1 Introduction

Heart failure is a serious malady and a principal cause of death and disability in children and adults. It can be viewed upon as the end-stage of various forms of heart disease. The annual case fatality was reported to be 10-20% in patients with mild-moderate symptoms requiring hospital admission and could be reached to 40-60% in patients with severe heart failure (Murray and Lopez, 1997). Left ventricular hypertrophy (LVH) is the single most powerful predictor for the development of heart failure. The idiopathic forms of LVH were found to be 0.2% in young adults (Maron *et al*., 1995), among which more than 50% are familial forms (Davies, 2000). Heart failure patients typically present the acute symptoms of clinical cardiac failure, e.g. fatigue dyspnea, anginal pain and palpitations. The failing heart differs from the normal heart in function as well as in structure. The morphology of the failing heart is remodeled, most often along with hypertrophy or dilation. Hypertrophy can be clinically recognized by cardiac imaging with the increases of the ventricular wall thickness and smaller ventricular chambers. Pathologic remodeling that increases cardiac chamber volumes is called dilation. Diminished contractile function is the critical hemodynamic feature of cardiomyopathy, which triggers complex neurohumoral responses, increases circulatory volumes and contributes the onset of heart failure.

With the recognition that many primary cardiomyopathies are familial and heritable, strategies to study these disorders are shifted to molecular genetic methods (Seidman and Seidman, 2001). The genetics of heart failure and cardiomyopathy are much more complex, and may include a number of genes, common genetic variants, i.e. single nucleotide polymorphisms and haplotypes, environmental factors, together with risk conferring behaviors. Experimental animal models play an essential role in the systematic investigation of genetic traits. Inbred strains represent unique fixed genotypes that can be repeatedly accessed as homogeneous experimental individuals, with predictable phenotypes and defined allelic composition. They can also be used to link a specific trait with a phenotype by producing genetic crosses. Global analysis of gene expression has proven to be a fruitful means to examine the molecular portrait of a particular event as well as to seek out novel candidate transcripts that may play a role in the phenotype of interest. Those strategies make it possible to simultaneously visualize multiple genes and pathways involved in the complex disorders.

1.1 Molecular genetics of heart failure and cardiomyopathy

Genetic contributions to heart failure can be broadly grouped into causative and modifier genes. Mutations in cytoskeletal protein lead to dilated cardiomyopathy and mutations in components of the sarcomere are frequently associated with hypertrophic cardiomyopathy (Towbin and Bowles, 2002). In contrast, modifier genes become active after the onset of the disease and thus influence the clinical course (Donahue *et al*., 2002). Most of the causative genes of cardiomyopathies were first identified by linkage analysis, and then confirmed by gene function study *in vivo* animal experiments or *in vitro* cell cultures (Table 1.1). Some modifier genes and other candidate genes for cardiomyopathies and heart failure have been identified by analyzing the gene expression in different status of the disease tissue compared with normal controls (Table 1.2).

Table 1.1 **Identified genetic causes of cardiomyopathies**

Gene name	**Chromosomal location**	**Pathway**	**Phenotype**
Dystrophin	Xp21	Cytoskeletal; Signal transduction	DCM
G4.5	Xq28	Unknown	DCM; LVNC
Actin	15q14	Contractile elements	DCM; HCM
Desmin	2q35	Cytoskeletal	DCM
δ-sarcoglycan	5q33	Signal transduction	DCM
β-sarcoglycan	4q12	Signal transduction	DCM
Troponin T	1q32	Contractile elements	DCM; HCM
β-myosin heavy chain	14q11	Contractile elements	DCM; HCM
α-tropomyosin	15q2	Contractile elements	DCM; HCM
Mitochondrial respiratory chain	MtDNA	Signal transduction	DCM; HCM
Lamin A/C	1q21	Membrane-associated; Signal transduction	CDDC
Troponin I	12q23	Contractile elements	HCM
Myosin-binding protein C	11p11	Contractile elements	HCM
Myosin essential light chain	3p21	Contractile elements	HCM
Myosin regulatory light chain	3p21	Contractile elements	HCM
Titin	2q31	Cytoskeletal	HCM
AMPK	7q3	Energy metabolism	HCM
α-dystrobrevin	18q12	Cytoskeletal; Signal transduction	LVNC

DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; CDDC, dilated cardiomyopathy with conduction disease; LVNC, left ventricular noncompaction; *AMPK*, AMP-activated protein kinase. *G4.5*, Gene encodes protein tafazzin.

1.1.1 Genetics of dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is characterized clinically by left ventricular wall thinning and dysfunction of the myocardium. DCM is the most common cause of congestive heart failure, affecting 40 persons in every 100,000 of the population (Codd *et al*., 1989). About 30-40% of individuals with DCM have a familial aggregation (Grunig *et al*., 1998). Seven genes are now known for pure DCM (Table 1.1): *actin* (15q14), *desmin* (2q35), *δ-sarcoglycan* (5q33), *β-sarcoglycan* (4q12), *cardiac troponin T* (1q32), *β-myosin heavy chain* (14q11), and *α-tropomyosin* (15q2) (Towbin and Bowles, 2002). Cardiac actin is a member of the sarcomeric thin filament that interacts with tropomyosin and troponin complex. DCM related actin mutations may affect directly its binding to dystrophin (Olson *et al*., 1998). Mutations of desmin cause abnormalities of force and signal transmissions, which are similar to mutations in actin. δ-sarcoglycan and β-sarcoglycan are involved in stabilizing the myocyte sarcolemma along with signal transduction, also can cause DCM when they mutated. The mutations in β-myosin heavy chain and cardiac troponin T are thought to cause reduced force generation by the sarcomere. Genes for dilated cardiomyopathy with conduction disease have been mapped on chromosomes 1p1-1q1, 2q14-21, 3p22-25 and 6q23. The only gene identified so far is *lamin A/C* (1q21) that encodes a nuclear envelope intermediate filament protein. *Dystrophin* was identified as the disease-causing gene for X-linked dilated cardiomyopathy (Towbin *et al*., 1993). Dystrophin is a cytoskeletal protein that provides structural support to myocytes by creating a lattice-like network to the sarcolemma. It has a principal role in linking the sarcomeric contractile apparatus to the sarcolemma and extracellular matrix. Dystrophin is also involved in cell signaling, particularly through its interactions with nitric oxide synthase (Kaprielian *et al*., 2000). Gene *G4.5* encodes the protein tafazzin, its function is unknown, but mutations in this gene can result in pure DCM, hypertrophic DCM, left ventricular noncompaction, and Barth syndrome (Towbin and Bowles, 2002).

1.1.2 Genetics of hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a diverse clinical course with unique pathophysiology, which is a principal cause of sudden death in young healthy individuals and athletes. The prevalence of echocardiographically defined HCM in the general population has been reported as high as 0.2% (Maron, 1997). In addition, a significant proportion of HCM patients develops congestive heart failure (CHF), which is caused either by diastolic

dysfunction or by the development of left ventricular dilation together with systolic dysfunction.

Linkage analyses provided the first evidence for allelic heterogeneity with sequential definition of familial HCM loci on chromosomes 14q11 (Jarcho *et al*., 1989), 15q2 (Thierfelder *et al*., 1993), 11q11.2 (Carrier *et al*., 1993), and 1q3 (Watkins *et al*., 1993). As listed in Table 1.1, gene encoding mutations in the *cardiac myosin heavy chain*, *cardiac troponin T*, *α-tropomyosin*, and *cardiac myosin binding protein C* led to the conclusion that hypertrophied cardiomyopathy resulted from defects in cardiac sarcomere proteins (Fatkin *et al*., 2000). Mutations in *cardiac actin* (Mogensen *et al*., 1999), *troponin I* (Kimura *et al*., 1997), the essential and regulatory *myosin light chains* (Poetter *et al*., 1996), and *titin* (Satoh *et al*., 1999) are also recognized as rare causes of hypertrophic cardiomyopathy (Table 1.1). Numerous mutations have been identified within each of these genes and none of them predominates in spite of some hot spots for codon mutation (Bonne *et al*., 1998; Komajda *et al*., 1999). AMP-activated protein kinase, an important enzyme in energy metabolism, may affect individuals to CHF along with sudden death in early lives or as adults. *α-Dystrobrevin* (18q12), encoding a dystrophin-associated protein, was identified to be linked with left ventricular noncompaction. α-Dystrobrevin helps to maintain the structural integrity of the muscle membrane, plays signaling functions through the nitric oxide synthase pathway, and is also a substrate for tyrosine kinases (Heydemann *et al*., 2001).

1.1.3 Modifier and candidate genes of heart failure

The sympathetic nervous system and the renin-angiotensin-aldosterone system are initially activated to compensate for the reduction of cardiac output because of CHF. Patients with high plasma levels of the sympathetic neurotransmitter noradrenaline have the worst prognosis compared with other patients. The activation of the sympathetic nervous system can induce vasoconstriction and cardiac vascular hypertrophy. It can also lead to metabolic changes in glucose metabolism and insulin resistance. Abnormalities in β-adrenergic signal transduction may be one of the earliest events in the transition from compensated hypertrophy to decompensated heart failure (Marin *et al*., 2000). Except for that, the renin-angiotensin-aldosterone system plays a pivotal role in the progression of CHF mediated by angiotensin II, which is increased when left ventricular dysfunction becomes overt CHF. Angiotensin II stimulates adrenal aldosterone secretion resulting in sodium accumulation and arteriolar

constriction, thereby increasing peripheral vascular resistance. It can also stimulate cardiac hypertrophy (Yin *et al*., 2003).

Plasma concentrations of brain natriuretic peptide (*BNP*) and its precursor, *proBNP* are biochemical markers of left ventricular function in patients with CHF. Myocyte stretching and neurohormonal activation both contribute to increased BNP expression in the failing heart (Cowie and Mendez, 2002). Increased levels of the N2-terminal fragment of atrial natriuretic peptide (*ANP*) is a good predictor for the development of heart failure (Marin *et al*., 2000).

Proinflammatory cytokines such as interleukin 1, interleukin 6, and tumor necrosis factor α are identified as contributors to the syndrome of chronic heart failure. Raised plasma levels of cytokines in heart failure are therefore more likely the result of extramyocardial production because of the altered tissue perfusion and tissue hypoxia (Paulus, 1999). Myocardial fibrosis and maladaptive extracellular matrix remodeling are pathognomonic findings in the end-stage CHF. Changes in myocardial total collagen content, collagen subtypes and collagen denaturation along with collagen cross-linking are important features of extracellular matrix remodeling together with diastolic and systolic dysfunction.

The increased levels of matrix metalloproteinase 3 in DCM can degrade a wide range of extracellular proteins and therefore activate other matrix metalloproteinases. Transgenic mice with cardiac specific overexpression of tumor necrosis factor α develop ventricular hypertrophy and dilation that are accompanied by significant increases in matrix metalloproteinase 2 and 9, collagen deposition, and collagen denaturation. Anti-tumor necrosis factor α treatment attenuated matrix metalloproteinases, prevented further collagen deposition and denaturation, and thus improved myocardial diastolic function. These results suggest a critical role of tumor necrosis factor α and matrix metalloproteinase in the remodeling of myocardial matrix and regulation of myocardial function (Li *et al*., 2002).

Intracellular Ca^{2+} concentrations and handling are also involved in the hypertrophic response. The Ca^{2+} /calmodulin-dependent protein kinase II and the Ca^{2+}/calmodulin-dependent protein phosphatase (calcineurin) are involved in the cardiac hypertrophic response. In dilated and failing human hearts, no differences in the protein levels of sarcoplasmatic reticulum Ca^{2+} ATPase, phospholamban and calsequestrin have been detected compared to the non-failing controls. Although no reduction in protein levels was detected, attenuation in the sensitivity of sarcoplasmatic reticulum Ca^{2+} ATPase was identified, which is probably caused by the phospholamban–sarcoplasmic reticulum calcium pump interaction (Lips *et al*., 2003).

Table 1.2 **Modifier and candidate genes of heart failure and cardiomyopathy**

Gene name and symbol	Pathway	Gene regulation
Bcl-2 / Bax (Kang and Izumo, 2003)	Apoptosis	down
Fas (Sabbah, 2000)	Apoptosis	up
Tumor suppressor p53 (MacLellan and Schneider, 1997)	Apoptosis	up
Cytochrome c (Narula *et al*., 2000)	Apoptosis	up
Tumor necrosis factor α (Steenbergen *et al*., 2003)	Apoptosis; Proinflammatory	up
Phosphoinositide 3 kinase (Vlahos *et al*., 2003)	β_2-adrenoceptors	up
Gelsolin (Yang *et al*., 2000)	Ca^{2+} pathways	up
Calcineurin (Lips *et al*., 2003)	Cardiac sarcoplasmic reticulum	up
Cyclins D (Busk *et al*., 2002)	Cell-cycle regulatory	up
Cyclin-dependent kinase-inhibitors p21 and *p27* (Monkawa *et al*., 2002)	Cell-cycle regulatory	down
Cyclin-dependent kinases-7, 9 (Sano *et al*., 2002)	Cell-cycle regulatory	up
Matrix metalloproteinases 2, 3, 9 (Li *et al*., 2002)	Extracellular matrix	up
Collagen I and III (Tan *et al*., 2002)	Extracellular matrix	up
CPT I and *II* (Taroni et, 1992)	Fatty acid metabolism	down
MCAD (Tanaka *et al*, 1992)	Fatty acid metabolism	down
VLCAD (Orii *et al*., 1997 Strauss *et al*)	Fatty acid metabolism	down
MTP (Den Boer, 2003)	Fatty acid metabolism	down
Cd36 (Tanaka *et al*., 2001)	Fatty acid metabolism	down
Transcription factor 2 (Vara *et al*., 2003)	G1/S transition	up
TGFβ1 (Monkawa *et al*., 2002)	Growth factor	up
TGFβ3 (Monkawa *et al*., 2002)	Growth factor	up
Neuronal-NOS1 (Champion *et al*., 2003)	Nitric oxide	down
Endothelial-NOS3 (Champion *et al*., 2003)	Nitric oxide	down
SLIM1 (Yang *et al*., 2000)	Probable like MLP; Cytoskeleton	down
Interleukin 1 and 6 (Paulus, 1999)	Proinflammatory cytokines	up
Ca^{2+} -dependent protein kinase C (Lips *et al*., 2003)	Protein kinase C	up
Multiprotein bridging factor 1 (Busk and Hinrichsen, 2003)	Protein kinase C; MAPK	up
C-fos (Sadoshima and Izumo, 1997)	Proto-oncogenes	up
Angiotensin-II (Yin *et al*., 2003)	Renin-angiotensin-aldosterone system	up
ANP (Busk *et al*., 2003)	Signalling pathway	up
BNP (Cowie and Mendez, 2002)	Signalling pathway	up
ProBNP (Cowie and Mendez, 2002)	Signalling pathway	up
β_2-adrenoceptors (Marin *et al*., 2000)	Sympathetic nervous system	down
Noradrenaline (Marin *et al*., 2000)	Sympathetic nervous system	up
p38-MAPKs (Lips *et al*., 2003)	The mitogen-activated protein kinase	up
Extracellular signal- regulated kinases (Lips *et al*., 2003)	The mitogen-activated protein kinase	up
c-Jun NH (2)-terminal kinases (Lips *et al*., 2003)	The mitogen-activated protein kinase	up

The agonists of heptahelical transmembrane G protein coupled receptors were involved in the hypertrophic response. Protein kinases are located downstream from these receptors and Ca^{2+}-dependent protein kinase C is strongly implicated in hypertrophic signaling. The small GTP-binding proteins play important roles in the hypertrophic response through mediating the activation of the mitogen-activated protein kinase (MAPK) super family cascades following protein kinase C activation. MAPKs are a widely distributed group of enzymes ending in three terminal MAPK branches: p38-MAPKs, the extracellular signal-regulated kinases, c-Jun NH (2)-terminal kinases. The family of MAPKs has been shown playing causal roles in the development of cardiac hypertrophy and the transition towards heart failure (Lips *et al*., 2003). High concentration of multiprotein bridging factor 1 was present in cardiomyocytes, and the protein level was upregulated both *in vitro* and *in vivo* models of hypertrophy. Multiprotein bridging factor 1 can enhance the hormonal regulation of the *ANP* gene to be considered a marker gene for the hypertrophic phenotype. The activity of protein kinase C is necessary for the multiprotein bridging factor 1 function, which was further identified as a coactivator of c-Jun in participating hormone-induced cardiomyocyte hypertrophy (Busk and Hinrichsen, 2003). Phosphoinositide 3-kinase is a conserved family of lipid kinases, whose isoforms are crucial in calcium regulation and contraction resulting in hypertrophy and cardioprotection. Phosphoinositide 3-kinase β affects L-type calcium channel function. Overt stimulation of phosphoinositide 3-kinase γ might restrict the effects of the β_2-adrenoceptors on myocyte contraction. Phosphoinositide 3-kinase α isoform and its downstream mediators modulate cardiac hypertrophy, organ size and cardiprotection (Vlahos *et al*., 2003).

Apoptosis plays a role in the pathophysiology of several cardiac diseases including hypertrophy and heart failure. The Bcl-2 protein family regulates the mitochondrial pathway that integrates the apoptotic signal. At least 18 members of the Bcl-2 family have been identified, which can be either anti-apoptotic (e.g. Bcl-2 and Bcl-x) or pro-apoptotic (e.g. Bad, Bak and Bax). The ratio of Bcl-2 to Bax is often used as the indicator of apoptosis; a decrease in the ratio signifies the exacerbation of the apoptotic process (Kang and Izumo, 2003). The tumor suppressor p53 protein is believed to induce apoptosis, which implicates cell cycle arrest through up-regulating of cyclin-dependent kinase inhibitor p21/WAF-1 (MacLellan and Schneider, 1997). The release of cytochrome c from mitochondria may be an important pathway for the activation of caspases with resulting apoptosis in the failing heart (Narula *et al*., 2000). A death ligand (e.g. Fas ligand) or tumor necrosis factor (TNF) initiate the death-receptor-mediated pathway. TNF-α can stimulate apoptosis; however, the increase in TNF-α signaling does not lead to myocyte apoptosis when the NF-kB pathway is activated

(Steenbergen *et al*., 2003). Other factors implicated as triggers of cardiomyocyte apoptosis include exposure to hypoxia, excess levels of AngII or norepinephrine, and increased cytosolic calcium concentration (Hasegawa *et al*., 2001; Sabbah, 2000).

Nitric oxide (NO) plays critical roles in the regulation of integrated cardiac and vascular function and homeostasis. The relative contribution of the three NO synthase isoforms (neuronal-NOS1, inducible-NOS2, and endothelial-NOS3) and their physiologic roles are imperative to understand derangement of the NO signaling pathway in heart failure. Both NOS1 (-/-) and NOS3 (-/-) mice develop age-related hypertrophy, and NOS3 (-/-) mice are also hypertensive. NOS1/3 (-/-) double knockout mice have an additive phenotype of marked ventricular remodeling. The independent contributions of NO synthase isoforms to the maintenance of cardiac architecture have important implications for the pathophysiology of heart failure. However there are many controversies in the literature regarding the differences in the effects of NO pathway in heart failure (Champion *et al*., 2003).

Cell-cycle regulatory proteins take part in developing hypertrophy. 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). Hypertrophic signals cause phosphorylation of the RNA polymerase II C-terminal domain required for transcription elongation. RNA polymerase II kinases include two basal transcription factors: cyclin-dependent kinases-7 (Cdk7) and Cdk9. In hypertrophy, Cdk7 and Cdk9 are triggered by signaling proteins (Gq, calcineurin) or chronic mechanical stress (Sano *et al*., 2002). The hypertrophic growth effects of TGF-β are reduced in the absence of both cyclin-dependent kinase-inhibitors p21 and p27 (Monkawa *et al*., 2002). The expressions and activities of transcription factor 2 and dimerization partner family members were more recently detected during the development of myocyte hypertrophy *in vitro* (Vara *et al*., 2003).

A dynamic adaptation is described by the re-expression of fetal genes in cardiac hypertrophy. This adaptation includes a switch of the structural proteins from the ‘adult ’ to the ‘fetal’ isoforms such as myosin heaven chain (MHC), Carnitine palmitoyltransferase I (CPT1); and glucose transporter, the induction of growth factor and the re-expression of proto-oncogenes such as c-fos (Sadoshima and Izumo, 1997; Schwartz *et al*., 1993). Re-expression of growth factors and proto-oncogenes, and a downregulation of the ‘adult’ isoforms was detected in the patterns of gene expression in the unloaded rat heart compared with those in hypertrophied rat heart (Depre *et al*., 1998).

1.1.4 Fatty acid metabolism in cardiac failure

Long chain fatty acids (LCFA) are the chief energy substrate in the normal adult mammalian heart. During cardiac hypertrophy and in the failing heart, a transition reminiscent of fetal energy substrate utilization occurs with the reduced fatty acid oxidation and increased glucose utilization (Christe and Rodgers, 1994; Marin-Garcia and Goldenthal, 2002). In the heart, fatty acids are oxidized primarily in the mitochondria via the ß-oxidation cycle. Inherited defects in almost every enzyme of the fatty acid ß-oxidation (FAO) pathway have been identified and are recognized as the important causes of inherited cardiac hypertrophy and cardiomyopathy (Kelly and Strauss, 1994). In the heart, lipid storage capacity is limited, and thus under normal conditions, most of the fatty acid entering the myocytes is oxidized. Accordingly, the fatty acid uptake must be tightly coupled with the FAO capacity. LCFA are presumed to enter the myocyte via one of two currently recognized transporters: fatty acid transport protein (Schaffer and Lodish, 1994) and/or fatty acid translocase (FAT)/Cd36 (Abumrad *et al*., 1993). Once in the cytoplasm, LCFA may be transported by heart-type fatty acid binding (Binas *et al*., 1999) and are rapidly esterified to acyl-CoA by the long-chain acyl-CoA synthetase. Transport of LCFA into the mitochondrion occurs via transesterification by carnitine palmitoyltransferase I. During the development of cardiac hypertrophy, a fetal metabolic gene program is initiated via the complicity of the transcription factors, which bind to regulatory elements and reduce the fatty acid oxidation gene expression. Specific heritable deficiencies in fatty acid metabolism are associated with cardiomyopathy and cardiac failure, such as deficiencies of the medium chain acyl-CoA dehydrogenase (MCAD) (Tanaka *et al*., 1992), CPTII (Taroni *et al*., 1992), very-long-chain acyl-CoA dehydrogenase (VLCAD) (Orii *et al*., 1997; Strauss *et al*., 1995), Mitochondrial trifunctional protein (MTP) (den Boer *et al*., 2003), and Cd36 (Tanaka *et al*., 2001). The postnatal cardiac expression of most nuclear genes encoding mitochondrial FAO enzymes is coordinately regulated by the transcription factor, peroxisomal proliferating activating receptors α (PPARα). PPAR has been identified playing a key role in the transcriptional regulation of genes that take part in the intracellular lipid and energy metabolism involving those fatty acid oxidation enzymes. Pressure overload hypertrophy results in the de-activation of PPARα with the lower expression of fatty acid oxidation, abnormal cardiac lipid homeostasis and reduced energy production (Barger *et al*., 2001). PPAR also plays a pivotal role in mediating the effect of hypoxia on the mitochondrial fatty acid oxidation in cardiac myocytes, resulting in diminished CPT-I β mRNA levels. This

is accomplished via PPAR transcriptional regulation and its obligate partner retinoid X receptor α (RXRα) binding to a DNA response element residing within the CPT-I β promoter (Huss *et al*., 2001).

1.1.5 Cd36 in fatty acid transport

Cd36 is one of the members of glycoprotein gene family expressed both at cell surface and within lysosomes. All family members share a hairpin membrane topology with two transmembrane domains, and with both termini in the cytoplasm. The extracellular domain of Cd36 has over 10 potential glycosylation sites. Glycosylation increases the apparent mass of protein from 53 kDa to 88 kDa (Tao *et al*., 1996). The corresponding cDNA was isolated from a rat adipose tissue library, which revealed a high homology with human platelet Cd36 (Abumrad *et al*., 1993; Greenwalt *et al*., 1992).

The primary roles of Cd36 vary with the cell type on which it is expressed. As an adhesion molecule, Cd36 reflects its ability of binding the extracellular matrix proteins thrombospondin-1 and collagens type IV and I (Febbraio *et al*., 2001). As a scavenger receptor on macrophages, Cd36 is important for the recognition and the phagocytosis of apoptotic cells as well as for the internalization of the oxidized low-density lipoproteins. Cd36 was identified as a candidate fatty acid transporter based on its binding of both DIDS (4,4'-di-isothiocyanostilbene-2-2'sulphonate) and reactive sulpho-N-succinimidyl fatty acid esters (Abumrad *et al*., 1999; Harmon and Abumrad, 1993). The role of Cd36 in fatty acid transport is further confirmed in transgenic mice. Mice with the over expression of Cd36 perform better than wild type mice to enhance the muscle ability to oxidize fatty acids in response to contraction. The increased ability of oxidizing fatty acid is accompanied by the decreases of triacylglycerols, fatty acid, cholesterol and body fat in plasma, combined with an increase in blood glucose levels (Ibrahimi *et al*., 1999). The fatty acid uptake is 60% reduced by heart, red skeletal muscle and adipose tissue in *Cd36* knockout mice upon fasting, while *Cd36* null mice show opposite to the phenotype of the *Cd36*-overexpressing mice in triacylglycerols, fatty acid, cholesterol and blood glucose (Febbraio et al., 1999). *Cd36* re-expressed in heart and skeletal muscle on the *Cd36* null background showed almost complete normalization of the fasted plasma fatty acid, triacylglycerols, and glucose levels compared to those of the wild type (Brinkmann *et al*., 2002).

Cd36 is expressed abundantly in tissues active in fatty acid metabolism, such as heart, skeletal muscle, adipose tissue and intestine (Luiken *et al*., 1999). *Cd36* expression is regulated by

agonists of PPAR. The increased *Cd36* expression induced by PPAR activation was observed with hyperlipidemia (Greenwalt *et al*., 1995). Two functional PPAR binding sites are present in the murine *Cd36* promoter, and these two functional binding sites were shown strong effect on the *Cd36* promoter activity in adipose cells (Teboul *et al*., 2001). Membrane recruitment of Cd36 in muscle is influenced by leptin, therefore it may play a role in the peripheral effects of this hormone (Steinberg *et al*., 2002). Cd36 regulation was shown to be present at the translational level in the human monocyte-derived macrophages. The 5'-untranslated region of *Cd36* is more than 200 bp long and contains three upstream open reading frames. Reporter assays indicated that re-initiation followed by the translation of the first ORF is responsible for the increased translational efficiency under hyperglycaemic conditions (Griffin *et al*., 2001). Cd36 regulation is also found at the protein level, which is mediated by post-translational modifications such as glycosylation, palmitoylation and phosphorylation (Asch *et al*., 1993). Fatty acid transport is acutely regulated by translocation of Cd36 from an intracellular pool to the plasma membrane, which is analogous to the manner in glucose transport mediated by the glucose transporter 4 (Bonen *et al*., 2000).

Oxidized fatty acid analogue 15-(p-iodophenyl)-3-(R, S)-methylpentadecanoic acid was used to identify significant defects in myocardial fatty acid uptake in Cd36-deficient humans, *Cd36* (-/-) mice and spontaneously hypertensive rats (SHR/NCrj) (Coburn *et al*., 2000; Fukuchi *et al*., 1999; Hajri *et al*., 2001). *Cd36* (-/-) mice show heart hypertrophy and the tolerance to ischaemia was significantly impaired compared with wild-type mice (Nicholson *et al*., 2000). Supplementation of the diet with short-chain fatty acids eliminated heart hypertrophy in SHR/Ncrj, which indicates the lack of metabolic energy consequent to deficient fatty acid uptake is the primary defect behind heart hypertrophy in SHR/Ncrj (Hajri *et al*., 2001). Cd36 deficiency has a prevalence of 3-10% in Asian populations (Kashiwagi *et al*., 1995; Kashiwagi *et al*., 2001), 5-18.5% in African populations (Aitman *et al*., 2000; Curtis and Aster, 1996), and less than 0.3% in Caucasians. The pathophysiology of Cd36 deficiency in humans is currently unclear and has been studied only sporadically. More data are needed in order to establish the role of Cd36 deficiency in the pathogenesis of cardiomyopathies in humans.

1.2 The rat as experimental model for studying heart failure

Rat models in studying heart failure and cardiomyopathy have been proven particularly valuable. They closely resemble human complex syndromes and help to dissect a complex disease into discrete genetic factors.

1.2.1 The SHHF strain used for studying heart failure

The hypertensive heart failure prone rat (SHHF/Mcc-fa^{cp}) is a genetically inbred strain that develops hypertension, left ventricular hypertrophy, congestive heart failure, and exhibits the clinical signs of dyspnea, cyanosis, piloerection, lethargy, and cold tails (Doggrell and Brown, 1998). This strain was originated from a cross between the spontaneously hypertensive rat and Koletsky obese rats with corpulent gene (*cp*), and then bred at the National Institutes of Health (NIH) followed by brother and sister mating. The strain is maintained by matings of proven cp/- heterozygote. In some cases, cp/cp homozygous males develop obesity and manifest hyperinsulinemia together with diabetes or abnormal glucose tolerance. The obese rats develop dilated cardiomyopathy between the months of 10 and 12 (male rats) or 14 and 16 (female rats). Lean male SHHF animals develop hypertension and left ventricular hypertrophy by the age of 3 to 5 months and eventually develop overt congestive heart failure at the age of 16 to 20 months (Sack *et al*., 1996). In the study of gender-related differences in myocyte remodeling during progression to heart failure, dramatic increase in cell length was observed in the left ventricular myocytes during the progression to heart failure in both male and female SHHF rats (Tamura *et al*., 1998).

The SHHF rat demonstrates a natural progression from cardiac hypertrophy, via a stage of compensated left ventricular dysfunction to overt decompensated heart failure. The expression of the enzymes controlling mitochondrial fatty acid ß-oxidation was reduced both in heart failure stages of SHHF rat and in human heart failure. This model shows many similarities to the human disease when it is slowly developing a mix of hypertension, hyperinsulinemia and diabetes. The initial stage of cardiac remodeling in response to elevated blood pressure is coincident with increased TNF-α secretion from the left ventricle in both SHR and SHHF rats. SHR and SHHF rats become stably hypertensive at the age of 3-4 months. During this relatively early appearance of the stable hypertension, TNF-α expression is increased. There is a general decline in bioactive TNF-α release from the left ventricle with age, independent of strain or blood pressure. However, the older SHHF rats and the SHHF in overt CHF retain

or reacquire the ability to synthesize TNF-α, which maybe important in cardiac remodeling occurrence later in the process of the disease (Bergman *et al*., 1999). Lean male SHHF rats at the onset of overt heart failure have reduced chronotropic response to β-adrenergic stimulation. Manifestation of cardiac dysfunction in vivo is coincident with the reduced β-adrenergic receptor density and importantly, preceded by an elevated β-adrenergic receptor kinase 1 levels and activity, which suggests that elevated β-adrenergic receptor kinase 1 may be a precipitating factor in the transition from hypertension-induced compensatory cardiac hypertrophy to heart failure. The above results provide strong evidence in the relevance of SHHF rat model to the study of human heart failure (Anderson *et al*., 1999).

1.2.2 Characteristics of the rat strains SHR, SHRSP, and WKY

The spontaneously hypertensive rat (SHR) is a model of human insulin-resistance syndromes because of the presence of essential hypertension, hyperinsulinemia, glucose intolerance, hypertriglyceridemia and visceral obesity. The advantage of this model is that it closely mimics the phenotype of humans with high risk of coronary heart disease, thus representing a multiple risk factor syndrome (Gerdes *et al*., 1996). A deletion mutation in the fatty acid transporter gene *Cd36*, found in one SHR strain (SHR/NCrlBR), was proposed as the underlying cause of the insulin-resistant phenotype in this model (Aitman *et al*., 1999). The quantitative traits loci were mapped to the tip of chromosome 4. Sequence analysis revealed that the *Cd36* gene is truncated in the SHR/NCrlBR strain, while the truncation fully explained the low level of Cd36 in SHR rats. Over-expression of wild-type *Cd36* in the SHR rat strain was shown to ameliorate its insulin resistance (Pravenec *et al*., 2001). However, the *Cd36* mutation is absent in the original SHR strain, which still present insulin resistance (Gotoda *et al*., 1999), suggesting that other insulin resistance susceptibility genes might exist in these SHR strains.

The stroke-prone spontaneously hypertensive rat (SHRSP) was developed following a separation of the SHR into three substrains, A, B, and C. The substrain A with a higher incidence of cerebrovascular disease was followed by selective mating of the offspring with at least one parent with spontaneous stroke. The SHRSP are hypertensive at week 5 and systolic blood pressure rise to at least 250 mmHg in males (Doggrell and Brown, 1998). Genotype analysis showed that the *Cd36* deletion mutation in SHR/NCrlBR was absent in SHRSP, Wistar-Kyoto (WKY) and Brown Norway (BN) rats. The molecular causation of abnormal

insulin action on glucose and fatty acid metabolism is not identical in SHR and SHRSP (Collison *et al*., 2000).

The WKY strain was established as the normotensive controls at the NIH by inbreeding of the wistar kyoto colony via intercross. The degree of genetic difference among SHR, SHHF, SHRSP, and WKY strains and within different colonies of each strain is substantial and comparable to the maximum divergence possibility among the unrelated humans (Doggrell and Brown, 1998; Johnson *et al*., 1993; St Lezin *et al*., 1992).

The SHHF rats had significantly greater left ventricular mass compared with the normotensive control group. Although left ventricular mass was not different between SHHF and SHR strains, significant differences were seen in the pattern of left ventricular remodeling as determined by the relative wall thickness. These differences in left ventricular remodeling may explain the earlier development of heart failure in SHHF. The different patterns of left ventricular hypertrophy in SHHF and SHR suggest that heart failure in SHHF is not mediated by hypertension alone (Haas *et al*., 1995). Despite a number of common risk factors present in both models, resultant heart failure only occurs in SHHF, but not in heart failure resistant SHR rats, even though SHHF is derived from SHR. The heart failure susceptibility in SHHF rats is heritable and therefore genetically determined, but the underlying genetic defects and molecular causes are unknown. The main phenotypes of four different strains used in this study are listed in Table 1.3 (Collison *et al*., 2000; Doggrell and Brown, 1998; Pravenec *et al*., 2001).

Table 1.3 **Phenotypes of four inbred strains**

Phenotypes	**SHHF**	**WKY**	**SHRSP**	**SHR**	**Test**
Hypertension	Yes	No	Yes	Yes	Blood pressure
Congestive heart failure	Yes	No	No	Some*	Dyspnea, cyanosis, piloerection, lethargy and cold tails
Cardiomyopathy (LVH, DCM)	Yes	No	Some*	Some*	Echocardiography
Insulin resistance (Type II Diabetes, Obesity, Hyperlipidemia	?	No	Yes	Yes	Oral glucose intolerance test; Blood glucose; Triglycerides
Defective fatty-acid	?	No	No	Yes	Non-esterified fatty acid
Stroke	No	No	Yes	No	Piloerection, hyperkinesis, hyperirritability, Aggressiveness and motion disturbance

*, Some animals of this strain exist these phenotypes

1.3 Expression profiling for the analysis of heart failure

1.3.1 Expression profiling using cDNA array

Gene expression profiling technologies have enabled the large-scale analysis of gene expression changes during disease progression. RNA profiling is based on hybridization of transcripts to arrays of DNA molecules bound to a solid support. The support-bound DNA is in excess, so that the amount of probe hybridized to a particular DNA spot is a measure of the abundance of that transcript in the mRNA population. The advantage of arrays is that they give quantitative information on the abundance of hundreds or thousands (depending on the array design) of specific genes simultaneously. High-density arrays can be generated by robotics. PCR products amplified from the cDNA library are removed from 96- or 384-well plates and arranged in reproducible fashion, in patterns determined by the user. Blots are hybridized with ^{33}P-labeled first-strand cDNA made from polyA+ RNA isolated from test and control tissues. Hybridizations are carried out in duplicate for each sample. Data is captured on Phosphor Imager screens and analyzed with ImageQuant software or Visual Grid (Baldwin *et al*., 1999; Chen *et al*., 2001; Hughes and Shoemaker, 2001; Kozian and Kirschbaum, 1999). Expression analysis using a nonredundant 10848-element human cardiovascular-based expressed sequence tag (EST) glass slide cDNA microarray has been performed to obtain a genomic portrait of heart failure derived from end-stage dilated cardiomyopathy. More than 100 transcripts were differentially expression in DCM. ANP, Cardiac troponin, tropomyosin, HSP40, HSP70 and CCAAT box binding factors were detected to be upregulated. Downregulation was observed with cell-signaling channels and mediators, particularly those involved in Ca^{2+} pathways such as Ca^{2+} ATPase, 1,4,5-triphosphate receptor. A number of novel expressed sequence tags were also identified related to dilated cardiomyopathy from this study (Barrans *et al*., 2002; Barrans *et al*., 2001). One expression profiling using pharmacological models of cardiac hypertrophy in mice was performed, which contained more than 4000 genes to characterize and contrast the expression changes during induction and regression of hypertrophy. Twenty-five genes have been identified as hypertrophy-associated genes, which were detected previously through the analysis of cellular and physiologic models, meanwhile 30 hypertrophy-associated genes were first identified (Friddle *et al*., 2000). Approximate 5000 unique known genes represented by cardiac ESTs in hypertrophic heart cDNA library was analyzed and up to half of the good candidate genes were shown potentially involved in stress response (Hwang *et al*., 2000). Gene expression

profiling was further set up in dilated and hypertrophic cardiomypathic end-stage heart failure using cDNA microarray with 10272 unique clones from various cardiovascular cDNA libraries. 192 highly expression genes were identified in both DCM and HCM, as well as 51 downregulated genes were detected in both conditions (Hwang *et al*., 2002).

1.3.2 Expression profiling using Affymetrix chips

The Affymetrix GeneChip features a cassette enclosing the oligomer microarray and a 250-µl chamber for hybridization, washing and staining. The single stranded DNA oligonucleotide, with the length of 25 bp and complementary to a specific sequence, is synthesized directly on the surface of the array using photolithography and combinatorial chemistry (Fig 1.1). mRNA is converted to cDNA by reverse transcription from a primer that incorporates the T7 promoter, which allows subsequent *in vitro* transcription using T7RNA polymerase to amplify each cDNA into a cRNA population. This amplification by transcription boosts the sensitivity for rare mRNAs while maintaining the original relative ratio of each message in the population, and also allows for the incorporation of biotinylated CTP and UTP. The cRNA is fragmented, hybridized to the chip and stained with streptavidinphycoerythrin, which attaches fluorescent labels through high affinity interaction with the biotin tags. A scanning confocal microscope detects laser-excited fluorescence from hybridized cRNA. The sensitivity is sufficient enough to detect rare transcripts present at less than 0.1 (on average) copies per cell in yeast (Wodicka *et al*., 1997).

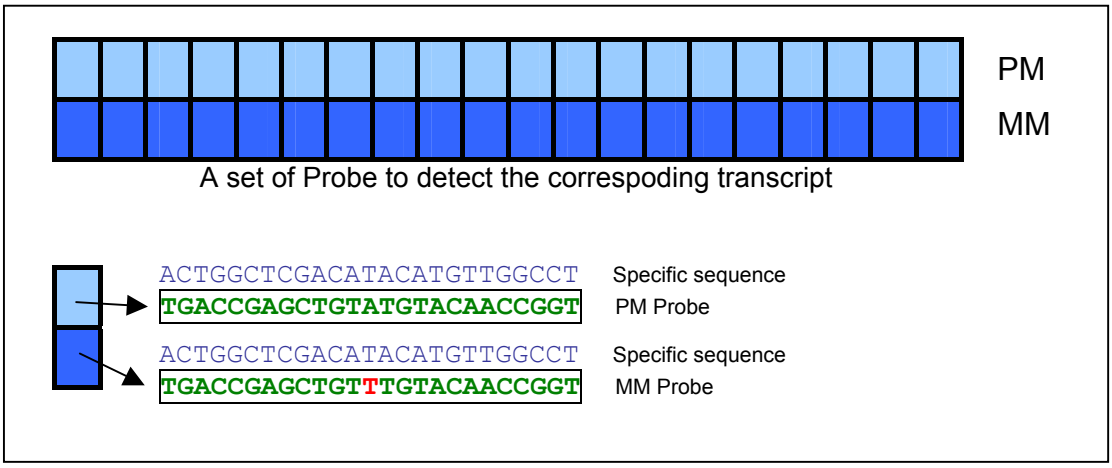

Fig 1.1 One probe set designed on Affymetrix chip

Expression profiles from the end stage heart failure patients suffering from ischaemic cardiomyopathy and dilated cardiomyopathy were compared to non-diseased myocardium using Affymetrix chips. Shared expression profiles between heart failure with different underlying aetiologies may be fundamental to the failing myocardium. 12 of 7000 arrayed genes were identified with similar expression changes in both types of heart failure. Downregulation of the striated muscle LIM protein-1 and upregulation of the gelsolin protein especially in failing heart were detected in the study listed in Table 1.2 (Yang *et al*., 2000). A mouse oligonucleotide array containing more than 12 000 genes has ever utilized to identify altered expression in the hearts of the transgenic α_{1b} adrenergic receptor mice. It is a mouse model of hypertrophy but not heart failure. A unique profile of altered genes set up in hypertrophy only model may be helpful to identify genes for preventing progress to heart failure (Yun *et al*., 2003). Oligonucleotide microarrays of 6606 genes was used to profile human hearts with an end-stage DCM to nonfailing hearts, 103 genes in 10 functional groups were differentially expressed in failing heart. Among them 19 genes showed the same changes as their past study (Yang *et al*., 2000). Additionally, genes involved in fatty acid metabolism, e.g. apoliprotein D, fatty acid synthase, phospholipid transfer protein were exclusively downregulated, while genes related to glucose metabolism, such as fructose 1-6 biphophatase and mitochondrial NADP (+) –dependent malic enzyme were upregulated. These observations may indicate the fact that the primary myocardial energy source switches from fatty acid to glucose in heart failure (Tan *et al*., 2002).

1.4 Objectives

Cardiomyopathy and heart failure have family inheritable tendency, and the mutations of some genes are found link to family aggregation of DCM or HCM. Since the genetics of heart failure and cardiomyopathy are more complex, the genes responsible for the development of the diseases should be further addressed using inbred animal models combined expression profiling. The SHHF have been generated to simulate human heart failure and cardiomyopathy, and proven valuable in the genetic studies on these diseases. The control rats are from WKY and SHRSP. In order to identify candidate genes associated with cardiomyopathy and heart failure, both cDNA array and Affymetrix chip are used in gene expression profiling. Gene differential expressions are analyzed in heart tissue of the SHHF rat compared with WKY and SHRSP.

Cd36, an important glycoprotein in fatty acid transport, has been linked to insulin resistance, cardiac hypertrophy, tolerance to ischaemia, and diastolic dysfunction in rodent models. *Cd36* deficiency is associated with a diversity of heart disease in human and rat models, further exploration of the different rodent models might yet produce valuable information about the links between *Cd36* expression and heart function or failure. *Cd36* was also found significantly downregulation in SHHF from expression profiling in this study. Therefore the second goal of this study is to explore the molecular basis and function of Cd36 in SHHF strain. The gene locus structure and linkage analysis of *Cd36* in SHHF strain may help to understand its relation to cardiomyopathy and heart failure in SHHF. The hypothesis on the linkage between Cd36 deficiency and hypertrophy or failure are tested using linkage analysis with F2 animals of the cross SHHF strain with WKY.