blue color shows the triplicate samples that were treated by growth hormone. Brown points display control samples.

3.2 Grouping genes according to biology function

The result of this part mainly indicated that the different groups of significant genes based on biology functions.

Gene ontology analysis (GO analysis) was used to explore gene ontology and displayed the percent of the genes which have similar function. "After ANOVA gene group" had been used to analyze by GO analysis. There were three main functions had been found: molecular function, biological process and cellular component. Table 3.1 showed the functional sub-groups of biological process. The functional sub-groups of cellular component was displayed in Table 3.2. Table 3.3 indicated the functional sub-groups of molecular function.

"Count" in GO analysis tables (Table 3.1, Table 3.2 and Table 3.3) refers to the number of genes which have similar gene ontology term. "Percent" refers to the percentage of genes which have similar gene ontology term in the "After ANOVA gene group".

Table 3.1 Gene ontology chart of biological process

GO Term	Count	Percent(%)
GO:009987|GO:008151 celluar process	990	24.42
GO:008152 metabolic process	623	15.37
GO:0065007 biological regulation	615	15.17
GO:0032502 developmental process	320	7.89
GO:0032501|GO:0050874 multicelluar organismal process	316	7.79
GO:0051179 localization	293	7.23
GO:0051234 establishment of localization	256	6.31
GO:0050896|GO:0051869	219	5.4
GO:002376 immune system process	77	1.9
GO:0022610 biological adhesion	69	1.7
GO:0051704|GO:0051706 multi-organism process	61	1.5
GO:00000003|GO:0019952|GO:0050876 reproduction	60	1.48
GO:0043933|GO:0034600 macromolecular complex subunit organization	59	1.46
GO:0022414 reproductive process	41	1.01
GO:0040011 locomotion	26	0.64
GO:0040007 growth	12	0.3
GO:0048511 rhythmic process	8	0.2
GO:0016032 viral reproduction	6	0.15
GO:0043473 pigmentation	2	0.05
GO:001906 cell killing	1	0.02

Table 3.2 Gene ontology chart of cellular component

GO Term	Count	Percent(%)
GO:0005623 cell	1210	28.39
GO:0044464 cell part	1210	28.39
GO:0043226 organelle	782	18.35
GO:0044422 organelle part	393	9.22
GO:0032991 macromolecular complex	219	5.14
GO:0031974 membrane-enclosed lumen	145	3.4
GO:0005576 extracellular region	128	3
GO:0031975 envelope	71	1.67
GO:0044421 extracellular region part	57	1.34
GO:0045202 synapse	23	0.54
GO:0044456 synapse part	14	0.33

GO:0044420 extracellular matrix	8	0.19
GO:0044423 virion part	1	0.02
GO:0019012 virion	1	0.02

Table 3.3 Gene ontology chart of molecular function

GO Term	Count	Percent(%)
GO:0005488 binding	1061	50.55
GO:0003824 catalytic activity	439	20.91
GO:0060089 molecular transducer activity	188	8.96
GO:0030528 transcription regulator activity	138	6.75
GO:0005215|GO:0005478 transporter activity	100	4.76
GO:0030234 enzyme regulator activity	73	3.48
GO:0005198 structural molecule activity	49	2.33
GO:0045182 translation regulator activity	16	0.76
GO:0009055|GO:0009053|GO:0009054 electron carrier activity	15	0.71
GO:0016530 metallochaperone	3	0.14
GO:0031386 protein tag	1	0.05
GO:0016209 antioxidant activity	1	0.05
GO:0015457 auxiliary transport protein activity	1	0.05

3.3 Identification of target genes by Volcano plot

The result of this part mainly identified the up and down regulated genes by comparing the treatment (BA and GH) data and control data.

"After ANOVA gene group" had two treatment groups (beta agonist and growth hormone) and control group. Used volcano plot to filter BA or GH group versus control group, the results showed in Figures 3.4 and Figures 3.5. These volcano plot's corrected p-value cutoff were 0.05, fold change cutoff were 1.5. Used unpaired T Test to produced. Therefore, in Figures 3.4, there were 236 genes in the green pane of right side up-regulated by beta-agonist (BA) relative to the control group (p value < 0.05, fold change > 1.5), and 209 genes in the green pane of left side down-regulated by beta-agonist (BA) relative to the control group (p value < 0.05, fold change < -1.5). This result showed that BA had a clear effect on gene expression with approximately equal effects on increased and decreased expression. These up/down-regulated genes as the target genes for this project had been mapped in GeneSpring and IPA database to

identify pathways. The top 10 highest fold change genes of these up/down-regulated genes showed in Table 3.4.

Similarly, in Figures 3.5, there were 147 genes in the green pane of right side up-regulated by growth hormone (GH) relative to the control group (p value < 0.05, fold change > 1.5), and 139 genes in the green pane of left side down-regulated by growth hormone (GH) relative to the control group (p value < 0.05, fold change < -1.5). This result also showed that GH had a clear effect on gene expression with approximately equal effects on increased and decreased expression. These up/down-regulated genes as the target genes for this project had been mapped in GeneSpring and IPA database to identify pathways. The top 10 highest fold change genes of these up/down-regulated genes showed in Table 3.5.

There were 236 genes up-regulated by BA relative to the control group and 209 genes down-regulated by BA relative to the control group, while there were 147 genes up-regulated by growth hormone (GH) relative to the control group, and 139 genes down-regulated by growth hormone (GH) relative to the control group. Hence, BA tended to change gene expression of more genes than GH.

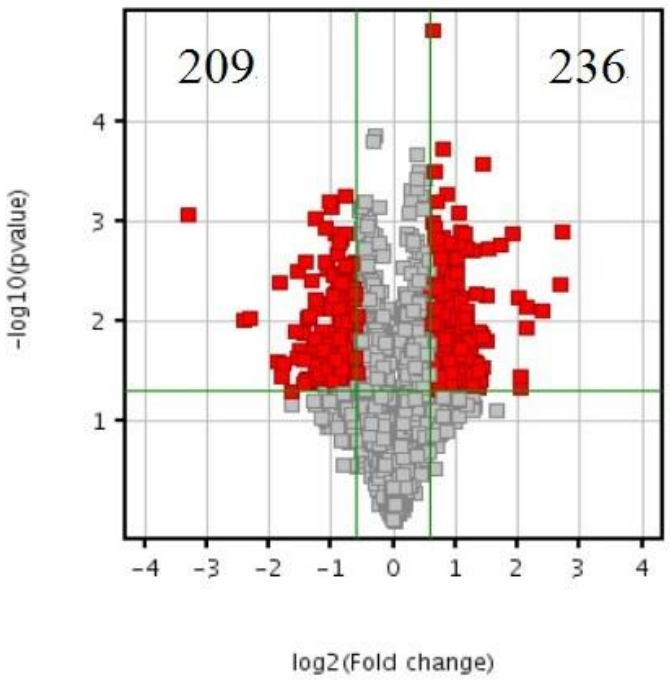

Figure 3.4 Volcano plot for the data treated by beta agonist (BA) versus the data treated by control.

The X axes shows the negative log10 of p-value and the Y axes displays the log (base2.0) of fold change. The red points in the green pane of right side indicate the genes satisfy p value less than 0.05 and fold change more than 1.5. The red points in the green pane of left side indicate the genes satisfy p value less than 0.05 and fold change less than negative 1.5. The rest genes appear in gray color. 209 means the number of red points in left side and 236 means the number of red points in right side. The green lines means the cutoff of p-value is 0.05 and the cutoff of fold change is 1.5.

Table 3.4. The top 10 highest fold change genes up-regulated by BA relative to control and top 10 highest fold change genes down-regulated by BA relative to control

Probe set ID	p-value	Fold Change	regulation	Gene symbol	Gene title
206667_s_at	0.00124	6.5635695	up	SCAMP1	secretory carrier membrane protein 1
44783_s_at	0.004238	6.372318	up	HEY1	hairy/enhancer-of-split related with YRPW motif 1
209432_s_at	0.00792	5.2507415	up	CREB3	cAMP responsive element binding protein 3
1569871_at	0.007322	4.411797	up	LOC650392	Hypothetical protein LOC650392
201825_s_at	0.011707	4.358996	up	SCCPDH	saccharopine dehydrogenase (putative)
212857_x_at	0.045971	4.122034	up	SUB1	SUB1 homolog (S. cerevisiae)
234865_at	0.036119	4.0604424	up	TRBV25-1	T cell receptor beta variable 25-1
205132_at	0.005813	4.0215974	up	ACTC1	actin, alpha, cardiac muscle 1
232006_at	0.001719	3.2433836	up	STK35	Serine/threonine kinase 35
218140_x_at	0.00188 4	2.8869574	up	SRPRB	signal recognition particle receptor, B subunit
204173_at	0.000861	9.872075	down	MYL6B	myosin, light chain 6B, alkali, smooth muscle and non-muscle
207318_s_at	0.009622	5.3139696	down	CDC2L5	cell division cycle 2-like 5 (cholinesterase-related cell division controller)
1552777_a_at	0.009273	5.024218	down	RAET1E	retinoic acid early transcript 1E
203543_s_at	0.024992	3.6362588	down	KLF9	Kruppel-like factor 9

213301_x_at	0.004001	3.5485144	down	TRIM24	tripartite motif-containing 24
229060_at	0.035918	3.5231614	down	YPEL2	yippee-like 2 (Drosophila)
228856_at	0.026676	3.4488096	down	ZNF747	zinc finger protein 747
224357_s_at	0.04984	3.123486	down	MS4A4A	membrane-spanning 4-domains, subfamily A, member 4
223179_at	0.003176	2.9623978	down	YPEL3	yippee-like 3 (Drosophila)
212977_at	0.023291	2.9376898	down	CXCR7	chemokine (C-X-C motif) receptor 7

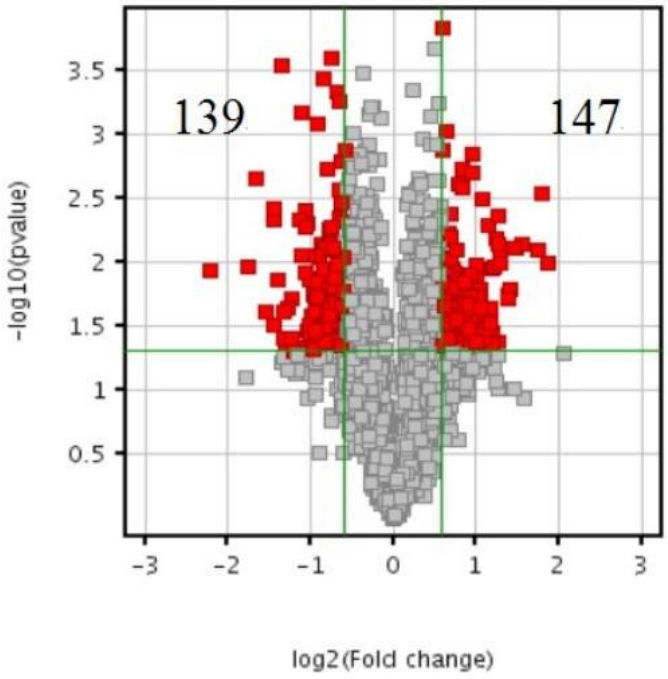

Figure 3.5 Volcano plot for the data treated by growth hormone (GH) versus the data treated by control.

The X axes shows the negative log10 of p-value and the Y axes displays the log (base2.0) of fold change. The red points in the green pane of right side indicate the genes satisfy p value less than 0.05 and fold change more than 1.5. The red points in the green pane of left side indicate the genes satisfy p value less than 0.05 and fold change less than negative 1.5. The rest genes appear in gray color. 139 means the number of red points in left side and 147 means the number of red points in right side. The green lines means the cutoff of p-value is 0.05 and the cutoff of fold change is 1.5.

Table 3.5 The top 10 highest fold change genes up-regulated by GH relative to control and top 10 highest fold change genes down-regulated by GH relative to control

Probe set ID	p-value	Fold Change	regulation	Gene symbol	Gene title
204204_at	0.01009	3.6135406	up	SLC31A2	solute carrier family 31 (copper transporters), member 2
218387_s_at	0.00283	3.469396	up	PGLS	6-phosphogluconolactonase
201825_s_at	0.00788	3.3515317	up	SCCPDH	saccharopine dehydrogenase (putative)
230464_at	0.0071	2.91482	up	S1PR5	sphingosine-1-phosphate receptor 5
210691_s_at	0.00771	2.760892	up	CACYBP	calcyclin binding protein
1562411_at	0.00766	2.718481	up	MYLK3	myosin light chain kinase 3
217316_at	0.01627	2.631674	up	OR7A10	olfactory receptor, family 7, subfamily A, member 10
205509_at	0.0176	2.5984006	up	CPB1	carboxypeptidase B1 (tissue)
208790_s_at	0.00999	2.4406958	up	PTRF	polymerase I and transcript release factor
1557987_at	0.00819	2.418115	up	LOC641298	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase pseudogene
1552777_a_at	0.01145	4.648777	down	RAET1E	retinoic acid early transcript 1E
244684_at	0.01076	3.375558	down	PGGT1B	Protein geranylgeranyltransferase type I, beta subunit
212924_s_at	0.02454	2.90002	down	LSM4	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)
218533_s_at	0.00454	2.749083	down	UCKL1	uridine-cytidine kinase 1-like 1
220093_at	0.031	2.7295175	down	ANTXR1	anthrax toxin receptor 1
221580_s_at	0.00371	2.7195306	down	TAF1D	TATA box binding protein (TBP)-associated factor, RNA polymerase I, D, 41kDa
202233_s_at	2.85E-04	2.5676868	down	UQCRH	ubiquinol-cytochrome c reductase hinge protein
235252_at	0.02406	2.5175514	down	KSR1	Kinase suppressor of ras 1
202681_at	0.03915	2.5004194	down	USP4	ubiquitin specific peptidase 4 (proto-oncogene)

226465_s_at	0.0223	2.4167554	down	SON	SON DNA binding protein

3.4 Exploring commonality and differences of gene expression between two growth promoters by Venn diagram

The up and down regulated genes had been found in "Filtering on volcano plot" step, the results of this part mainly indicated that the common and different genes between the two growth promoters.

From the results of volcano plot analysis, it was easy to see that there were a total of 445 (209 plus 236) genes up-regulated or down-regulated by BA relative to control (CK) and a total of 286 (139 plus 147) genes up-regulated or down-regulated by GH relative to control (CK). In Figure 3.6, there were 84 genes up-regulated or down-regulated by both treatments. These 84 genes made up of "common group" that had been used to map in pathway analysis of GeneSpring and IPA database to identify pathways. In order to show the expression of genes more clearly, the Venn diagrams of up-regulated genes and down-regulated genes were displayed in Figure 3.7 and Figure 3.8 respectively.

"Filter on volcano plot analysis" step showed that there were a total of 236 genes up-regulated by BA relative to control (CK) and 147 genes up-regulated by GH relative to CK (Figure 3.7), with 39 genes up-regulated by both treatments. Similarly, there were a total of 209 genes down-regulated by BA relative to CK and 139 genes down-regulated by GH relative to CK (Figure 3.8), with 44 genes down-regulated by both treatments. Hence, BA tended to alter gene expression of more genes than GH.

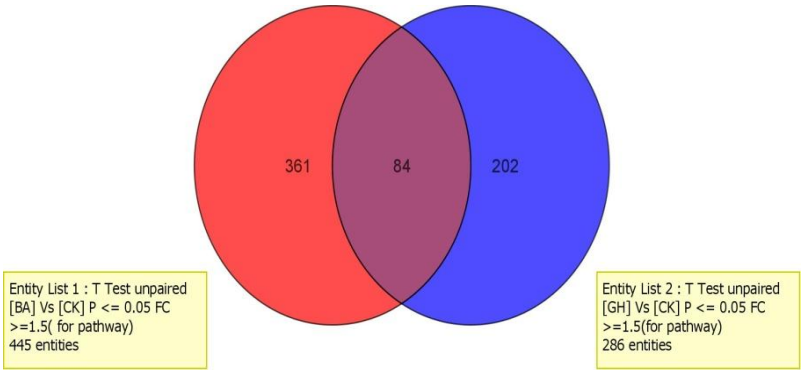

Figure 3.6 Venn diagram of numbers of genes up-regulated or down-regulated by BA and/or GH relative to control (CK).

The Red pie shows the numbers of genes up-regulated or down-regulated by beta-agonist (BA) relative to the control group (CK); while the blue pie shows the numbers of genes up-regulated or down-regulated by Growth Hormone (GH) relative to the control group (CK), with the intersect indicating the numbers of genes up-regulated or down-regulated by both BA and GH.

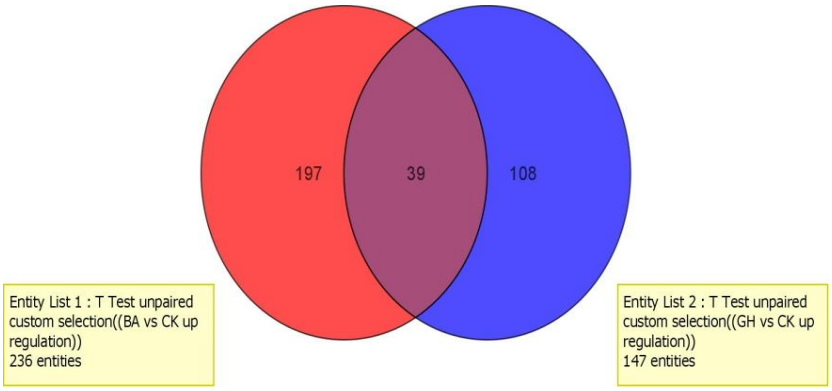

Figure 3.7 Venn diagram of numbers of genes up-regulated by BA and/or GH relative to control (CK).

The Red pie shows the numbers of genes up-regulated by beta-agonist (BA) relative to the control group (CK); while the blue pie shows the numbers of genes up-regulated by Growth Hormone (GH) relative to the control group (CK), with the intersect indicating the numbers of genes up-regulated by both BA and GH.

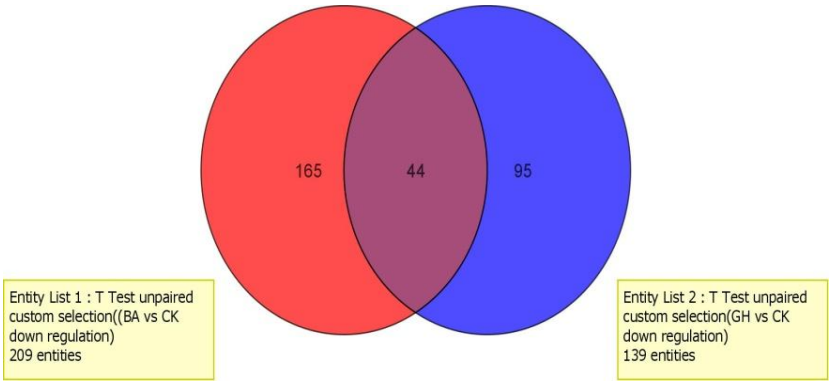

Figure 3.8 Venn diagram of numbers of genes down-regulated by BA and/or GH relative to control (CK).

The Red pie shows the numbers of genes down-regulated by beta-agonist (BA) relative to the control group (CK); while the blue pie shows the numbers of genes down-regulated by Growth Hormone (GH) relative to the control group (CK), with the intersect indicating the numbers of genes down-regulated by both BA and GH.

3.5 Pathway analysis

3.5.1 All pathways identified by Ingenuity Pathways Analysis

Three gene groups had been mapped in IPA, "Up or down-regulated by BA group", "Up or down-regulated by GH group" and "common group". All of the IPA pathway results of each group had been showed in Table 3.6, Table 3.7 and Table 3.8. These tables included scores and the main functions of each network. The scores had been got by counting how many inputted gene can been mapped in the pathway. Compared Table 3.6 with Table 3.7, there were more networks which had high scores (more than 10) had been found in the group treated by BA than the group treated by GH. This trended to prove that BA had more predominantly effects to sheep skeletal muscle than GH.

In all of the IPA networks identified form "Up or down-regulated by BA group", top NO.3 network (Figure 3.16) and top NO. 5 (Figure 3.12) network included PSAT1 and AKT1 respectively, which were the core genes of PSAT1 pathway (Figure 3.15) and AKT1 pathway (Figure 3.9) generated by GeneSpring. In addition, the top number 15 and top number 16 networks in Table 3.6 had direct relationship with skeletal development (colored by gray).

Table 3.6 All IPA networks identified from "Up or down-regulated by BA group"

The pathway had direct relationship with skeletal development had been colored by gray.

Network NO.	Score	Top Functions of networks
1	41	Cardiovascular Disease, Cellular Development, Connective Tissue Development and Function
2	30	Cancer, Respiratory Disease, Infection Mechanism
3	29	Cardiovascular System Development and Function, Cell Morphology, Cellular Movement
4	26	Cardiovascular Disease, Developmental Disorder, Cell Morphology
5	25	Genetic Disorder, Neurological Disease, Psychological Disorders
6	25	Cell Death, Cardiovascular System Development and Function, Organ Development
7	20	Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function
8	19	Hematological Disease, Cellular Assembly and Organization, Protein Degradation
9	18	Cancer, Hair and Skin Development and Function, Organ Development

10	17	Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry
11	17	Small Molecule Biochemistry, Cell Cycle, Cellular Development
12	17	Cellular Growth and Proliferation, Lipid Metabolism, Molecular Transport
13	15	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Hypersensitivity Response
14	15	Antimicrobial Response, Inflammatory Response, Infection Mechanism
15	15	Neurological Disease, Skeletal and Muscular System Development and Function, Tissue Morphology
16	15	Genetic Disorder, Skeletal and Muscular Disorders, Cellular Assembly and Organization
17	13	Cellular Assembly and Organization, Cellular Development, Cellular Growth and Proliferation
18	12	Gastrointestinal Disease, Cancer, Reproductive System Disease
19	2	Gene Expression, Infection Mechanism, Cancer
20	2	Hematological System Development and Function, Hematopoiesis, Tissue Morphology
21	2	Cellular Development, Developmental Disorder, Genetic Disorder
22	2	Cellular Development, Reproductive System Development and Function
23	2	Cellular Assembly and Organization, Cellular Compromise, DNA Replication, Recombination, and Repair
24	2	Developmental Disorder, Dental Disease, Genetic Disorder
25	2	Cardiovascular Disease, Genetic Disorder, Cancer

Table 3.7 All IPA networks identified form "Up or down-regulated by GH group"

Network NO.	Score	Top Functions
1	45	Tumor Morphology, Tissue Morphology, Cancer
2	38	Small Molecule Biochemistry, Endocrine System Development and Function, Energy Production
3	26	Cell-mediated Immune Response, Cellular Assembly and Organization, Cellular Development
4	24	Genetic Disorder, Respiratory Disease, Cellular Function and Maintenance
5	18	Lipid Metabolism, Small Molecule Biochemistry, Endocrine System Development and Function
6	18	Cellular Growth and Proliferation, Drug Metabolism, Endocrine System Development and Function
7	16	Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cellular Assembly and Organization

8	16	Cell Death, Cell Morphology, Cellular Assembly and Organization
9	16	Cellular Growth and Proliferation, Connective Tissue Development and Function, Cardiovascular System Development and Function
10	14	Cell-To-Cell Signaling and Interaction, Embryonic Development, Hematological System Development and Function
11	13	Developmental Disorder, Renal and Urological Disease, Cellular Compromise
12	7	Immunological Disease, Respiratory Disease, Cancer
13	2	Hematological System Development and Function, Hematopoiesis, Tissue Morphology
14	2	Cell Cycle, Embryonic Development, Organismal Development
15	2	Reproductive System Development and Function, Tissue Development, Reproductive System Disease
16	2	Unknown
17	2	Unknown
18	2	Cancer, Reproductive System Disease, Cellular Development
19	2	Unknown
20	2	Unknown
21	2	Cancer, Genetic Disorder, Respiratory Disease
22	2	Nervous System Development and Function, Tissue Development, Genetic Disorder
23	2	Genetic Disorder, Metabolic Disease, Small Molecule Biochemistry
24	2	Unknown
25	1	Cell-mediated Immune Response, Cellular Movement, Dermatological Diseases and Conditions

Table 3.8 All IPA networks identified form "common group"

NO.	Score	Top Functions
1	35	Small Molecule Biochemistry, Endocrine System Development and Function, Carbohydrate Metabolism
2	34	Cellular Function and Maintenance, Cell Cycle, Cellular Assembly and Organization
3	29	Cellular Compromise, Cell Morphology, Cell-To-Cell Signaling and Interaction
4	2	Gene Expression, Infection Mechanism, Cancer
5	2	Hematological System Development and Function, Hematopoiesis, Tissue Morphology
6	2	Cellular Assembly and Organization, Cellular Compromise, DNA Replication, Recombination, and Repair
7	2	Cancer, Reproductive System Disease, Cellular Development

3.5.2 Examining potential interactions between the up and down regulated genes by GeneSpring

There were many pathways had been found by GeneSpring. Two of them have been considered as key pathways to do further analysis. The key pathways will be showed below.

3.5.2.1 AKT1 pathway

Mapped "Up or down-regulated by BA group" in GeneSpring to explore direct interactions between each target gene, the AKT1 pathway (Figure 3.9) which included most interactions had been found.

AKT1 (V-akt murine thymoma viral oncogene homolog 1) down-regulated by BA relative to the control. It was the core of this pathway and there were 26 genes had direct or indirect relation with it. As recent research indicated that the AKT1or Atrogin-1(MAFbx)/MuRF1 signaling network is an vital route to the hypertrophy and differentiation of skeletal muscle (Coolican et al., 1997; Jiang et al., 1998; Glass, 2003; Nader. 2005; Chang 2007). Hence, this pathway was an important result of this project. And in the further work, qReal-Time PCR can be used to confirm the expression of these genes.

When the "Up or down-regulated by BA group" had been mapped in IPA, AKT1 also showed in the top number 5 network of all the IPA pathways. This network had been named "AKT1 network from IPA" (Figure 3.12). The main functions of this network was about Genetic Disorder, Neurological Disease and Psychological Disorders (showed in Table 3.6). This result proved the presence of AKT1 pathway and provided more information of it. Compared AKT1 pathway from GeneSpring with AKT1 network from IPA, there were much more genes linked to AKT1 in GeneSpring's result, thereby GeneSpring was better to find the relationships between each target gene than IPA.

Similarly, mapped "Up or down-regulated by GH group" in GeneSpring to explore direct interactions between each target gene, Figure 3.14 had been found. From this figure it was easy to see that the number of genes in Figure 3.9 was much more than the

number of genes in Figure 3.14 when used the same method to explore the pathway. This trended to prove that beta-agonist had stronger effect to skeletal muscle than growth hormone.

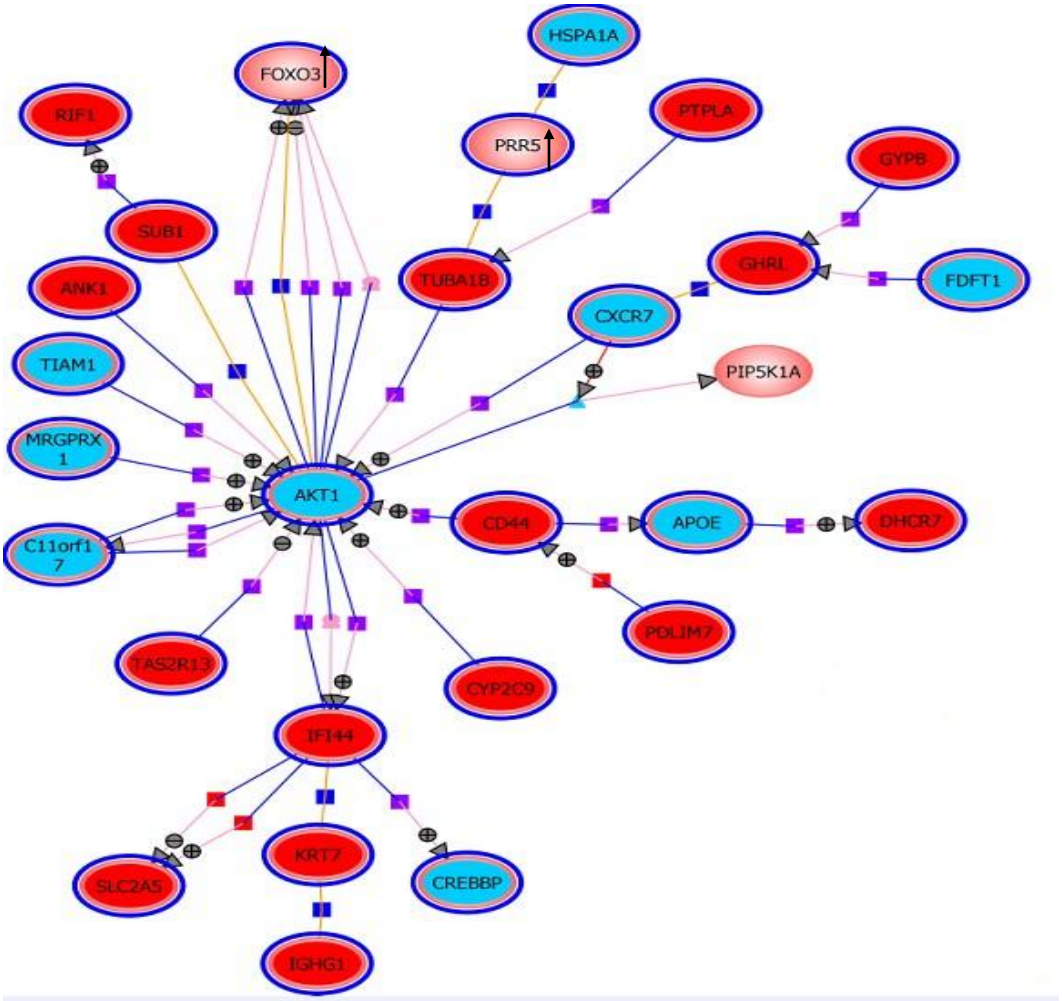

Figure 3.9 AKT1 pathway generated from GeneSpring analysis

The red ellipses indicate the genes up-regulated by beta-agonist (BA) relative to the control group, blue ellipses indicate the genes down-regulated by beta-agonist (BA) relative to the control group. The gene with blue ring means this gene is in "Up or down-regulated by BA group". The gene does not have blue ring and was showed pink color, this means it is in the GeneSpring database and has relationship with this pathway, but not in the "Up or down-regulated by BA group", therefore it is not up or down regulated gene and colored by pink. Sometimes, one gene has to via more than one probe sets to express, however GeneSpring cannot map more than one probe sets in one ellipse, as a result this kinds of gene has blue ring but colored by pink. For example, FOXO3 and PRR5. There are up-regulated genes in this pathway. The lines between each ellipse show different biology relationship and processes, these were described in Figure 3.10 and Figure 3.11.

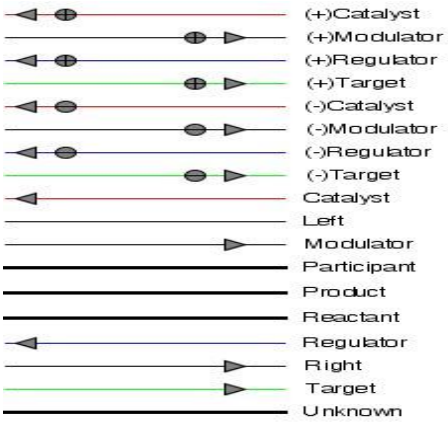

Figure 3.10 Edges legend of GeneSpring pathway analysis

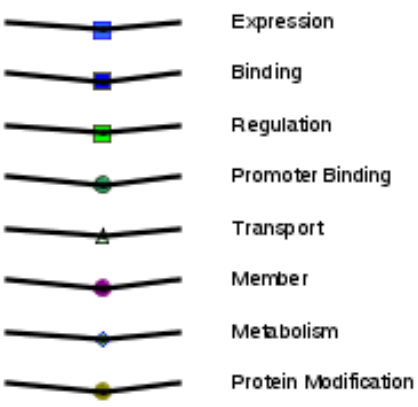

Figure 3.11 Relations legend of GeneSpring pathway analysis

Network 5 : ba vs c-7-0k : ba vs c-7-0k.xls : ba vs c-7-0k

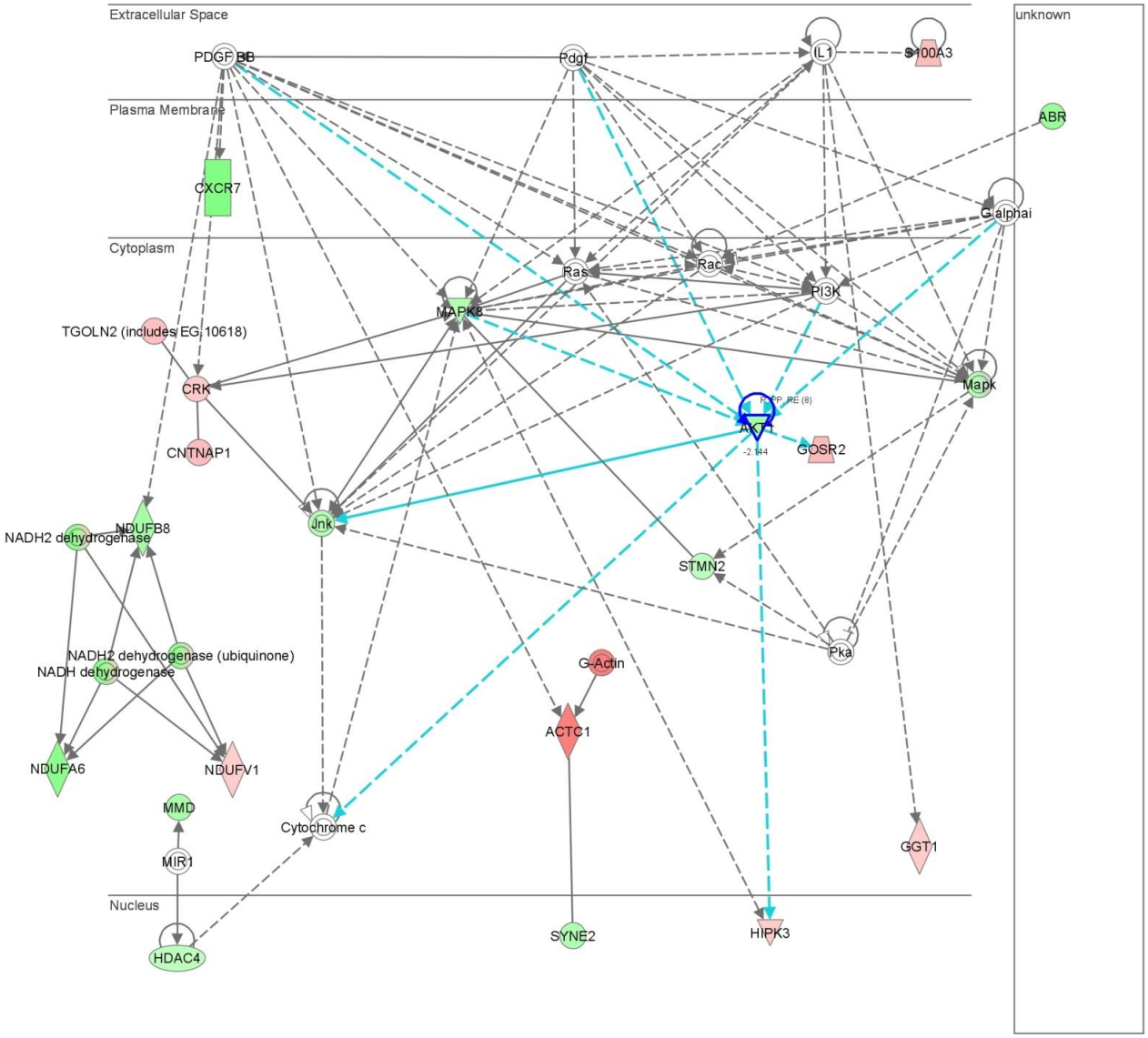

Figure 3.12 AKT1 network generated from IPA

Red shows the gene or protein up-regulated by beta-agonist (BA) relative to the control group, green shows the gene or protein down-regulated by beta-agonist (BA) relative to the control group. White means the molecule is not in "Up or down-regulated by BA group", it was added from Ingenuity Knowledge Base. AKT1 and its relationship lines were colored by blue. Blue dashed lines show indirect interactions and the blue solid lines display direct interactions. The meaning of various shapes were indicated in Figure 3.13. Different kinds of arrows and lines indicate the directionality of the interaction and specific molecular relationships, the details of them are showed in Figure 3.13. This network was displayed in Toggle Subcellular Layout (mapped molecules' spatial location into their relevant subcellular locations).

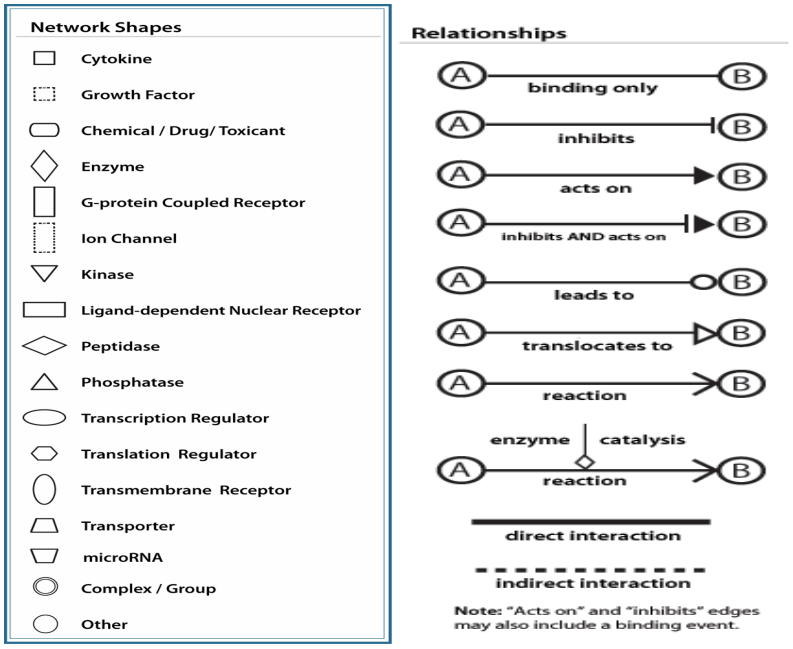

Figure 3.13 The legend of IPA network shapes and relationship types

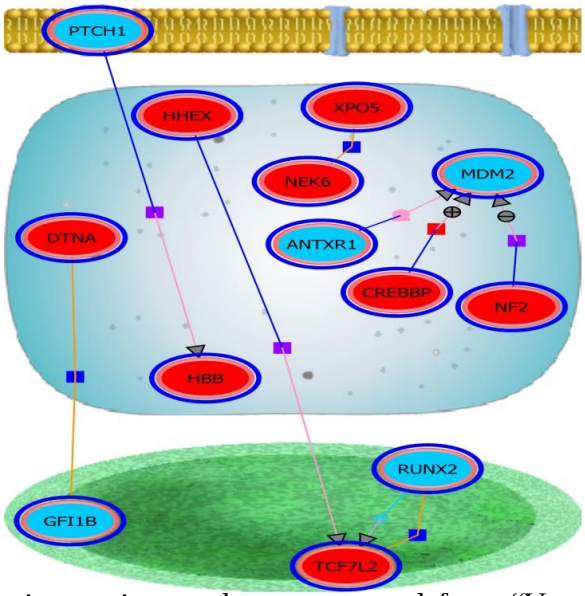

Figure 3.14 GH-direct interactions pathway generated from "Up or down-regulated by GH group".

This figure shows the pathway in cellular layout. According to the information from the cellular component of Gene Ontology, genes have been located in the correlative cellular parts. Top yellow and middle blue parts respectively means plasma membrane and cytoplasm, while nucleus was displayed by bottom green part. The red ellipses in this figure indicate the genes up-regulated by growth hormone relative to the control group, blue ellipses indicate the genes down-regulated by growth hormone relative to the control group. The gene with blue ring means this gene is in "Up or down-regulated by GH group". The lines between each ellipse show different biology relationship and processes, these were described in Figure 3.10 and Figure 3.11.

3.5.2.2 PSAT1 pathway

Mapped "Up or down-regulated by BA group" in GeneSpring to explore direct interactions, the PSAT1 pathway (Figure 3.15) had been found. PSAT1 (Phospho Serine Amino Transferase 1) was associated with synthesis of serine and showed up-regulated by BA relative to the control. There were some studies proved that PSAT over expression in cells in culture was associated with growth and cell proliferation (Baek. et.al 2003; Vie. et.al. 2008).

When the "Up or down-regulated by BA group" had been mapped in IPA, PSAT1 also showed in the top number 3 network. This network had been named "PSAT1 network from IPA" (Figure 3.16). The main functions of this network were about Cardiovascular System Development and Function, Cell Morphology and Cellular Movement (showed in Table 3.6). Compared PSAT1 network from GeneSpring with PSAT1 network from IPA, there were more gene related to PSAT1 in PSAT1 network from GeneSpring, also proved that the GeneSpring was good at finding the relationship between target genes than IPA.

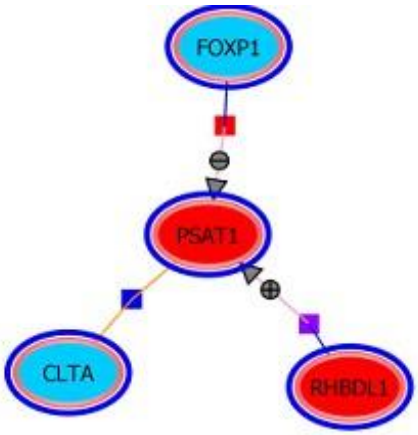

Figure 3.15 PSAT1 pathway generated from GeneSpring analysis

The red ellipses in this figure indicate the genes up-regulated by beta-agonist (BA) relative to the

control group, blue ellipses indicate the genes down-regulated by beta-agonist (BA) relative to the control group. The gene with blue ring means this gene is in "Up or down-regulated by BA group". The lines between each ellipse show different biology relationship and processes, these were described in Figure 3.10 and Figure 3.11.

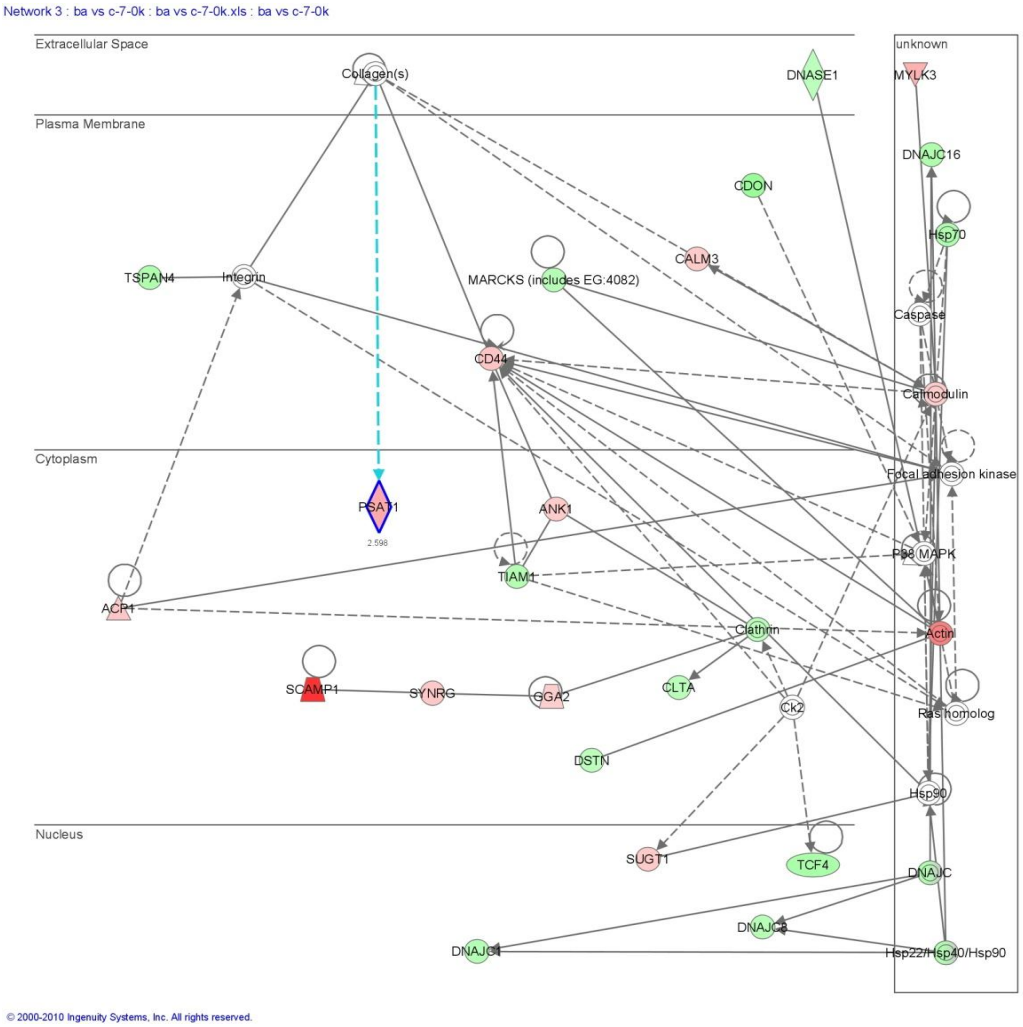

Figure 3.16 PSAT1 network generated from IPA

Red shows the gene or protein up-regulated by beta-agonist (BA) relative to the control group, green shows the gene or protein down-regulated by beta-agonist (BA) relative to the control group. White means the molecule is not in "Up or down-regulated by BA group", it was added from Ingenuity Knowledge Base. PSAT1 and its relationship lines were colored by blue. Blue dashed lines show indirect interactions and the blue solid lines display direct interactions. The meaning of various shapes were indicated in Figure 3.13. Different kinds of arrows and lines indicate the directionality of the interaction and specific molecular relationships, the details of them is showed in Figure 3.13. This network was displayed in Toggle Subcellular Layout (Mapped molecules' spatial location into their relevant subcellular locations).

3.5.3 Specific pathways which maybe involved in mediating the response of the growth agents

3.5.3.1 IPA Canonical pathway-citrate cycle pathway

Mapped "Up or down-regulated by BA group" in IPA, the Citrate Cycle (TCA cycle) pathway (Figure 3.17) as the top one significant Canonical pathway had been found. Its P value was 2.32E-04 and the ratio of IPA was 5/58 (0.086). The rank accorded to the number of IPA ratio. The calculated method of IPA ratio was: molecules' number in a given pathway that meet cutoff criteria, divided by total number of molecules that make up that pathway (www.ingenuity.com).

In this pathway, five genes showed down-regulated and one gene showed up-regulated by BA. Therefore, these genes maybe decreased the biology function of TCA cycle. In other words, this figure suggested that action of beta-agonists trended to repress the citrate cycle to make more carbon.

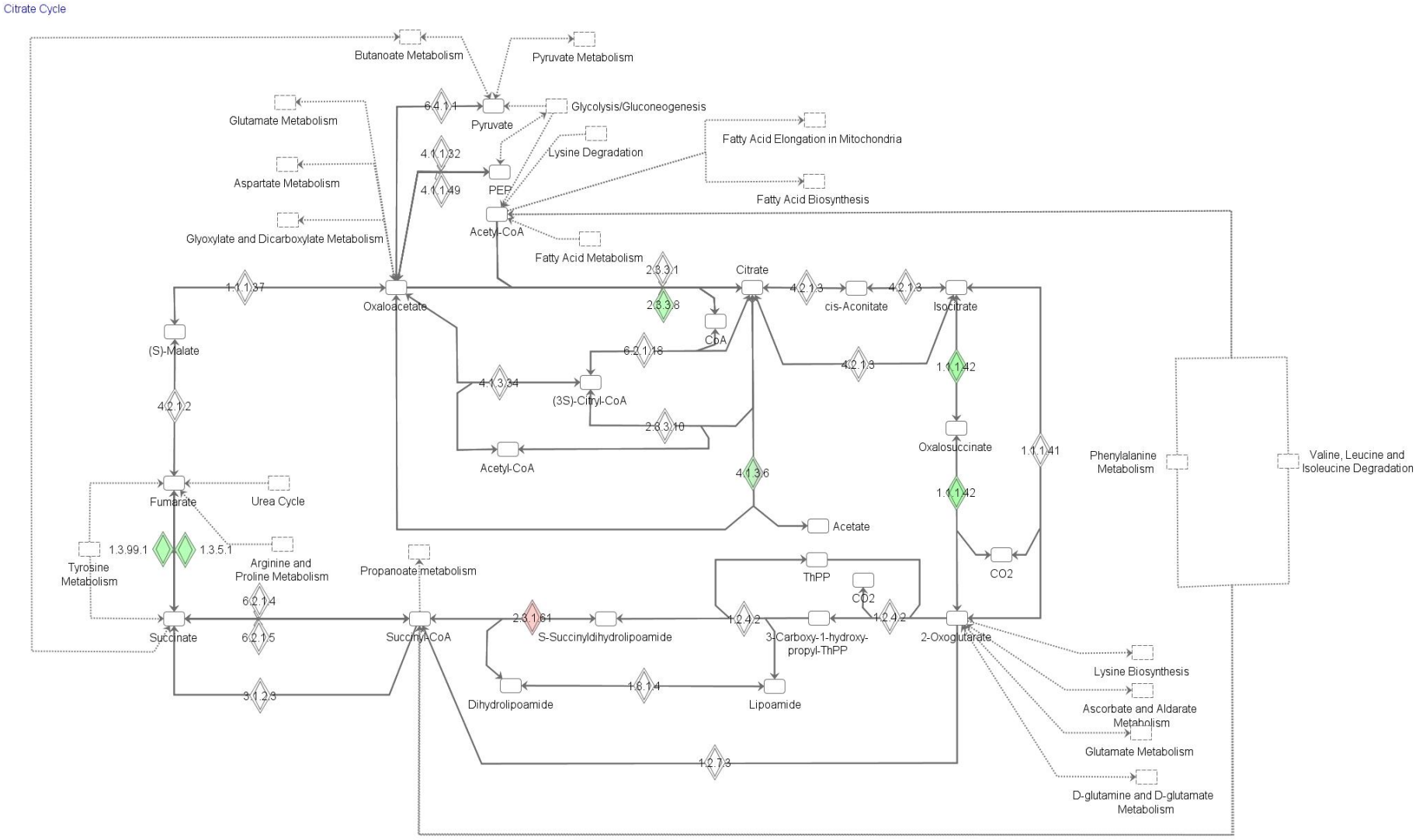

Figure 3.17 IPA Canonical pathway-citrate cycle pathway

Red shows the gene or protein up-regulated by beta-agonist (BA) relative to the control group, green shows the gene or protein down-regulated by beta-agonist (BA) relative to the control group. White means the molecule is not in "Up or down-regulated by BA group", it was added from Ingenuity Knowledge Base. The meaning of various shapes were indicated in Figure 3.13. Different kinds of arrows and lines indicate the directionality of the interaction and specific molecular relationships, the details of them is showed in Figure 3.13. The meaning of important symbols are showed in Table 3.9. The names of all symbols will display in Appendix 4.

Table 3.9 The meanings of important symbols in citrate cycle pathway

Symbol	Meaning
1.1.1.42	isocitrate (NADP) dehydrogenase,
1.3.5.1	complex II, fumarate reductase complex, menaquinol:fumarate oxidoreductase,
1.3.99.1	Complex II, Succinate INT Dehydrogenase
2.3.1.61	dihydrolipoamide succinyltransferase,
2.3.3.8	acetyl-CoA:oxaloacetate acetyltransferase (isomerizing, ADP-phosphorylating),
4.1.3.6	citratase, citrate aldolase, citrate oxaloacetate-lyase,

3.5.3.2 IPA Canonical pathway-Oxidative Phosphorylation pathway

Mapped "Up or down-regulated by BA group" in IPA, the Oxidative Phosphorylation pathway (Figure 3.18) had been found.

This photo indicated that five genes down-regulated but only one gene up-regulated by BA. Therefore, these genes trended to decrease the whole process of oxidative phosphorylation associated with energy metabolism. In other words, this pathway suggested that action of beta-agonists tended to repress the electron transport chain to produce ATP.

Oxidative Phosphorylation

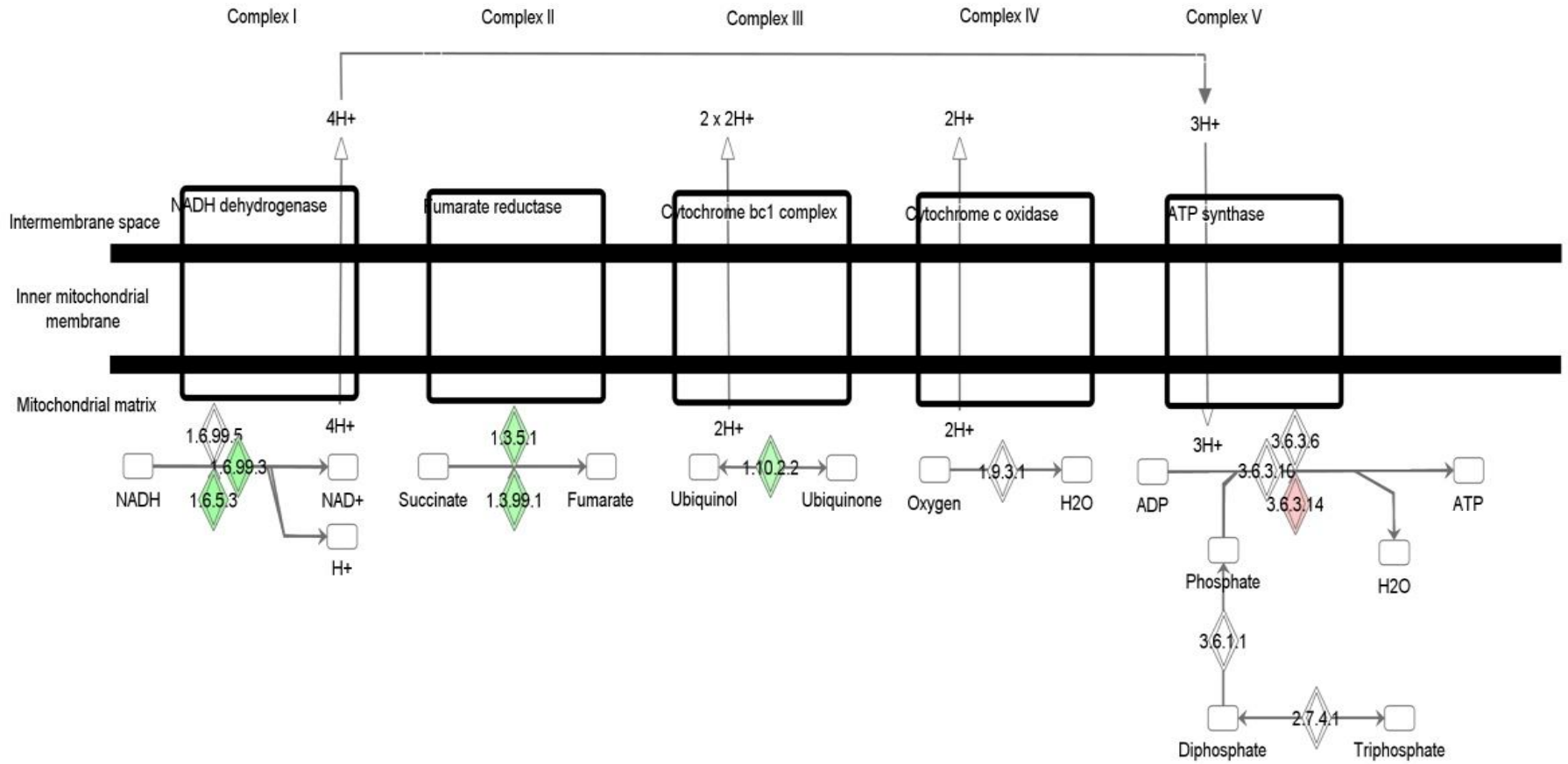

Figure 3.18 IPA Canonical pathway- Oxidative Phosphorylation

Red shows the gene or protein up-regulated by beta-agonist (BA) relative to the control group, green shows the gene or protein down-regulated by beta-agonist (BA) relative to the control group. White means the molecule is not in "Up or down-regulated by BA group", it was added from Ingenuity Knowledge Base. The meaning of various shapes were indicated in Figure 3.13. Different kinds of arrows and lines indicate the directionality of the interaction and specific molecular relationships, the details of them is showed in Figure 3.13. The meaning of important symbols are showed in Table 3.10. The names of all symbols will display in Appendix 5.

Table 3.10 The meanings of important symbols in oxidativephosphorylation pathway

Symbol	Synonym(s)
1.10.2.2	coenzyme Q-cytochrome c reductase, coenzyme QH2-cytochrome c reductase,
1.3.5.1	complex II, fumarate reductase complex, menaquinol:fumarate oxidoreductase,
1.3.99.1	Complex II, Succinate INT Dehydrogenase
1.6.5.3	complex 1 dehydrogenase, complex I (electron transport chain),
1.6.99.3	beta-NADH dehydrogenase dinucleotide, cytochrome c reductase,
3.6.3.14	ATP phosphohydrolase (H+-transporting), ATP synthase,

3.5.3.3 Glycolysis network generated form KEGG database

Used GeneSpring to map "Up or down-regulated by BA group" into KEGG pathway database. Through this analysis several pathways were identified which had gene who's expression was affected. As indicated in Figure 3.19 a pathway that had several genes influenced by BA treatment was glycolysis.

This glycolysis is responsible for anaerobic respiration the product of the pathway is pyruvate which can reduced to lactic acid, as is the case in anaerobic respiration, or can be converted to Acetyl CoA and then this enters the TCA cycle and associated with energy metabolism. It also showed the PSAT and enolase were up-regulated by BA using Q-PCR (Tim Parr unpublished observations). PSAT is part of a pathway which utilizes intermediates of glycolysis to synthesize the non-essential amino acid serine whilst enolase is a enzyme which is part of glycolysis. As indicated in the Figure 3.19 and Table 3.11 the other enzymes within glycolysis that were affected were Fructose 1,6-bisphosphatase, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Phosphoglycerate mustase. Of these Fructose 1,6-bisphosphatase is of particular interest as it is involved in promoting the reverse of glycolysis Phosphofructokinase being involved in the movement of substrates "down" glycolysis whilst Fructose 1,6-bisphosphatase is involved in moving substrates "up"

glycolysis. Therefore this step is a key regulatory step in the control of substrate flux through glycolysis.

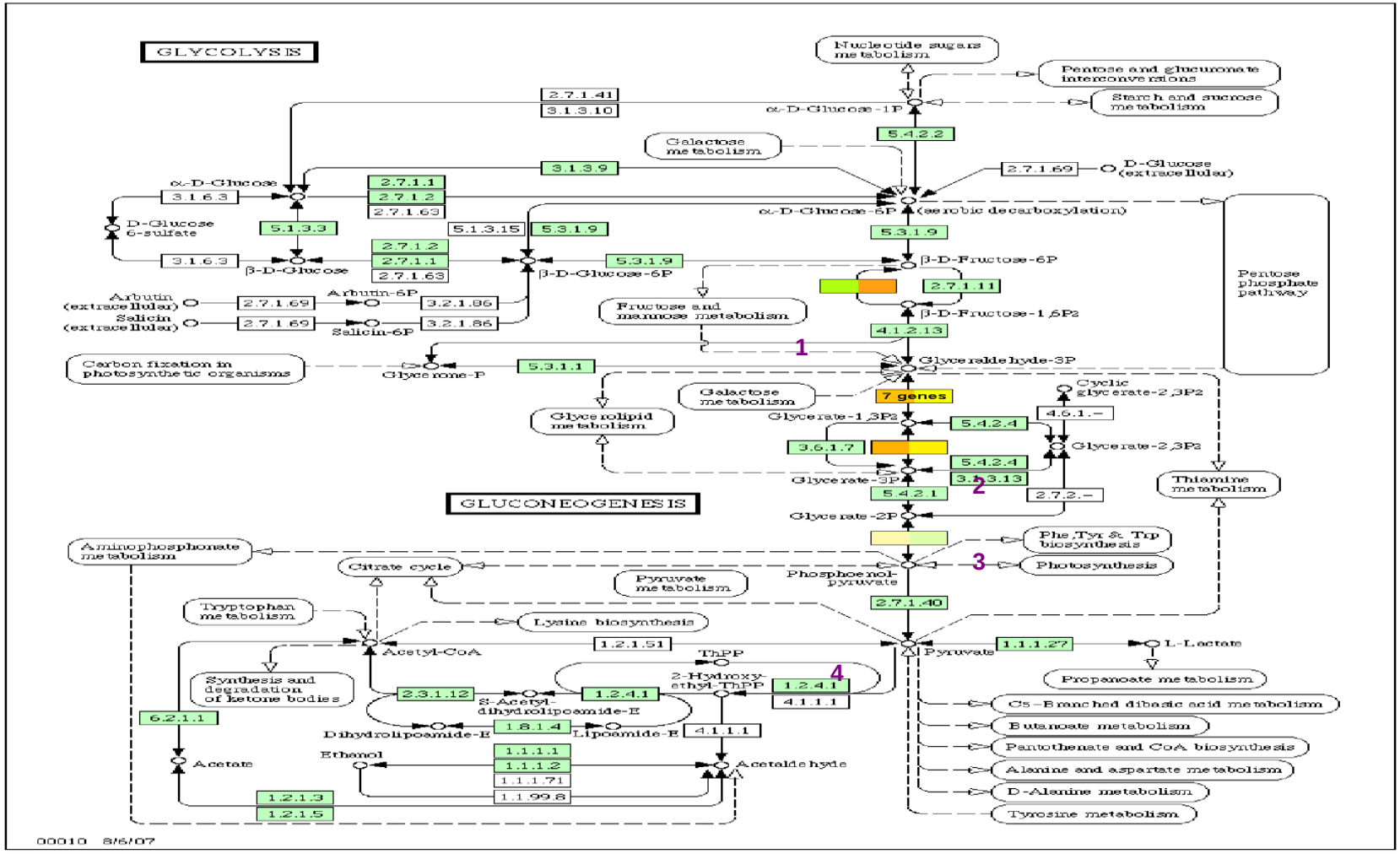

Figure 3.19 Glycolysis network generated from GeneSpring

Yellow shows the gene or protein up-regulated by beta-agonist (BA) relative to the control group, green shows the gene or protein down-regulated by beta-agonist (BA) relative to the control group. White means the molecule is not in "Up or down-regulated by BA group", it was added from KEGG database. The meaning of main symbols is showed in Table.3.11.

Table 3.11 The meaning of main symbols in Glycolysis pathway

Symbol	name
4.1.2.13	fructose-bisphosphate aldolase; fructose-1,6-bisphosphate triosephosphate-lyase; diphosphofructose aldolase;
2.7.1.11	6-phosphofructokinase;phosphohexokinase;phosphofructokinase I; phosphofructokinase(phosphorylating);6-phosphofructose 1-kinase;
5.3.1.9	glucose-6-phosphate isomerase;phosphohexose isomerase; hexosephosphate isomerase;phosphosaccharomutase; glucose phosphate isomerase;
5.4.2.4	bisphosphoglycerate mutase; glycerate phosphomutase;bisphosphoglycerate synthase;bisphosphoglyceromutase; 2,3-diphosphoglycerate mutase;
3.6.1.7	acylphosphatase;acetylphosphatase;1,3-diphosphoglycerate phosphatase;acetic phosphatase;Ho 1-3;GP 1-3
3.1.3.13	bisphosphoglycerate phosphatase;2,3-diphosphoglycerate phosphatase;diphosphoglycerate phosphatase;
5.4.2.1	phosphoglycerate mutase;phosphoglycerate phosphomutase;phosphoglyceromutase;
5.4.2.2	phosphoglucomutase;glucose phosphomutase;phosphoglucose mutase
2.7.1.40	pyruvate kinase;phosphoenolpyruvate kinase;phosphoenol transphosphorylase
1	Fructose 1,6-bisphosphatase
2	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
3	Phosphoglycerate mustase
4	Enolase

3.5.4 Real-time PCR

3.5.4.1 Sequences searching and primer design results

According as the flow chart of searching process showed above (Figure 2.2), the sequences of main target genes from AKT1 pathway, PSAT1 pathway and citrate cycle pathway had been searched.

As less ovine cDNA sequences had been found, therefore most of the genes used similar bovine cDNA for primer design. If some gene no ovine and bovine sequence, the similar human cDNA had been used for primer design (showed in Table 3.12).

The CLUSTAL score and the number of EMBOSS Similarity indicated the similarity of bovine cDNA/human cDNA compared with Ovine EST sequence. These scores and numbers were showed in Table 3.13 and Table 3.14. By comparing the CLUSTAL score and the number of EMBOSS Similarity in Table 3.13 and Table 3.14, it is easy to see that the similarity of ovine versus bovine was higher than the similarity of ovine versus human, this suggested that ovine and bovine had higher homology than ovine and human.

Table 3.12 Sequences summary for primer design

In this table, "YES" means the gene has Human cDNA/Bovine cDNA/Ovine cDNA/Ovine EST , or the cDNA/EST can be used for primer design. "NO" means the gene no Human cDNA/Bovine cDNA/Ovine cDNA/Ovine EST .

Gene name	Human cDNA	Bovine cDNA	Ovine cDNA	Ovine EST	Use for primer design
FOXP1	YES	YES	NO	YES	YES
CLTA	YES	YES	NO	YES	YES
RHBDL1	YES	YES	NO	YES	YES
CD44	YES	YES	NO	YES	YES
CXCR7	YES	YES	NO	YES	YES
FOXO3	YES	YES	NO	YES	YES
SUB1	YES	YES	NO	YES	YES
ANK1	YES	YES	NO	YES	YES
TIAM1	YES	YES	NO	YES	YES
IFI44	YES	YES	NO	YES	YES

AKT1	YES	YES	YES	---	YES
TUBA1B	YES	NO	NO	YES	YES
C11orf17	YES	NO	NO	YES	YES
CYP2C9	YES	NO	NO	YES	YES
CLYBL	YES	YES	NO	YES	YES
DLST	YES	YES	NO	YES	YES

Table 3.13 The similarity of bovine cDNA and ovine EST

Gene name	Bovine cDNA (1) Full Length	length Bp	Ovine EST (length: Bp) (2)	CLUSTAL score (1 and 2)	EMBOSS Similarity (1 and 2)
FOXP1	YES	3309	682	97	666/3312 (20.1%)
CLTA	YES	1110	874	87	787/1176 (66.9%)
RHBDL1	YES	2309	499	96	483/2309 (20.9%)
CD44	YES	1355	540	94	510/1365 (37.4%)
CXCR7	YES	1745	841	96	810/1746 (46.4%)
FOXO3	YES	2050	376	98	370/2050 (18.0%)
SUB1	YES	3384	728	90	679/3384 (20.1%)
ANK1	YES	1750	795	85	682/1844 (37.0%)
TIAM1	YES	2821	661	97	646/2821 (22.9%)
IFI44	YES	1959	826	91	753/1986 (37.9%)
CLYBL	YES	1046	641	66	563/1047 (53.8%)
DLST	YES	2221	895	96	872/2221 (39.3%)

Table 3.14 The similarity of human cDNA and ovine EST

Gene name	Human cDNA (1)		Ovine EST (length: Bp) (2)	CLUSTAL score (1 and 2)	EMBOSS Similarity (1 and 2)
	Full Length	length Bp			
TUBA1B	YES	1356	577	81	473/1436 (32.9%)
C11orf17	YES	1427	876	75	719/1438 (50.0%)
CYP2C9	YES	1273	493	76	381/1274 (29.9%)

3.5.4.2 PSAT1 pathway confirmed by qReal-time PCR

The every target gene of PSAT1 pathway had been confirmed by qReal-time PCR. The qReal-time PCR result of PSAT1 showed high significant (p value less than 0.01, supplied by Zoe Daniel), the other three genes which had direct relationship with PSAT1 hadbeen checked by qReal-time PCR experiment. The results of CLTA (p-value = 0.026) and FOXP1(p-value = 0.009) were significant (Figure 3.20**)**. These proved that the PSAT1 indeed up-regulated by BA, CLTA and FOXP1 really down-regulated by BA. This real-time PCR result was agree with microarray data.

In the PSAT1 pathway, 3 of 4 genes had been confirmed. Only REBDL1 had not significant result, this may because microarray was too sensitive and actually REBDL1 was not up-regulated by BA. However, since in this experiment the human array had been used to hybridize sheep sample to get this pathway result and the bovine cDNA of REBDL1 had been used to primer design, these effects also worth to be considered.

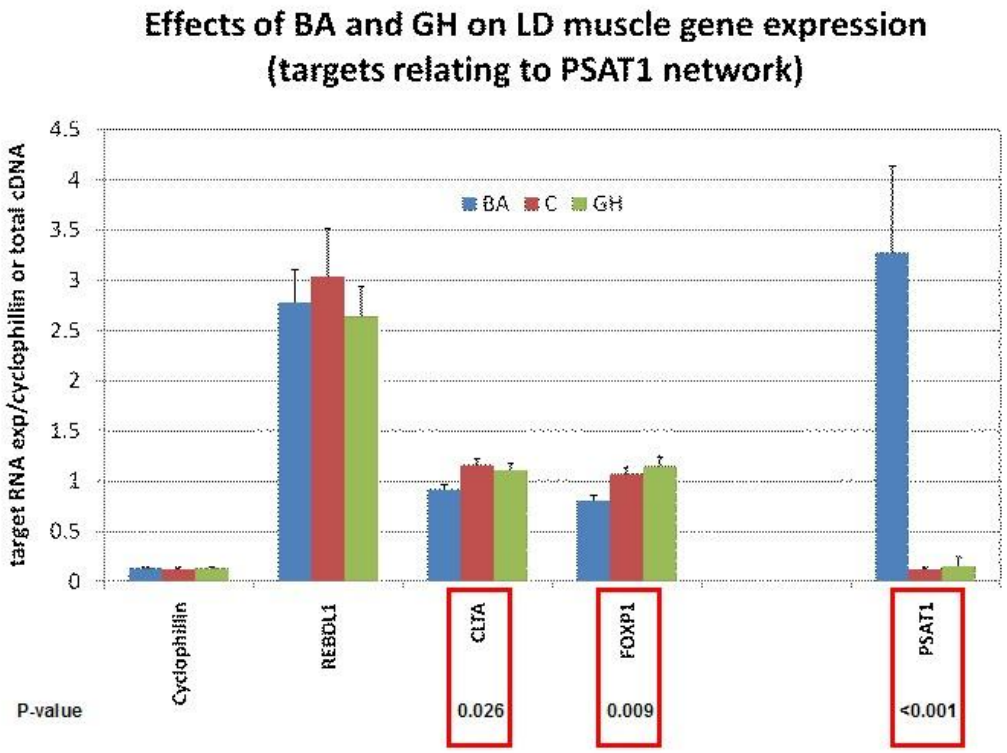

Figure 3.20 Effects of BA and GH on LD muscle gene expression (targets relating to PSAT1 pathway) (supplied by Zoe Daniel).

The X axes means there were four genes (REBDL1, CLTA, FOXP1 and PSAT1) and cyclophillin had been used to do qReal-time PCR. Y axes shows the number of target RNA expression is divided by the number of cyclophillin expression or total cDNA expression (only for PSAT1). Blue displays the qReal-time PCR result form the samples which treated by beta-agonist (BA). Red displays the qReal-time PCR result form the control samples (C). Green displays the qReal-time PCR result form the samples which treated by growth hormone (GH). The number of p-value is showed in the red boxes.

4 DISCUSSION

In this investigation the following hypothesis was investigated.

Hypothesis of this study: Growth promoter administration to sheep will lead to the effects on muscle gene expression which when detected by microarray analysis will indicate the specific pathways and target genes which mediate this response.

From the examination of the data there were some target genes and pathways had been identified, these included AKT1 pathway (Figure 3.9) and PSAT1 pathway (Figure 3.15) associated with protein synthesis, and citrate cycle pathway (Figure 3.17), oxidative phosphorylation pathway (Figure 3.18) and glycolysis network (Figure 3.19) correlated with energy metabolism. These pathways were predominantly associated with beta-agonists response in comparison there were only relatively minor effects of growth hormone .

As indicated in the introduction specific objectives were investigated. Indicated below are those objectives and the principal outcomes which were determined.

1. To analyze the data produced from a microarray examination of the effects of growth promoters on sheep skeletal muscle. This will include:

a. *Identify up and down regulated genes and commonality and differences between the two growth promoters.*

Used the "Filtering on volcano plot" and "Venn diagram" functions of GeneSpring, the up and down regulated genes and the intersections of them have been found. In the analysis of the microarray there was a clear effect on gene expression with approximately equal effects on increased and decreased expression. However it was noticeable that more genes were affected in the beta-agonists treated.

b. *Examination of potential interactions between genes whose expression had been altered to try to identify signaling pathways that are effected by the agents.*

Used pathway analysis of GeneSpring and IPA software, there were

several pathways had been identified. By comparing the identified pathways which effected by BA with GH, again indicated that the effects of BA were stronger than GH's. Focused on the pathways which were effected by BA and through examination of the literature, two predominant pathways had been identified. One was AKT1 pathway had the most associate/interactions, another one was PSAT1 pathway. It included PSAT which associated with the synthesis of serine.

c. The identification gene expression alterations which are associated with specific pathways which maybe involved in mediating the response of the growth agents

Further analysis was carried out using pathway based program IPA and GeneSpring which allowed identification of the predominant pathways influenced by the agents. The most clear and significant observation was that there were a group of genes affected that were associated with oxidative metabolism. This included the TCA cycle, the electron transport chain found in mitochondria (Oxidative Phosphorylation pathway) and glycolysis network.

2 On selected genes associated with specific pathways that respond to the agents confirm changes in gene expression identified by microarray using quantitative Real Time PCR

One special group of genes associated with the serine synthesis pathway (PSAT1 pathway) was analyzed. From this analysis were able to confirm the changes observed by the microarray. Thereby placing a high level of confidence that the expression of genes associated with serine synthesis are affected by beta-agonists indicating that serine synthesis may be increased in this treatment.

In today's world, to increase feed conversion efficiency (FCE) is very popular and welcomed by producers, consumers and environmentalists. Because it decreases production costs, while increase product quality and profit for producers. It also can provide meat to more people under the conditions of limited resources. And the other benefits are reduced grazing pressure and decreased nitrogenous excretion into the environment. Improvements in the ratio of lean to fat deposition or increases in FCE can be achieved by using some growth promoters, like beta-agonist (BA) and growth

hormone (GH). These growth promoters can increase muscle mass by rising fibre number and/or fibre size. We already know that IGF can activate PI3K-Akt1 pathway mediating this effect (Chang. 2007). But we still need to know more in order to identify potential targets for manipulation.

It was clear that pathways which under the beta-agonists treatment were affected significantly than GH, and BA can influence muscle growth and switch fibre type (Enright, 1989; Buttery and Dawson, 1990). Myosin changes and potential AKT1 changes that are associated with growth (Nader, 2005). Some indications of these changes can be found from this microarray data. As AKT1 can cause changes in protein synthesis. But in the AKT1 pathway of this project, AKT1 goes down. This is puzzling. But we know that predominant regulation of AKT is at post-translational level with the control of the activity of this enzyme being through its phosphorylation. This post-translational modification activates the enzyme thereby leading to increased activation of downstream proteins in this signaling pathway which is associated with increases in protein synthesis. What needs to be done is to look at all the other genes associated which we found in the analysis to see if the changes seen are associated with a protein synthesis down or up regulation. Also do experiments to conform by Real-time PCR. In this project we used sheep sample to hybridize with human array to get this pathway result and most target genes used bovine or human cDNA to design primers, these reasons may effect the accuracy of Real-time PCR. However when we looked at PSAT pathway by Real-time PCR, it clearly indicated that this approach of cross-species array worked as the data confirmed the microarray data. PSAT associated with synthesis of serine. Serine is an amino acid so would be expected to be require in growth. Indeed data on PSAT shows that over expression in cells in culture is associated with growth and cell proliferation (Baek. et.al 2003; Vie. et.al. 2008). Interestingly to note that the substartes which are used for the synthesis of serine originate from glycolysis.

Another major outcome from pathway analysis was that the energy production pathways were affected by BA. In the introduction part has indicated that fast fibres were induced by BA. Data from microarray indicated that there was a metabolic response which reflected or perhaps induced this change as oxidative pathway was decreased. Decrease in genes associated with production of energy associated with

mitochondria (the down-regulated genes in citrate cycle pathway and Oxidative Phosphorylation). This suggested that the release of glucose from glycogen was not being oxidized. Therefore we could speculate that the effect of BA is to cause a degradation of glycogen which enters glycolysis as we have indication that enzymes (like enolase) in glycolysis are up regulated as well as PSAT. The effect of decreasing oxidative metabolism may force the carbon skeletons entering glycolysis to be utilized for synthesis of none essential amino acids which then help to induce the increase in muscle protein synthesis. In other words, there is a new hypotheses has been advanced that result on the action of beta-agonists is that the citrate cycle is repressed making more carbon available to go into amino acids (Figure 4.1).

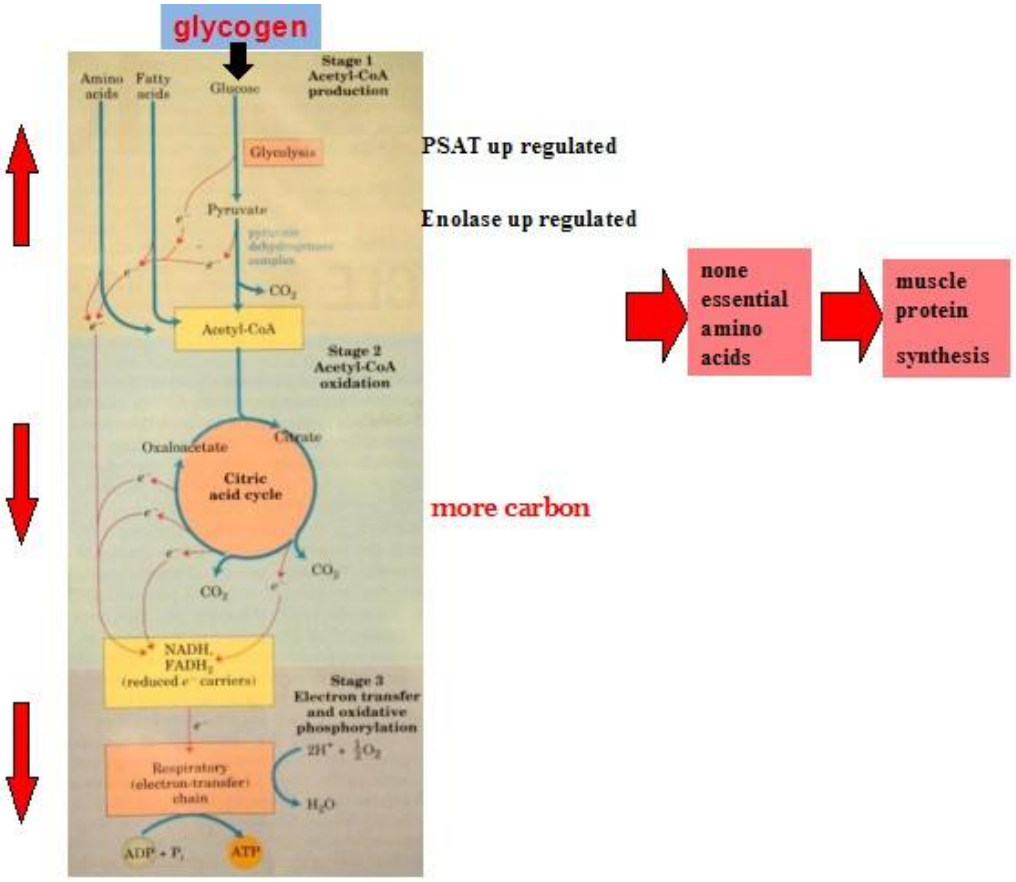

Figure 4.1 Affected energy production pathways (original photo supplied by Nelson and Cox.2005)

There were many genes down-regulated by BA in citrate cycle pathway and oxidative phosphorylation pathway. This suggested that the whole processes of TCA and oxidative phosphorylation had been decreased by BA, and the release of glucose from glycogen was not being oxidized. Enolase and PSAT were up regulated and PSAT was associated with synthesis of serine. Hence, these suggested that carbon entering glycolysis was not oxidized, but utilized for synthesis of none essential amino acids (like serine). This trended to induce the increase in muscle protein synthesis and influenced muscle growth.

5 FUTURE WORK

Since every kinds of high-throughput technology has error-prone nature. Microarray data always need to be confirmed by another independent method, for example qReal-time PCR. The aim is to validate that the changes of expression explored by microarray can be repeated by other methods (Clarke and Zhu 2006). For this project, the cross-species microarray has been used, therefore using qReal-time PCR to confirm the expression of the other target genes is very necessary. Besides this, what needs to be done is through doing qReal-time PCR to look at all the other genes of AKT1 pathway to see if the changes seen are associated with a protein synthesis down or up regulation. Also can use western blot to analyze the changes of specific proteins in post-translational level. After these, the metabolomic methods can be considered to confirm the pathways about energy metabolism. Such as metabolomic fingerprinting method (Liquid chromatography-mass spectrometry) and metabolomic profiling method. Finally, we should nearly observe that whether the improvement of microarray can supply a new kind of sheep array which suit to analysis ovine skeletal muscle.

6 CONCLUSION

Recently, the control of lean and fat tissue deposition in livestock is widespread concerned by producers, consumers and environmentalists. Since increasing the ratio of lean to fat deposition can improve feed conversion efficiency and increase meat quality, thereby earn more profit to producers. Beside this, to improve feed conversion efficiency also can reduce grazing pressure, decrease nitrogenous excretion into the environment and supply more meat to the people with limited resources. Growth promoter (beta agonists and growth hormone) administration results in muscle fibre hypertrophy and changes in muscle fibre type composition, responses that are important in terms of human ageing and disease (such as sarcopenia and type 2 diabetes), as well as animal growth efficiency.

In order to study the reason of these effects, in this project, growth signaling pathways in skeletal muscle need to be identified. As current available sheep microarrays are more targeted to study genes involved in immune response, not suitable for studies on muscle growth and metabolism. And also the ovine does not have accomplished genome arrays available. Thereby, for this project the cross-species microarray had been used. Microarray data was analyzed using Ingenuity Pathway Analysis software and GeneSpring. The microarray analysis results were conformed by real-time quantitative RT-PCR.

As the results of this project, there were 236 target genes up-regulated by beta-agonist relative to the control group, 209 target genes down-regulated by beta-agonist relative to the control group, 147 target genes up-regulated by growth hormone relative to the control group and 139 target genes down-regulated by growth hormone relative to the control group. These numbers also trended to suggest that the effect of beta-agonist to sheep skeletal muscle was stronger than the growth hormone's. Used pathway analysis of GeneSpring and IPA software, two predominant pathways which associated with the effects of beta-agonist had been identified. One was AKT1 pathway included most target genes or proteins, another one was PSAT1 pathway. It included PSAT which associated with the synthesis of serine. Other three important pathway results, citrate cycle pathway, oxidative phosphorylation pathway and glycolysis pathway suggested a new hypotheses. The whole processes of TCA

and oxidative phosphorylation had been decreased by BA, and the carbon entering glycolysis was not oxidized, but used for synthesis of none essential amino acids (like serine). This trended to induce the increase in muscle protein synthesis and influenced muscle growth. The quantitative real-time PCR had been used to conform the target genes in PSAT1 pathway, the significant result provided us a high confidence to these cross-species microarray results.

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8 APPENDICS

Appendix 1 The summary of finally designed primers

Gray shows exon boundaries.

Target name	Target sequence	Exon boundaries	Amplicon length(bp)	Sequences	Penalty NO.	Primer length(bp)
FOXP1	Ovine EST	YES	71	Forward primer: GGAGCATACCAACAGCAACGA Reverse primer: GTACAGGATGCACAGCTTGCA Probe: CGACAGCAGTCCGGGCAGATCC	107	FOXP1F: 21 FOXP1R: 21
CLTA	Ovine EST	YES	91	Forward: GAAAGTATCCGTAAATGGAGAGAAGAG Reverse: TTTCTTTCCACTCCGCTTCCT Probe: ACGCTTGGAAGCCCTTGATGCCA	213	CLTA F: 27 CLTA R: 21
RHBDL1	Ovine EST	YES	111	Forward: CCTGACCGTCTCCATTACTGACA Reverse: ATCCCAGCCCAGTTCATGAC Probe: CTGTGTTCTGCACACCTGGCCAATG	308	RHBDL1F: 23 RHBDL1R: 20
AKT1	Ovine cDNA	YES	113	Forward: GAGGTCATCGTGGCCAAAGA Reverse: TCGTGCGTCTGGAAGGAGTA Probe: ACCCGTTCCTGACGGCCCTGAA	315	AKT1F: 20 AKT1R: 20
CD44	Ovine EST	YES	75	Forward: CCTGCAGGTACGGGTTCATAG Reverse: TGTTCGCAGCACAGATGGAA Probe: TGATTCCCCGGATCCACCCCA	126	CD44F: 21 CD44R: 20
TUBA1B	Ovine EST	YES	99	Forward: ACCATGCGTGAGTGCATCTC Reverse: GGGCTGAATGCCGTGTTC Probe: AATGCCTGCTGGGAGCTCTACTGCC	247	TUBA1BF: 20 TUBA1BR:18
SUB1	Ovine EST	YES	87	Forward: GAAATGAAACCAGGAAGAAAAGGTATA Reverse: TGCATCATCAATGTCAGAAATCTG Probe: CTTTAAATCCTGAACAATGGAGCCAGCTGA	196	SUB1F: 27 SUB1R: 24
ANK1	Ovine EST	YES	94	Forward: GGTGACGGAGGAGCATTTCA Reverse: GGCTCCGGACGGGTCTAT Probe: CAACGTCGTCACCAAGAAGATCATTCGC	222	ANK1F: 20 ANK1R:18
C11orf17	Ovine EST	YES	77	Forward: TCTCCCAGGAAAGAGAGAAGACA Reverse: TGTAGTTCATGTATTCAGCCATTGTTC Probe:	145	C11orf17F: 23 C11orf17R: 27

				ACACCCGACCCTCAGTGCTTCCTTC		
IFI44	Ovine EST	YES	87	Forward: TTGTGCCTGCCTAGCTGTCA Reverse: CTGCCATACTTCTCAACCTGTTTG Probe: AGAGCAGCTACCCTCAGCTTCAGCCCT	189	IFI44F: 20 IFI44R:24
CYP2C9	Ovine EST	YES	118	Forward: AATCAGAGGCATGGCAAAGG Reverse: CTTCTGCTAGCTTGCTTATGGATTT Probe: TGCCACCTGGGCCCACTCCTCT	345	CYP2C9F: 20 CYP2C9R: 25
CXCR7	Ovine EST	NO	73	Forward: CGCCTCCTACAACGAGACCTA Reverse: CATGCTGATGAGCCACTCCTT Probe: TGCCGGGCCTTCTACCCCG	117	CXCR7F: 21 CXCR7R: 21
FOXO3	Ovine EST	NO	71	Forward: TCGCAGTGACCCAATGATGT Reverse: GGAGCAAGTTCTGATTGACCAAA Probe: CTTTGCCGCCCAGCCTAACCAGG	108	FOXO3F: 20 FOXO3R: 23
TIAM1	Ovine EST	NO	62	Forward: AGCCTCCGTGCAGAGCAT Reverse: ACGTCGCGTCATCCCTGTA Probe: CCAGACTCGGAAGACAGCCGGC	63	TIAM1F: 18 TIAM1R: 19
CLYBL	Ovine EST	YES	107	Forward: GGCAATGATGAAAAGAAGATAAACAAG Reverse: AATCTAGCTTCATCCTTTTTGTTCACA Probe: TTCCCTCCCTGAATGTAGACTGTGCGG	299	CLYBLF: 27 CLYBLR: 27
DLST	Ovine EST	YES	79	Forward: GAACATCGGGAGAAAATGAACAG Reverse: GCATTGCGCAGGTGTTCTG Probe: CAGCGCATCGCTCAGCGTCTGA	149	DLSTF: 23 DLSTR: 19

Appendix 2 The meanings of all symbols in citrate cycle pathway

Green shows down-regulated genes or proteins in the citrate cycle pathway. Red shows up-regulated genes or proteins in the citrate cycle pathway.

Symbol	Synonym(s)
1.1.1.37	(S)-malate:NAD oxidoreductase, L-malate dehydrogenase, L-malate-NAD+ oxidoreductase,malate (NAD) dehydrogenase, malic acid dehydrogenase, malic dehydrogenase, MDH, NAD-dependent malate dehydrogenase, NAD-dependent malic dehydrogenase, NAD-L-malate dehydrogenase, NAD-linked malate dehydrogenase, NAD-malate dehydrogenase, NAD-malic dehydrogenase, NAD-specific malate dehydrogenase
1.1.1.41	beta-ketoglutaric-isocitric carboxylase, isocitrate:NAD oxidoreductase (decarboxylating), isocitric acid dehydrogenase, isocitric dehydrogenase, NAD dependent isocitrate dehydrogenase, NAD isocitrate dehydrogenase, NAD isocitric dehydrogenase, NAD-linked isocitrate dehydrogenase,NAD-specific isocitrate dehydrogenase
1.1.1.42	isocitrate (NADP) dehydrogenase, isocitrate (nicotinamide adenine dinucleotide phosphate) dehydrogenase,Isocitrate dehydrogenase (NADP), isocitrate dehydrogenase (NADP-dependent), isocitrate:NADP oxidoreductase (decarboxylating), NADP isocitric dehydrogenase, NADP+-linked isocitrate dehydrogenase, NADP-dependent isocitrate dehydrogenase, NADP-dependent isocitric dehydrogenase,NADP-linked isocitrate dehydrogenase, NADP-specific isocitrate dehydrogenase,oxalosuccinate decarboxylase, oxalsuccinic decarboxylase
1.2.4.2	2-ketoglutarate dehydrogenase, 2-oxoglutarate dehydrogenase, 2-oxoglutarate:lipoamide 2-oxidoreductase (decarboxylating and acceptor-succinylating), 2-oxoglutarate:lipoate oxidoreductase, AKGDH, alpha-ketoglutarate dehydrogenase, alpha-ketoglutaric acid dehydrogenase, alpha-ketoglutaric dehydrogenase, alpha-oxoglutarate dehydrogenase, ketoglutaric dehydrogenase, OGDC, oxoglutarate decarboxylase, oxoglutarate dehydrogenase
1.2.7.3	2-oxoglutarate-ferredoxin oxidoreductase, 2-oxoglutarate:ferredoxin 2-oxidoreductase (CoA-succinylating), alpha-ketoglutarate synthase, alpha-ketoglutarate-ferredoxin oxidoreductase, oxoglutarate synthase
1.3.5.1	complex II, fumarate reductase complex, menaquinol:fumarate oxidoreductase, succinate dehydrogenase complex, succinate:ubiquinone oxidoreductase, succinic dehydrogenase

1.3.99.1	Complex II, Succinate INT Dehydrogenase
1.8.1.4	dehydrolipoate dehydrogenase, diaphorase, dihydrolipoamide:NAD oxidoreductase, dihydrolipoic dehydrogenase, dihydrolipoyl dehydrogenase, dihydrothioctic dehydrogenase, LDP-Glc, LDP-Val, lipoamide dehydrogenase (NADH), lipoamide oxidoreductase (NADH), lipoamide reductase, lipoamide reductase (NADH2), lipoate dehydrogenase, lipoic acid dehydrogenase, lipoyl dehydrogenase
2.3.1.61	dihydrolipoamide succinyltransferase, dihydrolipoic transsuccinylase, dihydrolipolyl transsuccinylase, dihydrolipoyl transsuccinylase, lipoate succinyltransferase (Escherichia coli), lipoic transsuccinylase, lipoyl transsuccinylase, succinyl-CoA:dihydrolipoamide S-succinyltransferase, succinyl-CoA:dihydrolipoate S-succinyltransferase
2.3.3.1	(R)-citric synthase, acetyl-CoA:oxaloacetate C-acetyltransferase [thioester-hydrolysing, (pro-S)-carboxymethyl forming], citrate condensing enzyme, citrate oxaloacetate-lyase [(pro-3S)-CH2COO-acetyl-CoA], citrate oxaloacetate-lyase, CoA-acetylating, citrate synthase, citrate synthetase, citric synthase, citric-condensing enzyme, citrogenase, condensing enzyme, oxalacetic transacetase, oxaloacetate transacetase
2.3.3.8	acetyl-CoA:oxaloacetate acetyltransferase (isomerizing, ADP-phosphorylating), acetyl-CoA:oxaloacetate C-acetyltransferase [(pro-S)-carboxymethyl-forming, ADP-phosphorylating], adenosine triphosphate citrate lyase, ATP citrate (pro-S)-lyase, ATP-citric lyase, ATP:citrate oxaloacetate-lyase [(pro-S)-CH2COO->acetyl-CoA] (ATP-dephosphorylating), ATP:citrate oxaloacetate-lyase [(pro-S)-CH2COO-acetyl-CoA] (ATP-dephosphorylating), citrate cleavage enzyme, citrate-ATP lyase, citric cleavage enzyme
2.8.3.10	acetyl-CoA:citrate CoA-transferase
3.1.2.3	succinyl coenzyme A deacylase, succinyl coenzyme A hydrolase, succinyl-CoA acylase
4.1.1.32	GTP:oxaloacetate carboxy-lyase (transphosphorylating), PEP carboxylase, phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxylase, phosphoenolpyruvic carboxykinase, phosphoenolpyruvic carboxykinase (GTP), phosphoenolpyruvic carboxylase (GTP), phosphopyruvate (guanosine triphosphate) carboxykinase, phosphopyruvate carboxylase, phosphopyruvate carboxylase (GTP)
4.1.1.49	ATP:oxaloacetate carboxy-lyase (transphosphorylating), PEP carboxykinase, PEP carboxylase, PEPCK, PEPCK (ATP), PEPK, phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxylase (ATP), phosphoenolpyruvic carboxykinase, phosphoenolpyruvic carboxylase, phosphopyruvate

	carboxykinase, phosphopyruvate carboxykinase (adenosine triphosphate), phosphopyruvate carboxylase (ATP)
4.1.3.34	(3S)-citryl-CoA oxaloacetate-lyase
4.1.3.6	citrase, citratase, citrate aldolase, citrate lyase, citrate oxaloacetate-lyase, citrate oxaloacetate-lyase [(pro-3S)-CH2COO-acetate], citric aldolase, citridesmolase, citritase
4.2.1.2	(S)-malate hydro-lyase, fumarase, L-malate hydro-lyase
4.2.1.3	Acon, Aconitate hydratase, cis-aconitase, citrate(isocitrate) hydro-lyase
6.2.1.18	citrate:CoA ligase (ADP-forming)
6.2.1.4	succinate:CoA ligase (GDP-forming), succinyl-CoA synthetase (GDP-forming)
6.2.1.5	succinate:CoA ligase (ADP-forming), Succinic Thiokinase, succinyl-CoA synthetase (ADP-forming)
6.4.1.1	pyruvate:carbon-dioxide ligase (ADP-forming), pyruvic carboxylase

Appendix 3 The meanings of all symbols in oxidativephosphorylation pathway

Green shows down-regulated genes or proteins in the oxidative phosphorylation pathway. Red shows up-regulated genes or proteins in the oxidative phosphorylation pathway.

Symbol	Synonym(s)
1.10.2.2	coenzyme Q-cytochrome c reductase, coenzyme QH2-cytochrome c reductase, CoQH2-cytochrome c oxidoreductase, dihydrocoenzyme Q-cytochrome c reductase, mitochondrial electron transport complex III, QH2:cytochrome c oxidoreductase, reduced coenzyme Q-cytochrome c reductase, reduced ubiquinone-cytochrome c oxidoreductase, reduced ubiquinone-cytochrome c reductase, complex III (mitochondrial electron transport), ubihydroquinol:cytochrome c oxidoreductase, ubiquinol-cytochrome c oxidoreductase, ubiquinol-cytochrome c-2 oxidoreductase, ubiquinol-cytochrome c1 oxidoreductase, ubiquinol-cytochrome c2 reductase, ubiquinol:ferricytochrome-c oxidoreductase, ubiquinone-cytochrome b-c1 oxidoreductase, ubiquinone-cytochrome c oxidoreductase, ubiquinone-cytochrome c reductase
1.3.5.1	complex II, fumarate reductase complex, menaquinol:fumarate oxidoreductase, succinate dehydrogenase complex, succinate:ubiquinone

	oxidoreductase, succinic dehydrogenase
1.3.99.1	Complex II, Succinate INT Dehydrogenase
1.6.5.3	coenzyme Q reductase, complex 1 dehydrogenase, complex I (electron transport chain), complex I (mitochondrial electron transport), complex I (NADH:Q1 oxidoreductase), dihydronicotinamide adenine dinucleotide-coenzyme Q reductase, DPNH-coenzyme Q reductase, DPNH-ubiquinone reductase, electron transfer complex I, mitochondrial electron transport complex 1, mitochondrial electron transport complex I, NADH coenzyme Q1 reductase, NADH-coenzyme Q oxidoreductase, NADH-coenzyme Q reductase, NADH-CoQ oxidoreductase, NADH-CoQ reductase, NADH-Q6 oxidoreductase, NADH-ubiquinone oxidoreductase, NADH-ubiquinone reductase, NADH-ubiquinone-1 reductase, NADH2:ubiquinone oxidoreductase, NADH:ubiquinone oxidoreductase complex, reduced nicotinamide adenine dinucleotide-coenzyme Q reductase, type 1 dehydrogenase, ubiquinone reductase
1.6.99.3	beta-NADH dehydrogenase dinucleotide, cytochrome c reductase, diaphorase, dihydrocodehydrogenase I dehydrogenase, dihydronicotinamide adenine dinucleotide dehydrogenase, diphosphopyri3633se, DPNH diaphorase, NADH cytochrome C reductase, NADH diaphorase, NADH hydrogenase, NADH oxidoreductase, NADH-menadione oxidoreductase, NADH2:(acceptor) oxidoreductase, NADH:cytochrome c oxidoreductase, reduced diphosphopyridine nucleotide diaphorase, type 1 dehydrogenase
1.6.99.5	D-diaphorase, DPNH-menadione reductase, NADH-quinone oxidoreductase, NADH2:(quinone-acceptor) oxidoreductase, reduced nicotinamide adenine dinucleotide (quinone) dehydrogenase
1.9.3.1	complex IV (mitochondrial electron transport), COX, Cytochrome Aa3, Mitochondrial Complex IV, respiratory chain complex IV
2.7.4.1	ATP:polyphosphate phosphotransferase, polyphosphoric acid kinase
3.6.1.1	diphosphate phosphohydrolase
3.6.3.10	(K+ + H+)-ATPase, ATP phosphohydrolase (H+/K+-exchanging), H+-K+-ATPase, H,K-ATPase
3.6.3.14	ATP phosphohydrolase (H+-transporting), ATP synthase, bacterial Ca2+/Mg2+ ATPase, chloroplast ATPase, coupling factors (F0, F1 and CF1), F1-ATPase, FoF1-ATPase, H+-transporting ATPase, mitochondrial ATPase
3.6.3.6	ATP phosphohydrolase, ATP phosphohydrolase (H+-exporting), proton-translocating ATPase, yeast plasma membrane ATPase, yeast plasma membrane H+-ATPase

ADP	20398-34-9, 58-64-0, 9-beta-D-arabinofuranosyladenine 5'-diphosphate, adenosine 5'-(trihydrogen diphosphate), adenosine diphosphate, C10H15N5O10P2, [(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl phosphono hydrogen phosphate
ATP	56-65-5, 9-beta-D-arabinofuranosyladenine 5'-triphosphate, adenosine 5'-(tetrahydrogen triphosphate), adenosine 5'-triphosphate, adenosine triphosphate, ATP4-, C10H16N5O13P3, [[(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy-hydroxyphosphoryl] phosphono hydrogen phosphate
Diphosphate	14000-31-8, diphosphate, diphosphate(4-), inorganic pyrophosphate, O7P2-4, phosphonato phosphate, PPi, pyrophosphate ion
Fumarate	(E)-but-2-enedioic acid, 110-17-8, 2-butenedioic acid (2E)-, C4H4O4, fumarate
H+	12408-02-5, 12586-59-3, H+, hydrogen ion, hydron, proton
H2O	7732-18-5, H20, H2O, oxidane
NAD+	53-84-9, adenosine 5'-(trihydrogen diphosphate), P'-5'-ester with 3-(aminocarbonyl)-1-beta-D-ribofuranosylpyridinium, inner salt, beta-NAD+, beta-nicotinamide adenine dinucleotide+, C21H28N7O14P2+, NAD, [[(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy-hydroxyphosphoryl] [(2R,3S,4R,5R)-5-(3-carbamoylpyridin-1-ium-1-yl)-3,4-dihydroxyoxolan-2-yl]methyl hydrogen phosphate
NADH	58-68-4, adenosine 5'-(trihydrogen diphosphate), P'-5'-ester with 1,4-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide, beta-NADH, C21H29N7O14P2, dihydronicotinamide-adenine dinucleotide, NADH2, nicotinamide dinucleotide, [[(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy-hydroxyphosphoryl] [(2R,3S,4R,5R)-5-(3-carbamoyl-4H-pyridin-1-yl)-3,4-dihydroxyoxolan-2-yl]methyl hydrogen phosphate
Oxygen	7782-44-7, dioxygen, molecular oxygen, O2
Phosphate	14265-44-2, inorganic phosphate, O4P-3, phosphate, phosphate ion, phosphate(3-), Pi
Succinate	1,2-ethanedicarboxylic acid, 1,4-butanedioic acid, 110-15-6, 56-14-4, amber acid, asuccin, butanedioate, butanedioic acid, C4H6O4, ethylenesuccinic acid, katasuccin, potassium succinate, succinate, wormwood acid
Triphosphate	14127-68-5, O10P3-5, triphosphate, [oxido(phosphonatooxy)phosphoryl] phosphate

Ubiquinol	56275-39-9, C14H20O4(C5H8)n, CoQH2, QH2, ubihydroquinone, ubiquinol, ubiquinone hydroquinone
Ubiquinone	1339-63-5, C14H18O4(C5H8)n, coenzyme Q, ubiquinones