2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Agarose	Invitrogen, US
Agarose, low melting point	Biozyme, Germany
Ambion Message amp	Ambion, US
Ampicillin	Sigma,US
AMV reverse transcriptase	Promega, US
β-actin cDNA probe	Clontech, US
Baker's yeast t-RNA	Roche, Germany
BM purple	Roche, Germay
BSA	Sigma, US
Cell culture plates	Greiner, US
Chaps	Sigma, US
Coverslips	Roth, Germany
Deionised Formamide	Roth, Germany
Dextran Sulfat	Sigma, US
Dietylpyrocarbonate (DEPC)	Sigma, US
Dig Labeled UTP	Roche, Germay
DNA molecular weight standards 25 bp, 50 bp, 100 bp, 1 kb	Fermentas,US
dNTPs	Amersham, Germany
DPX Mountant for histology	Fluka, Germany
Dulbecco's Modified Eagle Medium	Gibco, US
Entellan	Sigma, US
Ethanol	Merck, Germany
Ethidiumbromide	Sigma, US
Expresshyb hybridisation solution	Clontech, US
Fetal bovine Serum, FBS	Gibco, US
Ficoll	Sigma, US
Formaldehyde	Sigma, US
Formamide	Roth, Germany
γ^{33P}-dATP	Amersham, UK
Glutaraldehyde	Sigma, US
HotStarTaq DNA Polymerase	Qiagen, Germany
Human Unigene SetII array membranes	Amersham, UK
Kodak-developer D-19	Kodak, US

Kodak-fixer	Kodak, US
Maleic acid	Sigma, US
MMLV Rnase H - point mutant reverse transcriptase	Promega, US
MOPS	Sigma, US
NBT/BCIP	Sigma, US
O.C.T., optimal cutting temperature compound	Tissue-Tek
Oligo dT	Invitrogen, US
Paraffin-paraplasts	Sherwood Medical Co, US
Penicillin-Streptomycin	Sigma, US
Paraformaldehyde, PFA	Merck, Germany
Phenol:Chloroform:Isoamylalcohol	Invitrogen, US
Photoemulsion (autoradiography emulsion type NTB2)	Kodak, US
Polynucleotide kinase	New England Biolabs, US
Proteinase K	Sigma, US
Redivue α^{32}P-dCTP	Amersham, UK
Redivue α^{33}P-dCTP	Amersham, UK
RNA ladder	Invitrogen, US
RNAse A	Sigma, US
RNasin ribonuclease inhibitor	Promega, US
Schneider's Drosophila medium	Invitrogen, US
Sheep serum	Sigma, US
Superfrost plus slides	Menzel-Glaeser, Germany
SYBR Green I PCR Master Mix	Applied Biosystems, US
T7 RNA-polymerase	Roche, Germany
Taq-DNA polymerase	Qiagen, Germany
Toluidine Blue	Sigma, US
TRIzol reagent	Invitrogen, US
Tween20 (nuclease free)	Sigma, US
Unfertilised chicken (White leghorn) eggs	Tierzucht Lohman, Germany
Xylene	Roth, Germay

2.1.2 Kits

DNA easy kit	Ambion, US
Fast plasmid purification mini	Eppendorf, Germany
Micro spin S-200 HR columns	Amersham, UK
MiniElute PCR and gel purification	Qiagen, Germany
NucleoTrap mRNA mini kit	Macherey-Nagel GmbH, Germany

PaqLab RNA probe purification kit	PaqLab, Germany
QIAquick PCR purification	Qiagen, Germany
Phase Lock Gel columns	Eppedorf, Germany
ProbeQuant G-50 micro columns	Amersham, UK
Rediprime-II random prime labelling system	Amersham, UK
Topo TA cloning with M13,T7,SP6 promoters	Invitrogen, US
Wizard SV gel and PCR clean up	Promega, US

2.1.3 Devices

Bas cassette 2325	Fuji photo film, Japan
Bas- IP MS 2325 Imaging plate	Fuji photo film, Japan
Fuji Film Bas-1800 array scaner	Fuji photo film, Japan
Gel documentation system	Herolab GmbH, Germany
Gel electrophoresis equipment	Amersham, UK
Gene Quant RNA/DNA calculator	Amersham UK
Heating oven	MRP Binder, US
Homogenisator	Kinematika, Switzerland
Hybridisation oven	Oncor, Appligene, UK
Hybridisation oven	Grant Boekel, US
Nanadrop Spectrophotometer	Nanodrop technologies, US
Q-Fill	Genetix, UK
Thermocycler	PTC100, MJ Research Inc, US
Thermomixer	Eppendorf AG, Germany
UV crosslinker	Stratagene, USA
Water bath	Köttermann, Germany

2.1.4 Software

ABI PRISM 7900HT Sequence Detection System	Applied Boisystems, US
Chromas v1.43	Technelysium, Australia
Primer Express	Applied Biosystems, US
SDS 2.0 software	Applied Biosystems, US
Tina-v2.10i	Raytest Isotopenmessgeraete GmbH, Germany
Vector NTI	Informax-Invitrogen, US
Visual Grid microarray image processing software	GPC-Biotech, Germany
X-Digitise microarray image processing software	MPI-MG, Germany
Zeiss Axio Vision	Zeiss, Germany

2.1.5 Solutions

50x Denhardt's reagent: 50g Ficoll / 5g polyvinylpyrrolidone/ 5g BSA/ H_2O to 500 ml	Compounds from Sigma, US
20x SSC (standard saline citrate): 300 mM Sodiumcitrate /3 M NaCl; pH 7.0 or 9.5	Compounds from Sigma, US
PBS (Phosphat Buffered Saline): 1.5 mM KH2PO4/ 140 mM NaCl/ 3 mM Kcl; pH 7.4 or 6.5	Compounds from Sigma, US

2.1.6 Oligonucleotides

Primers used for Conventional PCR, Generation of Riboprobe-and Northern-Templates

Gene symbol	Primer name	Primer sequence 5'→ 3'
	Human	
DPF3	HDPF3_NOR_F2_B	ACTGTCATTCACAACCCCCTG
DPF3	HDPF3_NOR_R2_B	TTCCTGGATGCTTTCCTCCTC
DPF3	HDPF3_E10_F1	AGGTCCGCCTGGAATTACTCT
DPF3	HDPF3_E10_R1	TTACCACATCCAACTCGCAGA
DPF3	HDPF3_E12_F1	GGACAGGACTAGAGGACGGG
DPF3	HDPF3_E12_R1	GCCACTGTTGCTTCCCACTT
DPF3	HDPF3_E9_F1	TTGGTCATTGTGCTGGTTCTG
DPF3	HDPF3_E9_R1	CGGCTTGCCTCTAAACAGGAT
DPF3	HDPF3_F5	CCTGCAGTTTACCCTGAACATG
DPF3	HDPF3_R5	CTTTGAGCAGTTCCCAGCATA
	Mouse	
MLC2V	MLC2V_F	TGTTCCTCACGATGTTTGGG
MLC2V	MMLC2V_R	CTCAGTCCTTCTCTTCTCCG
MLC2V	MMLC2V_T7F1	TAATACGACTCACTATAGGTGTTCCTCACGATGTTTGGG
MLC2V	MMLC2V_T7R1	TAATACGACTCACTATAGGCTCAGTCCTTCTCTTCTCCG
NKX2.5	MNKX-2.5_T7F1	TAATACGACTCACTATAGGCAGTGGAGCTGGACAAAGCC
NKX2.5	MNKX-2.5_T7R1	TAATACGACTCACTATAGGTAGCGACGGTTCTGGAACCA
NKX2.5	MNKX-2.5_F	CAGTGGAGCTGGACAAAGCC
NKX2.5	MNKX-2.5_R	TAGCGACGGTTCTGGAACCA
CFL2	MCFL2_EF2	TCATACACCTCACCTGCCTT
CFL2	MCFL2_ER2	GGGTAGGGAGTTTGATCTCA
CFL2	MCFL2_IR2	TAATACGACTCACTATAGGGGCTCTTTTATACAGAAATTGCCAT
CALU	MCALU_EF2	GGTAAAAGCCCAGTTGTGGTG
CALU	MCALU_ER2	TGCTCACTGCCTCAGACTTGA
CALU	MCALU_IR2	TAATACGACTCACTATAGGCACACCCAAACCCCAACACTAT
TNNI3	MTNNI3_EF1	AAGTCTAAGATCTCCGCCTCCA
TNNI3	MTNNI3_ER1	TTCTTCACCTGCTTGAGGTGG

TNNI3	MTNNI3_T7IR1	TAATACGACTCACTATAGGCCTCAGGTCCAAGGATTCCTT
CALU	MCALU_F1	TTCACAGCTTTCCTGCACCCT
CALU	MCALU_R1	ACTAAGGCCTCCCCGAAATCTG
CALU	MCALU_T7IR1	TAATACGACTCACTATAGGATGAAAGACATAGTCGTGCAGG
CFL2	MCFL2_F1	TTGTTGCCTCTGAATGATTGCC
CFL2	MCFL2_R1	CATGGAACCAGTTTGTTTTGGC
CFL2	MCFL2_T7_IR1	TAATACGACTCACTATAGGTCATGGCAATACTGACAGGCTT
DPF3	MDPF3_E1_13F	GGTAGCAAAATGGCGACTGTC
DPF3	MDPF3_E1_13R	TCTGGGTTGAACAGGGCTTC
DPF3	MDPF3_E111213F	TCATCCAACTTGCCTGCAGTT
DPF3	MDPF3_E111213R	TTTCTCTTTGAGCAGCTCCCA
DPF3	MDPF3_E111213F_T7	TAATACGACTCACTATAGGTCATCCAACTTGCCTGCAGTT
DPF3	MDPF3_E111213R_T7	TAATACGACTCACTATAGGTTTCTCTTTGAGCAGCTCCCA
DPF3	MDPF3_E1-5_F1	AGGAAGCCATTGAGCACTGC
DPF3	MDPF3_E1-5_R1	CTACGGGTTTGATTTCCAGGA
DPF3	MDPF3_E1-5_T7F1	TAATACGACTCACTATAGGAGGAAGCCATTGAGCACTGC
DPF3	MDPF3_E1-5_T7R1	TAATACGACTCACTATAGGCTACGGGTTTGATTTCCAGGA
DPF3	MDPF3_E9_F1	ACTTATTCGGTTCCACGTCAGAAAGT
DPF3	MDPF3_E9_R1	TCAGAACTAGAACAATGACCAGAGTC
DPF3	MDPF3_E9_F2	CGGTTCCACGTCAGAAAGTG
DPF3	MDPF3_E9_R2	CTGTCCCATGTGCTCAGCAG
DPF3	MDPF3_E9_T7F2	TAATACGACTCACTATAGGCGGTTCCACGTCAGAAAGTG
DPF3	MDPF3_E9_T7R2	TAATACGACTCACTATAGGCTGTCCCATGTGCTCAGCAG
DPF3	MDPF3_E10-12_F1	CATCCAACTTGCCTGCAGTTC
DPF3	MDPF3_E10-12_R1	GATAGCCACGATCGCAGTCAT
DPF3	MDPF3_E10-12_F2	CAACTTGCCTGCAGTTCACTC
DPF3	MDPF3_E10-12_R2	CGATCGCAGTCATCACAGAAG
DPF3	MDPF3_E10-12_T7F2	TAATACGACTCACTATAGGCAACTTGCCTGCAGTTCACTC
DPF3	MDPF3_E10-12_T7R2	TAATACGACTCACTATAGGCGATCGCAGTCATCACAGAAG
DPF3	MDPF3_EF1	AAGAGCTGGTGTCCTGTGCAGA
DPF3	MDPF3_ER1	AGGCAGAAGGAAAAGGCAAGG
DPF3	MDPF3_T7IR1	TAATACGACTCACTATAGGGGAAATGCCCAAGTTGTCTTTC
DPF3	MDPF3_NOR_F2_B	ACTGTCATTCACAACCCCCTG
DPF3	MDPF3_NOR_R2_B	GCAAGGCTTCCAGTGTGGTAC

Chicken

DPF3	CDPF3_COM_F1	TCGAACACTGTCGCAGCTACA
DPF3	CDPF3_COM_R1	AGCAGCTTCAGCCTGGAATC
DPF3	CDPF3_COM_R1T7	TAATACGACTCACTATAGGAGCAGCTTCAGCCTGGAATC
DPF3	CDPF3_E11_13F1	GCCTGCAGTTCACCACAAACAT
DPF3	CDPF3_E11_13R1	GAAGCCAAAAGCTGATGCTCTC
DPF3	CDPF3_E11_13R1_T7	TAATACGACTCACTATAGGGAAGCCAAAAGCTGATGCTCTC
DPF3	CDPF3_E9_F1	ATTTGGGAAGAGAAGGCAGGA
DPF3	CDPF3_E9_R1	TGGACAGCAAATTGCTCAGAGT
DPF3	CDPF3_E9_R1_T7	TAATACGACTCACTATAGGTGGACAGCAAATTGCTCAGAGT
DPF3	CDPF3_INS1_F1	GCCCCAGGTCAGCTGTACAC
DPF3	CDPF3_INS1_R1	ACGTAGCAAGGCCTCCAGTG
DPF3	CDPF3_INS2_F1	CCTTGCTACGTGGAGAGGGA
DPF3	CDPF3_INS2_R1	GGGAGGCAGCATCATTTCGT

	Zebrafish	
DPF3	ZDPF3_COMF1	CCAGTTCTACAGGGAGGCCA
DPF3	ZDPF3_COMR1	ACGGAGCTGAGGGTCAAGAG
DPF3	ZDPF3_COMR1_T7	TAATACGACTCACTATAGGACGGAGCTGAGGGTCAAGAG
DPF3	ZDPF3_E9F1	CTGATCGGGTCGGTGGG
DPF3	ZDPF3_E9R1	GAAAGGTGGATGCCTCGCT
DPF3	ZDPF3_E9R1_T7	TAATACGACTCACTATAGGGAAAGGTGGATGCCTCGCT
DPF3	ZDPF3_E11-13F1	CCTGCAGTTCACAGACAACATGA
DPF3	ZDPF3_E11-13R1	CAGATGACAACTCCAACTTCCCT
DPF3	ZDPF3_E11-13R1_T7	TAATACGACTCACTATAGGCAGATGACAACTCCAACTTCCCT
NKX2.5	ZNKX-2.5F1	GTGCTTCAGGCTTTTACGCG
NKX2.5	ZNKX-2-5R1	CCATCTCCAGGGTCTGATCCT
NKX2.5	ZNKX-2-5R1_T7	TAATACGACTCACTATAGGCCATCTCCAGGGTCTGATCCT

Table 2.1. Hugo names used for genes symbols. First character of the primer name corresponds to the species. H-human, M-mouse, C-chicken, Z-zebrafish. An E-corresponds to the exon if it is indicated. F-forward, R-reverse primer. Numbers following to the primer name correspond to the design number of primer. T7 indicates that primer is attached to T7 sequences, NOR indicates the use of the primer for generation of northern blot probes. I- indicates that primer is hemi-nested.

Primers Used in Real-time PCR

Gene symbol	Primer name	Primer sequence 5'→ 3'
	TOF in RV	
DIA1	DIA1_F	GCCCAGCTCAGCACGTTG
DIA1	DIA1_R	GGAGCGCTGGAACAGCTT
DPF3	CERD4_F1	GGCTGCTGGAGATAAAACCTGA
DPF3	CERD4_R1	TTCCTGGATGCTTTCCTCCTC
DPF3/2	H1_h_F6	CGAGGCTGTCAAGACCTACAAG
DPF3/2	H1_h_R6	CGCAGAAGAGTAGCTGGTCATC
DPF3/1	H1_h_F7	GACGATTTGGAAGAGCCTCG
DPF3/1	H1_h_R7	GAGTCTGTTCCGTGGGTTTAGC
FLJ10350	FLJ10350_F	CTCAGTGGAGTCTCCCAAGCAA
FLJ10350	FLJ10350_R	TGTTCGGCTCAGACTCTTGTCC
LOC51189	LOC51189_F	AGAGAGAGCAGGCTGAAGAGGAA
LOC51189	LOC51189_R	GAACGATTTCTTCTTCATGGTGTTT
NDUFB10	NDUFB10_F	CAGACATCACTGAGTGCAAGGAG
NDUFB10	NDUFB10_R	TCTTGGTCGACTTTGTAGTCCCT
NDUFS7	NDUFS7_F	GGAGTTCTCTGTGGCCCATG
NDUFS7	NDUFS7_R	CGGAAGACCACGCCAAAG
NHP2L1	NHP2L1_F	AAGCTACTGGACCTCGTTCAGC
NHP2L1	NHP2L1_R	GTGGCCTCATTGGCTCCTTT
RPL37A	RPL37A_F	CCTCCGGAAAATGGTGAAGA
RPL37A	RPL37A_R	GGTTTTGCCACAGAAAGAGCA

S100A13	S100A13_F	TCACCACCTTCTTCACCTTTGC
S100A13	S100A13_R	CTCTTTGAACTCGTTGACGCTG
SDH4	SDH4_F	TCCTCCCCACCGTGCATTATA
SDH4	SDH4_R	TCAGGACCTGCCCCTTGTAGTT
SYTL2	SYTL2_F	GCGACTCGGAGGTAGGTGAT
SYTL2	SYTL2_R	TGCACCTGTCGTCAATTCATAA
TNNI1	TNNL1_F	TGGATGAGGAGCGATACGACAT
TNNI1	TNNL1_R	GGTCCTTAATCTCCCTGGTGTTG
TNNI3	TNNL3_F	AGAACATCACGGAGATTGCAGA
TNNI3	TNNL3_R	CCGCTTAAACTTGCCTCGAA
VPS35	VPS35_F	CCCAAGGCTTTACCTTTTGATCA
VPS35	VPS35_R	CGGCACATTTCTACCAAATCTTTC
VWF	VWF_F	ACAACAGCCTTGTGAAACTGAAGC
VWF	VWF_R	GCGGAGGTCACCTTTCAGGA
	VSD in RA	
CALU	CALU_F	CGGCTACGTTTTAGATGATCCAG
CALU	CALU_R	TCTCCATCCTTGTCTGCCATTT
CFL2	CFL2_F	CCTCTGAATGATTGCCGATATG
CFL2	CFL2_R	GCACTTTCAGGAGCCCAGAATA
COX6B	COX6B_F	GCTGGCAGAACTACCTGGACTT
COX6B	COX6B_R	CCATTCGCACACAGAGATATCG
GABARAPL1	GABARAPL1_F	GGAAGAGAATCCACCTGAGACCT
GABARAPL1	GABARAPL1_R	TGTCCTCATACAGTTGGCCCAT
GSN	GSN_F	TGAGGAACGGAAATCTGCAGTA
GSN	GSN_R	CACTCATTGCCCAGCCAGTAGT
NDUFB9	NDUFB9_F	ACGATTGCTACAAGGTCCCAGA
NDUFB9	NDUFB9_R	GCTTTCCCTCCGCAGTTTCTT
PIPPIN	PIPPIN_F	ACCAGGACCTATTCAGCGACA
PIPPIN	PIPPIN_R	AACTGCTTACAGACGCCCTTG
	Housekeeping genes	
B2M	B2M_F	TGCTGTCTCCATGTTTGATGTATCT
B2M	B2M_R	TCTCTGCTCCCCACCTCTAAGT
HPRT	HPRT_F	AGGAAAGCAAAGTCTGCATTGTT
HPRT	HPRT_R	GGTGGAGATGATCTCTCAACTTTAA

Table 2.2. Primers were used in Real-time assays. Gene symbol corresponds to HUGO symbols. F-forward, R-reverse.

2.2 Total RNA and mRNA Extraction

Total RNA was extracted from a variety of human heart tissue biopsies and HEK293 cells using TRIzol reagent, which is based on the acidic phenol and guanidium method (Chomczynski et al. 1987), according to the manufacturer's protocol with minor changings. Maximum yield was reached by 2 x 2 minutes homogenisation of tissue samples. For Real-time PCR assays, northern blotting and cloning experiments, additional DNase I treatment was performed and documented by agarose gel electrophoresis. All samples were quantified using OD260/280nm measurement. For mRNA purification total RNA samples were used as input for the NucleoTrap polyA RNA purification kit according to the recommended protocol.

2.3 Cloning of DNA Fragments

For *in situ* hybridisation and northern blot assays, gel-purified PCR products were cloned into the TOPO TA vector containing M13, T7 and SP6 promoters, transformed into electrocompetent TOP10 cells according to the manufacturers´ protocol and all clones were sequenced following plasmid isolation.

2.4 HEK293 Cell Culture

The HEK293 cell line was derived from human embryonic kidney cells. First, the D-MEM Medium/10 % FBS was pre-warmed at 37°C and filled into two culture flasks (25 ml for 150 cm^2, 5 ml for 25 cm^2 flasks). Secondly, rapidly thawed cells (at 37°C) were distributed in two concentrations in the flasks, medium was changed after 12 hrs or once the cells had attached. When confluency had being reached after 2-3 days, cells were passaged to new flasks with fresh medium after trypsinisation. HEK293 cells were used for total RNA isolation following aspiration of the medium from the cells and washing with PBS.

2.5 Complex Hybridisation with cDNA Membranes

2.5.1 Oligo Hybridisation

Complex-hybridisations were performed with the Human Unigene Set II array membranes (RZPD). Two separate PCR batches were generated and products spotted as duplicates to three separate membranes. To check the quality of the array membranes,

hybridisation with M13 oligonucleotides common for each spot were performed. M13 oligos were 5´ labelled using the following protocol: 6.7 µl ddH_2O, 1 µl M13F primer (100 pmol/µl), 1.2 µl 10x T4 Polynucleotide kinase buffer, 2.5 µl γ^{33}P-dATP 10mCi/ml , 0.6 µl 10 U/µl T4 Polynucleotide kinase were incubated for 30 min. at +37 °C. The reaction solution containing the labelled oligos was added to 10 µl SSARC (4x SSC/ 7.2 % Sarcosyl/ 4 mM EDTA pH=8) solution for an overnight hybridisation at +4 °C. The following day, membranes were rinsed twice and washed 2x 20 min with SSARC buffer.

2.5.2 Complex Hybridisation

To gain optimal intensity values, different hybridisation conditions were tested using 5 µg and 8 µg total RNA, several overnight incubation and exposure times. Pre-hybridisations and hybridisations were performed in custom-made hybridisation bottles (h=29 cm, r=4 cm) to ensure optimal wetting of the membrane. Direct labelling and first strand synthesis was carried out using AMV reverse transcriptase (AMV-RT). Initially 8 µg total RNA were denatured with 1 µl (0.5 µg/µl) $pd(T)_{12-18}$ in a final volume of 10 µl DEPC treated H_2O at +70 °C for 10 min., chilled on ice, mixed with 5 µl 5x AMV-RT buffer, 1.25 µl 10 mM dATP:dTTP:dGTP mix, 1 µl dCTP 12.5 µM, 0.6 µl 40 U/µl recombinant RNasin ribonuclease inhibitor, 3 µl 10 mCi/ml α^{33}P-dCTP and 3 µl 10 U/µl AMV-RT in a total volume of 25 µl. This mix was incubated for 10 min +30 °C, 40 min +42 °C and 10 min +51 °C. Following the labelling reaction unincorporated nucleotides were removed using ProbeQuant G-50 micro columns.

The labelled cDNA was denatured at +95 °C for 10 min, chilled on ice. Hybridisation solution was prepared simultaneously and consists of denatured 50 µl 10 mg/ml salmon sperm DNA, 1 µl 1µg/µl placenta DNA and 10 µl 500 ng/µl $pd(A)_{40}$ in a total volume of 10 ml pre-warmed (+65 °C) hybridisation buffer (1 M NaCl:1 % SDS:10 mM Tris-HCl). Following 2-3 h of pre-hybridisation of the array membrane at +65 °C, the pre-hybridisation solution (1M NaCl:1 % SDS:10 mM Tris-HCl) was replaced by the hybridisation solution containing labelled probe and blocking components and incubated 16 h at +65 °C in a hybridisation oven. Then array membranes were first rinsed and washed ones with 150 ml 2x SSC:0.1 % SDS in hybridisation bottles at +65 °C, followed by two washes using 200 ml 0.1x SSC:0.1 %SDS in horizontal boxes at +65 °C in a shaking oven. Finally, the membranes were partially dried on Whatman

paper and exposed for 24 hours to phosphoimaging screens. The Phosphoimaging screens were scanned in a Fuji Film Bas-1800 with 16-bit/pixel resolution, converted to 'tiff' file format by Tina-v2.10i and processed employing the X-Digitise microarray image processing software.

2.5.4 Statistical Analysis

Data Pre-processing

Data normalisation was performed essentially according to (Beissbarth et al. 2000). After local background subtraction, all intensity values below 200 were set to 200. The intensities of each hybridisation were then scaled to a constant sum. As the hybridisations were performed on arrays from two different production batches, which significantly affected the measurements, a correction for the influence of the production batch was necessary. We considered two virtual reference hybridisations defined as the spot-wise medians of 10 hybridisations from the respective array batch. These two sets of hybridisations shared equal phenotype profiles of the tissue samples. The natural logarithms of ratios between the intensities of each hybridisation and those of the respective batch-specific virtual reference hybridisation were shifted such that the median over the 40 % of spots with highest average log-intensity became 0. Finally, the obtained log-ratio values were averaged over duplicate spots per clone. All further statistical analysis was performed on the data from 8069 clones whose intensities were among the 15 % highest in at least 4 hybridisations and whose log-ratios had a standard deviation over the samples of at least 0.5.

Identification of Differentially Expressed Genes

To identify genes whose transcription is associated with particular phenotypes, we subjected the normalised expression levels of each clone to a linear model of the following form:

$y_{hijk} = \mu + A_h + S_i + T_j + D_k$, where A_h is the effect of age h (young or old), S_i the effect of the patient's sex, T_j the effect of tissue j (right or left ventricle, right or left atrium) and D_k the effect of disease status k (ASDII, TOF, VSD+PHT, VSD+PAS, DORV, normal). The models were fitted with the least squares method using the function lm() in the statistical software R, with the constraints that the coefficients for the different levels of each factor sum up to 0. For all coefficients of interest, the null hypothesis of their being equal to 0 was tested under the assumption of normally distributed errors.

To assess the significance of the results without making distributional assumptions, we used a permutation scheme (Tusher et al. 2001): The linear models were fitted for 500 random permutations of the sample labels. For each coefficient of interest, the mean number of significant genes ($P < 0.01$) per randomised data set provides an estimate of the number of false positives among the significant genes. Furthermore, the proportion of randomised data sets leading to larger sets of significant genes than the original one gives a p-value for the global null hypothesis that the factor level has no influence on the gene expression patterns.

For the comparison between normal right and left ventricle tissue, where samples from the same patients were analysed, t-tests for paired observations were performed.

Multivariate Analysis

To analyse which phenotypical differences among the tissue samples are most pronounced in terms of gene expression patterns, we used the class discovery method ISIS (von Heydebreck et al. 2001) with default parameters. ISIS searches for binary class distinctions among the set of tissue samples, which are characterised by clear differential expression of a corresponding subset of genes.

Correspondence analysis (Fellenberg et al. 2001) was used in order to visualise the association of gene categories with certain tissue phenotypes. The analysis was performed on the contingency table of numbers of significantly differentially expressed genes ($P < 0.05$) per gene category and phenotype comparison.

2.6 Real-time PCR Assays

Real-time PCR assays were carried out using SYBR Green I PCR Master Mix and the ABI PRISM 7900HT Sequence Detection System. Since SYBR Green I is an intercalating dye binding to double stranded DNA in an unspecific manner (Figure 2.1), intron-spanning primers were designed in order to exclude amplification of genomic DNA by using Primer Express software according to the considered specifications recommended by Applied Biosystems (Table 2.2).

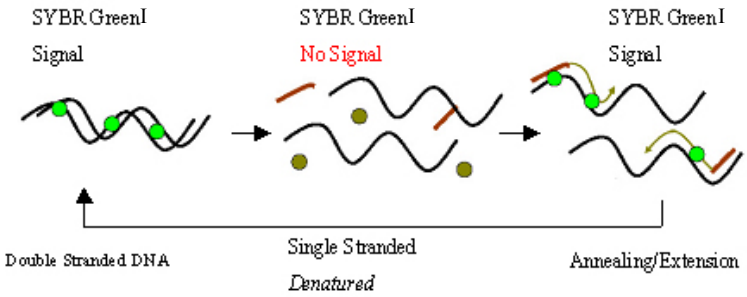

Figure 2.1. SYBR Green I read out during the Real-time PCR reaction.

For Real-time PCR assays, RT reactions were carried out using AMV-RT with random hexamers. Reaction was as follows: Initially, 1 µg total RNA was denaturated with 4 µl 5 mM dNTPs, 1µl 1.1925 µg/µl $pd(N)_6$ in the end volume of 10 µl DEPC treated dH_2O for 10 min at +65 °C, chilled on ice for 5 min, then 4 µl 5x AMV-RT buffer, 4 µl 25 mM $MgCl_2$, 0.5 µl 40 U/µl Recombinant RNasin Ribonuclease Inhibitor and 1.5 µl 10 U/µl AMV-RT were added to the end volume of 20 µl, incubated 1 h at 42 °C.

Real-time PCR reaction was performed in a final volume of 25 µl containing 12.5 µl 2x SYBR Green I PCR master mix (Applied Boisystems), 1.5µl 5 mM forward primer, 1.5 µl 5 mM reverse primer and cDNA corresponding to 50 ng RNA.

To normalise the different cDNA samples we used HPRT and calculated standard curves using dilution series of cDNA corresponding to 50 ng, 25 ng, 12.5 ng and 6.25 ng RNA. The following PCR conditions were used: 2 min +50 °C, 10 min +95 °C; 40/45 cycles of 15 sec +95 °C and 1 min +60 °C. Ct (threshold cycle) values of amplification plots were collected using SDS 2.0 software. A dissociation curve was performed by a temperature gradient from 15 sec +60 °C to 15 sec +95 °C with a ramp rate of 2 % to determining nonspecific amplification and primer dimers which may effect the specificity of amplification data. Relative quantification was done using the $\Delta\Delta C_T$ method (Livak et al. 2001). Exported C_T values were processed by Excel. Fold changes of diseased versus normal hearts and the statistical power using one-tailed student t-test were calculated.

2.7 Northern Blot Hybridisation

Labelling of DNA for Northern blot hybridisation: Random labelling was performed using the Rediprime-II random prime labelling system (Amersham biosciences). 25 ng of target DNA were diluted in 45 µl dH_2O, denatured for 7 minutes at 96 °C, snap

cooled on ice for 10 minutes. Denatured DNA was added to the Amersham labelling reaction mix^{R} (Klenow fragments/dNTPs/random hexamers), then 5 µl Redivue α^{32}P-dCTP [3000 Ci/mmol] were added and the mix was incubated for 1 hour at 37°C. The reaction was stopped using 2 µl 0.5 M EDTA. Unincorporated nucleotides were removed using either ProbeQuant G-50 micro columns or micro spin S-200 HR columns. Columns were vortexed, prespinned 1 min at 720 g, samples were loaded to the columns and spinned again 2 at 800x g. The elute was collected, 1 µl sample was dried on a 1 cm^2 membrane which was used for measurement of specific activity in a scintillation counter. Probes were diluted if their concentrations were higher than 2-10 ng/ml or 1-2x10^{6}cpm/ml (BD biosciences ref: PT1200-1). Then each probe and sheared Salmon sperm DNA were denatured at 96 °C for 5 min and chilled on ice for 5 minutes prior to hybridisation.

Blots were first prehybridised at 68 °C for 1 hour in 5 ml Expresshyb hybridisation solution (Clontech), then hybridised at 68°C (5 ml prewarmed Expresshyb hybridisation solution, 10 µl mg/ml sheared Salmon sperm DNA and 50 µl labelled cDNA probe with concentration of 1-2x 10^6 cpm/ml) for 3 hours to overnight with continuous rolling.

After hybridisation membranes were rinsed 4x and washed 3x 10 minutes with wash-I solution (2x SSC:0.05 % SDS) with continuous rolling at room temperature; then washed 2 x 20 minutes with wash-II solution (0.1x SSC:0.1 % SDS) at 50-55 °C in boxes with a continuous shaking and first exposed overnight to a phosphoimager screen and afterwards to a radiographic film. After imaging, blots were stripped with 0.5%SDS for 10 minutes at 95°C, cooled down for 10 minutes at room temperature in the same solution, semidried on 3M filter and re-exposed to a phosphoimager screen to check the stripping efficiency. Finally, until reuse the membranes were stored at 4 °C. To control for RNA loading blots were hybridised with β-actin cDNA probe (Clontech) after target hybridisations.

2.8 *In situ* Hybridisation of Mouse, Chicken and Zebrafish Embryos

2.8.1 Preparation of Embryos

Mouse and chicken embryos were dissected in 1x PBS and fixed in 4 % PFA in PBS overnight. Zebrafish embryos were dechorionated either manually or enzymatically with 0.5 mg/ml pronase (Westerfield 1993), washed 3 times with PBS, then fixed overnight

as mouse and chicken embryos. The next day, they were dehydrated in a series of methanol:PBS and stored in 100 % methanol at –20°C until use.

2.8.2 Preparation of Riboprobes

Riboprobes were synthesised from the DNA template either from a heart specific cDNA or vector-cloned fragment. Templates were sequenced using the BigDye Terminator Chemistry (PE Biosystems) prior to riboprobe synthesis. To obtain sufficient amounts of template, first round PCRs were carried out with gene specific primers for the desired region of the gene and purified via gel, the second round of PCR was performed using either T7 attached to the same primers or T7 attached to heminested primers as illustrated in Figure 2.2. The riboprobes were purified by NaAc precipitation (Sambrook et al. 2001).

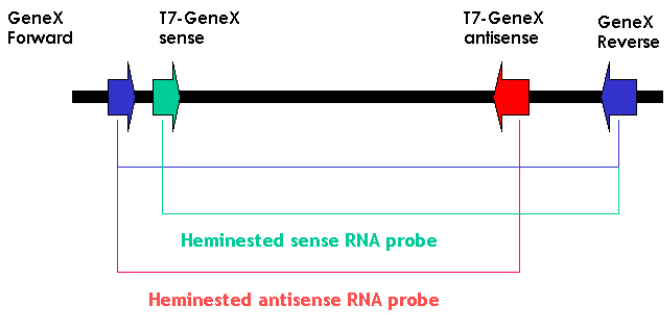

Figure 2.2. Strategies for preparation of fast and high yield ISH probes.

Reagents	Volume
DNA template	1 µg
10x Transcription Buffer	2 µl
10 mM ATP	1µl
10 mM CTP	1µl
10 mM GTP	1µl
10 mM UTP	0.6 µl
10 mM UTP*labelled	0.4 µl
T7 RNA Polymerase	2µl
Nuclease free H_2O to	20 µl

Table 2.3. Typical in vitro transcription reaction

After in vitro transcription (Table 2.3), products were precipitated using 3M LiCl and 100 % ethanol overnight at –80 °C, spun for 20 minutes at 15000x g, washed 2 times

with 70 % ethanol, resuspened in 100µl of either 1 mM sodiumcitrate pH 6.4 or 50% deionised formamide for stabilizing the RNA and quantified using an agarose gel prior to storage at -20°C.

2.8.3 Whole Mount *in situ* Hybridisation

First day: Embryos were rehydrated at 4 °C shaking through a 80 %, 60 %, 40 %, 20 % methanol/PBT series, spending 20 min in each methanol concentration and washed 3x in PBT at room temperature. Zebrafish embryos rehydrated through 75 %, 50 %, 25 % methanol/PBT. Embryos were bleached with 6 % H_2O_2 for 1 h at room temperature, and then washed 3x with PBT. Furthermore they were treated with 10 µg/µl Proteinase K in PBT with appropriate duration (Table 2.4), washed in 2 mg/ml glycine:PBT solution for 10 minutes, post fixed in 4 % PFA:02 % glutaraldehyde in PBT for 1 hour and rinsed 3x 5 minutes and washed again 3 times 20 minutes in PBT.

Mouse		**Chick**		**Zebrafish**	
7.0-7.5 dpc	4-5 min	HH4	4-5 min	5hhpf	4 min
8.0-8.5 dpc	7 min	HH8	7 min	up to 12 hpf	6 min
9.0-9.5 dpc	9 min	HH10	9 min	up to 25 hpf	8 min
10.0-10.5 dpc	12 min	HH16	12 min	up to 33 hpf	10 min
11.0-11.5 dpc	16 min	HH18	14 min	up to 55 hpf	13-15 min
12.0-12.5 dpc	20 min	HH20	16 min		

Table 2.4. Prot-K [10 µg/µl] treatment for whole mount *in situ* hybridisations..

After fixation, embryos were transferred to cryo tubes and incubated for 2-3 hours at 68 °C in 1 ml hybridisation buffer. After prehybridisation, the buffer was replaced with fresh prewarmed hybridisation buffer containing 5 µl denatured RNA probe and embryos were incubated overnight at 68 °C with gentle shaking.

Second day: Mouse and chicken embryos were washed 3 x 1 hour with prewarmed hybridisation buffer at 68 °C and transferred to cell culture plates with a membrane insert for easy handling in further steps, washed with prewarmed hybridisation (Table 2.5) solution for 30 minutes and exchanged with hybridisation buffer mixture containing 1:1 MABT for another 30 minutes with continuous shaking at room temperature to cool down. Embryos were then rinsed 4 x 5 minutes and washed 3 x 1 hour with MABT. Following post hybridisation washes, embryos were blocked in 3%

BBR in MABT for 1 hour, replaced with 3 % BBR/ 20 % heat inactivated sheep serum in MABT for another 2 hours. In the meantime, 1/2000 to 1/3000 diluted anti-digoxigenin- alkaline phosphatase antibody -depending on the stage of embryos- was absorbed in 3 % BBR/ 20 % heat inactivated sheep serum in MAB solution at 4 °C, then 0.1 % Tween20 was added to a final solution and exchanged for overnight incubation with slow shaking.

Third day: Embryos were rinsed 3x in MABT and washed 8x 1 hour with MABT on a rocking shaker with Tween20 concentration increased to 0.5 %. For mouse whole mounts, washes were carried on the fourth and fifth day with two changes; for chick whole mounts washes were limited to the fourth day and zebrafish washes were limited to the third day. After overnight washes with MABT at 4 °C, embryos were moved from MABT to NTMT with two changes of MABT:NTMT and washed 4x with NTMT 30 minutes each to reach the appropriate pH for the alkaline phosphatase reaction during staining. Embryos were then transferred into staining buffer. After appropriate staining, the reaction was stopped by fixing with 4 % PFA/ 0.1 % glutaraldehyde in PBT pH=6.5 for 1 hour and embryos were stored in 4 % PFA in PBS at 4 °C.

Reagents	**Final volume 10 ml**	**Final concentration**
100% Formamide	5 ml	50 %
10xSSC pH4.5	2.5 ml	2.5x
10µg/µl tRNA	50 µl	50 µg/ml
10%SDS	1 ml	1 %
50mg/ml Heparin-Li salt	50 µl	250 µg/ml
CHAPS	50 mg	0.5%
EDTA 0.5M EDTA pH8.0	5 µl	0.5 mM
Tween20	10 µl	0.1 %

Table 2.5. Whole mount *in situ* hybridisation buffer

2.8.4 *In situ* Hybridisation on Sections

Non-Radioactive *in situ* Hybridisation on Sections

After dissection of stage HH25 (Sanes 1992) embryos, they were directly embedded to OCT compound (Tissue-Tek), frozen and sectioned at 15µm thickness.

In situ hybridisation was carried out using the Tecan *in situ* hybridisation robot with the recommended commercial hybridisation buffer. The hybridisation protocol was programmed as in Table 2.6.

Cycles	Volume µl	Time	Reagent	Temperature
First day				
5	250	6 min	0.6 %H_2O_2 in MeOH	24 °C
8	300		PBS	↓
2	300	4 min	0.2 M HCl	↓
4	300		PBS	↓
2	300	8 min	Proteinase K	↓
8	250		PBS	↓
2	300	10 min	4% PFA	↓
8	300		PBS	↓
2	200		Hybridisation mix	65 °C
1		6 h	Hybridisation mix + riboprobe	66 °C
Second day				
4	300	5 min	5x SSC	63 °C
6	350	8 min	Formamide I	64 °C
4	350	15 min	Formamide II	↓
5	300	6 min	0.1x SSC	↓
3	300	2 min	NTE	22 °C
3	300	5 min	20 mM iodoacetamide	↓
4	300	2 min	NTE	↓
4	250	20 min	4% sheep serum	↓
9	200	9 min	TNT	↓
3	250	20 min	TNB blocking buffer	↓
2	200	45 min	AntiDIG-POD (1:600)	↓
8	200		TNT	↓
1	250	30 min	Tyramide-biotin	↓
8	300		Maleate wash buffer	↓
2	200	30 min	NeutrAvidine (1:750)	↓
8	300		Maleate wash buffer	↓
8	200		TNT	↓
2	200	2 min	TMN	↓
2 or 3	200	20 min	NBT/BCIP	↓
4	250		H_2O	↓
2	200	1 min	TNT	↓
2	200	7.5 min	4% PFA + 1% Gluteraldehyde	↓
2	300		PBS	↓
4	200		H_2O	↓

Table 2.6. Section *in situ* hybridisation with chick DPF3 probe with Tecan *in situ* robot

Radioactive *in situ* Hybridisation on Sections

Embryos were fixed overnight at 4 °C in 4 % PFA following dissection. The next day, they were processed through 70 % and 100 % ethanol at 2 hours each, then embedded into paraplast with 56 °C melting and sectioned 12 µm.

Reagents	Final volume10 µl
template DNA [1µg/µl]	0.5 µl
10x Transcription buffer	1 µl
NTPs (A,C,G) 5 mM	1 µl
RNAse-Inhibitor (RNAsin)	0,5 µl
T7 RNA-Polymerase	0,5 µl
α^{33}P-UTP (Amersham)	2 µl

Table 2.7. Typical radioactive in vitro transcription reaction

The labelling reaction was carried out as described in table 2.7. The reaction was then incubated for 1 hour at 40 °C, DnaseI treated for 20 minutes at 37 °C, and precipitated with the following mixture: 80 µl H_2O, 10 µl 4M $LiCl_2$, 1 µl Glycogen, 250 µl 100 % ethanol), incubated at –20 C° for 30 minutes to overnight, spun for 20 min at 14000 rpm at 4 °C, washed with 80 % ethanol, dried, resuspended in 25 µl H_2O. 0.5 µl of riboprobe was added to 1 ml hybridisation buffer, then the mixture was denatured and chilled on ice.

Reagents	Final volume 10 ml	Final concentration
100 % formamide	5 ml	50 %
5 M NaCl	1.8 ml	3 M
1 M Tris-HCL pH 7.4	0.2 ml	20 mM
0.5M EDTA pH8.0	0.1 ml	5 mM
1 M NaH_2PO_4-H_2O pH 8.0	0.1 ml	10 mM
40 % Dextran sulphate	2.5 ml	10 %
50 % Denhardts	0.2 ml	1x
10 mg/ml Yeast RNA	0.5 ml	0.5 mg/ml

Table 2.8. Radioactive section *in situ* hybridisation buffer

Sections were incubated 2x 5 min in Xylene, 1 min each in the following ethanol series: 100 %, 100 %, 90 %, 70 %, 50 %, 30 %, washed with H_2O (Millipore), washed 2x 2 minutes PBS, treated with Proteinase K-Sol. (10µg/ml in PBS) 3 minutes, fixed 10 minutes 4 % PFA in PBS), washed again 2x 2 minutes in PBS, then dehydrated in an ethanol series 30 %, 50 %, 70 %, 90 %, 100 %, 100 % for 2 minutes at each step and air dried. Then 50 µl riboprobe were applied to each section covered with coverslips and hybridised (Table 2.8) overnight at 69 °C in moist chamber containing paper towels soaked with formamide/4x SSC (v/v).

Second day: Sections were first washed for 30 minutes at 55°C in 5x SSC, then washed 30 minutes at 55°C in 2x SSC. Sections were subsequently treated with 0.02mg RNAse A for 30 minutes at 37 °C: 150 µl RNAse A in 150 ml RNAse-buffer (RNAse-Stock: c = 10 mg/ml), washed 30 minutes at 55 °C in 2x SSC/50 % Formamid and 2 x 30 minutes with 2x SSC at 55 °C. Sections were dehydrated in an ethanol series, air dried and exposed overnight to an X-ray film. The next day, slides were dipped in prewarmed Kodak fotoemulsion solution in a dipping-cuvette for 30-60 minutes at 42 °C in water bath in darkness. Subsequently, they were incubated for one week in lightproof boxes, developed for 5 min with cooled Kodak developer at 14°C, washed briefly with H_2O and fixed in Kodak fixer for 20 minutes. After that, slides were washed overnight with H_2O, fotoemulsion was scratched off from back of the slides and stained for 5 minutes in Toluidine Blue (0.5% TB in H_2O). After counterstaining, slides were dehydrated in an ethanol with: 30% 30 seconds, 50% 20 seconds, 70 %, 90 % und 2 x 100 % (each ca. 10 seconds), treated with xylene for 20 minutes and mounted with Entellan to coverslips.

2.9 Retinoic Acid Treatment of Cultured Chicken Embryos

Fertile unincubated chicken (white leghorn) eggs were obtained from commercial sources (Tierzucht Lohman GmbH). Eggs were incubated at 37 °C and embryos were harvested at indicated stages. Culture medium was prepared as described in (Chapman et al. 2001). Dissections were carried out very carefully using a filter paper technique – in case only 0.9 % NaCl has been used for cleaning- and incubated first for 2 hours without any treatment. After the initial incubation, fresh 200 µl 10^{-4} M all-*trans*-RA was indirectly introduced to embryos (Hochgreb et al. 2003) in darkness. After 12 hours overnight incubation, a second all-*trans*-RA treatment was made as mentioned above and embryos were incubated another 12 hours. Then they were removed from the medium, briefly cleaned and fixed in 4 % PFA in PBS overnight and dehydrated to 100 % methanol and stored in -20 °C.