5 Discussion

The progression of congestive heart failure is characterized by diverse cellular abnormalities associated with decreased ventricular function. Initially, the heart undergoes compensatory adaptations including cardiac hypertrophy and ventricular remodeling. These changes increase the oxygen consumption and vascular resistance, which ultimately impair the ability of the ventricles to pump blood. Exploring the genetic mechanisms of cardiomyopathies and the molecular basis of heart failure due to chronic hemodynamic overload is the way to better understand the pathophysiology of heart failure.

Multiple molecular pathways are believed to be responsible for transferring these stimuli into changes in gene expression during the process of heart failure. To obtain a global portrait of gene expression in the hypertrophy and failing heart, expression profiling with rat heart cDNA microarrays and rat genome Affymetrix chips were applied to heart failure model SHHF and none-heart failure controls. Expression differences of candidate genes were further analyzed with Real Time PCR. Gene *Cd36*, which owns multiple functions especially of a crucial role on fatty acid metabolism, was found significantly downregulation in SHHF compared with nonfailure controls. Molecular basis and linkage analysis of *Cd36* in SHHF were also studied in this thesis.

5.1 Candidate genes selected with cDNA arrays

In this study, rat heart cDNA microarray expression profiling was applied to detect the candidate genes related to cardiomyopathy and heart failure at first. Of the16 genes showing differential expression in the SHHF/WKY or SHHF/SHRSP comparison, the genes of cytochrome oxidase subunit I, II, IV, *Cd36*, and NADH dehydrogenase were related to energy metabolism. One *titin* similar gene and phospholamban gene were downregulated in SHHF rat. Titin may cause HCM in patients via altered affinity to alpha-actin (Satoh *et al*., 1999) and changes in titin isoform expression may be involved in diastolic dysfunction in heart failure (Wu *et al*., 2002). Phospholamban is one of the important genes related to cardiac contractility and Ca^{2+} handling (Lips *et al*., 2003). Previous reports have identified some differentially expressed genes in various models of heart failure and hypertrophy by using the approach of cDNA glass slides. Candidate genes were mainly involved in Ca^{2+} signaling pathway, stress

response, energy metabolism, and extracellular matrix (Barrans *et al*., 2002; Friddle *et al*., 2000; Hwang *et al*., 2002; Tyagi *et al*., 1996).

5.2 Candidate genes explored with Affymetrix chips

To further explore genes involved in cardiomyopathy and heart failure, Affymetrix chip RG_U34 A and B were used for expression profiling in the heart failure model SHHF and the controls of WKY and SHRSP. Genes expressed in SHHF were first compared respectively with nomortensive control WKY or hypertensive control SHRSP. From candidate lists of the above two groups, genes expressed differentially only in heart failure rat SHHF were selected. The SHRSP contains some similar syndromes like SHHF and both of them were developed from SHR strain. Thus genes showing differential expression in the comparisons of the SHHF/WKY and SHRSP/WKY were also analyzed.

5.2.1 Genes with differential expression in SHHF train versus WKY or SHRSP

Within the genes showing differential expression in SHHF strain compared with WKY or SHRSP (Appendix Table 11.1-2), functions for 81 genes of them were obtained in Affymetrix and NCBI websites. These genes were clustered into 9 functional groups (Table 5.1) under the headings of extracellular matrix/cytoskeleton, signal transduction, metabolism, apoptosis/ proinflammatory, cell cycle regulatory, transcription factor, calcium signaling, growth factor/hormone activity, and others. Among of them, thirty-one genes were downregulated (Table 5.2) whereas 50 genes were upregulated (Table 5.3). ANP related gene natriuretic peptide precursor type B showed upregulation in heart failure rat SHHF, confirming hormonal regulation of the *ANP* gene to be considered a marker gene for the hypertrophic phenotype (Busk *et al*., 2003). Bcl2-like 1 on apoptosis pathway showed downregulation in the SHHF in the study. Apoptosis plays a role in the pathophysiology hypertrophy and heart failure. Bcl-2 is one of the anti-apoptotic factors and the ratio of Bcl-2 to Bax is often used as the indicator of apoptosis; a decrease in the ratio signifies exacerbation of the apoptotic process (MacLellan and Schneider, 1997).

Table **5.1 Functional clusters of potential genes related to heart failure**

Function	Upregulated genes	Downregulated genes
Extracellular matrix / Cytoskeleton	*Acta1*, *Actb*, *Il2rb*, *Tagln, Timp2*, *Col1a1*, *Col3a*	*Timp1*
Signal transduction	*Uncl19*, *Penk-rs*, *Ptpn16*	*Ppap2a*, *Btnl2*
Metabolism: Energy metabolism	*Ucp1*, *Fbp2*, *Eno1*, *Hk1*, *Thrsp*, *SC2*, *Ca3*, *Rxrg*, *G6pt1*	*Cd36*, *Hmgcs2*, *Pdk4*
Transport, electr transport	*Maoa*, *Z49858*, *Mt1a*, *Kcnd2*, *Rab10*, *Scn5a*, *Ywhah*, *Kcnj11*	*Abcd3*, *Cyp26b1*, *Cyp2e1*, *Fdx1*, *Xdh*, *Rmt7*, *Hba1*
Enzymes	*Adam17*, *Enpep*, *Wfdc1*, *Serpinh1*, *Psmb9*, *Slc3a1, Mir16*	*B4galt6*, *Slc3a2*, *Sult1a1*, *Sod1*, *Cst3*, *Pla2g4a, Daf*
Apoptosis/ Proinflammatory		*Bcl2l1*, *Spp1*
Cell-cycle regulatory	*AF045564*, *Ccnd2*, *Ccnd1*	*Csrp2*, *Vdup1*
Transcription factor	*Nr3c1*, *Nr1d2*, *Dbp*, *Nrld1*	*Nupr1*
Calcium signaling	*Fstl*, *Thbs4*, *Canx*	*S100a9*
Growth factor and hormone activity	*Vegf*, *Nppb*, *Hspa4, Hspa1a*	
Others	*Ephx2*, *LOC290985*	*RT1Aw2*, *Lgals3*, *Ifi27l*, *LOC362850, Amd1*

5.2.1.1 Candidate genes showing the consistent expression change as the previous studies

Candidate genes selected in this study, such as genes of collagen type I and III, thrombospondin 4, heat shock 70, cyclin D, and cysteine rich protein have been also found as the candidate genes for cardiomyopathy or heart failure in the previous studies (Table 5.2-3, in bold). The increased expression of the Collagen type I, III, and thrombospondin 4 in SHHF rat were also found in human heart failure by gene expression fingerprint (Tan *et al*., 2002). Changes in myocardial total collagen content, collagen subtypes and collagen denaturation are important features of extracellular matrix remodeling together with diastolic and systolic dysfunction. Myocardial fibrosis and maladaptive extracellular matrix remodeling are pathognomonic findings in end-stage CHF (Li *et al*., 2002). Stress response protein heat shock 70, which was upregulated in dilated cardiomyopathy by using a human cardiovascular-based cDNA microarray (Barrans *et al*., 2002), was also increased in heart failure rat SHHF. D-type cyclins and cyclin-dependent kinases 4 (CDK4) were found to be involved in cardiac hypertrophy (Busk *et al*., 2002; Tamamori-Adachi *et al*., 2002). The expression of cell-cycle regulatory proteins cyclin D was also increased in SHHF.

Table 5.2 **Downregulated genes in SHHF strain compared with WKY or SHRSP**

Probe Set ID	Gene symbol	Chromosomal location	Gene title	Molecular function
rc_AA942718_at[3]	*Bcl2l1*	3q41.2	Bcl2-like 1	apoptosis; anti-apoptosis
AF072411_at[3]	***Cd36***	**4q11**	**cd36 antigen**	**fatty acid metabolism; long-chain fatty acid transport; cell adhesion**
rc_AA946532_at[2]	***Abcd3***	**2q41**	**ATP-binding cassette, sub-family D (ALD), member 3**	**transporter activity; ATPase activity**
L18948_at[2]	*S100a9*	2q34	S100 calcium-binding protein A9 (calgranulin B)	calcium ion binding
rc_AA818593_at[1]	*Ppap2a*	2q14	phosphatidate phosphohydrolase type 2a	ceramide metabolism; integral to plasma membrane; signal transduction
K02815_s_at[1]	*Btnl2*	--	butyrophilin-like 2 (MHC class II associated)	Neural cell adhesion molecule; Signal transduction mechanisms
M33648_at[3]	*Hmgcs2*	2q34	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	cholesterol biosynthesis
rc_AA957564_at[1]	*Cyp26b1*	4q34	cytochrome P450, family 26, subfamily b, polypeptide 1	electron transport
M20131cds_s_at[3]	*Cyp2e1*	--	Cytochrome P450, subfamily 2E, polypeptide 1	electron transport
D50436_at[1]	*Fdx1*	8q24	ferredoxin 1	electron transport
rc_AI172247_at[2]	*Xdh*	6q12	xanthine dehydrogenase	electron transport
AF039583_s_at[1]	*Daf*	13q13	decay-accelarating factor	enzyme inhibitor activity
U44948_at[3]	***Csrp2***	**7q13**	**cysteine rich protein 2**	**myoblast differentiation**
M14656_at[2]	*Spp1*	14p22	secreted phosphoprotein 1	Ossification; cell adhesion
J02962_at[1]	*Lgals3*	15p14	lectin, galactose binding, soluble 3	protein binding
rc_AI014169_at[1]	*Vdup1*	2q34	upregulated by 1,25-dihydroxyvitamin D-3	regulation of cell proliferation
AF014503_at[1]	*Nupr1*	1q36	nuclear protein 1	transcription factor activity
AF048687_s_at[2]	*B4galt6*	18p12	betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6	transferase activity
rc_AA955151_at[1]	*Rmt7*	4q33	hypothetical protein RMT-7	transport
rc_AI178971_at[1]	*Hba1*	10q12	hemoglobin, alpha 1	transport; globin; oxygen transport
AF034577_at[3]	*Pdk4*	4q13	pyruvate dehydrogenate kinase 4	pyruvate dehydrogenase kinase activity; ATP binding
X89225cds_s_at[2]	*Slc3a2*	1q43	solute carrier family 3, member 2	alpha-amylase; alpha-amylase activity
rc_AI008131_s_at[1]	*Amd1*	20q12	S-adenosylmethionine decarboxylase 1	adenosylmethionine decarboxylase activity
L19998_g_at[3]	*Sult1a1*	--	sulfotransferase family 1A, phenol-preferring, member 1	aryl sulfotransferase activity; sulfotransferase activity
Y00404_s_at[2]	*Sod1*	11q11	superoxide dismutase 1	copper, zinc superoxide dismutase activity
rc_AI231292_g_at[3]	*Cst3*	3q41	cystatin C	cysteine protease inhibitor activity
U38376_s_at[2]	*Pla2g4a*	13q21	phospholipase A2, group IVA (cytosolic, calcium-dependent)	lysophospholipase activity
rc_AI169327_at[2]	***Timp1***	**Xq12**	**tissue inhibitor of metalloproteinase 1**	**metalloendopeptidase inhibitor activity**
M10094_g_at[1]	*RT1Aw2*	20p12	RT1 class Ib gene(Aw2)	immune response
rc_AA945204_at[3]	*Ifi27l*	6q32	interferon, alpha-inducible protein 27-like	---
rc_AA944481_at[2]	*LOC362850*	7q12	angiopoietin-like protein 4	---

1, The genes showed downregulation in the SHHF/WKY comparison. 2, The genes showed downregulation in the SHHF/SHRSP comparison. 3, Candidate genes showed downregulation in both the SHHF/WKY comparison and SHHF/SHRSP comparison. The genes in bold have been selected as candidates for hypertrophy or heart failure from previous studies. --, No hit on the chromosome.

SmLIM protein was downregulated in the heart failure model SHHF compared with two none failure controls. Striated muscle LIM protein-1 (SLIM1) was measured downregulated in failing human hearts by high-density oligonucleotide arrays (Yang *et al*., 2000). Proteins of the LIM family are critical regulators of development and differentiation in various cell types. The cysteine-rich protein (CRP) family is a subclass of the LIM-only protein, which includes CRP1, CRP2/smooth muscle LIM protein (SmLIM), and CRP3/muscle LIM protein (MLP). CRP/MLP-deficient animals exhibited profound defects in cardiac and CRP/MLP-deficient mice develop heart failure soon after birth, most likely because of a disruption of the cardiomyocyte cytoarchitecture (Arber and Caroni, 1996). Differentiated, quiescent vascular smooth muscle cells assume a dedifferentiated, proliferative phenotype in response to injury, one of hallmarks of arteriosclerosis (Yet *et al*., 1998).

Table 5.3 **Upregulated genes in SHHF strain compared with WKY or SHRSP**

Probe Set ID	Gene Symbol	Chromosomal location	Gene title	Molecular function
D17445_at[1]	*Ywhah*	14q21	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	protein domain specific binding
J00692_at[1]	*Acta1*	19q12	actin alpha 1	actin; structural constituent of cytoskeleton
V01217_at[2]	*Actb*	12p11	actin, beta	actin; structural constituent of cytoskeleton
rc_AI029058_s_at[2]	***Ccnd1***	**1q42**	**cyclin D1**	**adipocyte differentiation; regulation of cell cycle**
M80804_s_at[1]	*Slc3a1*	6q12	solute carrier family 3, member 1	alpha-amylase; alpha-amylase activity
L20913_s_at[2]	*Vegf*	9q12	vascular endothelial growth factor	angiogenesis; neurogenesis; positive regulation of cell proliferation
rc_AA799498_at[2]	*Nppb*	--	natriuretic peptide precursor type B	ANP; hormone activity
X89963_at[1]	***Thbs4***	**2q12**	**thrombospondin 4**	**calcium ion binding**
rc_AI010725_g_at[2]	*Canx*	10q22	calnexin	calcium ion storage activity; calcium ion binding
M27207mRNA_s_at[1]	Col1a1	--	collagen, type 1, alpha 1	cell adhesion
AF045564_at[1]	*AF045564*	19p13	development-related protein	cell differentiation
D00688_s_at[1]	***Maoa***	**--**	**monoamine oxidase A**	**electron transport; synaptic transmission**
A04674cds_s_at[3]	*Ucp1*	19p11-q11	uncoupling protein 1	energy pathways; mitochondrial transport
rc_AA799340_at[1]	***Timp2***	**10q32.3**	**tissue inhibitor of metalloproteinase 2**	**metalloendopeptidase inhibitor activity**
X60328_at[2]	*Ephx2*	15p12	cytosolic epoxide hydrolase	epoxide hydrolase activity; hydrolase activity
M21354_s_at[1]	***Col3a1***	**9q22**	**collagen, type III, alpha 1**	**extracellular matrix structural constituent**
AJ005046_g_at[3]	*Fbp2*	17p14	fructose bisphosphatase 2	fructose-bisphosphatase activity; phosphoric ester hydrolase activity
D16308_at[1]	***Ccnd2***	**--**	**cyclin D2**	**G1/S transition of mitotic cell cycle; regulation of cell cycle**
rc_AA901006_at[1]	*Mir16*	--	membrane interacting protein of RGS16	glycerophosphodiester phosphodiesterase activity
X02610_at[2]	*Eno1*	5q36	enolase 1, alpha	glycolysis

Table 5.3 continued

rc_AI012593_at[1]	*Hk1*	20q11	hexokinase 1	glycolysis
L16764_s_at[2]	***Hspa1a***	**20p12**	**heat shock 70kD protein 1A**	**heat shock protein activity; ATP binding**
M55050_at[1]	*Il2rb*	7	interleukin 2 receptor, beta chain	Hematopoietin; interferon-class cytokine receptor activity
rc_AI010449_at[1]	*Fstl*	2	follistatin-like	heparin binding; calcium binding
S74351_s_at[3]	*Ptpn16*	10q12	protein tyrosine phosphatase, non-receptor type 16	intracellular signaling cascade
Z49858_at[2]	*Z49858*	19p12	plasmolipin	ion transport; response to wounding; myelination
D86039_at[1]	*Kcnj11*	1q22	potassium inwardly rectifying channel, subfamily J, member 11	inward rectifier potassium channel activity
K01934mRNA#2_at[3]	*Thrsp*	1q32	thyroid hormone responsive protein	lipid metabolism
S45663_at[1]	*SC2*	19q11	synaptic glycoprotein SC2	lipid transport
rc_AI009666_at[2]	*Enpep*	2q42	aminopeptidase A	membrane alanyl and glutamyl aminopeptidase activity
rc_AI102562_at[2]	*Mt1a*	19p12	Metallothionein	metalthio; metal ion binding
M83107_at[1]	*Tagln*	8q24	transgelin	muscle development
AF037072_at[1]	*Ca3*	2q23	carbonic anhydrase 3	one-carbon compound metabolism
S49491_s_at[2]	*Penk-rs*	5q12	preproenkephalin, related sequence	Opiods_neuropep; neuropeptide signaling pathway
M59980_s_at[2]	*Kcnd2*	4q22	potassium voltage gated channel, Shal-related family, member 2	potassium ion transport
rc_AI230406_at[2]	*Rab10*	6q12	ras-related protein rab10	ras;small monomeric GTPase activity
rc_AA893618_s_at[2]	*Nr3c1*	18p12	nuclear receptor subfamily 3, group C, member 1	regulation of transcription, DNA-dependent
J03179_g_at[2]	*Dbp*	--	D site albumin promoter binding protein	regulation of transcription, DNA-dependent; rhythmic behavior
AJ012603UTR#1_g_at[2]	*Adam17*	6q16	a disintegrin and metalloproteinase domain 17	Reprolysin; metalloendopeptidase activity
AF077354_at[1]	***Hspa4***	**10q22**	**heat shock 70 kDa protein 4**	**response to heat**
AF037272_at[3]	*Wfdc1*	19q12	wap four-disulfide core domain 1	serine-type endopeptidase inhibitor activity
M69246_at[3]	*Serpinh1*	1q32	serine (or cysteine) proteinase inhibitor, clade H, member 1	serine protease inhibitor activity
U40999_at[2]	*Uncl19*	10q25	UNC-119 homolog (C. elegans)	signal transduction
M27902_at[1]	*Scn5a*	8q32	sodium channel, voltage-gated, type V, alpha polypeptide	sodium ion transport
AJ223083_at[3]	*Rxrg*	13q24	retinoid X receptor gamma	steroid binding,; retinoid-X receptor activity
M25804_at[1]	*Nr1d1*	10q31	nuclear receptor subfamily 1, group D, member 1	transcription factor activity
U20796_at[1]	*Nr1d2*	15p16	nuclear receptor subfamily 1, group D, member 2	transcription factor activity
AF080468_g_at[2]	*G6pt1*	8q22	glucose-6-phosphatase, transport protein 1	transporter activity
rc_AI012340_s_at[2]	*Psmb9*	20p12	proteosome (prosome, macropain) subunit, beta type 9	ubiquitin-dependent protein catabolism
rc_AA946469_at[2]	*LOC290985*	17p14	HesB protein	---

1, The genes showed upregulation in SHHF strain compared with WKY. 2, The genes showed upregulation in SHHF strain compared with SHRSP. 3, The genes were upregulated in SHHF strain compared with both WKY and SHRSP. Genes in bold have been selected as candidates for hypertrophy or heart failure from the previous studies. --, No hit on the chromosome.

5.2.1.2 Candidate genes related to metabolism

Fourty-one genes encoding proteins related in metabolism were differentially expressed in SHHF (Table 5.1). Of them 12 genes are related to energy metabolism, 15 genes encode transporters or electro transporters, the other 14 genes are enzymes. Genes involved in fatty acid metabolism, e.g., *Cd36* and pyruvate dehydrogenate kinase 4 were exclusively downregulated. Genes related to glucose metabolism, such as fructose bisphosphatase 2, uncoupling protein 1, and retinoid X receptor were upregulated. These findings further indicated the fact that the primary myocardial energy source switches from fatty acid to glucose in heart failure. Fatty acid and associated lipids play an important role in cardiomyocytes structure and function. Fatty acid β oxidation is the preferred pathway for the energy utilization required for efficient cardiac pumping. Specific defects in mitochondrial fatty acid metabolism may cause cardiomyopathy leading to cardiac failure (Marin-Garcia and Goldenthal, 2002).

Specific heritable deficiencies in fatty acid metabolism are associated with cardiomyopathy and cardiac failure, such as deficiencies of *Cd36*, MCAD, MTP, VLCAD, and CPTII. Here, *Cd36* showed significantly downregulation in SHHF strain compared with two none heart failure controls. In this study, *Cd36* was also found deletion on chromosome DNA and normal Cd36 protein could not be detected in SHHF strain by immunoblot analysis. Downregulation of PDK4 in SHHF strain may be resulted from the decrease of free fatty acids. It has been reported that the upregulation of PDK4 causes inactivation of the pyruvate dehydrogenase complex, which blocks pyruvate oxidation and conserves lactate and alanine for gluconeogenes in many tissues of the body during starvation. This enhanced PDK4 expression may be caused by the increase in free fatty acids, which can activate PPARα, and the latter can promote PDK4 expression subsequently (Wu *et al*., 2000).

Ligand activation of PPAR leads to obligate heterodimerization with members of the 9-cis retinoic acid-activated nuclear hormone receptor (RXR) sub-family, and subsequent binding to cognate DNA response elements within target gene promoter regions (Kliewer *et al*., 1992). RXRγ showed three to seven folds increase in the comparisons of SHHF/WKY and SHHF/SHRSP. The RXR agonist has a potent effect on increasing glucose uptake, GS activity and palmitate oxidation in human skeletal muscle, which suggests direct effects on an important insulin sensitive tissue and may reduce hyperglycemia and hyperlipidaemia when combined with PPARγ agonists (Cha *et al*., 2001). The RXRα has been shown to activate the

transcription of the genes encoding MCAD and muscle –type CPT-1. The role of RXRα in cardiac failure has been further probed in the transgenic mouse (Ruiz-Lozano *et al*., 1998). But RXRα showed no expression change in the heart failure model SHHF in this study.

The uncoupling protein UCP1 showed great upregulation (10 to 15 folds) in heart failure model SHHF compared with the two controls. The uncoupling proteins are transporters present in the mitochondrial inner membrane, which mediate a regulated discharge of the proton gradient generated by the respiratory chain (Ledesma *et al*., 2002). The thermogenic mechanism by UCP1 is centered on the brown fat, which allows dissipation of the proton electrochemical potential gradient and therefore uncouples respiration from ATP synthesis. Nucleotides and fatty acids affect the regulation of UCP1. Free fatty acids are the substrate for brown fat thermogenesis and act as the cytosolic second messengers by noradrenaling activity (Gonzalez-Barroso *et al*., 1998; Rial and Gonzalez-Barroso, 2001). UCP2 and UCP3 are thought to be active in uncoupling electron transport from oxidative phosphorylation; thus, increased UCP protein expression would be predicted to reduce cardiac energy efficiency. These findings suggest that changes in cardiac UCP protein expression are linked with changes in cardiac metabolic fuel selection, from fatty acids to glucose during heart failure.

5.2.1.3 Protein tyrosine phosphatase and PS20 were upregulated in SHHF

Protein tyrosine phosphatase and PS20 protein were upregulated in SHHF compared with WKY and SHRSP. Although the implications of these two increased proteins in heart failure are unclear, their molecular function analysis suggested that they might be involved in hypertropy or failure. Protein tyrosine phosphatase has MAP kinase phosphatase activity, the family of MAPKs has been shown as the cause in the development of cardiac hypertrophy and the transition towards heart failure (Lips *et al*., 2003). PS20 was originally identified as secreted growth inhibitor (Rowley *et al*., 1995). Biological activity of PS20 is consistent with the biological activity of other WAP family proteins, which exhibit fundamental roles in growth control, differentiation and tissue remodeling in development and maintenance of homeostasis in adult tissue.

5.2.2 Genes with differential expression in SHHF and SHRSP strains versus WKY

Among the genes showing differential expression in the SHRSP/WKY comparison, twenty-eight also showed similar expression change in the SHHF/WKY comparison (Appendix Table 11.3). The genes, e.g., synaptic glycoprotein, atrial myosin light chain 1 (ALC1), skeletal muscle α-actin, and monoamine oxidase were upregulated in SHHF and SHRSP. Reexpression of ALC1 in human ventricle during hypertrophy is related to the degree of hemodynamic load (Ritter *et al*., 1999; Sutsch *et al*., 1992). ALC1 enhances cross-bridge kinetics that force generation is increased at a given cytosolic Ca^{2+}-concentration. In addition, covalent protein modification by reversible phosphoryla-tion may affect contractility (Morano, 1999; Schaub *et al*., 1998). Skeletal muscle α-actin gene accumulate relative to cardiac α-actin during cardiac hypertrophy (Schwartz *et al*., 1993). Monoamine oxidase (MAO) is well suited for regulatory and protective actions in most organ in the body (Fowler *et al*., 2003). The level of catecholamine is increased in left ventricles of SHR treated with ACE inhibitors or/and AT1 receptor antagonist, and is paralleled by a reduction of left ventricular MAO activity (Raasch *et al*., 2002). The genes, e.g., lectin, interferon induced protein, and ASM15 were also found downregulation in SHHF and SHRSP strains.

5.3 Conclusion for gene expression profiling

Global analysis of gene expression in the cardiomyopathy and heart failure model SHHF was performed using expression profiling in Affymetrix chips. More than 91% of retested genes obtained from Affymetrix chip analysis showed consistent change when reconfirmed with Real Time PCR, which was used to validate the reliability of the global gene expression portrait of the SHHF model.

Extracellular matrix/cytoskeletal proteins (e.g., collagen type I and III, thrombospondin 4), cell-cycle regulator (e.g. cyclin D, cysteine rich protein 2), and stress response protein (heat shock 70) that have been found related to cardiomyopathy or heart failure in the previous studies, were further confirmed in the SHHF in this study. Protein tyrosine phosphatase and PS20 were first found upregulation in SHHF. The striking result from this study is that a group of genes related to energy metabolism showed expression changing in heart failure model, compared with the two none heart failure controls. Although energy metabolism changes from fatty acid to glucose in heart failure is already clear, the underlying mechanism

and possible related genes remain unknown. The gene *Cd36* and pyruvate dehydrogenate kinase 4 on fatty acid metabolism were downregulated, meanwhile the genes fructose bisphosphatase 2, uncoupling protein 1, and retinoid X receptor on glucose metabolism were upregulated in SHHF strain. Expression changes of these genes simultaneously detected in the comparisons of SHHF/WKY and SHHF/SHRSP provide more chance to explore the key genes involved in hypertrophy and heart failure. From both cDNA arrays and Affymetrix chips, *Cd36*, which is an important receptor for long chain fatty acid and has other multiple functions, was detected significantly downregulated in SHHF model compared with two different controls, and also confirmed further by Real Time PCR.

Since two different controls (Hypertensive and Normotensive controls) were applied to Affymetrix chip analysis, candidate genes could be compared in different groups. This greatly facilitated comparison analysis of hundreds of candidate genes selected in normal DNA arrays. The two comparison methods with individual chip and mean chip were applied to analyze data from Affymetrix chip. Only genes showed consistent expression change by these two methods were set up as candidate genes. It greatly reduce fault positive genes. The limitation of this study is the smaller control samples may influence the selecting of the candidate genes. However, all animals used in this study are inbred strain, diversity among animals should be very limited.

5.4 Molecular basis of *Cd36* locus in SHHF rat

The molecular basis of changes at the *Cd36* locus in SHHF strain that underlie the differential expression and two different transcripts (about 3.5 kb and 5.0 kb) in heart tissue was determined. Three and a half quite similar homologue fragments with three corresponding genes: LOC360376 (hypothetical gene), LOC362310 (similar to fatty acid translocase/*Cd36*), and LOC296786 (similar to fatty acid transport protein) were found at the *Cd36* locus on rat genome chromosome 4q11. Analysis of genomic DNA and cDNA sequences revealed a genomic deletion in the region between the first intron 4 of the gene LOC360376 and intron 4 of the gene LOC296786. This chromosomal fragment deletion was caused by unequal recombination events, which was likely mediated by regions of homology and strong homology among the three model genes. The chromosomal deletion at the *Cd36* locus in SHHF strain is quite similar as that detected in SHR/Ncr1BR (Aitman *et al*., 1999; Glazier *et al*., 2002). *Cd36* deletion breakpoint in SHHF was between nt 9 and nt 630 away from the

first exon 4/intron 4 boundary of the gene LOC360376, while *Cd36* deletion point in the SHR/Ncr1BR was between nt 539 and nt 628 away from the exon 4/intron 4 boundary.

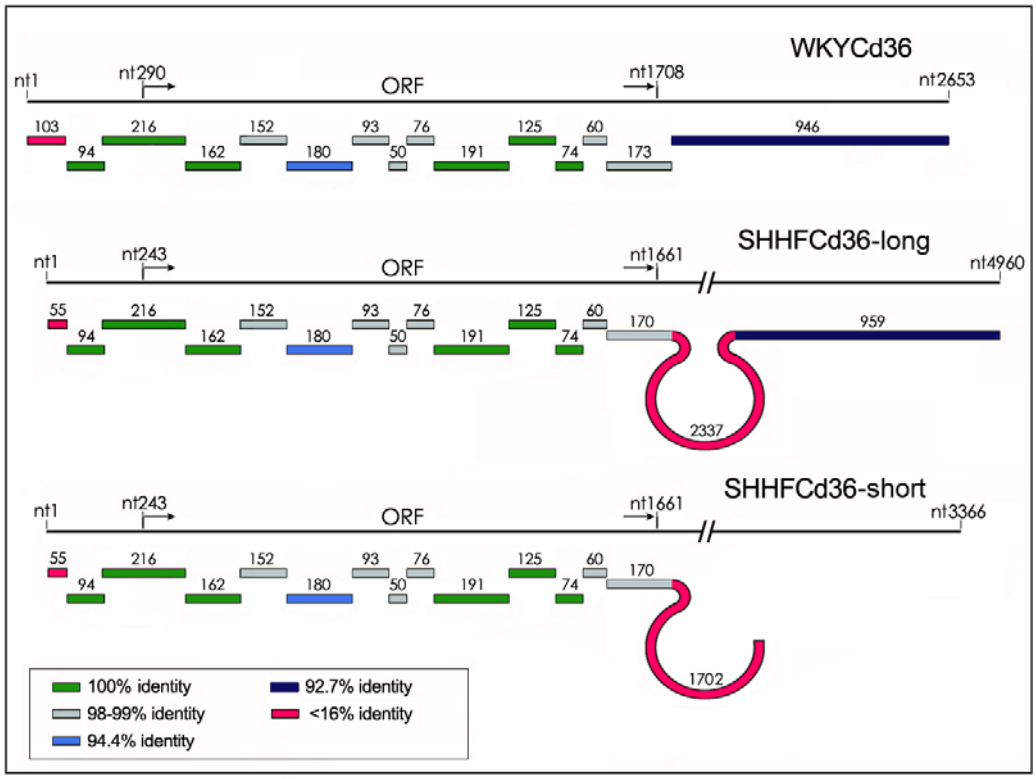

Fig 5.1 Comparison of *Cd36* cDNA structures between SHHF and WKY strains

The colored rectangles represent the different exons, exon 1 to 15 for WKY and 1 to 14 for SHHF, which rank from the left to right.

The full length of *Cd36* cDNA from WKY (1>2653 bp) and SHHF were first acquired in this study (Fig 5.1). Two transcripts for *Cd36* (1>3366 bp, and 1>4960 bp) were found in SHHF heart tissue and their first 3366 bp are the same. SHR/Ncr1BR *Cd36* cDNA (1>1867 bp, GenBank accession number AF111268) are 100% identical to the corresponding part of the SHHF (nt 203-1890), where includes complete ORF region. In Glazier's study, upstream copy of *Cd36* was normal *Cd36* gene, the other two copies of *Cd36* were two pseudogenes (Glazier *et al*., 2002). The physical positions of normal *Cd36* gene and two pseudogenes corresponded to the loci of the genes LOC360376, LOC362310 and LOC296786. From this and previous studies, WKY *Cd36* cDNA were transcribed from the transcription units in the region of the gene LOC360376. SHHF or SHR/Ncr1BR exons 2 to 4 were transcribed from the same transcription units as in WKY, and SHHF exons 5 to 14 were from the transcription units in the gene LOC296786. These were confirmed by the blast analysis of SHHF *Cd36* cDNA

against rat genome sequence in this study. *Cd36* exon 1 of WKY and SHHF were first detected in this study (Fig 5.1). Many sequence variants were observed within exon 1 between WKY and SHHF (Fig 4.12). WKY exon 1 was 92% identical to the mouse musculus *Cd36* gene upstream promoter, but their transcription units are still not found in the *Cd36* locus of currently published rat genome sequence due to the existing gaps, thus the transcription mechanisms of *Cd36* in the WKY and SHHF are still unclear.

Much effort has been invested to gain insight into the mechanisms of *Cd36* expression. Study on regulation of *Cd36* at the level of translation in human suggested it might rely on *Cd36* 5'-untranslated regions, which contains three upstream ORFs. The reporter assays indicated that re-initiation following translation of the first of ORFs is responsible for the increase in translational efficiency under hyperglycaemic conditions (Griffin *et al*., 2001). SHHF *Cd36* cDNA untranslated region (243 bp), which is shorter than WKY *Cd36* cDNA (Fig. 5.1), lacks the first upstream ORF compared with WKY *Cd36*. This may be the reason that affects the *Cd36* translational efficiency in this strain. Cd36 can also be regulated by translocational mechanism, combined by post-translational modifications such as glycosylation, palmitoylation and phosphorylation (Asch *et al*., 1993).

The Cd36 predicted amino acid sequence of SHHF is identical to that of SHR/Ncr1BR. It contains multiple amino acid substitutions compared to WKY *Cd36* sequence, and none of them locates within the putative transmembrane domains. Asn102Ser alters a putative glycosylation site and Ala401Pro may produce a major conformational change (Aitman *et al*., 1999). Cd36 protein was undetectable in SHHF heart tissue using Cd36 antibody in this study and also undetectable in SHR adipocyte plasma membrane (Aitman *et al*., 1999). It is possible that the Cd36 antibody did not bind to the mature Cd36 protein of SHHF because of the multiple sequence variants in this strain. It needs to be anchored if *Cd36* transcripts in SHHF can translate functional protein. From evidence viewer of *Cd36* locus at NCBI, exons of the human *Cd36* mRNAs are located at the locus of the gene LOC296786 where exons (5 to15) of SHHF or SHR mRNA located (Fig 4.13). The triplication of *Cd*36 gene is only present in rats and there is only one copy of the *Cd36* gene in human or mouse. Mechanisms of *Cd36* regulation in rat and evolution on its special triplicates that are different from mice and human are unclear.

5.5 Function of Cd36 in SHHF rat

During cardiac hypertrophy and heart failing, a transition reminiscent of fetal energy substrate utilization occurs with reduced fatty acid oxidation and increased glucose utilization (Christe and Rodgers, 1994; Marin-Garcia and Goldenthal, 2002). Long chain fatty acids (LCFA) are the chief energy substrate in the normal adult mammalian heart. Cd36 is one of important transport proteins for fatty acid and LCFA in the heart tissue. By microarray screening and molecular analysis, *Cd36* was found significant downregulation in the heart failure model SHHF, combined with multiple variants in mRNA sequence, which caused by chromosome deletion in SHHF *Cd36* locus. Linkage analysis between *Cd36* deletion and heart failure was performed by (SHHF×WKY) F2 animals. *Cd36* showed suggestive linkage with heart failure in F2 male rats reflexing on the cardiac output. So deletion of *Cd36* in SHHF may play important role on its hypertrophy and heart failure syndromes.

Through congenic mapping and microarray screening, *Cd36* on rat chromosome 4 was identified as a defective SHR/Ncr1BR gene at the peak of linkage to insulin resistance, defective fatty acid metabolism, hypertriglyceridaemia, reduced HDL phospholipid, and hypertensive (Aitman *et al*., 1999). The consequences of *Cd36* deficiency in the SHR/Ncr1BR were more directly analyzed by transferring a segment of the Brown Norway chromosome 4 (including *Cd36*) to the SHR/Ncr1BR background or a *Cd36* transgenic SHR. Insulin resistance and fatty acid metabolism were improved after expression of *Cd36* was rescued in these rats (Pravenec *et al*., 2001; Pravenec *et al*., 1999). *Cd36* was suggested to be a mediator on the development of cardiac hypertrophy in the SHR relying on a dietary rescue approach. After feeding a diet supplemented with short-and medium-chain fatty, not only hyperinsulinaemia but also cardiac hypertrophy were alleviated in the SHR (Hajri *et al*., 2001). Dilated cardiomyopathy was also developed in the hearts of *Cd36* null mice. *Cd36* deficiency has been linked to tolerance to ischaemia and diastolic dysfunction in *Cd36* null mice (Brinkmann *et al*., 2002).

The hemodynamic parameters in response to heart function showed clearly gender difference among the F2 animals. Normally, SHHF parental rats develop dilated cardiomyopathy at 10 to 12 months old in male rats, and 14 to 16 months old in female rats (Doggrell and Brown, 1998), afterwards heart failure were developed two or three months later after existing dilated cardiomyopathy. This difference may result in the different phenotypes between male and female rats in (SHHF×WKY) F2 animals

5.6 Conclusion and outlook for Cd36

Cd36 was confirmed downregulation in heart failure model SHHF, combined with two novel divergent mRNAs detected in heart tissue. A chromosomal deletion found in *Cd36* locus of SHHF strain was caused by unequal recombination events. The abnormal transcription of *Cd36* in SHHF may result in the nonfunctional product, or obviously lower translation of Cd36. From linkage analysis in (SHHF×WKY) F2 animals, Cd36 deficiency in SHHF strain may play a crucial role on pathophysiology of hypertrophy and heart failure in this model. The mechanism of Cd36 regulation in SHHF model and function of predicted Cd36 protein from the divergent mRNAs need to be further ascertained. The significant linkage of the Cd36 to heart failure may be facilitated by increasing animal numbers, and additional linkage analysis on F2 animals of the cross SHHF with SHRSP, which has been developed during this study. More detailed metabolic studies in SHHF may better understand the mechanism between Cd36 deficiency and heart failure. A few other genes showed differential expression on the microarray, and some of them lie on the same metabolic pathway. Characterization of these genes will be helped by whole genome gene scan, combined with analyses of their gene expression level on (SHHF×WKY) F2 animals. Elucidation of the molecular mechanism of Cd36, the possible involvement of additional proteins and the interactions between the regulatory mechanisms, is expected to provide valuable insight for fatty acid metabolism and to better understanding the aetiology of cardiac hypertrophy and heart failure.