3 Methods

3.1 Animal breeding and phenotyping

Rats from strain SHHF including age-matched animals from the control strains WKY, SHRSP, and SHR/mol were bred in a specified-pathogenic-free (SPF) environment. The F1 animals were developed from three male SHHF and six female WKY rats. The (SHHF×WKY) F2 animals were developed by intercross of the F1 animals (Fig 3.1). The morphology and function of the heart was determined by hemodynamic parameters. Hemodynamic parameters were determined with polygraph via femoral artery and intraventricular cannula. The ventricular and arterial catheters were separately connected with pressure transducer. After balancing with the air pressure and adjusting the electric mark, the ascending aortic systolic pressure, diastolic pressure and mean arterial pressure were recorded. Heart rate, and the maximum raising and dropping rate of inner ventricular pressure (±dp/dt) were recorded at 20 minutes (min) after inserting catheters. Subsequently, the sheath was retrograded into the left ventricle, and left ventricle systolic pressure, left ventricular end diastolic pressure was recorded.

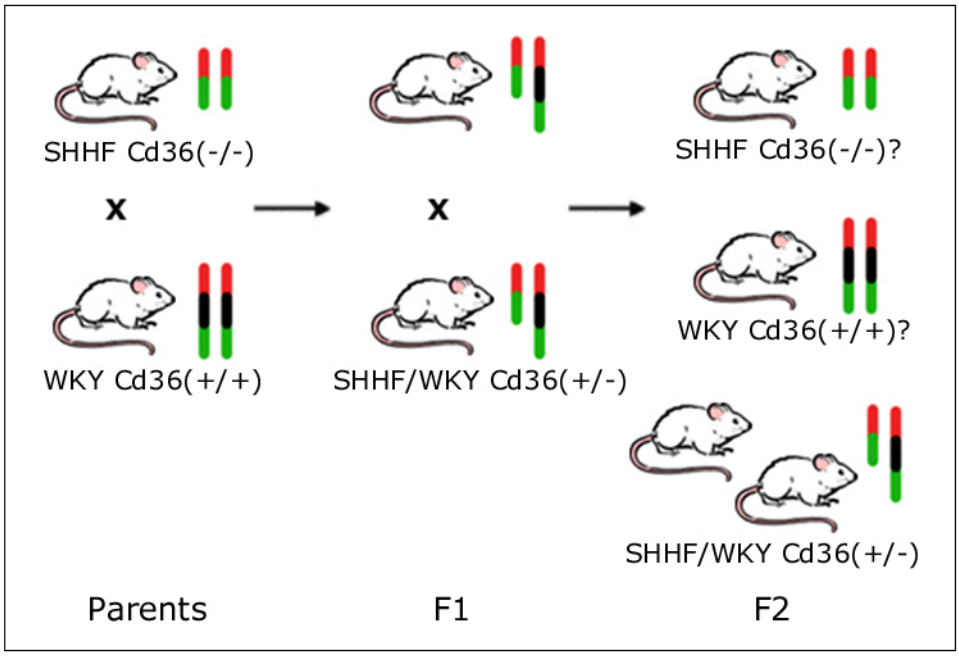


Fig 3.1 Development of the F2 animals of the cross SHHF with WKY rat

After the hemodynamic measurements were performed, the heart was dissected and quickly washed in 0.9% NaCl solution. The whole heart was weighed using an electronic balance and the tissue was immediately frozen in liquid nitrogen. The other tissues such as liver, kidneys, and brain were removed and frozen in liquid nitrogen after briefly washing in 0.9% NaCl. All samples were stored at -80°C.

3.2 Preparation of RNA from animal tissue

3.2.1 Isolation of total RNA

The whole hearts from above different animals were placed into a mortar and pulverized in liquid nitrogen. 50-100 mg of powder-like heart tissue was lysed in 1 ml of Trizol, vortexed thoroughly, and then incubated at room temperature for 5 min. The samples were mixed vigorously after 0.2 ml of chloroform was added to each sample, incubated at room temperature for 3 min and centrifuged at 12000 rpm for 15 min at 4°C. The upper colorless aqueous phase was removed carefully. The RNA was precipitated by mixing with 0.5 ml of isopropanol and incubating at room temperature for 10 min. Then the samples were centrifuged at 12000 rpm for 30 min at 4°C. The RNA pellet was washed with 75% ethanol. The pellet was air-dried for 5 to 10 min and dissolved in 30 µl of DEPC treated water. The total RNA was stored at -80°C.

3.2.2 RNA cleanup

43 µl of total RNA (20-30 µg) was mixed with 2 µl of DNAase (2 Units (U), from Promega) and 5 µl of 10× RQ1 DNase buffer and incubated at 37°C for 20 min. After digestion, cleanup was performed using RNeasy kit from QIAGEN. Samples were adjusted to a volume of 100 µl with RNase-free water. 350 µl of RNeasy lysis buffer was added to the sample and mixed thoroughly. 250 µl of absolute ethanol was added to the lysate and mixed well by pipetting, 700 µl of sample was applied to RNeasy mini spin column sitting in a collection tube. Purified RNA were eluted with 30 µl of RNase- free water after the RNeasy columns were washed for twice with buffer RPE from kit. The RNA yield was quantified by spectrophotometric analysis using the convention that 1 OD at 260 nm equals 40 µg RNA per ml. The integrity

and size distribution of total RNA was verified on 1.2% formaldehyde agarose gel according the method described in appendix C in RNeasy Mini Handbook.

3.2.3 Preparation of poly A^+ from total RNA

Poly A^+ was separated from the total RNA using Dynabeads oligo (dT_{25}) Kit. 100 µl (30 µg) of the total RNA mixed with 100 µl of binding buffer were added to the Dynabeads Oligo dT_{25} in 1.5-ml tube, the tube was placed in the Dynal MPC for 2 min and the supernatant was removed. The Dynabeads were washed twice with 0.5 ml of washing buffer A and once with 0.5 ml of washing buffer B at room temperature. 20 µl of 10 mM Tris·Cl was added to the tube and incubated at 65°C for 2 min. The supernatant containing the mRNA was transferred to a new RNase free tube. The freshly prepared Poly A^+ was directly used for preparation of first strand cDNA.

3.3 First strand cDNA synthesis

3.3.1 Preparation of the cDNA using Oligo $(dT)_{12-18}$ primer

Total RNA (8 µg) or poly A^+ (2 µg) from each sample was mixed with 1 µl of 0.5 µg/µl oligo dT primer in a final volume of 10 µl. The samples were incubated at 70°C for 10 min, cooled on ice for at least 1 min. Then 8 µl of mixture (4 µl 5× First strand buffer, 2 µl 0.1 M DTT, 1 µl 10 mM dNTP, and 1.0 µl 40 U/µl RNase inhibitor) were added to each sample and incubated at 42°C for 2 min in a thermocycler. The reaction was incubated for 50 min after 1 µl of Superscript II reverse transcriptase (SSII RT, 200 U/µl) was added. The reaction was inactivated by incubation at 70°C for 15 min and further incubated at 37°C for 20 min after 1 µl of RNase H (2 U/µl) was added in order to remove remaining RNA. cDNA was stored at -20 °C. The cDNA synthesis was verified by amplification of the gene *Gapdh* using PCR.

3.3.2 Preparation of the cDNA using Random Hexames

15 µl of total RNA (8 µg) from each sample, 3 µl of 50 ng/µl random hexames, and 1 µl of 10 mM dNTP were mixed. Samples were denatured at 70°C for 10 min and cooled on ice for 2 to 3 min. 10 µl of mixtures (6 µl 5× First strand Buffer, 3 µl 0.1 M DTT and 1 µl 40 U/µl RNase

inhibitor) were added to each sample. After incubating at 25°C for 2 min, 1 µl of SSII RT (200 U/µl) was added to each sample and the following incubation and inactivation were performed as described in 3.3.1.

3.3.3 Confirmation of cDNA free from genomic DNA

In order to confirm that the acquired cDNA was free from genomic DNA**,** the primer angi_intron (Table 2.1, angi _intron) was designed to amplyfy geonmic DNA without co-amplifying the cDNA according to the rat sequence of the gene *angiotensinogen* (accession number, L00094). Amplification conditions: the mixture was heated to 94°C for 3 min, 35 cycles were performed at 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec, then followed by an additional extension at 72°C for 5 min. PCR amplification was checked on 1.2% agarose gel.

3.4 Hybridization of cDNA arrays

About 27,600 PCR products amplified from a normalized cDNA library of the rat heart (RZPD library number: 619) were spotted in duplicate on 22 × 22 cm nylon filters in a 5 × 5 pattern. The filters were provided by the Resource Center in the German Human Genome Project (http://www.rzpd.de).

3.4.1 Preparation of probe

Two strategies were applied to identify clones that are differentially expressed in the different animal models. Hybridization of "complex probes" representing the whole mRNA population from heart tissue and hybridization of "subtracted probes" generated by the enrichment of differentially expressed transcripts by subtractive suppressive hybridization (SSH) performed according to the Clontech PCR-Select^{TM} cDNA Subtraction Kit user manual (PT1117-1).
Complex probes were prepared by directly labeling of cDNA while synthesized from total RNA. 8.5 µl total RNA (12 µg) from each sample was incubated with 2 µl oligo dT at 70°C for 10 min. The samples were cooled on ice for at least 2 min. Then 18.5 µl of labeling mixture (6 µl 5× RT buffer, 3 µl 0.1 M DTT, 1.5 µl 2.5 mM dATP/dGTP/dCTP, 1.0 µl 12.5 µM dCTP, 1.0 µl 40 U/µl RNase inhibitor, 1.0 µl 200 U/µl superscript II RT, 5 µl 10 µCi/µl

[α-^{33}P] dCTP) were added to each sample. The samples were mixed by votexing, then centrifuged briefly and incubated in a thermocycler according to the program: 30°C 10 min, 42°C 40 min, 52°C 10 min. Afterwards 1 µl RNase H (2 U/µl) was added and the samples were incubated at 37°C for 20 min.

Subtracted probes were prepared by labeling of secondary PCR products after subtractive suppressive hybridization. 14 µl of secondary PCR product from each subtracted sample was denatured at 95°C for 5 min; afterwards the samples were immediately chilled on ice. Then 25 µl labeling mixture (18 µl LS, 1.5 µl 10 mg/ml BSA, 3 µl 100 µM ATG, 0.5 µl 5 U/µl Klenow enzyme, 2.5 µl 10 µCi/µl [α-^{33}P] dCTP) were added and incubated at 37°C for 2 hours (h). 20 µl of salmon sperm DNA (40 ng/µl) was labeled and hybridized to the filter in order to visualize guide dots on the rat heart cDNA filter. After labeling, probes were purified using Microspin^{TM} G50 Column according to the manual.

3.4.2 Hybridization

Filters prewetted with hybridization buffer (1 M NaCl, 1% SDS, 10 mM Tris·Cl) were rolled and allowed to unfold in the hybridization tubes so that the filters adhere to the inside wall of the tube without trapping air bubbles. Filters were pre-hybridized at 65°C for 2 to 8 h. After prehybridization, the buffer was discarded. The purified labeled probes were denatured at 95°C for 10 min and cooled on ice for 2 min, the purified labeled probes were mixed with 50 µl unlabeled salmon sperm DNA (10 mg/ml) before denaturing if salmon sperm DNA was used as guide dot. Each hybridization probe was mixed with labeled guide dot DNA and immediately added to 15 ml warmed hybridization buffer. The hybridization buffer was added to the filters and hybridization was performed at 65°C overnight.

3.4.3 Washing and scanning of the filters

The hybridization solution was poured off. The filters were washed in 50 ml of washing buffer I in hybridization tubes for 20 min at 65°C. Subsequently, the filters were transferred to plastic boxes and washed in preheated washing buffer II for 20 min at 65°C in a water bath. The membranes were dried briefly on Whatman paper, wrapped in Saran wrap and exposed on phosphor storage screens overnight.

The exposed screens were scanned using the Molecular Dynamics Phosphor Imaging system. The phosphor Imager 445 SI scanned the screens using a helium-neon laser. The software

ImageQuant was used to produce an image of the original radioactive sample. The level of signal is proportional to the amount of radioactivity. cDNA filters can be reused after stripping using stripping buffer.

3.4.4 Analysis of cDNA arrays

The filters were analyzed with Visual Grid (GPC); the data that included the intensities of all clones on the filters were exported to Microsoft Excel, and imported to Access. Signals were normalized by the average signal intensities on each filter. Signal intensity ratios were calculated from the normalized signal intensities of each clone from the experiments versus controls. Clones with ratios > 10 and ratios < 0.1 using subtracted cDNA probes were considered to be differentially expressed, and clones with ratios > 2 and ratios < 0.5 using complex probes were selected. The candidate clones from the rat heart library were ordered from RZPD and checked by PCR using M13 primer. Then plasmid DNA was prepared using QIAGEN mini kit and sequenced using ABI PRISM BigDye^{TM} Terminator Cycle Sequencing ready reaction kit. After sequencing, blast analyses were performed using NCBI blast and candidate clones were selected.

3.5 Hybridization of Affymetrix chips

The Rat Genome U34 Set monitors the expression of more than 24,000 genes and EST clusters. The sequences include all rat sequence clusters from Build #34 of the Unigene Database (created from GenBank 107/dbEST 11/18/98) and are supplemented by additional annotated gene sequences from GenBank 110. The Rat Genome U34 Set contains 3 arrays (RG-U34A, RG-U34B and RG-U34C). The RG-U34A array contains probes derived from all full-length or annotated genes as well as thousands of EST clusters. The RG-U34B and RG-U34C arrays contain only EST clusters. RG-U34A and RG-U34B were used in this project. Fragmented, biotinylated anti-sense cRNA was prepared from mRNA, and hybridized to the probe array. The levels of hybridization were measured with the HP Gene Array scanner after the array was stained with streptavidin-phycoerythrin (SAPE). The simple procedure to prepare the target is shown in Fig 3.2.

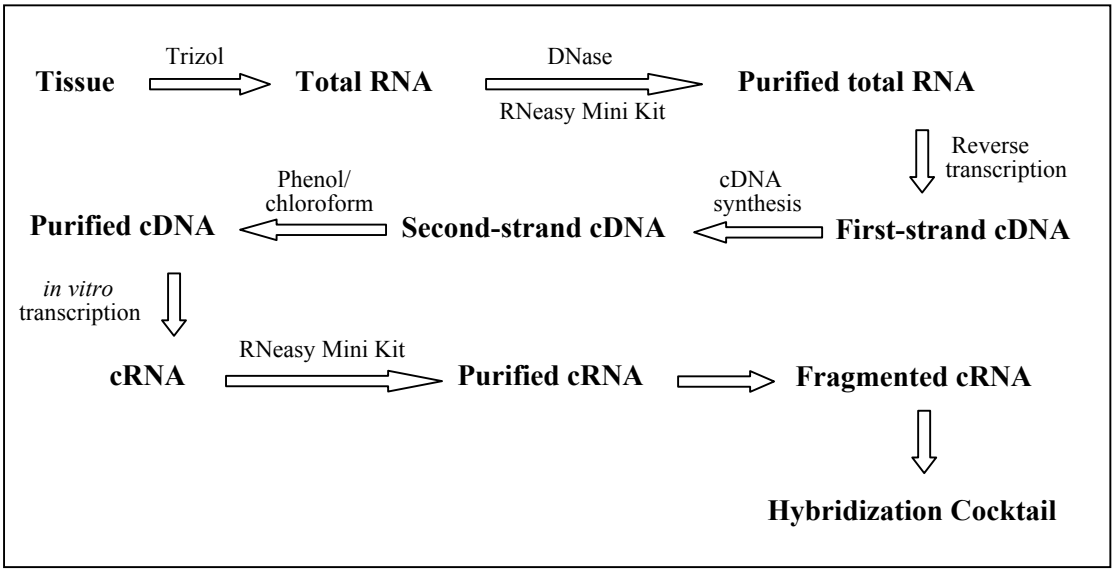


Fig 3.2 Procedures to prepare the target for hybridization with Affymetrix chip

3.5.1 Synthesis of double strand cDNA from total RNA

Total RNAs from rat heart tissue of strains SHHF, SHRSP and WKY (Table 3.1) were isolated and cleaned up according the method described in 3.2. 19 µl of high quality total RNA (17 µg) was incubated with 2 µl 100 pmol/µl T7 $(dT)_{24}$ oligomer at 70°C for 10 min. Then 19.6 µl of master mixture (8 µl 5× First strand cDNA buffer, 4 µl 0.1 M DTT, 4 µl 10 mM dNTP mix, 0.6 µl 40 U/µl RNase inhibitor, and 3 µl 200 U/µl SSII RT from Gibco) were added to each sample and the samples were incubated at 42°C for 1 h.

Second strand cDNA was synthesized using cDNA synthesis system from Roche. The following second strand reaction composition (30 µl 5× Second strand reaction buffer, 1.5 µl 10 mM dNTP, 6.5 µl second strand enzyme, and 72 µl of sterile water) was added to first strand cDNA to a final volume of 150 µl. The samples were incubated at 16°C for 2 h, continued by an incubation at 16°C for 5 min after 20 µl of 1 U/µl T4 DNA polymerase I were added. The samples were incubated at 37°C for 30 min after 17 µl of 0.2 M EDTA (pH 8.0) and 1.5 µl of 10 U/µl RNase I were added, and the incubation was continued for 30 min more after 5 µl (3 U) proteinase K were added to each sample.

Table 3.1 **Animals were used for Affymetrix chips**

Animals[1]	Age (Month)	Sex	Phenotypes[2]
C 2	17	Female	Hypertension, DCM (LVH), CHF
C 3	17	Female	Hypertension, DCM (LVH), CHF
C 4	11	Female	Hypertension, DCM (LVH)
C 5	11	Female	Hypertension, DCM (LVH)
C 6	11	Female	Hypertension, DCM (LVH)
W 18	11	Female	Normal
W 19	17	Female	Normal
W 22	17	Female	Normal
SP 24	11	Female	Hypertension, Stroke
SP 25	11	Female	Hypertension, Stroke

1, Heart tissues were used for Affymetrix chip from animals of SHHF strain (C), WKY strain (W), and SHRSP strain (SP). 2, Phenotypes were acquired according the literature (Doggrell and Brown, 1998).

3.5.2 cDNA purification using phenol/chloroform extraction

The final cDNA synthesis preparation was purified using phenol/chloroform according to the method described in gene chip expression analysis technique manual. 193.5 µl of synthesis preparation was mixed with 200 µl of phenol, vortexed for 10 seconds (sec). The upper water phase was transferred to a new tube after the tubes were centrifuged for 15 sec. 50 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) was added to the phenolic phase, vortexed for 10 sec and centrifuged for 15 sec. The upper water phase was transferred to the same tube. The water phase was extracted once with Phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform /isoamyl alcohol (24:1). 2 µl of 5 mg/ml glycogen, 120 µl 5 M NH_4AC and 500 µl 100% ethanol were added to each sample. The samples were stored at -60°C for 1 h and centrifuged at high speed for 10 min. The pellets were washed using 300 µl of 70% ethanol. The purified cDNA was dissolved in 1.5 µl of DEPC treated water.

3.5.3 Synthesis of Biotin-Labeled cRNA

cRNAs were prepared by an *in vitro* transcription reaction using T7 megascript Kit. 18.5 µl of labeling mixture (2 µl 75 mM ATP, 2 µl 75 mM GTP, 1.5 µl 75 mM CTP, 1.5 µl 75 mM UTP, 3.75 µl 10 mM Bio-11-CTP, 3.75 µl 10 mM Bio-16-UTP, 2 µl 10× T7 Buffer and 2 µl 10× T7 Enzyme) were added to 1.5 µl of purified cDNA acquired in 3.5.2. After the samples were

incubated at 37°C for 5 h in a thermocycler, the reaction mixtures were purified using RNeasy Mini kit according to method 3.2. 2 µl of cRNA from each sample was used measuring the concentration and purity of cRNA spectrophotometrically. The convention that 1 OD at 260 nm equals 40 µg/ml RNA was applied, maintain the A260/A280 ratio close to 2.0 for pure cRNA.

3.5.4 Fragmentation of the cRNA and preparation of the hybridization cocktail

The samples including 32 µl of cRNA (20 µg) with 8 µl of 5× fragmentation reaction were incubated at 94°C for 35 min in a thermocycler. Then 2 µl of the reaction was checked on 1.2% agarose gel. The standard fragmentation procedure should produce a distribution of RNA fragment sizes from approximately 35 to 200 bases. The fragmented cRNA was stored at -80°C until ready to perform the hybridization. Hybridization cocktails were prepared by mixing 30 µl (15 µg total cRNA) fragmented cRNA from each target, 2.5 µl control Oligonucleotide B2 (5 nM), 5.0 µl Herring sperm DNA (10 mg/ ml), 6.25 µl BSA (20 mg/ ml), 125 µl 2× MES, and 81 µl H_2O.

3.5.5 Targets cleanup and hybridization

Probe arrays were equilibrated to room temperature immediately before use. The arrays were wetted by filling it through one of the septa with appropriate volume of 1× MES hybridization buffer using a micropipettor (200 µl for a standard array and 80 µl for a test chip). The probe arrays were incubated at 45°C for 10 min with rotation.

Meanwhile the hybridization cocktail was heated at 95°C for 5 min, then cooled on ice and spun at maximum speed for 5 min to remove any insoluble material from the hybridization mixture. The buffer solution from the probe array cartridge was removed and the cartridge was filled with an appropriate volume of the clarified hybridization cocktail avoiding any insoluble matter in the volume at the bottom of the tube. The probe arrays were placed in rotisserie motor and loaded in a balanced configuration around the rotisserie axis. Samples were hybridized for 16 h at 45°C while rotating at 60 rpm.

3.5.6 Washing, staining, and scanning probe arrays

Affymetrix chips were washed and stained according to Affymetrix GeneChip expression analysis technical manual. Washing and staining of the chips were controlled by microarray suite software. After washing and staining, the probe array was removed and checked for large bubbles or air pocket. If bubbles were present, the array was refilled with non-stringent buffer. The probe arrays were kept at 4°C in the dark until scanning was performed.
The Affymetrix chip software also controlled the scanner. Laser was warmed up prior to scan by turning the laser on at least 15 minutes before use. If probe array was stored at 4°C, it was equilibrated to room temperature before scanning.

3.5.7 Analysis of Affymetrix chip

After the scan was completed, the microarray suite software displayed a picture of the image data file (*.dat) in the image window, the software represents the fluorescence intensity values for each pixel on the array in a grayscale and superimposes a grid on the image to delineate the probe cells. The software analyzed the image and derived a single intensity value for each probe cell on an array. These data were contained in the cell intensity file (*.cel).

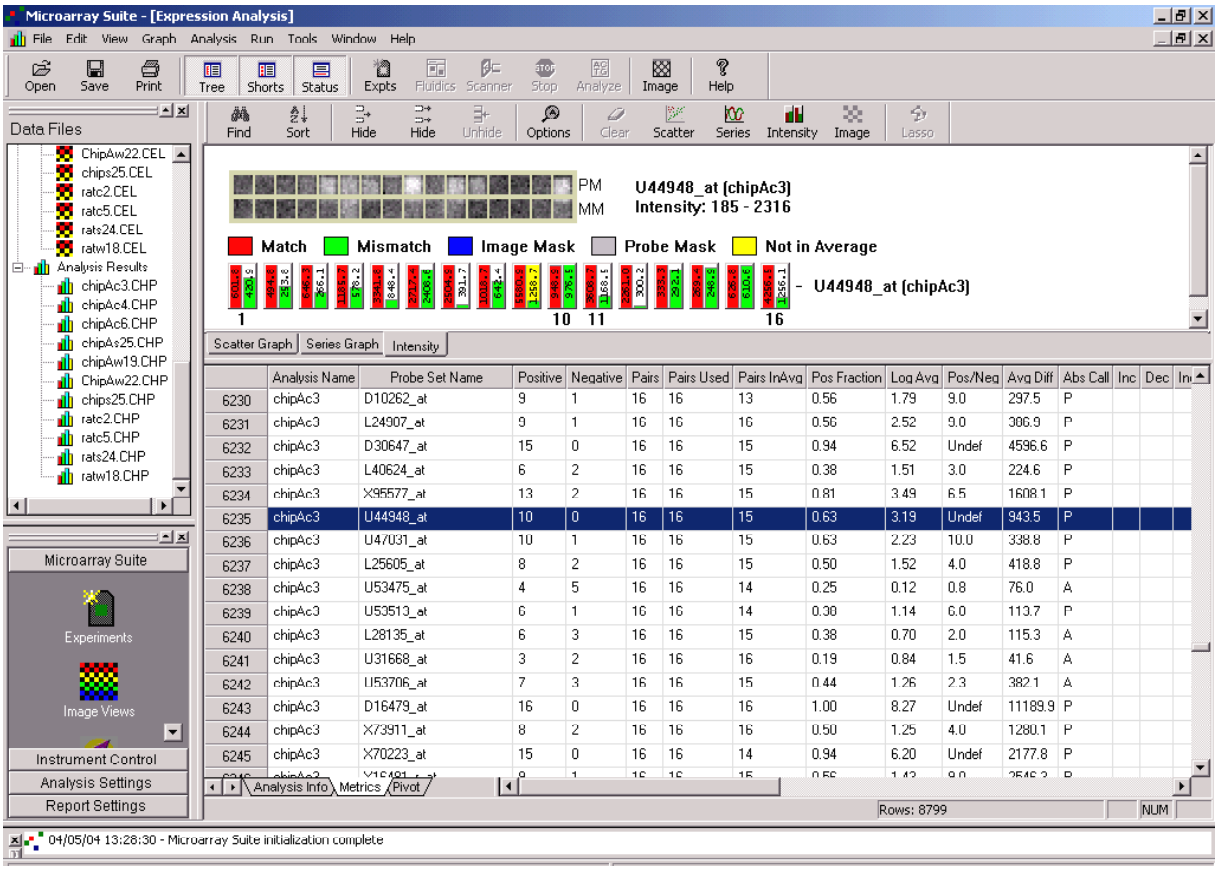
3.5.7.1 Normalization of Data

In order to compare data from multiple probe arrays, global scaling was used to minimize differences of non-biological origin in this study. In global scaling, the output of an experiment was multiplied by a factor (scaling factor) to make its average intensity equal to arbitrary target intensity. This scaling allows a number of experiments to become normalized to one target intensity, allowing comparison between any two experiments. Global scaling was first done before every absolute and comparison analysis was performed.

3.5.7.2 Absolute analysis

Absolute analysis by the microarray suite software was used to calculate a variety of metrics using the probe array' hybridization intensities (Affymetrix GeneChip Expression analysis technical manual). A table including Absolute Call Metrics was produced after absolute analysis was performed (Fig.3.3). Some metrics (e.g. **Average Intensity**) utilize intensity data

from the entire probe array and are used for **Background** and **Noise calculations**. Other metrics compare the intensities of the sequence-specific **Perfect Match** (PM) probe cells with their control **Mismatch** (MM), and then are used by a decision matrix to determine if a transcript is **Present** (P), **Marginal** (M), or **Absent** (A).



	Analysis Name	Probe Set Name	Positive	Negative	Pairs	Pairs Used	Pairs InAvg	Pos Fraction	Log Avg	Pos/Neg	Avg Diff	Abs Call	Inc	Dec	In
6230	chipAc3	D10262_at	9	1	16	16	13	0.56	1.79	9.0	297.5	P			
6231	chipAc3	L24907_at	9	1	16	16	16	0.56	2.52	9.0	306.9	P			
6232	chipAc3	D30647_at	15	0	16	16	15	0.94	6.52	Undef	4596.6	P			
6233	chipAc3	L40624_at	6	2	16	16	15	0.38	1.51	3.0	224.6	P			
6234	chipAc3	X95577_at	13	2	16	16	15	0.81	3.49	6.5	1608.1	P			
6235	chipAc3	U44948_at	10	0	16	16	15	0.63	3.19	Undef	943.5	P			
6236	chipAc3	U47031_at	10	1	16	16	15	0.63	2.23	10.0	338.8	P			
6237	chipAc3	L25605_at	8	2	16	16	15	0.50	1.52	4.0	418.8	P			
6238	chipAc3	U53475_at	4	5	16	16	14	0.25	0.12	0.8	76.0	A			
6239	chipAc3	U53513_at	6	1	16	16	14	0.30	1.14	6.0	113.7	P			
6240	chipAc3	L28135_at	6	3	16	16	15	0.38	0.70	2.0	115.3	A			
6241	chipAc3	U31668_at	3	2	16	16	16	0.19	0.84	1.5	41.6	A			
6242	chipAc3	U53706_at	7	3	16	16	15	0.44	1.26	2.3	382.1	A			
6243	chipAc3	D16479_at	16	0	16	16	16	1.00	8.27	Undef	11189.9	P			
6244	chipAc3	X73911_at	8	2	16	16	16	0.50	1.25	4.0	1280.1	P			
6245	chipAc3	X70223_at	15	0	16	16	14	0.94	6.20	Undef	2177.8	P			

Fig. 3.3 Absolute analysis of Affymetrix chip using Microarray Suite software

The analysis output file (*.chp) was displayed after absolute analysis, the data in table was exported to *.txt file as basic data, 20 files for all chips were produced after absolute analysis. Also the result from different experiments for the same array can be showed together in a Pivot tab, then 2 files (for RG_U34 A and B) including the results of 10 chips were exported.

For **Background** calculation, the array is divided into 16 sectors, the average of the lowest 2% of cells intensity values in each sector is as Background for this sector. The background was subtracted from the average intensities of all cells in this sector. **Noise (Q)** is measured by examining the pixel-to-pixel variations in signal intensities (see microarray suite software manual) and calculated by software. **Positive** is termed when the intensity of the PM probe cell is significantly greater than that of the corresponding MM probe cell. **Negative** is termed when the intensity of the MM probe cell is significantly greater than that of the corresponding PM probe cell. The significance is determined by calculating both the ratio (PM/MM) and the

difference (PM - MM) associated with each probe pair and compared against two threshold values: the Statistical Difference Threshold (SDT) and the Statistical Ratio Threshold (SRT). SDT is calculated by the software based on the noise (Q), $SDT = Q \times SDT_{mult}$ (SDT multiplier is set by default 2.0). SRT is set to 1.5 by default. Three additional Absolute Call Metrics, the **Positive Fraction**, the **Pos / Neg Ratio** and the **LogAvg Ratio** are derived from the intensities of PM and MM, as well as the numbers of positive and negative probe pairs (PP). The Absolute analysis metrics are expressed mathematically and thresholds values are set as default (Appendix Table 11.4). Each of the three metrics used to determine the **Absolute Call**. **Absolute Call** was employed to determine the **Present**, **Marginal** and **Absent** of each transcript.

3.5.7.3 Comparison analysis

Comparison analysis by the microarray suite software was used to perform additional calculation on data from two separate probe array experiments in order to compare gene expression levels between two samples. The comparison analysis begins with the user designating an absolute analysis of one probe array experiment as the source of baseline date and a second probe array experiment as the source of experimental data to be compared to the baseline. After comparison analysis, a table including comparison analysis metrics was produced by software. **Difference Call** as a decision matrix indicates whether a transcript has **Increased** (I), **Decreased** (D), **Marginally Increased** (MI), **Marginally Decreased** (MD), or exhibits **No change** (NC) in expression level. In addition, a **Fold Change** calculation was carried out as an indication of the relative change of each transcript represent on the probe array.

If the intensity difference between the PM and the MM probe cells in the experimental sample is significantly higher than in the baseline sample, a probe pair is considered to **Increase**. If the intensity difference between the PM and the MM probe cells in the experimental sample is significantly lower than in the baseline sample, a probe pair is considered to **Decrease**. Four comparison metrics, **Increase / Decrease Ratio, Max** (Increase / PP used, Decrease / PP used), **Log Average Ratio Change,** and **Dpos-Dneg Ratio** (difference positive - difference negative) are used to determine the **Difference Call** for every transcript. The comparison analysis metrics are expressed mathematically and thresholds values are set as default (Appendix Table 11.5).

Comparison analysis with individual chip

One comparison expression analysis was performed with every individual chip, which compared the cell intensity data (*.cel file) of an experiment (result from SHHF hybridization) and baseline probe array of the same probe array type (result from WKY or SHRSP). Thirty-one analysis output files (*.chp) of comparison analyses between SHHF to WKY (SHHF/WKY), SHHF to SHRSP (SHHF/SHRSP) or between SHRSP and WKY (SHRSP/ WKY) were produced for each type of chip (RG-U34A or RG-U34B). Six data files (SHHF/WKY, SHHF/SHRSP, and SHRSP/WKY of RG_U34A and RG_U34B) were exported in a Pivot tab to excel. Absolute call, difference call and fold change were selected for further analysis. In order to statistically analyze the hybridization result from each chip, the metrics of Difference call I, D, MI, MD, and NC were separately valuable with 1, -1, 0.5, -0.5 or 0. Total value of difference call for each transcript was summed. The maximum of absolute value for C_W was 15, 10 for C_SP, and 6 for SP_W, the transcripts with absolute value over half of total absolute value were selected. Then genes showing differential expression in the comparisons of SHHF/WKY, SHHF/SHRSP and SHRSP/WKY were acquired with each individual chip.

Comparison analysis with mean chip

Another comparison analysis was performed by mean chip method. The Mean chips of SHHF, WKY and SHRSP strains were produced with the cell intensity data (*. Cel file) hybridized with each kind of samples by a program designed by Dr. Schulz in our lab. Produced mean chips were analyzed by absolute and comparison analysis. Genes showing differential expression (I, MI, D and MD) in the SHHF/WKY, SHHF/SHRSP and SHRSP/WKY comparisons were selected with mean chips.

Genes selected from Affymetrix chips

The Microsoft excel Match analysis was used to compare the transcripts selected using mean chip and individual chip. In order to reduce the fault positive rates and stand out important genes, genes that were found differential expression using these two methods were selected.

3.5.7.4 Cluster

Clustering allows to group genes according to similarities in their expression profiles across multiple analyses. Data Mining Tool 2.0 provides two types of clustering methods: Self-Organizing Maps and a modified Pearson's Correlation Coefficient method. The clusters were visualized using line graphs that can help quickly find patterns in data leading to a greater understanding and more rapid interpretation of results. Clusters using Cluster Eisen were alternatively used for global analyses of data.

3.6 Quantitative Real Time PCR

The candidate genes selected from expression profiling were further investigated by quantitative Real Time PCR with the ABI prism 7700 Sequence Detection System. Two fluorescence-based methods (DNA binding dye SYBR Green I or TaqMan probe) were used for the detection of amplification products. The TaqMan probe contains a reporter dye (FAM) at the 5' end of the probe and a quencher dye (TAMRA) at the 3' end of the probe. During the reaction, the 5'-3' nucleolytic activity of the AmpliTaq Gold enzyme cleaves the probe between the reporter and quencher only if the probe hybridizes to the target. The reporter dye and quencher dye become separated, resulting in increased fluorescence of the reporter. TaqMan Probe provides a maximal specificity for product identification. Fluorescent dye SYBR Green I binds to the minor groove of the DNA double helix, and its fluorescence is greatly enhanced after DNA-binding. During elongation of PCR, more and more dye molecules bind to the newly synthesized DNA. Fluorescence measurement at the end of the elongation step of every PCR cycle was performed to monitor the increasing amount of amplified DNA. Together with a melting curve analysis performed subsequently to the PCR, the SYBR Green provided an excellent tool for measuring expression level of a specific gene.

3.6.1 Design of TaqMan probe and primer

TaqMan probe and two primers were designed together using Primer Express software. The shortest amplicons (50-150 bps) worked the best and consistent results. The following guidelines for TaqMan probe and primer were set after running the software. 1) The G-C content was kept in the 20-80% range; runs of an identical nucleotide, especially for guanine

were avoided. 2) For TaqMan probe, G was not put on the 5' end and the melting temperature (Tm) was estimated to be 65-67°C, the strand that contains more Cs than Gs was selected. 3) For primers, Tm should be 58-60°C and the five nucleotides at the 3' end should have no more than two G and/or C bases. 4) The forward and reverse primers were placed as close as possible to the probe without overlapping with the probe. Then a table of possible probes and primers were displayed, first the shortest probe was selected according above rule and a suitable primer pair was selected to get the shortest amplicon. If possible, a good amplicon was selected to ensure amplification of the target mRNA without co-amplifying the genomic sequence, pseudogenes and related genes. The primers can be independently used for SYBR Green detection.

3.6.2 Optimization of Real Time PCR condition

Primers were tested in a standard PCR with 50 ng cDNA as template using the following program: After initial denaturation at 95°C for 4 min, 30 cycles were performed at 95°C 15 sec, 60°C 1 min and 68°C 1 min. Then 8 µl of PCR products were loaded on a 2.5% agarose gel in 1 × TAE buffer and run for about 45 min to verify PCR amplification.

In order to get comparable results from experiment vs. control, the total RNA samples from different animal hearts were freshly isolated in parallel and cleaned up after DNase digestion as described in 3.2. Then cDNA was prepared from the same amount of total RNA (8 µg) for each sample using random hexamers and SSII in 30 µl of reaction as described in 3.3.2. cDNA synthesis and purity were verified by PCR using *Gapdh* primer and angi_intron primer as described in 3.3.3. In each quantitative RT-PCR, the expressions of the target gene and of the 18S rRNA control were measured in cDNA samples from at least three animals for each strain. Each sample was measured in three replicates: A "no template control" (NTC) was included to exclude potential contamination. In order to find a suitable concentration of cDNA for Real Time PCR, a standard curve was drawn for every batch of cDNA using an aliquot (80 ng of total RNA) of first–strand cDNA diluted serially on the order of twofold. Normally a diluted aliquot (2-5 ng of total RNA) of the first-strand cDNA was used for the quantification of the target genes.

3.6.3 Quantitative RT-PCR using TaqMan probe

TaqMan universal PCR master mix reagents provide a PCR mix that is used for designed primers and probe (Table 2.5) to detect any cDNA sequence. AmpErase uracil-N-glycosylase (UNG) as a component of the TaqMan master mix can prevent the reamplification of carryover-PCR products by removing any uracil incorporated into double-stranded DNA. Hot start PCR and Time Release PCR were performed to lower background and increase amplification of specific products. The passive reference included in the TaqMan master Mix provides an internal reference to which the reporter-dye signal can be normalized during data analysis. PCR reaction was prepared by mixing 12.5 µl TaqMan universal PCR master Mix (2×), 2.5 µl each of forward and reverse primer (10 µM), 2.5 µl TaqMan probe (2.5 µM), 2 µl diluted cDNA (2 ng total RNA), 3 µl H_2O in total volume 25 µl. The following thermal cycling parameters were used for TaqMan PCR: the reactions were incubated at 50°C for 2 min, and then heated at 95°C 10 min for activation of AmpliTaq Gold, afterwards 40 cycles were performed at 95°C for 15 sec, 60°C for 1 min.

3.6.4 Quantitative RT-PCR using SYBR Green

SYBR Green PCR Master Mix contains SYBR Green I Dye, AmpliTaq Gold DNA polymerase, and dNTP with dUTP, a passive reference and optimized buffer components. Hot start technique and UNG treatments are used to avoid potential contamination from DNA template and carryover PCR products. Due to the nonspecific nature of SYBR Green detection, any double stranded DNA will be detected. Nonspecific product formations are checked by dissociation curve or gel analysis. SYBR Green PCR reaction was prepared by mixing with 2 µl diluted cDNA (2 ng total RNA), 2.5 µl 10× SYBR Green PCR buffer, 0.75 µl each of forward and reverse primer (10 µM), 2 µl dNTP mix (2.5 mM dATP, dCTP, dGTP each and 5 mM dUTP), 3.5 µl $MgCl_2$ (25 mM), 0.25 µl AmpErase UNG (1 U/µl), 0.125 µl ApliTaq Gold polymerase (5 U/µl), and 13.125 µl sterile H_2O to total volume 25 µl. The thermal cycling parameters for SYBR Green PCR were the same as that for TaqMan PCR.

3.6.5 Analysis of quantitative Real Time PCR

Gene expression of candidate genes selected from microarray analysis was redetected using quantitative Real Time PCR by calculating relative quantification. Gene expression was measured by the quantitation of cDNA converted from messenger RNA from SHHF strain corresponding to the target gene relative to a calibrator sample (cDNA from WKY or SHRSP strain) serving as a physiological reference. All quantitations were normalized to the endogenous control 18S rRNA to account for variability in the initial concentration.

The threshold cycle or C_T value occurs when the sequence detection application begins to detect the increase in signal associated with an exponential growth of PCR product. Relative quantification of the target gene was calculated from C_T values for each well on the Real Time PCR plate. Wells with higher initial template concentrations reach the threshold value at lower cycle numbers during PCR than wells containing lower initial template concentrations, a change in threshold cycle number of one equates to a two-fold difference in initial template concentration.

The relative quantitation for a target gene was calculated following:

- The mean C_T value was the average of the replicate wells run for each sample.
- ΔC_T was the difference between the mean C_T values of target samples between endogenous controls (18s rRNA).
- $\Delta\Delta C_T$ was the difference between the mean ΔC_T values of the samples (such as cDNA samples from SHHF) for each target gene and the mean ΔC_T of the calibrator (cDNA samples from WKY or SHRSP) for the target.
- The relative quantitation value was expressed as $2^{-\Delta\Delta CT}$.

3.7 Preparation of DNA

3.7.1 Isolation of genomic DNA from rat tissue

Large amounts of gemomic DNA were prepared using common phenol/chloroform method. Rat liver was placed into a mortar and pulverized in liquid nitrogen. About 1.5 g of powder-like tissue from each sample were added to 20 ml of 1× TEN9 buffer in a 50-ml Falcon tube and mixed by shaking. 200 µl DNase free RNase (10 mg/ml) were added to each sample and the samples were incubated at room temperature for 10 min by gently shaking on a rocker. 0.5

ml proteinase K (20 mg/ml) and 2 ml 10% SDS were added to every sample and the samples were incubated at 55°C overnight by gently shaking on a rocker. DNA was extracted twice with phenol and once with chloroform/isoamylalcohol (23:1). The DNA was precipitated using equal volume isopropanol with 1/10 volume of 3 M ammonium acetate. Then DNA was fished out with sterile glass rod, washed with 70% ethanol once and dissolved in 1× TE buffer at 37°C for a few hours and then stored at 4°C.

3.7.2 Isolation of genomic DNA from rat tail

Genomic DNA from rat-tail was isolated using QIAGEN DNeasy kit following the manual instructions. 180 µl of buffer ATL was added to each tube containing about 0.8 cm lengths of tail, 20 µl of proteinase K was added and incubated at 55°C overnight. 40 µl of RNase (10 mg/ml) was added to each tube, mixed by vortexing and incubated for 2 min at room temperature. 400 µl of buffer AL-ethanol mixture was added to each tube after vortexing for 15 sec. After mixed vigorously by vortexing, the sample was purified using DNeasy mini column. 200 µl of elution buffer was used to elute genomic DNA for each sample. DNA was stored at 4°C.

3.7.3 Isolation of plasmid DNA

One colony picked up from Amp or Kan agar plate was transferred to 3 ml of LB (0.05 µg/ml Amp or 0.03 µg/ml Kan) in a 15-ml tube. The sample was incubated at 37°C for about 16 hours under 224 rpm rotation. The bacterial pellets were collected by centrifugation at 5000 rpm for 3 min. It was completely suspended in 0.3 ml of resuspension buffer (50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A) by vortexing according to the QIAGEN Plasmid mini handbook. 0.3 ml of lysis buffer (200 mM NaOH, 1% SDS) were added, mixed gently and incubated at room temperature for 5 min. 0.3 ml of chilled neutralization buffer (3.0 M potassium acetate, pH 5.5) were added, mixed immediately but gently and incubated on ice for 5 min. The sample was centrifuged at 14000 for 10 min and the supernatant was removed promptly. The supernatant was applied to the QIAGEN-tip 20 after equilibrated with 1 ml equilibration buffer. 0.8 ml of elution buffer was used to elute DNA. The DNA was precipitated with 0.56 ml of isopropanol and centrifuged immediately at more than 10000 rpm

for 30 min. The DNA pellet was washed twice with 70% ethanol, air dried for 5 min and redissolved in a 30 µl of TE. The plasmid was stored at -20°C.

3.7.4 Quantification of DNA and quality assessment

DNA was quantified by spectrophotometric analysis using the convention that 1 OD at 260 nm equals 50 µg DNA per ml. The A_{260}/A_{280} ratio between 1.9 and 2.1 is for pure DNA. DNA was also checked on agarose gel electrophoresis. Normally 0.8% agarose gel was used for analyzing genomic DNA and plasmids, 1% or 1.2% agarose gel were used to analyze PCR fragments or DNA fragments obtained by restriction enzyme digestion. 2% agarose gels were used for smaller size PCR fragments. 4% agarose gels were used for genotyping by *Hin*fI RFLP analysis. 0.5 µg of DNA Marker PhiX174/*Bsu*RI (*Hae*III), 1 kb of DNA Ladder or λ DNA were loaded in one slot of the gel as size standard. Before loading of samples, 1 µl of 6× DNA loading buffer was added to 5 µl of the DNA sample.

3.7.5 Analysis of plasmid DNA using restriction enzyme digestion

About 1 to 2 µg of plasmid DNA prepared by QIAGEN plasmid mini kit was digested with 1 µl *Eco*RI (20 U/µl) in 15 µl reaction solution containing 10% *Eco*RI buffer at 37 °C overnight. Afterwards, the samples were checked on 0.8% agarose gel.

3.8 PCR and purification of PCR products

3.8.1 Standard PCR

A standard PCR protocol was used for amplification of specific fragments from cDNA, genomic DNA and plasmid DNA. The amplification mixture (25 µl) was prepared by mixing 2.5 µl 10× PCR buffer (Promega), 1.5 µl 25 mM $MgCl_2$ (Promega), 0.25 µl dNTP (25 mM for each), 0.75 µl each of primer pairs (10 µM), and 0.2 µl Taq (5 U/µl, Promega or in house). 40 to 200 ng DNA were used as template, sterile water was added to final volume of 25 µl. For high-throughput screening, PCR reaction was prepared by mixing 3 µl 10× Leo's buffer, 0.75 µl 20 mM $MgCl_2$, 0.25 µl dNTP (25 mM for each), 0.75 µl each of primer pairs (10 µM), 0.2 µl Taq (5 U/µl) from house, 9 µl 5 M Bataine. Sterile water was added to a final volume of 30

µl. The PCR reaction was carried out using a standard PCR program or a touch down program. **Standard PCR program:** 94°C 3 min; 30 cycles at 94°C 30 sec, Tann 45 sec, 72°C 0.5-2 min; 72°C for 5 min.

Touch down PCR program: 94ºC for 2 min, 12 cycles at 94ºC 30 sec, gradient temperature with reducing 1ºC for each cycle till Tann for 1 min, 72ºC 2 min. 20 cycles at 94ºC 30 sec, Tann for 1 min, 72ºC for 2 min; 72ºC 5 min.

3.8.2 Colony PCR

The colony PCR protocol was used to amplify products from bacterial colonies on agar plates. Two or three colonies were suspended in 20 µl of sterile H_2O, and the bacterial suspension was heated for 5 min at 100°C in a thermocycler. 1 µl of the suspension was used for PCR amplification. The amplification mixture and the conditions were the same as the standard PCR protocol.

3.8.3 Purification of PCR products

PCR products ranging from 100 bp to 10 kb were purified from primers, nucleotides, polymerases and salts using QIAquick PCR purification kit. 5 volumes of buffer PB were added to 1 volume of PCR reaction and mixed. The sample was applied to the QIAquick column and centrifuged at 12000 g for 30 sec. The flow-through was discarded and 0.75 ml of buffer PE was used to wash the column by centrifugation at 12000 g for 30 sec. The column was centrifuged for an additional 1 min at 14000 g to completely remove the residual ethanol from buffer PE. Purified PCR products was eluted with 50 µl of buffer EB (10 mM Tris·Cl, pH 8.5) and stored in -20°C.

In order to purify single band from more than one-band PCR products, PCR products were separated on 1% low melt agarose gels. A specific single band was excised from the gel using a clean sharp scalpel. Following MinElute Gel Extraction Kit (QIAGEN) manual, 3 volumes of buffer QG were added to 1 volume of gel slice (100 mg ~ 100 µl) in a colorless tube. The sample was incubated at 55°C for 10 min, the gel was dissolved by vortexing every 2-3 min during the incubation. 10 µl of 3 M sodium acetate was alternatively added to the sample if the color of the mixture was orange or violet after the gel slice has been dissolved completely. One gel volume of isopropanol was added to the sample and mixed by inverting the tube several times. The samples were applied to the MinElute column. The maximum amount of

gel slice per spin column is 400 mg. After the columns were washed once with 500 µl of Buffer QG and once with 750 µl of buffer PE, the DNA was eluted with 10 µl of buffer EB in a new 1.5-ml microcentrifuge tube.

3.9 DNA sequencing

DNA sequencing was carried out using ABI PRISM^{TM} BigDye Terminator Cycle sequencing ready reaction Kit directly from purified PCR product or plasmid DNA. For each sequence reaction, 2 µl Terminator Ready Reaction Mix, 1 µl primer (10 µM), About 300-400 ng of plasmid DNA (10% of size) or 50-100 ng PCR fragments (10 ng per 100 bp), and sterile H_2O were mixed to final reaction volume of 10 µl in 8-tube strips. For sequencing plasmid DNA, the mixture was heated at 96°C for 5 min, followed by 30 cycles at 96°C for 20 sec, 50-60°C (depending on primer) for 10 sec and 60°C for 4 min. For sequencing PCR templates, the reaction was heated at 96°C for 3 min, followed by 25 cycles at 96°C for 10 sec, 50-60°C (depending on primer) for 5 sec and 60°C for 4 min. After the program was finished, the sequence reaction was precipitated using 25 µl 100% ethanol and mixed by vortexing. The sample was incubated at room temperature for 10 min and then centrifuged at room temperature at 2700 g for 60 min, ethanol solution was removed and the sample was washed twice with 200 µl 70% ethanol. The pellet was dried for 2 to 5 min and then stored at -20°C. Sequences were read on ABI 377 automatic sequencers.

3.10 Southern blot hybridization analysis

3.10.1 DNA gel electrophoresis and blotting

100 ml of 1% agarose gel with 1 µl ethidium bromide (10 mg/ml) were prepared with 1× TAE and poured into an 8.3× 18 cm electrophoresis platform. 5 µl of PCR products were mixed with 1 µl 6× loading buffer and loaded into the wells. The gel was run at 120 V for 1 h and then photographed before blotting. The gel was rinsed in sterile water and placed into a clean plastic box containing 300 ml denaturing buffer. The box was shaken slowly on a platform shaker for 20 min at room temperature. The DNA was transferred onto nylon membrane with denaturing buffer by the capillary transfer method (Dunn and Sambrook, 1980). After overnight transfer in denaturing solution, the membrane was neutralized by gently shaking in

0.05 M Na_2HPO4 for 5 min, air dried and DNA was fixed by exposing to UV light for 2 min in UV stratalinker 2400 (Stratagene).

3.10.2 Probe labeling by random hexamer priming

Purified PCR products were labeled by random hexamer priming (Feinberg and Vogelstein, 1984), which is based on a DNA polymerization reaction primed by random hexanucleotides binding to the template. About 120 ng of DNA (14.5 µl) were denatured at 95°C for 5 min and chilled on ice. Then 25.5 µl labeling mixture, 18 µl LS, 3 µl ATG (100 µM mix of dATP, dGTP, dTTP), 1.5 µl BSA (acetylated, 10 mg/ml), 0.5 µl Klenow enzyme (5 U/µl), and 2.5 µl [α-^{32}P] dCTP (10 µCi/µl), were added to the denatured samples and the labeling reaction was incubated for 2 h at 37°C. The probe was purified with Microspin^{TM} G50 Column (Pharmacia) before hybridization.

3.10.3 Hybridization

Filters were prehybridizated at 65°C for 2 h using 20 ml of hybridization buffer (1 M NaCl, 1% SDS, 10 mM Tris·Cl), then buffer was replaced with 10 ml hybridization buffer containing the denatured probe. Hybridization was performed at 65°C overnight. Filters were washed twice at 65°C for 20 min first with 100 ml of wash solution I, followed with 100 ml of wash solution II. Filters were dried briefly on Whatman paper, sealed in plastic foil (Saran wrap) and exposed to X-ray film (Kodak X-OMAT) for 1 to 2 days. The films were developed using Agfa curix 60.

3.11 Molecular analysis of *Cd36* in SHHF strain

3.11.1 cDNA sequencing

Five primer pairs (Table 2.3) were designed to amplify the *Cd36* cDNA from SHHF and WKY strains by PCR in five overlapping segments according to known published *Cd36* cDNA sequence (Genbank accession number L19658). The PCR fragments A, B, C, and E from SHHF and WKY strains were directly sequenced using BigDye Terminator (see 3.9). Fragment D was first cloned using AdvanTAge^{TM} PCR cloning kit and then the plasmids including fragment D from SHHF or WKY were sequenced at AGOWA.

The ligation reactions were prepared by mixing 2 μl of fresh PCR fragment D from SHHF (about 3.8 kb) or 1 μl from WKY (about 580 bp), 2 μl pT-Adv vector (25 ng/μl), 1 μl 10× ligation buffer, 1 μl T4 DNA ligase (4.0 weiss units), and suitable sterile H_2O for a final volume of 10 μl. After incubated at 14°C overnight, 2 μl of each ligation reaction was directly added to 50 μl of the competent cells (TOP 10F' E.coli) setting on ice and mixed by tapping gently. The tubes were incubated on ice for 30 min and then heat shocked for 30 sec in a 42 °C water bath. After the tubes were kept on ice for 2 min, about 250 μl of prewarmed SOC medium was added to each tube. The tubes were shaken at 37°C for 45 min at 225 rpm. Different amounts of transformation mixture (e.g. 50 μl and 200 μl) were plated on LB/X-Gal/IPTG plates containing 30 μg/ml of kanamycin. The plates were incubated at 37°C for at least 18 h, and then shifted to 4°C for 2-3 h to allow proper color development. At least 10 white colonies for each transformation were selected for colony PCR, plasmid isolation and restriction analysis as described in 3.7. The positive plasmids DNA were sequenced as described in 3.9.

3.11.2 Determination of 5' and 3' end of *Cd36* cDNA

Full-length cDNAs were generated in reverse transcription reactions using Clontech' SMART^{TM} (Switching mechanism at 5' end of RNA transcript) RACE (Rapid amplification of cDNA end) technology. Three to five residues of dCTP are added to the 3' end of the first-strand cDNA by PowerScript. This activity is harnessed by the SMART oligo whose terminal stretch of dG residues can anneal to the dC-rich cDNA tail and serve as an extended template for reverse transcriptase (RT). The SMART sequence is typically added only to complete

first-strand cDNAs (Fig. 3.4). The 3'-RACE cDNA is synthesized using a special cDNA synthesis primer (3'-CDS) that includes the lock-docking nucleotide positions as in the 5'-RACE cDNA Synthesis Primer (5'-CDS) (Fig. 3.4). The following methods are described in detail in the kit manual.

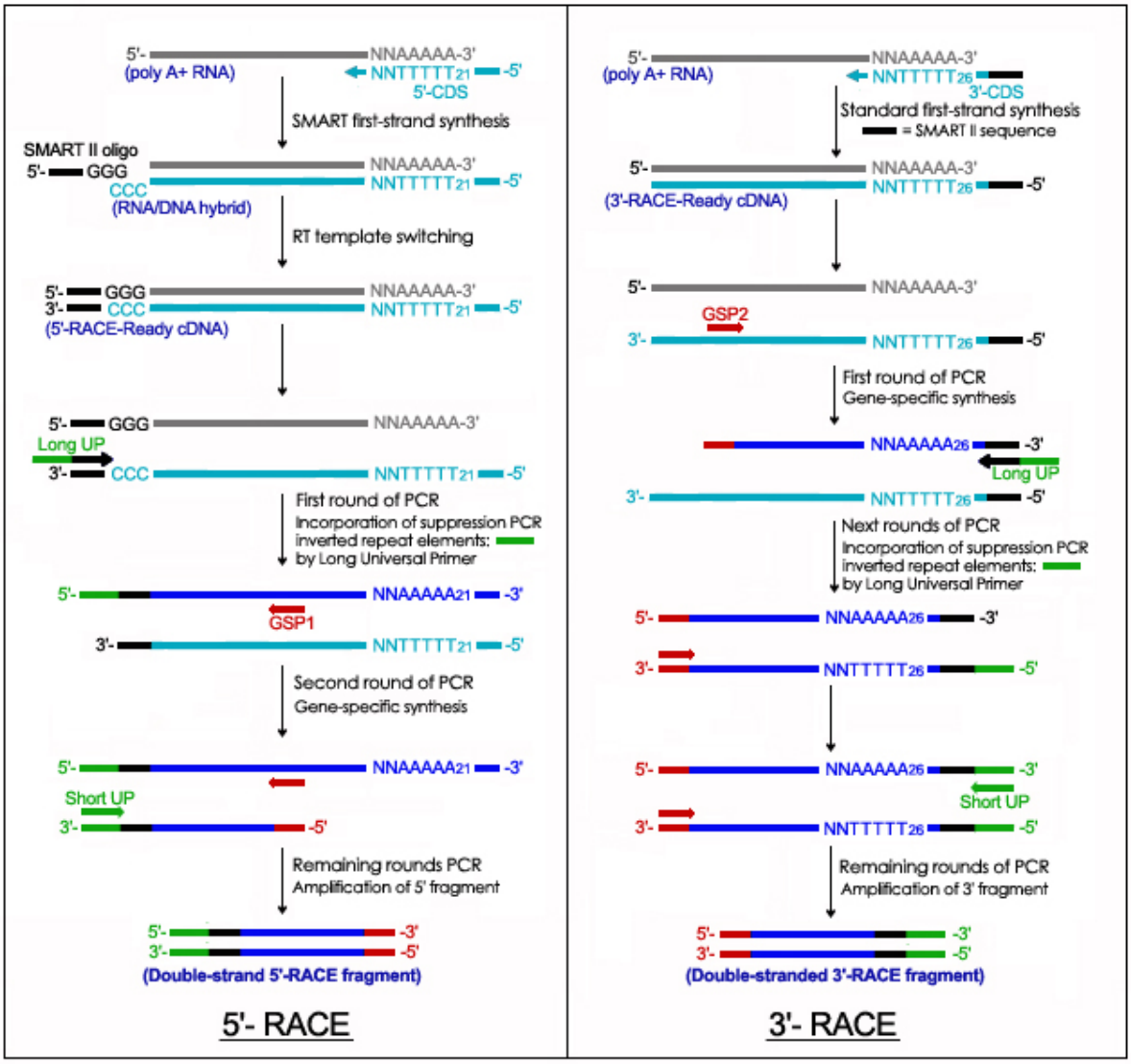


Fig. 3.4 Mechanism of the 5'-RACE and 3'-RACE

5' or 3'-Race-ready cDNA were prepared with 1 µg of total RNA isolated from strains SHHF or WKY by QIAGEN RNeasy kit as described in 3.2. For preparation of 5'-RACE-Ready cDNA, 1 µl 5'- CDS primer and 1 µl SMART II oligo were added. For 3'-RACE-Ready cDNA, 1 µl 3'- CDS primer was added. Sterile H_2O was added to each tube for a final volume of 5 µl. The tubes were incubated at 70°C for 2 min and then cooled on ice for 2 min. 10 µl of

following mix (2 µl 5× First- strand buffer, 1 µl DTT (20 mM), 1 µl dNTP Mix (10 mM), 1 µl PowerScript Reverse Transcriptase) were added to each reaction. The samples were incubated at 42°C for 1.5 h in an air incubator. The first strand reaction was diluted with 100 µl Tricine–EDTA buffer and heated at 72°C for 7 min, stored at -20°C.

The gene specific primers (GSPs, Table 2.4) were designed for amplifying the 5' and 3' RACE PCR products from SHHF or WKY strain. For preparing 5' RACE (or 3' RACE) PCR reaction, 41.5 µl of master mix (5 µl 10× advantage 2 PCR buffer, 1 µl dNTP Mix (10 mM), 1 µl 50× Advantage 2 Polymerase mix and 34.5 µl sterile H_2O) were added to 8.5 µl of the following mix (2.5 µl 5' or 3'-RACE-ready cDNA, 5 µl 10× universal primer mix, 1 µl GSP). A touch-down PCR program was performed for T_m over 70°C and a standard PCR program was performed for T_m lower than 70°C. Afterwards, 5 µl of 5' and 3' RACE PCR products were loaded on a 1.0% agarose gel, and further checked alternatively by southern blot as described in 3.10. The purified ready 5' or 3' RACE PCR products were cloned using QIAGEN PCR cloning kit. At least 2 plasmid DNAs representing 3' RACE PCR fragments and 5 plasmid DNAs representing 5' RACE PCR fragments were used for sequencing by BigDye Terminator as described in 3.9.

A touch-down PCR program (for GSP1, 3 and 4): 5 cycles at 94°C 5 sec, 72°C 3 min; 5 cycles at 94°C 5 sec, 70°C 10 sec, 72°C 3 min; 25 cycles at 94°C 5 sec, 68°C 10 sec, 72°C 3 min.

A Standard PCR program (for GSP2): 25 cycles at 94°C 5 sec, 68°C 10 sec, and 72°C 3 min.

3.11.3 Northern blot

12 µl (15 µg) of total RNA isolated from the heart tissue of four different animal models using TRIzol as described in 3.2.1 was mixed with 3 µl of 5× RNA loading buffer. The samples were incubated at 65°C for 5 min, chilled on ice for at least 2 min. The samples were loaded onto an equilibrated 1.2 % formaldehyde agarose gel. The gel was run at 5-7 V per cm (60 V for 8.3 × 10.6-cm gel) for about 2 h. After photographed, the gel was soaked in RNase-free water for 10 min. This step was repeated 3 times by changing new water. The nylon membrane was pretreated in sterile H_2O for 5 min before used. The RNA was transferred onto nylon membrane with 20× SSC by the capillary transfer method. After transferred overnight, the membrane was soaked in sterile 5× SSC for 5 minutes and then air dried. The RNA was

fixed by exposing to UV light for 2 min in UV stratalinker 2400. The membranes were stored at room temperature.

The filters prewetted with 5× SSPE were rolled and allowed to unfold in the hybridization tube. The filters were prehybridized at 42°C for 2 h in 10 ml hybridization buffer (5× SSPE, 5× Denhardts, 0.1% SDS, 100 μg/ml denatured salmon sperm, 50% deionized formamide). The filters were hybridized with 5-20 ng probe/ml labeled using 2.5 μl [α-^{33}P] dCTP (10 μCi/μl) in 10 ml hybridization buffer as described in 3.4.1 and hybridized at 42°C overnight.

The filters were washed with 2× SSPE/0.1% SDS for 15 min twice at room temperature, then washed with 0.1× SSPE/0.1% SDS buffer for 15 min at 37°C. The membranes were wrapped in Saran wrap after briefly dried on Whatman paper, then exposed to FuJi film for 1 to 2 h. The exposed screen was scanned using FuJi Film Bas-5000. The membranes were alternatively exposed to X-ray film for 1 to 2 days to get a clear picture.

3.11.4 Isolation of Cd36 protein from heart tissue

The removed hearts were washed in 0.9% NaCl solution and frozen in liquid nitrogen. The frozen hearts were pulverized in a mortar with liquid nitrogen. 200-400 mg of powder-like heart tissue were lysed with Buffer A (154 mM NaCl, 30 mM Tris·Cl, 0.35 mM SDS, 1 tablet/50 ml proteinase inhibitor complete, 0.1% Triton X-100) in the ratio 2:1 (v:w) to the weight of the hearts. The samples were homogenized for 1 min using the homogenizer while the tubes were kept on ice. The resting tissue and unbroken cells were eliminated by centrifugation at 3000 g for 30 min at 4°C. The supernatant was removed and further centrifuged at 100 000 g for 90 minutes at 4°C to sediment the membrane fraction. The supernatant was discharged. The pellet was suspended in Buffer B (50 mM Tris·Cl, 5 mM EGTA, 1 tablet/50 ml proteinase inhibitor complete, 1 % Triton X-100, pH 7.4), in the ratio 2:1 (v:w). The cell membranes were solubilized by shaking the suspension in capped tubes for 20 hours at 4°C. Insoluble material was removed by centrifugation at 100000 g for 30 minutes at 4°C. The supernatant prepared using Triton X100 was directly used for western blot.

3.11.5 SDS-PAGE and Western blot

The Cd36 protein prepared from rat heart tissue of strains SHHF, SHRSP, SHR and WKY were separated and transferred to nitrocellulose membranes using Bio-rad Mini-Protean®3 Cell and Mini Trans-Blot® Electrophoretic transfer Cell. 8% resolving gel and 5% stacking gel were prepared in a sandwich chamber (7 × 9 cm). The stacking gel solution was applied on top of the resolving gel and a 1.5-mm comb was inserted into the gel solution. 30 μg of protein for each sample in 20 μl of supernatant were mixed with 10 μl of protein loading buffer, denatured at 94°C for 5 min and cooled on ice. After removing the comb, the protein samples were loaded on the gel, and the gel was run for 45 min at 260 V. The gel was equilibrated for 30 min at room temperature in transfer buffer. A transfer sandwich consisting of the protein gel and a nitrocellulose membrane was assembled. The proteins were electrophoretically transferred from the gel to a nitrocellulose membrane for 1 h at 320 mA constant current in the precooled transfer buffer. The membrane was placed in a plastic box, washed for 5 min with wash buffer PBST (1× PBS, 0.05% Tween 20) and allowed to air dry at room temperature. The membrane was blocked in blocking solution (1× PBS, 5% Non-Fat milk) at 4°C overnight. The membrane was washed twice for 5 min with PBST. The diluted primary antibody (1:400) in 1% BSA/PBS-T was added and incubated for 1 hour at room temperature. The membrane was washed once for 15 min, and then four times for 5 min with PBS-T. The diluted HRP-labeled second antibody in 1% BSA/PBS-T (1:2000) was added and incubated for 1 h at room temperature. The membrane was washed once for 15 min, and then four times for 5 min with PBS-T. The membrane was incubated in the chemiluminescence reagent (0.125 ml of chemiluminescence regent per cm^2 of membrane) prepared by mixing equal volumes of the enhanced luminol reagent and oxidizing reagent for one minute while shaking. The excess chemiluminescence regent was removed by draining and the membrane was placed in a plastic sheet protector. The membrane was exposed to Kodak X-OMAT film for 30 sec and film was developed. An optimum exposure was determined according the quality of the developed film.

3.12 Linkage analysis of Cd36 in heart failure model SHHF

3.12.1 Pheotyping of F2 animals

The phenotypes of (SHHF×WKY) F2 animals in 13-14 months along with age matched F0 animals were measured using hemodynamic method as described in 3.1. The calculated hemodynamic parameters serve as indicators of cardiovascular performance in response to heart failure. Thirty-one parameters were selected for statistical analysis (Table 3.2).

Table 3.2 **Descriptions of parameters used for linkage analysis**

Abbreviation	Description	Abbreviation	Description
Pmax	Maximum pressure	Vmax	Maximum volume
Pmin	Minimum pressure	Vmin	Minimum volume
Pbd	Begin diastolic pressure	EDV	End diastolic volume
Ped	End diastolic pressure	ESV	End systolic volume
Pes	End systolic pressure	SV	Stroke volume
DP	Developed pressure	CO	Cardiac output
SEP	Systolic ejection period	EF	Ejection fraction
DFP	Diastolic filling period	SW	Stroke work
MSP	Mean systolic pressure	Ea	Effective arterial elastance
MDP	Mean diastolic pressure	HR	Heart rate
CT	Contraction time	ANIMALg	Animal weight
RT	Relaxation time	HGmg	Heart weight
dPmax	Max dPdt	LUNGmg	Lung weight
dPmin	Min dPdt	RNmg	Right kidney weight
CI	Contractility index	LNmg	Left kidney weight
Tau	Time constant of relaxation		

3.12.2 Genotyping of F2 animals

Genomic DNA from F0, F1 and F2 animals of the cross SHHF with WKY, were isolated from rat-tail with QIAGEN DNeasy kit described in 3.7.2. The F2 animals were first genotyped by a *Hin*fI restriction site detected in gene *Cd36* of SHHF strain. PCR reactions were prepared with Leo's buffer see in 3.8 and amplified using Hinf primers (Table 2.2). After amplifications were confirmed on 2% agarose gel, 5 µl of PCR products for each sample were mixed with 1 µl (10 U) *Hin*fI enzyme and incubated at 37°C overnight. The reactions were loaded on 4% small DNA agarose gel and the results were visualized by ethidium bromide.

The F2 animals were also genotyped by the marker D4Bro1 with normal PCR along with ethidium bromide staining on 4% small DNA agarose gel and genotyped by the marker D4Rat6, D4Rat221 with ^{32}P γ-ATP radio activating detection.

3.12.3 Expression level of *Cd36* in F2 animals

With the *Hin*fI RFLP analysis on gene *Cd36*, SHHF homozygotes were distinguished from rats that are either WKY homozygotes or SHHF/WKY heterozygotes. WKY homozygotes were further distinguished from SHHF/WKY heterozygotes according the genotype results of the marker D4Rat221 and D4Rat6. Expression levels of *Cd36* among SHHF homozygotes, WKY homozygotes and SHHF/WKY heterozygotes were detected using Real Time PCR with TaqMan probes (from AF072411 and AA799326, Table 2.3) as described in 3.6, *Cd36* cDNA from the three groups were also analyzed using the *Hin*fI RFLP method.

3.12.4 Statistical analysis

3.12.4.1 MAPMAKER/QTL

In order to find correlations between the inheritance of particular genetic markers and variation in the phenotype for each individual in the population, LOD scores for putative quantitative traits (QTLs) was calculated by MAPMAKER/QTL (Kruglyak and Lander, 1995; Lander *et al*., 1987). The based assume for using MAPMAKER/QTL is that the values of the QTL vary across the population follow a normal distribution. The Kolmogorov-Smirnov tests (KS-test) were performed to determine if the trait data fit this assumption. The LOD scores were calculated for the traits following normal distribution. If the traits data did not follow a normal distribution, they were transformed to “derived traits” with log (x), 1/div or sqrt transformation, “derived traits” that more closely fit the assumption were selected for MAPMAKER/QTL. Gender was confirmed as a weight factor for applied parameters of phenotype from Student t-test, and all rat data were normalized by gender after equality of variances for gender were confirmed by Levene's tests. So MAPMAKER/QTL was performed separately on male, female rats or all rats that normalized by gender.

4.12.4.2 T-test and anova

With the *Hin*fI RFLP analysis on gene *Cd36*, SHHF homozygotes were distinguished from rats that are either WKY homozygotes or SHHF/WKY heterozygotes. Student t-test was performed between above two groups on the data following a normal distribution and grouped by gender. SHHF homozygotes, WKY/SHHF heterozygotes and WKY homozygotes were separately divided according genotype results of the marker D4Rat221, D4Rat6 or D4Bro1, anova were further performed among SHHF homozygotes, WKY/SHHF heterozygotes and WKY homozygotes on the data following a normal distribution and grouped by gender.