Functional Analysis

of Heat Shock Protein HSPA4

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von
Amal Zohir Abo-Zeid Barakat
aus Kairo, Ägypten

Göttingen, 2010
D 7
Referent: Prof. Dr. med. Dr. h.c. W. Engel
Korreferentin: Prof. Dr. S. Hoyer-Fender
Tag der mündlichen Prüfung:
To my husband Mahmoud, for her patience and love

To my family for their moral support

To my friends for their support

To my supervisors for their valuable advices
# TABLE OF CONTENTS

## CONTENTS

ABBREVIATIONS

1. INTRODUCTION
   1.1 The heat shock protein family: the very short overview
   1.2 The heat shock protein family 110 (HSP110)
   1.3 Expression and function of \textit{Hspa4}
   1.4 Aims of the study

2. MATERIAL AND METHODS
   2.1 Materials
   2.1.1 Chemicals
   2.1.2 Solutions, buffers and media
   2.1.3 Laboratory materials
   2.1.4 Sterilisation of solutions and equipments
   2.1.5 Media, antibiotics and agar-plates
   2.1.5.1 Media for bacteria
   2.1.5.2 Antibiotics
   2.1.5.3 IPTG/X-Gal plate
   2.1.6 Bacterial strains
   2.1.7 Plasmids
   2.1.8 Synthetic oligonucleotides
   2.1.8.1 Primers used for generation of cDNA probes
   2.1.8.2 Genotyping primers
   2.1.8.3 Real time PCR primers
   2.1.9 Mouse strains
   2.1.10 Antibodies
   2.1.11 Enzymes
   2.1.12 Kits
   2.1.13 Equipment
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 Methods</td>
<td>18</td>
</tr>
<tr>
<td>2.2.1 Isolation of nucleic acids</td>
<td>18</td>
</tr>
<tr>
<td>2.2.1.1 Small-scale isolation of plasmid DNA</td>
<td>18</td>
</tr>
<tr>
<td>2.2.1.2 Isolation of genomic DNA from tissue samples</td>
<td>18</td>
</tr>
<tr>
<td>2.2.1.3 Isolation of total RNA from tissue samples</td>
<td>18</td>
</tr>
<tr>
<td>2.2.2 Determination of nucleic acid concentration</td>
<td>19</td>
</tr>
<tr>
<td>2.2.3 Gel electrophoresis</td>
<td>19</td>
</tr>
<tr>
<td>2.2.3.1 Agarose gel electrophoresis of DNA</td>
<td>19</td>
</tr>
<tr>
<td>2.2.3.2 Agarose gel electrophoresis of RNA</td>
<td>20</td>
</tr>
<tr>
<td>2.2.4 Purification of DNA fragments from agarose gel</td>
<td>20</td>
</tr>
<tr>
<td>2.2.5 Enzymatic modifications of DNA</td>
<td>21</td>
</tr>
<tr>
<td>2.2.5.1 Digestion of DNA using restriction enzymes</td>
<td>21</td>
</tr>
<tr>
<td>2.2.5.2 Ligation of DNA fragments</td>
<td>21</td>
</tr>
<tr>
<td>2.2.6 Transformation of competent bacteria</td>
<td>21</td>
</tr>
<tr>
<td>2.2.7 Polymerase chain reaction (PCR)</td>
<td>22</td>
</tr>
<tr>
<td>2.2.7.1 PCR amplification of DNA fragments</td>
<td>22</td>
</tr>
<tr>
<td>2.2.7.2 Genotyping of knockout mice by using PCR</td>
<td>22</td>
</tr>
<tr>
<td>2.2.7.3 Reverse transcription PCR (RT-PCR)</td>
<td>23</td>
</tr>
<tr>
<td>2.2.7.3.1 DNase I digestion</td>
<td>23</td>
</tr>
<tr>
<td>2.2.7.3.2 Reverse transcription technique</td>
<td>24</td>
</tr>
<tr>
<td>2.2.7.4 Quantitive Real-Time PCR</td>
<td>24</td>
</tr>
<tr>
<td>2.2.8 Protein and biochemical methods</td>
<td>25</td>
</tr>
<tr>
<td>2.2.8.1 Isolation of total protein from mouse tissues</td>
<td>25</td>
</tr>
<tr>
<td>2.2.8.2 Isolation of total protein from cell culture</td>
<td>25</td>
</tr>
<tr>
<td>2.2.8.3 Determination of protein concentration</td>
<td>25</td>
</tr>
<tr>
<td>2.2.8.4 SDS-PAGE gel for separation of proteins</td>
<td>25</td>
</tr>
<tr>
<td>2.2.9 Blotting techniques</td>
<td>26</td>
</tr>
<tr>
<td>2.2.9.1 Northern blotting of RNA</td>
<td>26</td>
</tr>
<tr>
<td>2.2.9.2 Western blotting of protein</td>
<td>26</td>
</tr>
<tr>
<td>2.2.9.3 Incubation of protein –bound membranes with antibodies</td>
<td>27</td>
</tr>
<tr>
<td>2.2.10 “Random Prime” method for generation of $^{32}$P labeled DNA</td>
<td>27</td>
</tr>
</tbody>
</table>
2.2.11 Hybridisation of nucleic acids ........................................... 28
2.2.12 DNA sequencing ............................................................. 28
2.2.13 Histological techniques ..................................................... 29
2.2.13.1 Tissue preparation for paraffin-embedding ....................... 29
2.2.13.2 Sections of the paraffin block ........................................ 29
2.2.13.3 Immunofluorescence staining ......................................... 29
2.2.13.4 Immunocytochemical staining of germ cell suspension ...... 30
2.2.13.5 Hematoxylin-eosin (H&E) staining of histological sections ... 30
2.2.13.6 Apoptosis detection ....................................................... 30
2.2.14 Masson's Trichrome staining ............................................. 31
2.2.15 Tissue preparation for electron microscopy ......................... 31
2.2.16 Echocardiogram .............................................................. 32
2.2.17 Microarray analysis ........................................................ 32
2.2.18 Computer analysis .......................................................... 33

3. RESULTS .................................................................................. 34

3.1 Analysis of Hspa4- deficient mice on the hybrid C57BL/6J x 129/SV genetic background ................................................................. 34
3.1.1 Impaired progression of the first wave of spermatogenesis in juvenile Hspa4-deficient mice ................................................................. 34
3.1.2 Immunohistochemical analysis of Hspa4 -/- testes ....................... 35
3.1.3 Increase of apoptotic germ cells in Hspa4 -/- testes ....................... 40
3.1.4 Expression analysis of germ cell marker genes in Hspa4-deficient testes ......................................................................................... 41
3.1.5 Expression analysis of other members of HSP110 family in Hspa4 -/- testis ......................................................................................... 41
3.2 Hspa4-deficient mice with the inbred 129/Sv genetic background display postnatal growth retardation ......................................................... 45
3.2.1 Metabolic state in Hspa4-deficient mice ..................................... 45
3.2.1.1 Measurement of glucose levels in sera of Hspa4-deficient mice ............................................................................................. 45
3.2.1.2 Expression of Pepck in liver of fasted Hspa4 -/- mice ............... 46
3.2.2 Is growth retardation due to malabsorption of lipids in intestine? . 47
3.2.3 Expression analysis of apolipoprotein B and AIV in intestine and liver during postnatal development of Hspa4<sup>−/−</sup> mice .................................................. 48
3.2.4 Growth hormone (GH) signaling is not affected in Hspa4<sup>−/−</sup> mice .... 48
3.2.5 Analysis of skeletal muscles of Hspa4- deficient mice ....................... 51
3.2.5.1 Hspa4-deficient mice display skeletal muscle myopathy ............. 51
3.2.5.2 Skeletal muscle myopathy develops during early postnatal development .............................................................. 51
3.2.6 Analysis of the heart in Hspa4-deficient mice ................................. 55
3.2.6.1 Development of cardiac hypertrophy in Hspa4-deficient mice....... 55
3.2.6.2 Echocardiographic measurements in wild type and mutant mice...... 60
3.2.6.3 Ultrastructural analysis of sections Hspa4<sup>−/−</sup> heart ................ 60
3.2.6.4 Analyses of molecular markers of cardiac hypertrophy ............... 62
3.2.6.5 Upregulation of fibrosis marker genes in heart of Hspa4<sup>−/−</sup> mice .... 62
3.2.6.6 Molecular pathways in the regulation of cardiac hypertrophy ....... 66
3.2.6.6.1 Analysis of STAT3 and MAPK signaling in heart of Hspa4<sup>−/−</sup> mice. 66
3.2.6.6.2 Genes involved in calcineurin/NFAT pathway are upregulated in heart of Hspa4<sup>−/−</sup> mice ................................................................. 69
3.2.6.7 Microarray analysis of cardiac gene expression ........................... 71
3.2.6.8 Quantitative real-time PCR analysis ....................................... 80
3.2.7 Expression analysis of HSPA4L and HSPH1 in Hspa4-deficiency ...... 84
3.2.8 The effect of aging and oxidative stress on expression of HSPA4 ..... 85

4. DISCUSSION ..................................................................................... 88

4.1 Overview of results of this study .................................................. 88
4.2 The role of HSPA4 for germ cell development ................................. 91
4.3 Potential role of Hspa4 in regulation of cell cycle ............................ 94
4.4 The cause of growth retardation in Hspa4-deficient mice ................. 96
4.5 Role of Hspa4 in skeletal muscle development ............................... 97
4.6 Development of cardiac hypertrophy in Hspa4-deficient mice .......... 99
4.6.1 MAPK/ERK signaling pathway ................................................ 101
4.6.2 IL-6-gp130-STAT3 signalling pathway ..................................... 103
4.6.3 Calcineurin/NFAT signalling pathway ....................................... 103
Table of contents

4.6.4 Cardiac fibrosis in Hspa4−/− mice ......................................................... 108
4.6.5 Role of heat shock in heart protection .................................................. 110
4.6.6 Expression profiles of Hspa4−/− heart .................................................. 110

5. SUMMARY ......................................................................................... 114

6. REFERENCES .................................................................................... 116

ACKNOWLEDGEMENTS ........................................................................

Curriculum vitae ......................................................................................
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI</td>
<td>Applied Biosystem Instrument</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosin triphosphate</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Tools</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Cy3</td>
<td>indocarbocyanine</td>
</tr>
<tr>
<td>dATP</td>
<td>Desoxyriboadenosintphosphate</td>
</tr>
<tr>
<td>dH2O</td>
<td>distil Water</td>
</tr>
<tr>
<td>DAPI</td>
<td>Diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>dCTP</td>
<td>Desoxyribo cytosintriphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotidetriphosphate</td>
</tr>
<tr>
<td>dpc</td>
<td>day post coitus</td>
</tr>
<tr>
<td>dT</td>
<td>deoxothymidinate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>gm</td>
<td>gram</td>
</tr>
<tr>
<td>HEPS</td>
<td>N-(-hydroxymethyl)piperazin, N'-3-propansulfoneacid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>hr(s)</td>
<td>hour(s)/IGL</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-B-thiogalactopyranoside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>JL</td>
<td>Jackson Laboratory</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>M</td>
<td>molarity</td>
</tr>
<tr>
<td>ML</td>
<td>Molecular layer</td>
</tr>
<tr>
<td>MoCo</td>
<td>Molybdenum cofactor</td>
</tr>
<tr>
<td>MOCS</td>
<td>Molybdenum cofactor synthesis step</td>
</tr>
<tr>
<td>MOPS</td>
<td>3 - [N-Morpholino] -Propanesilfate</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acid</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MHZ</td>
<td>Megahertz</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>NaAc</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleotidetriphosphate</td>
</tr>
<tr>
<td>OD</td>
<td>Optimal density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pH</td>
<td>Preponderance of hydrogen ions</td>
</tr>
<tr>
<td>pmol</td>
<td>picomol</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphatebuffer saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Phosphatebuffer saline + Tween 20</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rnase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecylsulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>Tag</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA-Electrophoresis buffer</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylene diamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Trihydroxymethylaminomethane</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-brom-4-chlor-3-indolyl-β-Dgalactopyranoside</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 The heat shock protein family: the very short overview

In both prokaryotes and eukaryotes, transcription of most genes for heat shock proteins (HSPs) is induced by environmental stress conditions and various agents, including heat shock, anoxia, heavy metals, and certain inhibitors of mitochondrial respiration (Lindquist and Craig, 1988; Welch, 1992). HSPs in mammalian cells are classified into several families based on their apparent molecular mass and degrees of structural homology. HSPs are subdivided into small HSPs (25-28 kDa), HSP40 (40kDa), HSP60, HSP70 (68-80 kDa), HSP90 (83-99 kDa), and high-molecular-weight HSPs (110 kDa). Many members of these subfamilies are in fact not heat inducible, but their expression is induced in a tissue-specific manner or during development. The number of genes encoding the different HSP members varies between organisms. The number of genes encoding the diverse HSP family members largely varies per organism. For HSP70, the number of genes varies from three in \textit{Escherichia coli}, 14 in \textit{Arabidopsis thaliana}, 12 in \textit{Drosophila melanogaster} and 13 in \textit{Homo sapiens}. For small HSP (sHSP), the number of genes is relatively high in plants and the same holds true for HSP40 (Table 1.1) (Vos et al., 2008). HSPs act as molecular chaperones that recognize unfolded or newly translated proteins and promote acquisition of the functional native state (Frydman, 2001). Therefore, molecular chaperones prevent the aggregation of unfolded and damaged proteins in cells. Severely damaged proteins are selected by a process termed protein quality control, in which the chaperone HSP70 in collaboration with other proteins such as E3 ubiquitin ligase target the damaged proteins for degradation via the ubiquitin-proteasome pathway (Connell \textit{et al.}, 2001; McDonough and Patterson, 2003).

1.2 The heat shock protein family 110 (HSP110)

The Hsp110 gene family includes two genes in \textit{Saccharomyces cerevisiae} known as \textit{SSE1} and \textit{SSE2} and four genes in the mammalian genome, namely, \textit{Hspa4l/Apg1}, \textit{Hspa4/Apg2}, \textit{Hsp1/Hsp105} and \textit{Hyou1/Grp175/orp150}. Except HYOU1 that is present in endoplasmic reticulum, all other members of mammalian and yeast HSP110 are found in the cytosolic compartment. Constitutive expression of \textit{Hspa4l} is high in testis and moderate in other tissues,
while *Hspa4* and *Hsph1* are expressed in various tissues (Morozov *et al*., 1995; Kojima *et al*., 1996; Kaneko *et al*., 1997, Kojima *et al*., 2004).

**Table 1.1. Number of Hsp genes in different species (Vos *et al*., 2008).**

<table>
<thead>
<tr>
<th>HSPA/H</th>
<th>HSP40</th>
<th>sHSP</th>
<th>genome size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. sapiens</em></td>
<td>13</td>
<td>41</td>
<td>11</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>12</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>14</td>
<td>89</td>
<td>19</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>14</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

Primary structure of HSP110 proteins is highly related to HSP70 and consists of a nucleotide-binding domain (NBD) and a peptide-binding domain (PBD) that are connected by a flexible linker region (Mayer and Bukau, 2005; Liu and Hendrickson, 2007). However, biochemical analyses revealed that HSP110 members serve as cochaperones of mammalian and yeast HSP70 chaperones and act as molecular exchange factors (NEF) during the ATP-hydrolysis cycle (Steel *et al*., 2004; Dragovic, 2006; Shaner and Morano, 2007). Binding of newly synthesized polypeptides to HSP70 chaperones and the subsequent release of folded proteins is regulated by a continuous cycle of ATP-hydrolysis and exchange of ATP to ADP (Fig. 1.1). In the ATP-bound state, PBD of HSP70 chaperone binds to polypeptides with low affinity. However, ATP-hydrolysis to ADP by HSP40 cochaperone leads to conformational changes that result in high affinity substrate binding by HSP70. To complete the protein folding cycle, binding of HSP110 NEF to HSP70 in the ADP-state stimulates the release of ADP. Subsequent binding of ATP induces the disassociation of HSP70-HSP110 complexes (Polier *et al*., 2008).

The cellular functions of the HSP110/SEE gene family members were determined in several species. In *S. cerevisiae*, deletion of SSE1 results in a reduction of cell proliferation and temperature sensitivity, whereas the depletion of SSE2 has no effect on proliferation. However
deletion of both SSE1 and SSE2 genes is reported to be lethal in some strain backgrounds, indicating a unique important cellular function of these proteins in yeast (Mukai, et al., 1993; Shaner et al., 2004; Raviol et al., 2006).

**Fig. 1.1** Model for the cooperation of HSP110 and HSP70 in protein folding. Recruitment of HSP70 (red) to unfolded substrate protein (green) is assisted by J-domain proteins (HSP40, orange; step 1). Complex formation between HSP70 and HSP110 (blue) displaces ADP from the HSP70 partner (step 2). Direct substrate binding to HSP110 may provide an anchor aiding the unfolding of kinetically trapped intermediates through thermal motions of the PBD of HSP70. Finally, upon binding of ATP to HSP70, the HSP70-HSP110 complex dissociates and the substrate protein is released for folding (step 3). The green circle indicates natively folded substrate protein (Polier et al, 2008).
The physiological function of the mammalian HSP110 gene family has been studied by analysis of knockout mice. Analysis of Hspa4−/− deficient mice revealed that Hspa4l is not essential for embryonic development and Hspa4l-deficient mice were indistinguishable from their wild-type littermates in appearance and gross behavior. However, approximately 42% of mutant males are infertile. The cause of the male infertility is due to reduction of sperm count and sperm motility. Further analysis revealed that the reduction of sperm number is due to the elimination of a significant number of developing germ cells via apoptosis. No defect in fertility was observed in Hspa4l-deficient females (Held et al., 2006). In addition, Held et al. (2006) found that Hspa4l−/− mice were preferentially susceptible to osmotic stress. Nakamura et al. (2008) have studied the physiological function of Hsph1/Hsp110, which is ubiquitously expressed. They found that the deletion of Hsph1 did not affect the embryonic development, viability and fertility of mutant mice. However, Hsph1/Hsp110 knockout mice are resistant to ischemic injury and that the protective effects of Hsph1 deficiency in cerebral ischemia may be mediated by an increase in the chaperone activity of HSP70.

1.3 Expression and function of Hspa4

In mouse, Hspa4 gene was mapped to chromosome 11, region B1.3 and consists of 19 exons. The Hspa4 cDNA has a length of 2832 bp, encoding for a polypeptide of 841 amino acids with a molecular weight of 94,1 kDa. The HSPA4 protein shows about 65% sequence identity with HSPA4L protein (Kaneko et al., 1997). Although HSPA4 protein is highly homologous in amino acid sequence to human HSP70RY (94%), HSPA4 is longer than HSP70RY by 140 amino acid sequence at its C-terminus. Expression analysis revealed that the transcript level of Hspa4 is not induced by heat shock (Kaneko et al., 1997; Nonoguchi et al., 1999; Okui et al., 2000). In the adult mouse, Hspa4 mRNA is detected in most tissues, with the highest expression in testis, ovary and spleen (Nonoguchi et al., 1999). Hspa4 transcripts were detected in cells of various origins, including embryonic fibroblasts, embryonal carcinomas, myelomonocytic leukemia, mastocytoma, Sertoli cells and bone narrow stromal cells (Kaneko et al., 1997).

In addition, the Hspa4 gene has been found as one of 250 genes, which are highly expressed in the pluripotent stem cells (Ramalho-Santos et al., 2002). Two Hspa4 transcripts of 3.2 and 4.8-kb
were found by in Northern blot analysis. However, protein analysis revealed that an anti-HSPA4 antibody only detect a 94-kDa protein in all studied tissues. These results suggest that both Hspa4 RNA isoforms result from alternative splicing of the 3’- untranslated region (Held, 2008). Analysis of HSPA4 expression during germ cell development revealed that the expression is highly enriched in male and female germ cells of prenatal gonad. Expression of HSPA4 in male gonocytes is gradually decreased after migration to basement layers of seminiferous tubules and differentiation to spermatogonia (Held, 2008). In brain, HSPA4 protein was found to be expressed constitutively in rat neuronal tissues throughout development (Ogita et al., 2001) and primary human articular chondrocytes (Dehne et al., 2010).

HSPA4 was identified as a new interaction partner of the protein zonula occludens (ZO-1), which is associated with tight junction proteins at cell membrane. This interaction between HSPA4 and ZO-1 regulates the function of ZO-1 in control of the cellular localization of the transcription factor ZO-1 associated nucleic acid binding protein (ZONAB/DbpA/Csda). Binding of ATPase domain of HSPA4 to the SH3 domain of ZO-1 competes with the binding of ZONAB to SH3 domain of ZO-1, resulting in the release the ZONAB from the complex with ZO-1, transport to the nucleus and stimulates the transcription of genes encoding cell cycle regulators such as cyclin D1 and PCNA. Cyclin D1 and PCNA regulate the G1/S phase transition (Balda et al., 2003; Tsapara et al., 2006). Both HSPA4 and ZONAB are required for normal proliferation and regulate entry to S-phase. Thus, only little HSPA4 is associated with ZO-1 under control conditions. On heat shock, however, HSPA4 is redistributed, not upregulated, resulting in accumulation in nucleoli and at intracellular junction. It is conceivable that HSPA4 stabilizes tight junctions during stress conditions (Tsapara et al., 2006). In hepatocellular carcinoma and pancreatic cancer cells, HSPA4 as well as human ZONAB homologue DbpA are overexpressed (Nakatsura et al., 2001; Hayashi et al., 2002; Gotoh et al., 2004) suggesting that ZONAB signaling becomes activated, because most of its interacting protein ZO-1 becomes associated with the HSPA4 (Tsapara et al., 2006).

Recently, it has been showed that overexpression of Hspa4 in chronic myelogenous leukemia cells (BaF3-BCR/ABL cells) increases cell proliferation and protects cells from oxidative damage, which may play an important role in chronic myelogenous leukemia carcinogenesis and progression (Li et al., 2010).
To analyze the function of HSPA4 in mammalian species, *Hspa4* was disrupted by homologous recombination in the mice (Held, 2008). Two lines of *Hspa4*-deficient mice were generated in hybrid C57BL/6J x 129/SV and inbred 129/Sv genetic background. Analysis of *Hspa4*-deficient mice in hybrid C57BL/6J x 129/SV genetic background revealed that approximately 62% of *Hspa4*-deficient males in F2 generation are infertile due to a significant reduction in sperm number and motility. No defects in fertility were observed in female mutants. Analysis of *Hspa4*-deficient mice in the inbred background showed that 94,1% of *Hspa4*-deficient mice display growth retardation and died between the third and fourth week after birth. Male and female deficient animals, which overcome the early lethality display impaired fertility. Kyphosis was developed in *Hspa4*-deficient mice of advanced age. Anatomical analysis of adult *Hspa4*+/− heart showed enlargement of heart size. In addition, deficiency of *Hspa4* gene causes skeletal muscle myopathy in adult *Hspa4*+/− mice (Held, 2008).

1.4 Aims of the study

In context of this work, several questions must be addressed. What are the underlying causes of male infertility, growth retardation and skeletal muscle myopathy in *Hspa4*-deficient mice? What are the signaling pathways that are disturbed in the cardiac hypertrophy in mutant mice? Therefore, the aim of my study was to determine the underlying causes of male infertility, growth retardation and cardiac hypertrophy in *Hspa4*+/− mice. To reach these goals, studies were performed and categorized as follows:

1. To determine the cause of disruption of spermatogenesis in *Hspa4*+/− mice, the progress of male germ cell development in mutant mice was studied at different stages of postnatal development.

2. To determine whether growth retardation is due to skeletal muscle myopathy, histological analysis of different types of skeletal muscle were performed at different postnatal development.

3. Development of cardiac fibrosis and hypertrophy in the *Hspa4*+/− heart was determined during postnatal life using different histological and molecular analyses.

4. To determine the molecular pathways, which are mediating the cardiac fibrosis and hypertrophy, a microarray assay was performed and the expression of differentially expressed genes in *Hspa4*+/− heart was confirmed by quantitative RNA and protein analysis.
2. Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Agar</td>
<td>Difco, Detroit, USA</td>
</tr>
<tr>
<td>Agarose</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>Fluka, Neu Ulm</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Ampiwa</td>
<td>Fresenius, Bad Homburg</td>
</tr>
<tr>
<td>Aqua Poly/Mount</td>
<td>Polysciences, Inc, USA</td>
</tr>
<tr>
<td>Bacto-tryptone</td>
<td>Difco, Detroit, USA</td>
</tr>
<tr>
<td>Bacto-Yeast-Extract</td>
<td>Difco, Detroit, USA</td>
</tr>
<tr>
<td>Blocking powder</td>
<td>Boehringer, Mannheim</td>
</tr>
<tr>
<td>BSA</td>
<td>Biomol, Hamburg</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Baker, Deventer, NL</td>
</tr>
<tr>
<td>Cardiotoxin</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Coomassie Blue G-250</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate (DEPC)</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Dimethyl sulfoxid (DMSO)</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>EDTA</td>
<td>ICN Biomedicalals, Eschwege</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Baker, Deventer, NL</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Eukitt-quick hardening mounting medium</td>
<td>Fluka, Neu Ulm</td>
</tr>
<tr>
<td>FBS</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Ficoll 400</td>
<td>Amersham Pharmalia, Freiburg</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Formamide</td>
<td>Fluka, Neu Ulm</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>Sigma, Deisenhofen</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Glycine</td>
<td>Biomol, Hamburg</td>
</tr>
<tr>
<td>Goat serum</td>
<td>PAN-Systems, Nürnberg</td>
</tr>
<tr>
<td>HCl</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>1 kb DNA Ladder</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>100 bp DNA Ladder</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>KCl</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Methanol</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>Serva, Heidelberg</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Roth, Karlsruhe</td>
</tr>
<tr>
<td>NaCl</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>NaOH</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>NuPAGE Novex Bis-Tris 4-12% Gel</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>NuPAGE MOPS SDS running buffer</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>NuPAGE SDS sample buffer</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Orange G</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Paraquat</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>PBS</td>
<td>PAN-Systems, Nürnberg</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>PAN-Systems, Nürnberg</td>
</tr>
<tr>
<td>Peptone</td>
<td>Roth, Karlsruhe</td>
</tr>
<tr>
<td>Phenol</td>
<td>Biomol, Hamburg</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Picric acid</td>
<td>Fulka, Neu Ulm</td>
</tr>
<tr>
<td>Protein marker</td>
<td>Biorad, Sigma</td>
</tr>
<tr>
<td>[$^{32}$P]-dCTP</td>
<td>Amersham Pharmacia, Braunschweig</td>
</tr>
<tr>
<td>RediprimeTM II</td>
<td>Amersham Pharmacia, Freiburg</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase A</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>RNase away</td>
<td>Biomol, Hamburg</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>Roche, Penzberg</td>
</tr>
<tr>
<td>RNA length standard</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Saccharose</td>
<td>Roth, Karlsruhe</td>
</tr>
<tr>
<td>Salmon sperm DNA</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>SDS</td>
<td>Serva, Heidelberg</td>
</tr>
<tr>
<td>SeeBlue Plus2 Pre-Stained</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>StandardSelect Peptone</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Gibco/BRL, Eggenstein</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>S.O.C Medium</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Sun flower oil</td>
<td>Sodium citrate</td>
</tr>
<tr>
<td>SuperScript II</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>Promega, Mannheim</td>
</tr>
<tr>
<td>Trisfast reagent</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Tris base</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Serva, Heidelberg</td>
</tr>
<tr>
<td>Trypsin</td>
<td>PAN-Systems, Nürnberg</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma, Deisenhofen</td>
</tr>
<tr>
<td>Vectashield (DAPI)</td>
<td>Vector, Burlingame</td>
</tr>
<tr>
<td>X-Gal</td>
<td>Biomol, Hamburg</td>
</tr>
<tr>
<td>Xylene</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Roth, Karlsruhe</td>
</tr>
</tbody>
</table>

#### 2.1.2 Solutions, buffers and media

All standard buffers and solutions were prepared according to Sambrook *et al.* (1989).

Bouin’s solution

- 15 volume of Picric acid (in H₂O)
- 5 volumes of 37% Formaldehyde
- 1 volume of Acetic acid
### Materials and Methods

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligation buffer (10x)</td>
<td>600 mM Tris/HCl (pH 7.5)</td>
</tr>
<tr>
<td></td>
<td>80 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>100 mM DTT</td>
</tr>
<tr>
<td>Loading buffer</td>
<td>15% Ficoll 400</td>
</tr>
<tr>
<td></td>
<td>10 mM EDTA (pH 8)</td>
</tr>
<tr>
<td></td>
<td>0.25% Orange G</td>
</tr>
<tr>
<td></td>
<td>1% Glycerol</td>
</tr>
<tr>
<td>Lysis buffer I</td>
<td>100 mM Tris/HCl (pH 8.0)</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>100 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>0.5% SDS</td>
</tr>
<tr>
<td>SSC (20x)</td>
<td>3 M NaCl</td>
</tr>
<tr>
<td></td>
<td>0.3 M sodium citrate (pH 7.0)</td>
</tr>
<tr>
<td>TBE buffer (5x)</td>
<td>450 mM Tris base</td>
</tr>
<tr>
<td></td>
<td>450 mM Boric acid</td>
</tr>
<tr>
<td></td>
<td>20 mM EDTA (pH 8)</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris/HCl (pH 8.0)</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
</tr>
</tbody>
</table>

#### 2.1.3 Laboratory materials

The laboratory materials, which are not listed here, were bought from Schütt and Krannich (Göttingen).

- Culture slides: BD Falcon, Heidelberg
- Disposable filter Minisart NMI: Sartorius, Göttingen
- Filter paper 0858: Schleicher and Schüll, Dassel
- Hybond C: Amersham, Braunschweig
- Hybond N: Amersham, Braunschweig
- HPTLC Aluminum folio: Merck, Darmstadt
Materials and Methods

Microcentrifuge tubes      Eppendorf, Hamburg
Petri dishes        Greiner, Nürtingen
Pipette tips        Eppendorf, Hamburg
RotiPlast paraffin   Roth, Karlsruhe
Transfection flasks   Lab-Tek/Nalge, Nunc, IL, USA
Superfrost slides     Menzel, Gläser
Whatman blotting paper Schleicher and Schüll, Dassel
(GB 002, GB 003 and GB 004)
X-ray films          Amersham, Braunschweig

2.1.4 Sterilisation of solutions and equipments

All solutions that are not heat sensitive were sterilised at 121°C, 105 Pa for 60 min in an autoclave (Webeco, Bad Schwartau). Heat sensitive solutions were filtered through a disposable sterile filter (0.2 to 0.45 μm pore size). Plastic wares were autoclaved as above. Glasswares were sterilised overnight in an oven at 220°C.

2.1.5 Media, antibiotics and agar-plates

2.1.5.1 Media for bacteria

LB Medium (pH 7.5)         1% Bacto-trypton
                          0.5% Yeast extracts
                          1% NaCl

LB-Agar                  1% Bacto-trypton
                          0.5% Yeast extracts
                          1% NaCl
                          1.5% Agar

The LB medium was prepared with distilled water, autoclaved and stored at 4°C.
2.1.5.2 Antibiotics

Stock solutions were prepared for the antibiotics. They were filtered through sterile disposable filters and stored at –20°C. When antibiotics were needed, in each case, they were added after the autoclaved medium has cooled down to a temperature lower than 55°C.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Stock solution</th>
<th>Working solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>50 mg/ml</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>25 mg/ml</td>
<td>50 µg/ml</td>
</tr>
</tbody>
</table>

2.1.5.3 IPTG/X-Gal plate

LB-agar with 50 µg/ml ampicillin, 100 µM IPTG and 0.4% X-Gal was poured into Petri dishes. The dishes were stored at 4°C.

2.1.6 Bacterial strains

*E. coli* DH5α

K-12 strain, F- Φ80d lacZΔM15 endA1 recA1 hsdR17 (rk-, mk+) sup E44 thi-1 d- gyrA96 (lacZYA-arg)

(Invitrogen, Karlsruhe)

2.1.7 Plasmids

*pGEMTeasy* (Promega, Wisconsin, USA)

2.1.8 Synthetic oligonucleotides

The synthetic oligonucleotide primers used in this study were obtained from OPERON and dissolved in dH₂O (Ampuwa) to a final concentration of 100 pmol/µl.
### 2.1.8.1 Primers used for generation of cDNA probes

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acr_F</td>
<td>5’-CTTCTCGACCGCTTTACCTG-3’</td>
</tr>
<tr>
<td>Acr_R</td>
<td>5’-AGCTGAGCGAGGGAGGATGTA-3’</td>
</tr>
<tr>
<td>Hsc70t_F</td>
<td>5’-GCTACAAAGCGAGGATGAG-3’</td>
</tr>
<tr>
<td>Hsc70t_R</td>
<td>5’-AGGATGGGTGACGTAGGCTTG-3’</td>
</tr>
<tr>
<td>Tnp2_F</td>
<td>5’-CATGGACACCAAGATGCAGA-3’</td>
</tr>
<tr>
<td>Tnp2_R</td>
<td>5’-CCTGTCACATCATCCAACA-3’</td>
</tr>
<tr>
<td>Anf_F</td>
<td>5’-CGGTAGAAGATGGAGTGTACG-3’</td>
</tr>
<tr>
<td>Anf_R</td>
<td>5’-CAAGACCCACTAGACCACCTC-3’</td>
</tr>
<tr>
<td>Bnf_F</td>
<td>5’-CAGAAGTGGTGGGAAGACC-3’</td>
</tr>
<tr>
<td>Bnf_R</td>
<td>5’-CAAAAGCAGAAATGCCTATG-3’</td>
</tr>
<tr>
<td>Apcs_F</td>
<td>5’-CTTCCATACCACGGACTGT-3’</td>
</tr>
<tr>
<td>Apcs_R</td>
<td>5’-CTCCACCTCTTTCATGTC-3’</td>
</tr>
<tr>
<td>Slco1b2_F</td>
<td>5’-TGCGATGGATCCAGATAT-3’</td>
</tr>
<tr>
<td>Slco1b2_R</td>
<td>5’-GCCCTTTTCACACCTTC-3’</td>
</tr>
<tr>
<td>Sycp3_F</td>
<td>5’-GTGCAGCAGTGGAACCTG-3’</td>
</tr>
<tr>
<td>Sycp3_R</td>
<td>5’-CTAAGAGCTGCCTCCTAGC-3’</td>
</tr>
<tr>
<td>Pkg2_F</td>
<td>5’-TCTCATGAGTCACCTCCGGTG-3’</td>
</tr>
<tr>
<td>Pkg2_R</td>
<td>5’-AAGCGAGGGATGTCAGG-3’</td>
</tr>
<tr>
<td>Pepck_F</td>
<td>5’-GGGAGACAGTGAGAGGAT-3’</td>
</tr>
<tr>
<td>Pepck_R</td>
<td>5’-AGGAGGCTACGGATGAGTAT-3’</td>
</tr>
<tr>
<td>ApoB_F</td>
<td>5’-AGCCACTGGAGGAGGTGAT-3’</td>
</tr>
<tr>
<td>ApoB_R</td>
<td>5’-AGCCACTGGAGGAGGTGAT-3’</td>
</tr>
</tbody>
</table>

### 2.1.8.2 Genotyping primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apg2-genotyping_F</td>
<td>5’-GATCACGGGAAGTGAGTGGT-3’</td>
</tr>
<tr>
<td>Apg2-genotyping_R</td>
<td>5’-GAGCGGGAGTGACAGTTTC-3’</td>
</tr>
<tr>
<td>Apg1-genotyping_F</td>
<td>5’-GGTCAGAAAGGCCTCAACAGA-3’</td>
</tr>
<tr>
<td>Apg1-genotyping_R</td>
<td>5’-ACTGAGGCCCCTGATTTGGCC-3’</td>
</tr>
<tr>
<td>PGK3</td>
<td>5’-TCTGAGCCCCAAAGCAGG-3’</td>
</tr>
</tbody>
</table>
2.1.8.3 Real time PCR primers

Myh7_F  
5'-AAGGGCCTGAATGAGGGA-3'

Myh7_R  
5'-TGCAAAAGGCTCCAGGCTGGA-3'

Acta1_F  
5'-TATGTGGCTATCCAGGCGGTG-3'

Acta1_R  
5'-CCAGAATCCACACGGATGC-3'

MCIP1.4_F  
5'- AGCTCCCTGATGCTGCTGTTG-3'

MCIP1.4_R  
5'-TTTGCCCTGGTCCTACTTTT-3'

Apg2_F  
5'- AGCTTCTGTGATGATGACATTG-3'

Apg2_R  
5'-CGAGGTTCCCTAAACTGA-3'

Hcn1_F  
5'-CAATGAAGACAGCCTGGAAGGAA-3'

Hcn1_R  
5'-ATGAAATGCACACGCGGCAAC-3'

Kcnd2_F  
5'-TTGCTGAGGTGAAGAAGTG-3'

Kcnd2_R  
5'-GGGGTCACCCAAATAACAC-3'

Mme_F  
5'-GAGTTTGCAGATGCTTTCCTCA-3'

Mme_R  
5'-AACCCGACATTTCCCTTTCTG-3'

Scn4a_F  
5'-CAGCATCGAGATGACCACCTAACT-3'

Scn4a_R  
5'-GAGGCTGTAGTGCTGTCTTGATGTC-3'

Ir4_F  
5'-GAACAGATGACCTGCACTTACGAA-3'

Ir4_R  
5'-GGGTGCAAGTCTTCCACTAAGTCAG-3'

Pt4a1_F  
5'-AGCAGCGACCTCTATGAGAAGTGA-3'

Pt4a1_R  
5'-GGCAATACAAAGGAAGTGCACTGAGG-3'

Maob_F  
5'-AAACCAGATGCGACCTATGAGC-3'

Maob_R  
5'-GCTTCTTGGGAGTTTACAGCAC-3'

Kcne1_F  
5'-AATGGTCTCCCTCCCCCT-3'

Kcne1_R  
5'-CTTGAGGAGGCTTTATAT-3'

Gncl_F  
5'-AGCTCAAAACACCTGCGATCC-3'

Gncl_R  
5'-TCACAACAGCCTGAGTGAGG-3'

Fktn1_F  
5'-TTTTTGCTGATGAGGAC-3'

Fktn1_R  
5'-TAGATGGGCCCATGACTCAGC-3'

Anf_F  
5'-CCATATTGGGCAAATCTGTG-3'

Anf_R  
5'-CAGGATCGGTCCTCCTCCAGG-3'

Bnf_F  
5'-TGGAAGCTCTAGCCAGTCTC-3'

Bnf_R  
5'-CTGCTTCCTGGCCATTTTC-3'
Materials and Methods

Sdha_F 5'–GCTTGCGAGCTGCATTTGG-3'
Sdha_R 5'–CATCTCCAGTTGCTCTTCCA-3'
Hprt_F 5'–AGCCCCAATAATGGTAAGGTGC-3'
Hprt_R 5'–TTGCAAGTTCAACCTGCGCTCAT-3'
Igfbp3_F 5'–CCAGGAAACATCAGTGAGTCC-3'
Igfbp3_R 5'–GGATGGAACCTTGAATCGGTCA-3'
ET-AR_F 5'–GGTTGGCTCTTGGGTTCT-3'
ET-AR_R 5'–GACGCTGTGGAGGTGCT-3'
ET-BR_F 5'–TGCGAAATGCTCAGGAAG-3'
ET-BR_R 5'–ACGAGGACCAGGCAGGAAG-3'
Collagen 1_F 5'–AGGCTTCAGTGTTGGATG-3'
Collagen 1_R 5'–CACCAACAGCACCATCGTA-3'
Calmodulin 1_F 5'–AGGGGTTTGGAGGTGACTT-3'
Calmodulin 1_R 5'–TTTCCTCGAGGTTAGGTT-3'
TGF-ß1_F 5'–TGAGTGCGCTGCTTTTGCAG-3'
TGF-ß1_R 5'–GGTTCATGTGATGGGTG-3'
Mef2C_F 5'–ATTTGGGAACCTGAGCTGC-3'
Mef2C_R 5'–CGCTCATCCATTATCGGCT-3'
Hdac 7a_F 5'–ATCTCTCTCTGCGAGGCTTA-3'
Hdac 7a_R 5'–TTCTGCTTACCACACTGCT-3'

2.1.9 Mouse strains

Strains C57BL/6J, 129/Sv, CD-1 and NMRI were initially ordered from Charles River Laboratories, Wilmington, USA, and kept at Animal Facility of Institute of Human Genetics, Göttingen, in air-conditioned and light-controlled rooms.

2.1.10 Antibodies

Rabbit anti-HSP110 polyclonal antibody Sigma, Steinheim
Rabbit anti-Apg1 polyclonal antibody Santa Cruz Biotechnology, Heidelberg
Rabbit anti-Apg2 polyclonal antibody Santa Cruz Biotechnology, Heidelberg
Mouse monoclonal anti-α-tubulin Sigma, Deisenhofen
Mouse anti-GCNA1 monoclonal antibody G. Enders, University of Kansas, USA
Materials and Methods

Rabbit anti-SCP3(Syp3) polyclonal antibody Abcam, Cambridge, UK
Goat anti-mouse IgG alkaline phosphatase conjugate Sigma, Deisenhofen
Goat anti-rabbit IgG alkaline phosphatase conjugate Sigma, Deisenhofen
Rabbit anti-mouse IgG Cy3 conjugate Sigma, Deisenhofen
Rabbit anti-mouse IgG FITC conjugate Sigma, Deisenhofen
Goat anti-rabbit IgG horse radish peroxidase conjugate Sigma, Deisenhofen
Rabbit anti-mouse IgG horse radish peroxidase conjugate Sigma, Deisenhofen
Rabbit anti-STAT3 polyclonal antibody New England Biolabs, Frankfurt
Rabbit anti-phospho-STAT3 polyclonal antibody New England Biolabs, Frankfurt
Rabbit Anti-MAP Kinase (ERK-1, ERK-2) polyclonal antibody New England Biolabs, Frankfurt
Rabbit Anti-phospho-ERK-1 and Anti-phospho-ERK-2 polyclonal antibody New England Biolabs, Frankfurt

2.1.11 Enzymes

Immolase DNA Polymerase (Bioline, Luckenwalde)
Proteinase K (Sigma, Deisenhofen)
Platinum Taq polymerase (Invitrogen, Karlsruhe)
Restriction enzymes (with supplied buffers) (Invitrogen, Karlsruhe)
RNase A (Qiagen, Hilden)
RNase inhibitor (Invitrogen, Karlsruhe)
Superscript-II (Invitrogen, Karlsruhe)
T4 DNA ligase (Promega, Mannheim)
Trypsin (Invitrogen, Karlsruhe)
DNase I Amplification Grade (Invitrogen, Karlsruhe)
2.1.12 Kits

| Labelling System                             | Qiagen, Hilden |
| Megaprime DNA Labeling Kit                  | Amersham Pharmacia, Freiburg |
| Mini Plasmid Kit                            | Qiagen, Hilden |
| PCR Purification Kit                        | Qiagen, Hilden |
| QIAquick Gel Extraction Kit                 | Qiagen, Hilden |
| Rediprime™ II Random Prime                  | Amersham Pharmacia, Freiburg |
| Masson's trichrome stain Kit                | Sigma, Deisenhofen |
| Periodic Acid Schiff Kit (PAS)              | Sigma, Deisenhofen |
| ApopTag® plus peroxidase                    | Qbiogene, Heidelberg, Germany |

2.1.13 Equipment

| Autoclave                                    | (Webeco, Bad Schwartau) |
| Centrifuge 5415D                             | (Eppendorf, Hamburg) |
| Centrifuge 5417R                             | (Eppendorf, Hamburg) |
| Biophotometer                                | (Eppendorf, Hamburg) |
| DNA Sequencer Modell Megabace 1000           | (Amersham, Freiburg) |
| Microscope BX60                              | (Olympus, München) |
| GeneAmp PCR System 9600                      | (Perkin Elmer, Berlin) |
| Histocentre 2 embedding machine              | (Shandon, Frankfurt aM.) |
| Microtiterplate-Photometer                  | (BioRad laboratories, München) |
| Molecular Imager FX                          | (BioRad laboratories, München) |
| Phosphoimager Screen                        | (BioRad laboratories, München) |
| Semi-Dry-Blot Fast Blot                     | (Biometra, Göttingen) |
| Spectrophotometer Ultraspec 3000             | (Amersham, Freiburg) |
| SpeedVac concentrator SVC 100H               | (Schütt, Göttingen) |
| Thermomixer 5436                             | (Eppendorf, Hamburg) |
| TurboblotterTM                               | (Schleicher & Schüll, Dassel) |
| UV StratalinkerTM1800                       | (Leica, Nußloch) |
2.2 Methods

2.2.1 Isolation of nucleic acids

2.2.1.1 Small-scale isolation of plasmid DNA
(adapted from Birnboim and Doly, 1979).

A single *E.coli* colony was inoculated in 5 ml of LB medium with the appropriate antibiotic and incubated in a shaker for 16 hrs at 37°C with a vigorous shaking. 0.5 ml of this culture was used for making glycerol stock (0.5 ml of culture and 0.5 ml of glycerol) and rest of the culture was centrifuged at 5000 x g for 10 min. The pellet was resuspended in 100 μl of solution P1. The bacterial cells were lysed with 200 μl of P2 solution and then neutralized with 150 μl of P3 solution. The precipitated solution was centrifuged for 10 min at 10,000 xg at 4 °C. The supernatant was transferred into a new tube and centrifugation was done again. The supernatant was transferred again into a new tube and 1 ml of 100% ethanol was added to precipitate the DNA. It was then stored in ice for 15 min, centrifuged at full speed for 20 min, and finally the pellet was washed with 70% ethanol and after air-drying was dissolved in 50 μl of Ampuwa water.

P1: 50 mM Tris/HCl, pH 8.0; 10 mM EDTA; 100 μg/ ml RNase A
P2: 200 mM NaOH; 1% SDS
P3: 3 M Potassium acetate, pH 5.5

2.2.1.2 Isolation of genomic DNA from tissue samples
(Laird et al., 1991)

Routinely 0.5 cm of the mouse tail was incubated in 700 μl of lysis buffer I containing 35 μl proteinase K (10 μg/μl) at 55°C overnight in thermomixer. The tissue lysate was centrifuged at 10,000 xg for 15 min. Then, DNA was precipitated from supernatant by adding an equal volume of isopropanol, mixed and centrifuged at 10,000 xg at RT for 15 min. DNA was washed with 1 ml of 70% ethanol, dissolved in 50-100 μl of dH2O and incubated at 60°C for 10 min.

2.2.1.3 Isolation of total RNA from tissue samples
(Chomczynski and Sacchi, 1987).

The composition of Trifast Reagent, which was used for RNA extraction contained phenol and guanidine thiocyanate in a monophase solution. In order to avoid any RNase activity, homogenizers which were used for RNA isolation were treated before with RNase away and DEPC water. 100 mg tissue sample was homogenised in 1 ml of RNA reagent by
using a glass-teflon homogenizer. The sample volume should not exceed 10% of the volume of reagent used for the homogenisation. The homogenate was vortexed and incubated on ice for 5 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, mixed and stored at 4°C for 10 min. After centrifugation at 12000 xg for 15 min at 4°C, the upper aqueous phase was transferred into a new tube. The RNA was precipitated by adding 0.5 ml of isopropanol. Finally, the pellet was washed twice with 75% ethanol and dissolved in 50-100 µl of RNase free water (DEPC-dH₂O). The RNA was stored at –80°C.

2.2.2 Determination of nucleic acid concentration

The concentration of nucleic acids was determined spectrophotometrically by measuring absorption of the samples at 260 nm. The quality of nucleic acids i.e. contamination with salt and protein was checked by the measurements at 230, 280, and 320 nm. The concentration was calculated according to the formula:

\[ C \cdot (E_{260} - E_{320}) = C \cdot f \cdot c \]

\[ C = \text{concentration of sample (µg/µl)} \]
\[ E_{260} = \text{ratio of extinction at 260 nm} \]
\[ E_{320} = \text{ratio of extinction at 320 nm} \]
\[ f = \text{dilution factor} \]
\[ c = \text{concentration (standard) / absorption (standard)} \]

for double stranded DNA : \( c = 0.05 \mu g/\mu l \)

for RNA : \( c = 0.04 \mu g/\mu l \)

for single stranded DNA : \( c = 0.03 \mu g/\mu l \)

2.2.3 Gel electrophoresis

2.2.3.1 Agarose gel electrophoresis of DNA

Agarose gels are used to electrophorese nucleic acid molecules from as small as 100 bp to more than 50 kb. For preparation of 1% agarose gel, 1 g of agarose was added in 100 ml 0.5 x TBE buffer, boiled in the microwave to dissolve the agarose, then cooled down to about 60°C before adding 3 µl ethidium bromide (10 mg/ml). This 1% agarose gel was poured into a horizontal gel chamber. 0.5x TBE buffer was used also as electrophoresis buffer. Before loading the samples, about 0.1 volume of loading buffer was added and mixed. The samples were then loaded into the wells of the gel and electrophoresis was carried out at a steady voltage (50 – 100 V). Size of the DNA fragments on agarose gels was determined using 1 kb
Materials and Methods

DNA ladder, which was loaded with samples in parallel slots. DNA fragments were observed and photographed under UV light.

2.2.3.2 Agarose gel electrophoresis of RNA

(Hodge, 1994)

Single-stranded RNA molecules often have complementary regions that can form secondary structures. Therefore, RNA was run on a denaturing agarose gel that contained formaldehyde. RNA was pre-treated with formaldehyde and formamide to denature. To prepare a denaturing agarose gel, 2 g of agarose was added to 20 ml of 10x MOPS buffer and 148 ml of DEPC water and dissolved by heating in microwave oven. After cooling it to about 50°C, 33.2 ml of formaldehyde (37%) was added, stirred and poured into a horizontal gel chamber. RNA samples were treated as follows:

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 – 20 μg</td>
<td>RNA</td>
</tr>
<tr>
<td>2 μl</td>
<td>10x MOPS Buffer</td>
</tr>
<tr>
<td>3 μl</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>7 μl</td>
<td>Formamide (40%)</td>
</tr>
<tr>
<td>1 μl</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>5 μl</td>
<td>Loading buffer</td>
</tr>
</tbody>
</table>

Samples were denatured at 65°C for 10 min and chilled on ice before loading into the gel. The gel was run at 40 V at 4°C overnight. To determine the size of the nucleic acid fragments on agarose gels, molecular weight ladder (0.24 – 9.5 RNA ladder) was loaded with samples in parallel slots.

2.2.4 Purification of DNA fragments from agarose gel

To purify DNA fragments from agarose gel, QIAquick gel extraction kit was used. The principle of this method depends on selective binding of DNA to uniquely designed silica-gel membranes. After running DNA in the agarose gel, agarose gel piece containing the DNA fragment was cut and incubated in 3 volumes of QG buffer at 50 °C for 10 min. Then, dissolved gel slice was applied to a QIAquick column and centrifuged for 1 min at 10,000 xg. The flow through was discarded and the column was washed with 0.75 ml of PE buffer. After drying, the column was placed into a fresh microcentrifuge tube. To elute DNA, 50 μl of dH₂O was added to the QIAquick membrane and the column was centrifuged for 1 min at 10,000 xg.
2.2.5 Enzymatic modifications of DNA

2.2.5.1 Digestion of DNA using restriction enzymes

Restriction enzymes are class of bacterial enzymes that recognizes and cut DNA at specific nucleotide sequence of 4 - 8 bp. Restriction enzyme digestions were carried out by incubating double-stranded DNA with an appropriate amount of restriction enzyme in its respective buffer as recommended by the supplier, and at the optimal temperature for that specific enzyme. Standard digestions include 2-10 U enzyme per microgram of DNA. Reactions were usually incubated for 1-3 hrs to ensure complete digestion at the optimal temperature for enzyme activity.

2.2.5.2 Ligation of DNA fragments

The ligation of an insert DNA into a vector, which was digested with appropriate restriction enzyme, was performed in the following reaction mix:

- 30 ng linearized vector DNA
- 50-100 ng insert DNA
- 1 µl ligation buffer (10x)
- 1 µl T4 DNA ligase (5U / µl)

in a total volume of 10 µl

Blunt-end ligations were carried out at 16°C for overnight, whereas overhang-end ligations were carried out at RT for 2-4 hrs. For cloning of PCR products, a pGEMTeasy vector system that has 5’T overhangs was used. The following substances were mixed:

- 50 ng of pGEMTeasy vector
- 150 ng PCR product
- 1 µl of T4 DNA Ligase buffer (x10)
- 1 µl of T4 DNA Ligase

The reactions were done in a total volume of 10 µl and incubated overnight at 4°C.

2.2.6 Transformation of competent bacteria

(Ausubel et al., 1994)

Transformation of the competent *E. coli* bacteria (invitrogen) was done by gently mixing one aliquot of competent bacteria (50 µl) with 10 µl of ligation reaction. After incubation for 30 min on ice, bacteria were heat shocked for 45 sec at 40°C and then cooled.
down for 2 min on ice. After adding 600 µl of S.O.C medium, bacteria were incubated at 37°C for 1 hr. Bacteria were plated out either on LB-agar plates containing appropriate antibiotic (50 µg/ml) or X-Gal plates.

2.2.7 Polymerase chain reaction (PCR)

2.2.7.1 PCR amplification of DNA fragments

The standard PCR assay contained the following components:

10 ng DNA
1 µl Forward primer (10pmol)
1 µl Reverse primer (10pmol)
1 µl 10mM dNTPs
5 µl 10x PCR buffer
1.5 µl 50mM MgCl2
1 µl Taq DNA polymerase (5U/µl)
Up to 50 µl H2O

The reaction mixture was added in a 200 µl reaction tube, vortexed slightly and placed in the thermocycler.

Standard PCR program:

\[
\begin{align*} 
\text{Initial denaturation} & : 95°C \text{ 5 min} \\
\text{Elongation} & : 95°C \text{ 30 sec (denaturation)} \quad 58°C \text{ 45 sec (annealing)} \quad 72°C \text{ 1-2 min (extension)} \\
\text{Final extension} & : 72°C \text{ 10 min} 
\end{align*}
\]

2.2.7.2 Genotyping of knockout mice by using PCR

All offspring of Hsp4a and Hspa41 mutant lines were genotyped by polymerase chain reaction (PCR). For amplification of the wild type and the mutant allele, the DNA was extracted from mouse tails as described in 2.2.1.2 and pipetted to the following reaction mixture:

0.5 µl DNA (300-500 ng)
0.5µl Forword primer (10 pmol/µl)
0.5 µl Reverse primer (10 pmol/µl)
Materials and Methods

0.5 μl Pgk_3 (10 pmol/μl)
0.5 μl dNTPs (10 mM)
0.5 μl Taq Platinum buffer (10x)
0.75 μl MgCl2 (25 mM)
0.25 μl Taq Platinum
Up to 25 μl H2O

The mixture was subjected to the following program in the thermocycler,

*Denaturation* 95°C for 7 min

Elongation
(for 35 cycle) 95°C for 30 sec (Denaturation)
58°C for 30 sec (Annealing)
72°C for 1 min (Elongation)

*Final extension* 72°C for 10 min

2.2.7.3 Reverse transcription PCR (RT-PCR)

2.2.7.3.1 DNase I digestion

RNA samples, which were used in RT-PCR assay, were treated firstly with DNase to eliminate the contaminated DNA. The reaction mixture contained the following components:

1-5 μg of RNA ≤8 μl
10X DNase I Reaction Buffer 1 μl
1 μl of DNase I, diluted 1:5 1 μl
DEPC-treated water to 10 μl

The reaction mixture was incubated for 30 min at 37°C. To inactivate the DNase I, 1 μl of 25 mM EDTA solution was added to the reaction mixture and incubated for 10 min at 65°C.
2.2.7.3.2 Reverse transcription technique

To determine the expression of genes in specific tissues or in different development stages, RT-PCR assay was performed. 1-5 µg of total RNA was mixed with 1 µl of oligo (dT) primer (10 pmol/µl) in a total volume of 12 µl. To disrupt the secondary structure of the RNA, which might interfere with the cDNA synthesis, the mixture was heated to 65°C for 5 min and then quickly chilled on ice. After a brief centrifugation the followings were added to the mixture:

- 4µl 5x First strand buffer
- 2µl 0.1 M DTT
- 1µl 10 mM dNTPs
- 1µl Rnase inhibitor (10U/µl)

The content of the tube was mixed and incubated at 42°C for 2 min. Then, 1µl of reverse transcriptase enzyme (Superscript II) was added and further incubated at 42°C for 50 min for the first strand cDNA synthesis. The reaction was then inactivated by heating at 70°C for 15 min. One µl of the first strand reaction was used for the PCR reaction.

2.2.7.4 Quantitive Real-Time PCR

Extraction of total RNA from tissues was performed using Trifast Reagent as described above (2.2.1.3). RNA was treated with DNase I and was then reverse-transcribed according to section 2.7.3. Serial dilutions of sample and standard DNA’s were made. To generate a standard curve, standard DNA was serially diluted to 20, 10, 5, 2.5, 1.25 and 0.625 ng/µl, while each RNA sample was diluted to a concentration of 10 ng/µl. To enhance the efficiency of PCR amplification, primers were designed to generate amplicons less than 200 bp. Real-Time quantitative PCR was performed using QuantiTect SYBR Green PCR Master mix (Quiagen) in an ABI Prism 7900HT sequence detection system. Each reaction was run in triplicate, repeated three times using three animals of each genotype. Levels of mRNA expression were normalized to those of the mouse housekeeping genes *Sdha* (succinate dehydrogenase) and *Hprt* (phosphoribosyl-transferase). 5 µl of 2x QuantiTect SYBR-Green PCR-Master-Mix, 1 µl Forward Primer (9µM), 1 µl Reverse Primer (9µM), 0.3µl MgCl2 (50mM) and 1µl of cDNA (in a 1/20 dilution) were mixed with RNase free water to a total volume of 10 µl. The following PCR program was used:

- 2 min 50°C
- 15 min 95°C
- 15 sec 95°C
Materials and Methods

30 sec 54°C        40x
30 sec 72°C
15 sec 95°C
15 sec 60°C       Dissociation stage
15 sec 95°C

2.2.8 Protein and biochemical methods

2.2.8.1 Isolation of total protein from mouse tissues

100 mg of tissue was homogenized in 500 µl of RIPA buffer containing protease inhibitors. Tissues extract was incubated for 30 min at 4°C and then sonicated two times for 1 min with Branson ultra-Sonifier. The lysates were centrifuged at 8000xg for 10 min. The supernatant containing total proteins was taken and aliquoted in e-cups. The protein samples were stored at -80°C.

2.2.8.2 Isolation of total protein from cell culture

5 x 10^6 cells/ml were washed with cold phosphate buffered saline (PBS) and resuspended in 50 - 200 µl of lysis buffer A. The cell lysate was incubated on ice for 30 min, treated with ultrasound on ice two times for 30 sec and centrifuged at 24000 x g for 15 min at 4°C. The supernatant with protein extract was stored at –80°C.

2.2.8.3 Determination of protein concentration

(Bradford, 1976)

Bio-Rad protein assay was used to determine the protein concentration. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomasie Blue G-250 shifts from 494 to 595 nm when it is associated to protein. In order to obtain standard dilutions in range of 10 µg/ml to 100 µg/ml, BSA stock solution of 1 mg/ml was diluted. The dye reagent was diluted 1:5 with H₂O and a 2 µl sample was added. The absorption of the color reaction was measured at 595 nm in a spectrophotometer.

2.2.8.4 SDS-PAGE gel for separation of proteins

(Laemmli, 1970)

NuPage 4-12% Bis-Tris gel (Invitrogen) was used for separation of proteins according to their molecular weight. To 15 µl of whole protein lysate, 5 µl of 4 x LDS sample buffer and 3 µl of 1 M DTT were added. Then, the samples were denatured in 95°C for 10 min and chilled in ice. The gel electrophoresis was run in 1 x MOPS buffer (Invitrogen). As a
weight marker, a pre-stained molecular weight standard (See Blue Plus2, Invitrogen) was loaded. The gel was run at 100 V for 2 – 3 hrs at RT.

2.2.9 Blotting techniques

2.2.9.1 Northern blotting of RNA

To transfer the RNA to the nitrocellulose membrane, we used a Turbo-Blot apparatus (Schleicher & Schuell, Dassel). About 25-28 Whatman filter papers (GB 003) were layered on a Stack Tray, followed by 4 Whatman filter papers (GB 002) and 1 Whatman filter paper (GB 002), which were soaked with 20 x SSC. The nitrocellulose filter, which was also soaked with 20 x SSC, was laid on the top. The agarose gel was placed on the nitrocellulose filter and was covered with 3 Whatman filter papers GB 002 soaked with 20 x SSC. The buffer tray was filled with 20 x SSC. Then, a wick that was soaked with 20 x SSC, was put on top of the blot. The transfer was performed for overnight. Finally, after disassembling of the blot, the RNA was fixed onto the filter by baking at 80°C for at least 2 hours.

2.2.9.2 Western blotting of protein

(Gershoni and Palade, 1982)

After electrophoresis of proteins on the SDS-PAGE, the nitrocellulose membrane Hybond-C (Amershan) was cut at the size of the gel and soaked with transfer buffer. Four pieces of Whatman filter paper were soaked in transfer buffer and placed on the semi dry transfer machine’s lower plate (Biometra, Göttingen). Then, the wet membrane and the gel were put over them. Another four soaked Whatman papers were placed over to complete the sandwich model. The upper plate of semi dry transfer machine was placed over this sandwich and the transfer was carried out at 10 W (150-250 mA, 39 V) for 1 hr. Next, the nitrocellulose membrane was blocked and incubated with antibodies. To assess transfer efficiency of proteins onto nitrocellulose membranes, the gel was stained for 30 min in Coomassie blue solution at RT. Finally, gel was destained in Comassie destaining solution for 3-8 hrs at RT.

Transfer buffer  pH 9.2  5.8 g Tris-HCl
2.9 g Glycine
3.7 ml 10% SDS
dH₂O to 1000 ml
Materials and Methods

Membrane staining

Membrane was stained with Coomassie blue at RT.

Coomassie blue: 0.1% Coomassie
90% Methanol
10% CH₂COOH

Destaining of the membrane

Destaining solution: 40% Methanol
10% CH₂COOH
up to H₂O

2.2.9.3 Incubation of protein–bound membranes with antibodies

The blotted membrane was first incubated with 5% non-fat milk in PBT for 1-2 hrs at RT and then it was incubated overnight at 4 °C with a primary antibody at the recommended antibody dilution in 2% non-fat milk in PBT. Then, the membrane was washed 3x 20 min with 2% non-fat milk in PBT and incubated with horse radish peroxidase conjugated secondary antibody diluted 1:10000 in PBT containing 2% non-fat milk in PBT for 1 hr at RT. After this step the membrane was washed 3x for 10 min at RT in PBT with 2% non-fat milk in PBT and one time for 5 min at RT in PBS. Finally, the proteins from the membrane were visualized by using SuperSignal® West Pico Chemiluminescent Substrate (Pierce, USA) . Membrane was incubated for 3-5 min with 1 ml of developing mixture (0.3 ml stable peroxidase solution and 0.3 ml Luminal/enhancer solution) and then was wrapped in saran foil and exposed to Roentgen films (Hyperfilm MP, Amersham, Braunschweig) for 0.5 to 10 min. The films were developed in X-Ray Automatic processor Curix.

2.2.10 “Random Prime” method for generation of ³²P labeled DNA

(Denhardt, 1966; Feinberg and Vogelstein, 1989)

For generation of ³²P labeled DNA RediprimeTM II Random Prime Labeling System (Amersham Pharmacia) was used. The method depended on the random priming principle developed by Feinberg and Vogelstein (1989). Firstly, 25-50 ng DNA were denaturated in a total volume of 46 μl at 95°C for 10 min and quickly chilled on ice for 5 min. After pipetting the denaturated probe into RediprimeTM II Random Prime Labelling System cup, 4 μl of [α-³²P] dCTP (3000 Ci/mmol) were added to the reaction mixture. The labelling reaction
incubated at 37°C for 45 min. The labelled DNA was purified from free [$\alpha^{-32}$P] dCTP by using illustra™Probe Quant™ G-50 Micro Columns (GE Healthcare).

### 2.2.11 Hybridisation of nucleic acids

(Denhardt, 1966)

The membrane to be hybridised was equilibrated in 2 x SSC and transferred to a hybridisation tube. After adding 12 ml of Rapid-hyb buffer (GE Healthcare) and sheared denatured salmon DNA, the membrane was incubated for 2 hrs in the hybridisation oven at 65°C. The $^{32}$P labeled DNA probe was denatured at 95°C for 10 min, chilled on ice for 5 min, and added to the hybridisation solution. The hybridisation was carried out overnight in the hybridisation oven. The membrane was washed for 10 min with 2 x SSC, and then with 2 x SSC containing 0.2% SDS at 65°C for 10 – 20 min. Finally, the membrane was washed with 0.2 x SSC containing 0.1 % SDS at the hybridisation temperature. After drying the filter, it was sealed in plastic foil and exposed to autoradiography overnight at -80°C. The film was developed in X-Ray Automatic Processor Curix 60. If the membrane has to be used again, it was stripped in 0.2 x SSC at 80°C until radioactive signal was no longer detected.

### 2.2.12 DNA sequencing

DNA sequencings was performed with the Dye Terminator Cycle Sequencing-Kit (ABI PRISM). The reaction products were analysed with automatic sequencing equipment, MegaBase DNA Sequencer. For the sequencing reaction, four different dye labelled dideoxy nucleotides were used (Sanger et al., 1977), which, when exposed to an argon laser, emit fluorescent light that can be detected and interpreted.

The reaction was carried out in a total volume of 10 μl containing 1 μg plasmid DNA or 100-200 ng purified PCR products, 10 pmol primer and 4 μl reaction mix (contained dNTPs, dideoxy dye terminators and Taq DNA polymerase). Elongation and chain termination took place during the following program in a thermocycler: 4 min denaturation followed by 25 cycles at 95°C, 30 sec; 55°C, 15 sec, annealing; 60°C, 4 min, elongation. After the sequencing reaction, the DNA was precipitated with 1/10 volume 3 M sodium acetate and 2.5 volume 100% ethanol and washed in 70% ethanol. The pellet was dissolved in 4 μl of loading buffer, denatured at 95°C for 3 min, and finally loaded on the sequence gel.
2.2.13 Histological techniques

2.2.13.1 Tissue preparation for paraffin-embedding

Tissues were isolated from mice and fixed in Bouin’s solution or 4% (w/v) paraformaldehyde for 6 - 24 hrs to prevent alterations in the cellular structure. The dehydration process was accomplished by passing the tissues through a series of increasing alcohol concentrations, i.e. 70%, 80%, 90%, 96%, 100% ethanol for 1 hr at RT and isopropanol overnight. The alcohol was removed from the tissues by incubation in 25%, 50%, 75% and 100% xylene. Tissues were then incubated in paraffin at 60°C for 12-24 hrs. The paraffin was changed at least three times. Finally, tissue was placed in embedding mold and melted paraffin was poured into the mold to form a block. The paraffin block was cooled at 4°C.

2.2.13.2 Sections of the paraffin block

Paraffin blocks were clamped into the microtome (Hn 40 Ing., Nut hole, Germany). The thickness of the sections was 5-7 μm. The sections were floated on 40°C water to allow actual spread. Then, they were put onto slides. After complete drying at 37°C, slides were stored at 4°C for further analysis.

2.2.13.3 Immunofluorescence staining

Tissue cross sections were incubated twice for 10 min in xylene to remove the paraffin. Then, they were rehydrated by descending ethanol concentrations. For immunofluorescence staining, sections were placed into a plastic staining dish containing the antigen retrieval buffer. This plastic staining dish was placed into boiling water bath for 15 min and then placed in ice for 10 min. Then slides were incubated with a blocking solution containing 10% goat or sheep serum in 0.02% Tween-20 in PBS for 2 hrs at RT. Slides were then incubated with primary antibodies for overnight in a humidified chamber at 4°C. Subsequently, they were rinsed three times for 5 min in PBS and incubated with secondary antibody for 1 hr. Finally, the slides were washed three times for 5 min in PBS and the nuclei were counterstained with DAPI. Immunostaining of the sections was examined using a fluorescence equipped microscope (BX60; Olympus). When alkaline phosphatase secondary antibody was used, immunostaining was detected by adding alkaline phosphatase substrate (Fast Red TR/Naphthol AS-MX®, Sigma) to the sections for 15 min. To stop the reaction, the slides were immersed in Copling jars filled with distilled water, slides were then covered with Aqua-Poly-Mount.
Materials and Methods

Antigen retrieval buffer:
Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)

2.2.13.4 Immunocytochemical staining of germ cell suspension

Germ cell suspension was prepared from mouse testes by using the collagenase/trypsin method according to published procedure (Romrell et al., 1976). Testes from 60 days old mice were collected in serum-free culture medium, rinsed in 0.1 M PBS, pH 7.2. After removal of the tunica albuginea, seminiferous tubules were enzymatically dissociated by the addition of 1ml collagenase (1mg/ml). The slurry maintained at 37°C for 30 min was triturated every 5 min. 5 ml of Hank’s solution was added and then spun at 500 xg to sediment the dissociated cells. The pellet was resuspended in 3 ml trypsin, incubated for 5 min and then trypsin (0.5 mg/ml) was inactivated by adding 2 ml FKS. The slurry was passed through 80µm nylon mesh. The filtrate was spun at 500 xg to sediment the cells. Cells were resuspended in PBS and spread onto superfrost slides, air-dried and fixed in 4% PFA for 10 min at RT. Next, they were washed twice in PBS and immunostained as described above (2.2.13.3).

2.2.13.5 Hematoxylin-eosin (H&E) staining of histological sections

Histological sections were incubated three times in xylene for 3 min each, followed by incubation in 100% for 3 min, 95% and 80% ethanol for 2 min each. Slides were then washed in dH₂O for 5 min and stained for 3 min in hematoxylin. The staining was followed by rinsing with deionised water and washing in tap water for 10 min. Slides were dipped in acid ethanol (1ml concentrated HCl in 400 ml 70% ethanol) for 8-12 times to destain, then in ammonium water (0.25%), rinsed in tap water for 2 min and in deionised water for 2 min. Thereafter slides were stained with eosin (0.1% + 2% acetic acid) for 1 min, then in dH₂O for 1 min and incubated in 50%, 70%, 80%, 90%, 96% and 100% ethanol for 2 min in each. Finally they were incubated two times in histoclear (Xylol) for 5 min and mounted with Eukitt-quick hardening mounting medium.

2.2.13.6 Apoptosis detection

The slides containing the thin (5 µm) sections of testis were processed for a TUNEL assay to assess the possible number of cells undergoing apoptosis by an ApopTag detection kit. The sections were firstly deparafinized, hydrated and washed 2 x 5 min in PBS. Slides
Materials and Methods

were then incubated for 15 min at RT in 20 μg/ml Proteinase K and washed 2 x 2 min in dH$_2$O. To block endogenous peroxidase, tissues were incubated in 3 % H$_2$O$_2$ for 5 min at RT. After 2 x 5 min washing in PBS, the tissues were covered for 10 sec with equilibration buffer. Slides were incubated with Working Strength TdT Enzyme (30% enzyme in reaction buffer) for 1 hr at 37°C in darkness. Slides were incubated for 10 min shaking at RT in Stop/Wash buffer (1:34 in dH$_2$O), washed 3 x 1 min with PBS. Thereafter, slides were incubated with anti-digoxigenin for 30 min at RT in darkness and then washed 4 x 2 min with PBS. Slides were stained with Working Strength Peroxidase Substrate (2% DAB Substrate in DAB Dilution buffer) for 6 min at RT, washed 3 x 1 min and 1 x 5 min with dH$_2$O. Finally, slides were covered by AquaPolyMount liquid. The percentage of cell death was determined by counting the cells exhibiting brown nuclei (TUNEL-positive) and compared with cells from wildtype littermate.

2.2.14 Masson's Trichrome staining

Masson's Trichrome stain identifies nuclei in black, cytoplasm, keratin and muscle fibers in red, and collagen, mucin in blue. Trichome stains are used primarily for distinguishing collagen from muscle tissue. Accustain trichome stains (Masson) from Sigma-Aldrich was used. Heart and skeletal muscle sections were stained with Masson's Trichrome stain to identify any fibrotic areas. Briefly, slides were deparaffinized and rehydrated before placing in Bouin's fixative overnight at RT. Slides were placed in Working Weigert's Iron Hematoxylin solution for 5 min, rinsed in running tap water for 10 min, placed in Beibrich scarlet-acid fuchsin solution for 5 min and then rinsed until clear. Next, slides were immersed in phosphomolybdic-phosphotungstic acid solution for 5 min, followed by aniline blue solution for 5 min, rinsed and then placed in 1% acetic water for 2 min. Finally, rinsed slides were dehydrated and protected with Eukitt-quick hardening mounting medium.

2.2.15 Tissue preparation for electron microscopy

The left ventricle of freshly isolated heart was cut in small pieces and treated with fixation solution for 8-12 hrs in 4°C. Tissues were then washed in washing buffer for a few hours and sent to Dr. C. Mühlfeld (Department of Anatomy and Cell Biology, University of Giessen), who did the electron microscopy analysis.

Fixation solution: 1% Paraformaldehyde
3% Glutaraldehyde
Materials and Methods

In 0.1 M Cacodylat buffer, pH 7.4
Washing solution 3.4 % Saccharose
in 0.1 M Cacodylat buffer, pH 7.4

2.2.16 Echocardiogram

Echocardiographic assessments were done in collaboration with Prof. Dr. Maier, Department of Cardiology and Pneumology, Göttingen University. Echocardiography was performed on a Toshiba Power Vision 6000 system with a 15-MHz ultrasound probe under general anesthesia with tribromoethanol/amylene hydrate (Avertin; 2.5% wt/vol, 6 _L/g body weight i.p.) under spontaneous respiration (Natalie. et al., 2007).

2.2.17 Microarray analysis

Microarray analysis was performed at the Göttingen Transcriptome Analysis Laboratory, Medical Faculty, University of Göttingen. RNA was isolated from heart as described in 2.2.1.3. 0.3 µg of total RNA were used as a starting material to prepare cDNA. The synthesis of double-stranded cDNA was done with the WT Target Labeling and Control Reagents (Affymetrix; Cat. N° 900652). The cleanup of double-stranded cDNA was done using the GeneChip® Sample Cleanup module (Affymetrix). The in-vitro transcription was conducted with the WT Target Labeling Kit (Affymetrix). The total amount of the reaction product was purified with the GeneChip® cRNA Samle Cleanup Module (Affymetrix) and quantified using the NanoDrop ND-1000. A cDNA synthesis (ss) were performed using the WT Target Labeling Kit (Affymetrix). 5.5 µg of ssDNA were cleaved into fragments of 35-200 bases by enzymatic processes. The degree of fragmentation and the length distribution of the ssDNA were checked by capillary electrophoresis using the Agilent 2100 Bioanalyzer. A terminal labeling reaction (Biotin) was performed after fragmentation using the WT Labeling Kit (Affymetrix; Cat. N° 900652). Biotinylated fragmented ssDNA was hybridized onto the GeneChip® Mouse Gene 1.0 ST Array (Affymetrix; Cat. N°901171) according to the manufacturer’s recommendation. The hybridization were performed for 16 hrs at 60 rpm and 45°C in the GeneChip® Hybridization Oven 640 (Affymetrix). Washing and staining of the arrays were done on the Gene Chip® Fluidics Station 450 (Affymetrix) according to the manufacturers recommendation. The antibody signal amplification, washing and staining protocol were used to stain the arrays with streptavidin R-phycoerythrin (SAPE; Invitrogen, USA). To amplify staining, SAPE solution were added twice with a biotinylated anti-
streptavidin antibody (Vector Laboratories, CA) staining step in between. Arrays were scanned using the GeneChip® Scanner 30007G.

2.2.18 Computer analysis

For the analysis of the nucleotide sequences, programs like BLAST, BLAST2, MEGABLAST and other programs from National Center for Biotechnology Information (NCBI) were used (www.ncbi.nlm.nih.gov). For protein studies ExPASy tools (www.expasy.ch) were used. Mouse genome sequence and other analysis on mouse genes, transcripts and putative proteins were done in Ensembl database (www.ensembl.org).
3. Results

3.1 Analysis of Hspa4-deficient mice on the hybrid C57BL/6J x 129/SV genetic background

Analysis of Hspa4-deficient mice in hybrid C57BL/6J x 129/SV genetic background showed that male infertility is the most apparent phenotype. However, the fertility was heterogeneous in male. Thus, 8 of 13 male mutants did not produce a single litter, whereas the remaining 5 males produced litter size similar to that of wild type males. Histological analysis of testes from adult Hspa4-deficient mice revealed that the disruption of spermatogenesis is the main cause of male infertility (Held, 2008).

3.1.1 Impaired progression of the first wave of spermatogenesis in juvenile Hspa4-deficient mice

To identify the spermatogenic stage at which spermatogenesis is affected by Hspa4-deficiency, testicular sections from different postnatal days (P5, P10, P15, P20 and P25) were stained with hematoxylin and eosin. Histological analysis of 5-day-old Hspa4−/− testis showed normally developed Sertoli cells and gonocytes at the basement membrane of seminiferous tubules (Fig. 3.1A, B). In wild-type testis, at postnatal day 10 spermatogonia proliferate and develop to primary spermatocytes. No apparent differences were found in histological structure of seminiferous tubules between wild type and mutant mice at postnatal days 5 and 10 (Fig.3.1A-D). These results suggest that the proliferation and differentiation of spermatogonia to primary spermatocytes did not require Hspa4, despite the high expression of Hspa4 in gonocytes (Held, 2008). At postnatal day 15, when spermatogenesis progresses to mid-and late pachytene spermatocytes in wild-type testis (Fig. 3.2A), the number of pachytene spermatocytes was found to be drastically reduced in Hspa4-deficient testes (Fig. 3.2B). By postnatal day 20, spermatogenesis has reached the stage of round spermatids in wild-type tubules (Fig.3.2C). In contrast, mutant tubules are almost completely devoid of round spermatids and contain a reduced number of pachytene spermatocytes (Fig. 3.2D). At day 25, when tubules of wild-type littermates showed elongated spermatids as most advanced germ cells (Fig. 3.2E), Hspa4−/− testis showed severe depletion of germ cells. Very few seminiferous tubules contained round spermatids as most advanced germ cells in Hspa4-deficient testes (Fig. 3.2 F).
Results

Fig 3.1. Histological analysis of testes from 5- and 10- day-old wild type (+/+ ) and Hsp4a-deficient mice (-/-) reveals normal histological structure of seminiferous tubules. (A, B) 5-day-old testis (C, D) 10-day-old testis. Sp – primary spermatocytes... Photos were taken in 20 x magnification.

These results suggest that spermatogenesis in Hspa4^-/- testis is arrested at first meiotic prophase.

3.1.2 Immunohistochemical analysis of Hspa4^-/- testes

To confirm the results of histological analysis, we performed immunohistochemical analysis using different germ cell markers. Using the anti-heat shock protein 110 (HSPH1) antibody to label gonocytes (Held, 2008), the Hspa4^+/+ and Hspa4^-/- tubules contained an equivalent number of gonocytes (Fig. 3.3A-C), suggesting that the Hspa4 deficiency does not
Results

impair the gonocytes. Using anti-germ cell nuclear antigen 1 (GCNA) antibody, which recognizes
the spermatogonia and primary spermatocytes (Enders and May, 1994), the number of spermatogonia per tubule was not significant different between 5-day-old wild-type and mutant testis (Fig. 3.3D-F). This result suggests that the differentiation of gonocytes to spermatogonia is not affected in 5-day-old $Hspa4^{-/-}$ testis. In wild type testis, at postnatal day 10 spermatogonia underwent mitotic division and differentiate to spermatocytes. The mean number of GCNA1-positive germ cells in mutant and wild-type tubules was not significantly different at postnatal day 10, suggesting that mitotic division in mutant testes is not affected (Fig. 3.3G-I). However, few seminiferous tubules of $Hspa^{-/-}$ testes lacked meiotic germ cells (pachytene spermatocytes) at postnatal day 15. By immunohistological staining for heat shock protein 4-like (HSPA4L) protein, which is highly expressed in pachytene spermatocytes (Held et al., 2006), a reduction was observed in the mean number of HSPA4L-immunopositive cells per tubule in testis of $Hspa4^{-/-}$ mice compared to wild-type mice (Fig. 3.4A-C). Such reduction of HSPA4L-immunopositive cells was also observed in 20-day-old $Hspa^{-/-}$ testis (Fig. 3.4D-H). To prove whether the arrest of meiotic division is due to defects in pairing of homologous chromosomes during prophase I, we examined chromosomal synapses in $Hspa4^{-/-}$ spermatocytes. Germ cell suspension was prepared from adult wild-type and mutant testes, spread on slides, fixed with paraformaldehyde and immunostained with an antibody against synaptonemal complex protein 3 (SYCP3). The SYCP3 is a part of the synaptonemal complex between synapsed chromosomes during pachytene and remains on the disynapsed axis during diplotene. Analysis of germ cell spreads revealed that the formation of synaptonemal complexes in the meiotic cells of mutant testis was indistinguishable from that in wild-type. This indicates the proper accumulation of the synaptonemal complex protein SYCP3 (Fig. 3.5A-F).
Fig. 3.2. Delayed and disrupted first wave of spermatogenesis in Hspa4 mutant mice. Testicular sections from wild-type and Hspa4-null mice of various postnatal days (P) were stained with H&E. At P15, spermatogenesis has progressed up to pachytene spermatocyte stage (black
Results

arrowhead) in wild-type mice (A), whereas very few pachytene spermatocytes are present in testes of mutant mice (B). At P20, spermatogenesis reached the stage of round spermatids (arrow) in wild-type mice (C), whereas germ cell development in Hspa4-null mice is mostly impaired, seminiferous tubules are filled with premeiotic germ cells (white arrowhead) and contain very few pachytene spermatocytes. At P25, spermatogenesis is progressed to stages of round and elongated spermatids in wild-type tubules (E). In contrast, meiotic germ cells are sloughing off and vacuolization is visible in seminiferous tubules of Hspa4<sup>-/-</sup> mice (F). Scale bar, 50 µm.

![Image of histological sections showing spermatogenesis in wild-type and mutant mice](image)

**Fig. 3.** Expression profile of premeiotic and meiotic markers in Hspa4<sup>+/+</sup> and Hspa4<sup>-/-</sup> testes. Histological sections of wild-type (A, D and G) and null-mice (B, E, I) at different postnatal days (P5 and P10) were immunostained with HSPH1 (A, B) and GCNA1 (D E, G, H) antibodies. No significant difference is observed in the mean number of HSPH1- (C) and GCNA1-positive cell at P5 (F) and P10 (I) per tubule between wild-type and mutant testes. Scale bar, 50 µm.
Fig. 3.4. Expression of HSPA4L in Hspa4+/+ and Hspa4−/− testes. Histological sections of wild-type (A, B) and null-mice (D, E) at different postnatal days (P15 and P20) were immunostained with HSPA4L antibody. A significant reduction in the mean number of HSPA4L-positive cells per tubule was found in Hspa4−/− testes at days 15 (C) and 20 (F). Scale bar, 50 µm.
Results

**Fig. 3.5** Immunofluorescent localization of SYCP3 on synaptonemal complexes of wild-type (A-C) and Hspa4-deficient (D-L) pachytene spermatocytes. Representative sections were obtained from 5-month old Hspa4−/− and WT. Cells stained with anti-SYCP3 (Red) and DAPI (blue). 60 fold magnification.

3.1.3 Increase of apoptotic germ cells in Hspa4−/− testes

To determine whether the observed absence of differentiated germ cells is the result of enhanced apoptosis in Hspa4-null mice, TUNEL assay was performed to identify apoptotic cells during first wave of spermatogenesis in testes of postnatal day 10, 15, 20 and 25 (Fig. 3.6A-H). There are no significant differences in the number of apoptotic cells between Hspa4−/− and Hspa4+/+ testes at P10 (Fig. 3.6A, B and Fig. 3.7). A significant increase of TUNEL-positive spermatocytes was found in Hspa4-null mice at P15, 20 and 25 (Fig.3.6 D- H and Fig. 3.7). The frequency of TUNEL-positive cells was variable among tubules, but overall there were significantly more apoptotic cells in the seminiferous tubules of infertile Hspa4−/− mice than in those of their wild-type littermates.
Most of TUNEL-positive cells were seen to be in meiotic prophase (Fig. 3.6 D-H). These results indicate that germ cells at meiotic stages appear to be the most affected cells in Hspa4-deficient testes.

### 3.1.4 Expression analysis of germ cell marker genes in Hspa4-deficient testes

To further identify the spermatogenic stage at which spermatogenesis is impaired in adult Hspa4-null mice, we analysed the expression of different meiotic and postmeiotic marker genes. Testicular RNA was isolated from 5-month old wild type, fertile and infertile Hspa4<sup>-/-</sup> mice, and probed subsequently with a cDNA probe for Sycp3, Pkg2, Acr, Hsc70t and Tnp2 genes. The amount of loaded RNA used in this experiment were proved by rehybridization of blots with a cDNA probe for β-actin (Fig. 3.8A). Expression of Sycp3 gene encoding synaptonemal complex protein-3, which is exclusively expressed in leptotene and zygotene spermatocytes, revealed that the expression levels of Sycp3 in testes of fertile and infertile Hspa4<sup>-/-</sup> mice are similar with that in wild-type testes. In contrast, expression of testis-specific phosphoglycerate kinase gene (Pgk2) and acrosin (Acr), which were reported to peak in pachytene spermatocytes, was significantly reduced in testes of infertile Hspa4-null mice. Similar results were also shown for the transcript level of postmeiotic-specific genes Hsc70t (Hsp70 homolog gene) and Tnp2 (transition nuclear protein 2). These results confirm that the spermatogenic arrest in Hspa4-deficient mice occurred late in meiotic prophase I.

### 3.1.5 Expression analysis of other members of HSP110 family in Hspa4<sup>-/-</sup> testis

The leaky phenotype in spermatogenesis of Hspa4-null mice may be due compensation by overexpression of other members of HSP110 family. Therefore, we analysed the expression of HSPA4L and HSPH1 in testes of fertile Hspa4<sup>-/-</sup> mice (Fig. 3.8B). Western blot analysis did not reveal a marked increase in the expression of HSPA4L and HSPH1 in testes of Hspa4-null mice. These results suggest that the deficient of HSPA4 expression in the testes is not compensate for by increased expression of other cytolic proteins of HSP110 family.
Results

Fig. 3.6. Enhanced apoptosis during germ cell differentiation of Hspa4-null mice. Histological sections of Hspa4<sup>+/+</sup> (A, B, E and G) and Hspa4<sup>-/-</sup> mice (B, D, F and H) at different postnatal days (P) were subjected to TUNEL staining. The proportion of TUNEL-stained apoptotic cells (brown nuclei) at P15, P20 and P25 is higher in testes of Hspa4-deficient than in testes of wild-type
mice. Apoptotic germ cells are mainly pachytene spermatocytes as indicated by their nuclear size and their position in the seminiferous tubules. Scale bar, 50 μm.

**Fig.3.** Quantification of TUNEL –positive germ cells in Hspa4- deficient mice and wild type mice at different postnatal days (P10, P15, P20 and P25). Mean (± SEM) number of apoptotic germ cells per seminiferous tubule. WT, Wild –type; KO, Hsp4a-deficient mice; *, p<0.05; **, p<0.001.
Results

Fig. 3.8. Expression profile of different germ cell markers and members of HSP110 family in Hspa4<sup>+/−</sup> testes. (A) Northern blot with total RNA from testes of Hspa4<sup>+/+</sup> (+/+), fertile (-/-F) and infertile Hspa4<sup>−/−</sup> (-/-IF) mice was sequentially hybridized with cDNA probes for the indicated genes. (B) Expression pattern of other members of the HSP110 family in testes of wild-type and Hspa4-null mice. Immunoblots were probed with the antibodies against proteins shown at the right margin.
3.2 *Hspa4*-deficient mice with the inbred 129/Sv genetic background display postnatal growth retardation

In contrast to the *Hspa4* deficiency in the hybrid C57BL/6J x 129/Sv genetic background, analysis of *Hspa4*-deficient mice with the inbred 129/Sv genetic background revealed that 85% of *Hspa4*-deficient mice display growth retardation and 94.1% of newborn animals died between the third and fourth week after birth (Held, 2008). The cause of postnatal growth retardation shown in *Hspa4*-deficient mice could be due either to:

1. Metabolic failure or malabsorption of nutrients in the intestine
2. Defect in growth hormone (GH)-signaling
3. Increased degeneration of skeletal muscle and/or failure in muscle regeneration

Several experiments are performed to differentiate between these possible causes.

3.2.1 Metabolic state in *Hspa4*-deficient mice

3.2.1.1 Measurement of glucose levels in sera of *Hspa4*-deficient mice

To determine whether growth retardation in *Hspa4*-deficient mice is due to impaired glucose homeostasis, we have determined the glucose levels in serum of 10-, 15-, (22-28) and 60-day-old wild-type and mutant mice. The presence of milk food in the stomach of mutant neonatal animals was also monitored. Blood glucose concentrations in 10-, 15-and 20-day-old *Hspa4*-/ mice were significantly lower than in control littermates (Fig. 3.9). Dissection of mutants at these postnatal stages revealed that these mice had milk in their stomach. Although these mutant mice have lower glucose levels, they did not suffer with hypoglycemia, which is characterized by decrease of glucose levels in blood to less than 60 mg/dl. In contrast to their siblings, all the mutant mice (between 22- to 28-day-old) had less or no food in their stomachs and their glucose levels were decreased to 56±17.3 mg/dl (Fig. 3.9). Blood glucose concentrations in adult mutants, which had overcome the neonatal lethality, were not significantly different from those of wild-type. These results suggest that the growth retardation shown in *Hspa4*-/ animals during postnatal life is not due to hypoglycemia. Hypoglycemia found in *Hspa4*-/ animals at age between 22 to 28 days could a result either of fasting or of impairment in glucose homeostasis.
Results

**Fig. 3.9.** Glucose levels in the blood of $Hspa4^{-/-}$ and $Hspa4^{+/+}$ mice at different postnatal stages. Blood glucose was determined in the tail veins of 10-, 15-, 20-, (22-28)-, and 60 day-old mice. The glucose levels are significantly lower in $Hspa4$-deficient mice at different postnatal stages. In contrast, adult mutants have normal glucose level. Data are presented as mean ±SD of at least 4 animals of each age; *, p<0.05, **, p<0.001.

### 3.2.1.2 Expression of Pepck in liver of fasted $Hspa4^{-/-}$ mice

The gluconeogenesis is the main source of liver glucose output upon long term fasting. There are several enzymes which are involved in gluconeogenesis (Irimia, et al. 2010). To determine whether the hypoglycemia found in $Hspa4^{-/-}$ mice is a result of impaired gluconeogenesis, four mutants and three wild-type mice were fasted for 24 hrs and glucose concentrations in their blood were measured before and after fasting. Total RNA was isolated from the liver of fasting animals and used to determine the expression profile of phosphoenolpyruvate carboxykinase ($Pepck$), which is a key enzyme of gluconeogenesis in liver (Rajas et al., 2000). In contrast to the fed animals, the glucose levels in blood of fasted $Hspa4^{-/-}$ mice were significantly lower than in of their control littermates (Fig. 3.10A and B). Northern blot analysis revealed that the expression of $Pepck$ is not downregulated in liver of mutant mice (Fig. 3.10C). These results suggest that hypoglycemia showed in fasted $Hspa4^{-/-}$ is not due to impaired gluconeogenesis.
Results

Fig. 3.10. (A, B) Glucose levels were measured in blood of 2-month-old wild-type (n=3) and Hspa4\(^{-/-}\) (n=4) animals before (A) and after fasting (B). Data are presented as mean ±SD; *, p<0.05. Northern blot with RNA isolated from liver of these fasted animals was hybridized with a Pepck cDNA probe (C). Blot was rehybridized with β-actin to determine the integrity and equal amount of RNA loading (C).

3.2.2 Is growth retardation due to malabsorption of lipids in intestine?

The decrease of glucose levels in blood despite the presence of normal amount of milk and food in stomach of Hspa4\(^{-/-}\) mice at age 10- to 25-day lead us to determine whether their growth retardation may be a result of malabsorption of lipids in the intestine. In newborn mice, dietary fat is absorbed in intestine, predominantly as triacylglycerol (TG). In the endoplasmic reticulum of enterocytes, the lipoproteins are assembled in chylomicrons, which transport the lipids to peripheral tissues. The assembly of these chylomicrons by intestinal epithelial cells is dependent on the presence of microsomal triglyceride transfer protein (MTP) and apolipoprotein B (ApoB) and apolipoprotein AIV (apoAIV). The B apolipoproteins, apo48 and apoB100, play important structural roles in the formation of lipoproteins in intestine and liver. In the intestine,
the microsomal triglyceride transfer protein (MTT) transfer triglyceride to ApoB and apoAIV proteins (Black, 2007).

### 3.2.3 Expression analysis of apolipoprotein B and AIV in intestine and liver during postnatal development of Hspa4<sup>-/-</sup> mice

To determine whether the growth retardation in Hspa4<sup>-/-</sup> mice is due to a defect in lipid absorption, expression of apoB and apoAIV was determined in liver and intestine of 10-, 15-, 20- and 25-day-old Hspa4<sup>+/+</sup> and Hspa4<sup>-/-</sup> mice. As shown in figure 11A and B, no difference between wild-type and Hspa4<sup>-/-</sup> mice was found in expression of ApoB in liver and intestine (10- and 15-day-old mice). Similar results were obtained for ApoAIV in intestine of both genotypes at these postnatal stages (Fig. 3.11A, B). Apo AIV is not expressed in liver of 10- and 15-day-old Hspa4<sup>+/+</sup> and Hspa4<sup>-/-</sup> mice. In contrast, overexpression of ApoB was found in liver and intestine of 20- and 25-day-old Hspa4<sup>-/-</sup> mice, compared to that of wild-type. Expression of apoAIV was also found upregulated in Hspa4<sup>-/-</sup> intestine of both ages (Fig. 3.11C, D). At postnatal day 20, expression of Apo AIV was higher in intestine of Hspa4<sup>-/-</sup> than in that of Hspa4<sup>+/+</sup> mice, while no expression of Apo AIV could be detected in 20-day-old liver of both genotypes (Fig. 3.11C). Expression of ApoAIV in liver of 25-day-old Hspa4<sup>-/-</sup> mice was higher than in Hspa4<sup>+/+</sup> liver (Fig. 3.11D). These results revealed that the ApoB gene is upregulated in intestine and liver of Hspa4<sup>-/-</sup> mice at postnatal day 20 and 25-day, while the levels of ApoAIV mRNA are higher in 20- and 25-day-old Hspa4<sup>-/-</sup> mice in intestine.

### 3.2.4 Growth hormone (GH) signaling is not affected in Hspa4<sup>-/-</sup> mice

It is known that postnatal growth, such as muscle and longitudinal growth, is mediated by growth hormone (GH) signaling. Furthermore, several lines of evidence reveal the crucial role of the hepatic glucocorticoid receptor (GR) to modulate the large set of GH-responsive genes (Tronche et al., 2004; Engblom et al., 2007). To determine whether the growth retardation of Hspa4<sup>-/-</sup> mice is due to defect in GH-signaling, we analyzed the expression of two GH-responsive genes, Amyloid P component (APCS) and solute carrier organic anion transporter family, member 1b2 (SLCO1B2) (Lin et al., 2008), in liver of wild-type and Hspa4<sup>-/-</sup> animals (Fig. 3.12). Northern blots with RNA isolated from 10- and 14-day-old wild-type and Hspa4<sup>-/-</sup> were hybridized with APCS and SLCO1B2 cDNA probe. The results of Northern blot show that
there are no differences in expression of APCS and SLCO1B2 in liver of Hspa4−/− and wild-type mice. This result suggested that GH-signaling is not impaired in Hspa4- deficient mice.

**Fig. 3.11.** Expression analysis of apolipoprotein B (apoB) and apolipoprotein A4(apoAIV) during the postnatal development of Hspa4+/+ and Hspa4−/− mice. Total RNA was extracted from liver and intestine at different postnatal developmental stages. Northern blots with total RNA from liver and intestine of 10- (A), 15- (B), 20 (C)- and 25- (D) day-old Hspa4+/+ and Hspa4−/− mice were hybridized with ApoB and ApoAIV cDNA probes. The blots were rehybridized with a mouse β-actin probe to determine the integrity of RNA and amount of RNA loading.
Fig. 3.12. Expression analysis of GH-responsive genes in Hspa4-deficient and wild-type liver. Total RNA was extracted from 10- and 14-day-old Hspa4\textsuperscript{+/+} and Hspa4\textsuperscript{−/−} liver and subjected to Northern blots hybridization using the Amyloid P component (APCS) and the solute carrier organic anion transporter (SLCO1B2) cDNA probe, respectively. For RNA quality control Northern blots were rehybridized with mouse \(\beta\)-actin.
3.2.5 Analysis of skeletal muscles of *Hspa4*-deficient mice.

3.2.5.1 *Hspa4*-deficient mice display skeletal muscle myopathy

The presence of kyphosis in *Hspa4*–deficient mice of advanced age and reduction of skeletal muscle mass in *Hspa4*–deficient mice compared with wild-type lead us to suggest that the skeletal muscle of *Hspa4*−/− mice is affected. In order to determine the effect of *Hspa4* deficiency on skeletal muscle, histological analysis of different skeletal muscles was performed. Tibialis anterior, Vastus intermedius, Soleus and diaphragm muscles were isolated from 4-month-old *Hspa4*-deficient and wild-type mice, fixed in Bouin solution and embedded in paraffin. Analysis of hematoxylin & eosin stained sections of Soleus muscle revealed clear differences between wild-type and *Hspa4*-deficient mice (Fig. 3.13 A-D). Soleus muscle of mutant mice displayed severe myopathic changes. The myopathic changes are characterized by a marked variation in myofibril size, percentage of muscle fibers with central nuclei, which represent regenerated muscle fibers, and accumulation of nuclei representing necrotic fibers (Fig. 13B and D). Vastus intermedius (Fig.3.13F and H) and Tibialis anterior (Fig 3.13 J and L) also displayed myopathic changes. No myofibers with centrally located nuclei and variation in size of myofibers was detected in the diaphragm muscle of *Hspa4*−/− mice at 4-month of age (data not shown). To determine whether the skeletal muscle myopathy represents the major cause for observed kyphosis in *Hspa4*−/− mice of advancing age, histological analysis of paraspinal muscle of 18-month-old wild type and *Hspa4*−/− mice was performed and an abundant pathology was found (Fig. 3.14A-G). These results suggest that myopathy of the paraspinal muscle is a cause of kyphose development in aging *Hspa4*−/− mice.

3.2.5.2 Skeletal muscle myopathy develops during early postnatal development

The growth retardation of *Hspa4*−/− mice was observed after postnatal day 10. Therefore, histological sections of the Soleus muscle of 13- and 19-day-old wild-type and *Hspa4*−/− mice were analyzed (Fig 3.15A-F). Careful analysis of cross-section areas showed a significant increase in the percentage of myofibers with central nuclei in 13-day-old Soleus muscle of *Hspa4*−/− mice compared to wild-type (Fig.3.15C). The decrease of fiber size and the presence of myofibers with central nuclei are associated with skeletal muscle myopathy (Buj-Bello et al., 2008; Guo et al., 2006; Joya et al., 2004). This result further support that the growth retardation in *Hspa4*−/− mice is a result of myopathy of skeletal muscle. Similar muscle histopathology was
shown with histological sections in the Soleus muscle from 19-day-old \(Hspa4^{-/-}\) and wild-type mice (Fig. 3.15D-F).

**Fig. 3.13.** Histological evidence of myopathy in 4-month-old \(Hspa4^{-/-}\) mice. Histological sections of a region of Soleus (A-D), Vastus intermedius (E-H) and Tibialis anterior (I-L) muscles of 4-month-old wild-type (+/+) and mutant (-/-) mice. The boxed areas in B, F, J are shown in higher magnification in D, H and L. The white arrows and white head arrows indicate fibers with centrally located nuclei and accumulation of nuclei, respectively. In the boxed area show also the
variation in myofiber size. Sections were photographed at 20x (A, B, E, F, I, J) and at 60x (B box area, C, D, G, H, K, L) magnification.

Fig. 3.14. Myopathy in the paraspinal muscle is a cause of kyphosis in Hspa4<sup>−/−</sup> mice. (A) Development of kyphosis in 18-month-old Hspa4<sup>−/−</sup> mice. H&E-stained section from paraspinal muscle of wild-type (+/+), and mutant mice (-/-) at 18-months of age. Myofibers with central nuclei (white arrows). B, C: 10x magnification; D, E: 20x magnification; F, G: 60 x magnification.
Fig. 3.15. Histological studies of 13- (A-C) and 19-day-old (D-E) muscle in Hspa4-mutant (−/−) and wild type (+/+). (B and E) Sections of skeletal muscle from Hspa4-mutant mice showed marked increase of myofibers with central nuclei (white arrows) compared to control. Histogram comparing the percentage of fibers with centrally located nuclei in Soleus from 13- (C) and 19-day (F) Hspa4−/− and wild-type mice. *; P< 0.05.
3.2.6 Analysis of the heart in Hspa4-deficient mice

3.2.6.1 Development of cardiac hypertrophy in Hspa4-deficient mice

To determine whether myopathy of skeletal muscle is also realized in heart as hypertrophic cardiomyopathy, we analyzed hearts from 12-month-old wild-type and Hspa4-mutant mice. Heart showed marked enlargement in comparison to that of wild-type control (Fig. 3.16A). Histological examination revealed prominent myocyte degeneration and loss and intracellular vacuoles in cardiomyocytes surrounded by fibrotic tissue (Fig. 3.16B, C). This histopathology are features of sustained hypertrophy. To confirm that loss of myocytes is accompanied by extensive fibrosis, heart sections were stained with Massion's trichome staining. As shown in figure 3.16A and D, the increase of Hspa4<sup>−/−</sup> heart size is consistent with the thickened interaventricular septum and ventricular wall and decreased left ventricular cavity size. Significant levels of fibrosis are indicated by accumulation of collagen compared to heart of wild-type mice (Fig. 3.16, E, F). The fibrosis is more sever in septum and left ventricular wall than in right ventricular (Fig. 3.16D). These results reveal that Hspa4 disruption in the inbred 129/Sv genetic background causes cardiac hypertrophy and fibrosis. To determine the development of cardiac hypertrophy in Hspa4<sup>−/−</sup> mice, histological analysis of hearts and heart weight to body weight ratios (HW/BW) were performed in wild-type and Hspa4-mutant mice at different postnatal stages. In animals between 7- to 25-days of age, heart weight to body weight ratios (HW/BW) were not significant different from that of wild-type littermates (Fig. 3.17). In contrast, Hspa4<sup>−/−</sup> mice at 2- and 6- months of age showed a significant increase in HW/BW ratio compared to wild-type littermates (Fig. 3.17). Analysis of cross-sectional cardiomyocyte areas revealed no overt differences in morphology and size of myocytes between wild-type and mutant hearts isolated from mice at age between postnatal days 7 to 25 (Fig. 3.18A-F, 3.19O). Histological sections of hearts from these animals were stained with Massion's trichome staining to detect the extent of fibrosis. As shown in figure 3.16 G-L, the fibrosis is not yet realized in mutant hearts at these postnatal stages. Histological analysis of hearts from 2-3 and 6-month-old mice revealed that the size of left ventricular myocytes was markedly increased in Hspa4<sup>−/−</sup> mice compared with wild-type mice (Fig. 3.19M, N, O). Vacuoles in cardiomyocytes and development of fibrosis were also prominent in hearts of 2-, 3- and 6-month-old Hspa4<sup>−/−</sup> mice (Fig. 3.19D-F, J-N). Increase of fibrosis areas was found in hearts of 2-, 3- and 6-month-old Hspa4<sup>−/−</sup> mice. These areas were mostly marked in left ventricle and septum.
Results

Fig. 3.16. Hspa4 deficiency causes cardiac hypertrophy and fibrosis. (A) External view of representative hearts from 12-month-old Hspa4<sup>+/+</sup> (left) and Hspa4<sup>-/-</sup> (right) mice. (B and C) Histological sections of wild-type (B) and Hspa4<sup>-/-</sup> hearts (C) stained with H&E show degeneration and vacuoles in cardiomyocytes (white arrows). (D-F) Representative Massion's trichome–stained heart sections showing collagen-distribution (blue) in heart of Hspa4<sup>-/-</sup> mice, indicative for fibrosis (D). In sections of wild-type heart (E), no fibrosis was found. Right ventricle (RV); left ventricle (LV); Septum (S). Optical magnifications: 4x in E, F; 60x in B, C.
Results

Fig. 3.17. Heart weight to body weight (HW/BW) ratios in wild type and Hspa4-null mice. There was no significant difference in HW/BW ratio before 2 months of age between the Hspa4-null mice when compared with littermate controls. HW/BW ratios of Hspa4−/− mice were significantly greater in 2-3-, 6-, 12-, 24 month-old compared with those of wild-type mice. Data are mean ± SD from 4-15 mice for each group; *, p < 0.001, HW: heart weight; BW: body weight.
Results

Fig. 3.18. Cardiac hypertrophy and fibrosis were not observed in 7 to 25 day-old Hspa4-mutant mice. Hematoxylin and eosin-stained cross sections of hearts obtained from wild-type (A, B, C)) and Hspa4-mutant (D, E, F) mice at postnatal day 7 (A, D), 16 (B, E), 25 (C, F) are shown. At P7 (D), P16 (E) and P25 (F) heart sections did not show differences in morphology and size of cardiomyocytes between wild-type and mutant mice. (G-L) Representative Massion’s trichome – stained heart sections at postnatal day 7 (G, J), 16 (H, K), 25 (I, L) without fibrosis in Hspa4/− mice (J, K, L). Black lines indicate cardiomyocyte diameters in the region of the cell nucleus. Optical magnifications: 4 x in G-L; 60 x in A-F.
Fig. 3.19. Cardiac hypertrophy and fibrosis were first observed in hearts of 2-month-old Hspa4⁻/⁻ mice. Hematoxylin and eosin-stained cross sections of hearts obtained from wild type (+/+) (A, B, C) and Hspa4-mutant (-/-) (D, E, F) mice at month 2 (A, D), 3 (B, E), 6 (C, F) are shown. The 2-, 3- and 6-month-old Hspa4⁺/⁻ (D, E, F) hearts display severe signs of hypertrophy including increase of myocyte size, degeneration and loss of myocytes compared to control littermates. (G-N) Representative Massion’s trichome heart sections from 2- (G, J), 3- (H, K) and 6- (I, L, M, N) month-old Hspa4⁺/+ and Hspa4⁻/⁻ mice showing increased fibrotic areas (blue) in Hspa4⁻/⁻ heart (J, K, L, N). Myocyte diameter of mutant and wild-type hearts (O). Black lines indicate cardiomyocyte diameters in the region of the cell nucleus, which were significantly increased in hearts of 6-month mutant mice. Collagen-containing fibrotic region is stained with blue color (white arrow). Optical magnifications: 4x in G-L; 60x in A-F, M, N. *, p < 0.05; **, p < 0.001.
3.2.6.2 Echocardiographyic measurements in wild type and mutant mice

To further confirm the cardiac hypertrophy phenotype in *Hspa4*-mutant mice, echocardiogram was performed in 4-month-old wild-type (n=7) and *Hspa4*-null mice (n=10) (Fig. 3.20A, B). Two-dimensional directed M-mode echocardiograms were recorded to measure left ventricle end-diastolic and end-systolic dimensions, right ventricle and septal thickness. As shown in figure 3.20A-C, *Hspa4*<sup>-/-</sup> mice exhibit significant hypertrophy with increase of intraventricular septal thickness, left ventricle posterior wall thickness during diastole compared with age-matched wild-type control mice. The lumen of *Hspa4*<sup>-/-</sup> left ventricle was thinner than wild-type control (Fig. 3.20A, B). Left ventricular mass (LVM) as well as the left ventricular mass to body weight ratio (LVM/BW) were higher than in wild-type mice. However, the increase of LVM/BW ratio was not significant (Fig. 3.20E, F). Furthermore, the calculated ratio of wall thickness to heart radius at diastole (H/R) was significantly increased compared to that in wild type mice, suggesting a concentric pattern of enlargement (Fig. 3.20G). At 17 days of age, the *Hspa4*- null mice showed a small degree of hypertrophy, but with a significant increase in septal wall thickness compared with wild-type controls (Fig.3. 20D).

3.2.6.3 Ultrastructural analysis of sections *Hspa4*<sup>-/-</sup> heart

To further evaluate the morphological changes in cardiomyocytes of *Hspa4*<sup>-/-</sup> mice more precisely, we performed ultrastructural analysis on sections from left ventricular wall of 2-month-old *Hspa4*<sup>++</sup> and *Hspa4*<sup>-/-</sup> mice (Fig. 3.21A-F). Ultrastructural analysis revealed myofibrillar disarray and disassembly in *Hspa4*<sup>-/-</sup> heart. The structure of the myocyte nuclei was also markedly abnormal, with redundancy of vacuoles in the nuclei. Nuclei have bizarre shapes (Fig 3.21E). Furthermore, increased collagen accumulation (fibrosis) was frequently observed in *Hspa4*<sup>-/-</sup> hearts (Fig 3.21C, F). In contrast, ultrastructure of wild-type heart did no show these pathological changes (Fig. 3.21A, D).
Results

Fig. 3.20. Transthoracic echocardiography revealed affected dimensions and functions of Hspa4-/- hearts. (A and B) Shown two representative M mode images from heart of wild type (A) and null-mice (B). (C-G) Quantitative analysis of the diameter of diastolic left ventricular wall and septal of heart from 4-month- (C) and 17-day-old mice (D), left ventricular mass (LVM) (E) and LVM/BW ratio (F) of 4-month-old animals. (G) The H/R (left ventricular thickness/radius) ratio of 4-month-old heart shown marked increase of the H/R ratio in Hspa4-null mice. Data are mean ± SEM of 7 wild type and 10 Hspa4-/- mice; *, p <0.05; **, p <0.001, ventricular end-diastolic (right arrows) and end-systolic (left arrows). Ventricular cavity (double head arrows).
3.2.6.4 Analyses of molecular markers of cardiac hypertrophy

Ventricular hypertrophy induced by pressure or volume overload is known to lead to reactivation of several specific embryonic genes, such as atrial natriuretic factor (Anf), Brain natriuretic peptide (Bnf), β-myosin heavy chain 7 (Myh7) and skeletal α actins 1 (Acta1) (Komura and Yaakov, 1993; Chine et al., 1991). In order to show at which postnatal stage the development of heart hypertrophy starts, we studied the expression of hypertrophic marker genes in the heart from different postnatal stages. Northern blots with RNA isolated from hearts at P10, P14, P15, P17, P19, P37 and 3.5-month-old Hspa4+/+ and Hspa4-/- mice were hybridized with 32P-labeled Anf coda probe (Fig. 3.22). To check the integrity and equal amounts of RNA, the blots were rehybridized with β-actins coda probe. Northern blot analysis showed no difference in expression levels of Anf in hearts of wild-type and null-mice at postnatal days 10 and 14. Expression level of Anf was increased in RNA of Hspa4-null hearts at postnatal day 15 (P15) compared with the control littermates. Thereafter, a significant increase in the level of Anf expression was observed through the postnatal developmental stages of Hspa4-null hearts. To further confirm these results, expression levels of Anf, Bnf, Acta1 and β-Myh7 were determined at different postnatal stages by quantitative real-time RT-PCR (Fig. 3.23A-D). The results revealed that the expression levels of hypertrophic marker genes are significantly higher in Hspa4-/- hearts at postnatal days 15-25 than in wild-type hearts. The expression levels of Anf, Bnf, Acta1 and β-Myh7 were increased 7.0-, 4.7-, 3.8- and 2.8-fold in Hspa4-/- mice, respectively, compared with wild type. These results suggest that reactivation of the fatal gene program in the Hspa4-null hearts. Furthermore, the expression pattern of hypertrophy marker genes indicates that the development of hypertrophy in Hspa4-null mice starts in early postnatal life.

3.2.6.5 Upregulation of fibrosis marker genes in heart of Hspa4-/- mice

The development of cardiac fibrosis in Hspa4-/- lead us to determine at molecular level the progression of fibrosis in heart of Hspa4-/- mice at different postnatal ages. We determined the expression levels of fibrosis marker genes collagen I, collagen III and transforming growth factor- β1(TGF- β1). Quantitative real-time PCR was used to characterize expression level of fibrosis marker genes.
Fig. 3.21. Ultrstructural analysis by transmission EM of Hspa4^{+/+} (A, D) and Hspa4^{-/-} mice (B, C, E, F) reveals disarray and disassembly of myofibrils (white arrows) in Hspa4^{-/-} (B) heart versus the normal architecture of wild-type heart (A, D). Nuclei of myofibrils from Hspa4-null mice exhibit irregular morphology with multiple inclusions (white arrow head) compared to the normal nuclear shape of control myocyte (D). Collagen accumulations are frequently observed in mutant myocytes (black arrows).
Fig. 3.22. Expression of the Anf gene in heart of wild-type and null mice at different postnatal stages. Total RNA was extracted separated in 1% formaldehyde-agarose gel (15μg/lane), transferred onto nylon membrane and hybridized with $^{32}$P-labelled Anf and β-actin cDNA probes, respectively. Northern blot showing an increase in Anf mRNA expression in heart of Hspa4$^{-/-}$ mice at postnatal day 15 and advancing ages.
Fig. 3.23. Expression of hypertrophy genes in hearts of wild-type and null-mice at different postnatal ages. The mRNA levels of Anf (A), Bnf (B), Myh7(C) and Acta1 (D) were quantified by real time RT-PCR (n=3 per group). The relative mRNA level of each gene was normalized to that of house keeping succinate dehydrogenase (Sdha). Values indicate relative expression to wild-type littermate.
Results

As expected from the results of histological analysis for development of fibrosis in $Hspa4^{-/-}$ heart, no significance different in expression levels of collagen I, collagen III and TGF-β1 in heart of 20-day-old wild-type and $Hspa4^{-/-}$ mice (Fig. 3.24 A-C), while significant increase in expression of collagen III and TGF-β1 were observed in heart of 50-day-old $Hspa4^{-/-}$ mice at compared to that of littermate control (Fig 3.24. B, C). Expression levels of all three genes were markedly increased in 3-month old $Hspa4^{-/-}$ heart compared to that of wild-type. These results reveal that the cardiac fibrosis is developed in $Hspa4^{-/-}$ heart at early postnatal age.

3.2.6.6 Molecular pathways in the regulation of cardiac hypertrophy

Several molecular pathways have been implicated in the molecular response of cardiomyocytes to external stimuli or stress and in the development of cardiac hypertrophy. Gp130/STAT3, MAPK, Calcineurin-NFAT and PI3K/Akt/GSK-3-dependent signalings are among the best established mediators of cardiac hypertrophy. Alteration in the activity of one of these molecular signalings leads to development of cardiac hypertrophy (Frey and Oslon, 2003). To determine the signal pathway that is affected in $Hspa4^{-/-}$ mice and responsible for the development of cardiac hypertrophy, we determined the expression of some genes and proteins involved in Gp130/STAT3, MAPK and Calcineurin-NFAT signalings in heart of wild-type and $Hspa4^{-/-}$ mice.

3.2.6.6.1 Analysis of STAT3 and MAPK signaling in heart of $Hspa4^{-/-}$ mice

Several studies have reported that IL-6-related cytokines such as LIF induced hypertrophy in cardiac myocytes through gp130 receptor. Activation of gp130 receptor leads to downstream activation of two signaling pathways, one is the JAK/STAT-, the other the MAPK pathway (Kishimoto et al., 1994; Kunisada et al., 1996). The transcription factor STAT3 is essential for gp130-mediated hypertrophy in heart. To study whether the STAT pathway is affected in $Hspa4^{-/-}$ mice and responsible for the development of cardiac hypertrophy, we determined the expression of some genes and proteins involved in Gp130/STAT3, MAPK and Calcineurin-NFAT signalings in heart of wild-type and $Hspa4^{-/-}$ mice.
Fig. 3.24. Increase of fibrosis marker gene expression in Hspa4-mutant adult mice. Relative levels of collagen I (A), collagen III (B), and TGFβ1 (C) mRNA expression in heart of Hspa4-null and wild type mice during cardiac postnatal development (15-20-, 50-and 90-day-old) are illustrated. Expression levels were normalized to the expression of endogenous control (Sdha). RNA from hearts of three mice was used for each stage and genotype.
MAPK signaling pathway consists of a sequence of successively acting kinases that result in dual phosphorylation and activation of terminal effector kinases such as ERK1/2 (Widmann et al., 1999). To determine whether the MAPK signaling is altered in heart of Hspa4−/− mice, expression of ERK1/2 and activated form (phospho-ERK1/2) was studied by Western analysis. Blots with heart protein extracts from wild-type and Hspa4-null mice at different ages were probed with ERK1/2, phospho-specific ERK1/2 and anti-α-tubulin antibodies. Western blot analysis revealed that the ERK1/2 and phospho-related ERK1/2 protein levels are similar in heart of wild-type and mutant mice at different ages (Fig. 3.25B). These results suggest that the both signaling pathways are not responsible for the development of cardiac hypertrophy in Hspa4-null mice.

**Fig. 3.25.** Expression of inactive and active STAT3 and Erk1/2 proteins in heart. Western blots with protein extracts from heart of 3-week, 3-, 6- and 9-month-old Hspa4+/+ and Hspa4−/− mice were probed with STAT3, phospho-specific STAT3 and α-tubulin antibodies (A). Blots with the same proteins as in A were probed with anti-ERK1/2, anti phosph-ERK1/2 and anti-α-tubulin (B).
3.2.6.6.2 Genes involved in calcineurin/NFAT pathway are upregulated in heart of Hspa4⁻/⁻ mice

Several studies have shown that the angiotensin-endothelin system induce the activity of calcineurin-NFAT signaling pathway (Abbasi, et al., 2006). Therefore, we determined the expression of genes coding for receptors that regulate the activity of downstream proteins in the calcineurin/NFAT pathway in heart of wild-type and Hspa4-null mice. These receptors include endothelin A (ETₐ) and B (ETₐ), angiotensin II type 1 (AT₁) and type 2 (AT₂) receptors. We measured the expression of these genes by real time PCR in heart at two postnatal ages. At P18, expression of most genes in heart of Hspa4⁻/⁻ was slightly higher than that in wild-type littermates (Fig.3.26 A, B, D, E). Only expression of the gene coding for ETₐ endothelin receptor did not show any significant difference between heart of Hspa4⁻/⁻ and control mice at this postnatal age. In contrast, expression of the genes coding for these receptors was significantly up-regulated (at least 2-fold) in heart of 3-month-old Hspa4⁻/⁻ compared to heart of control mice (Fig.3.26 A, B, D, E). The expression of angiotensin II gene that codes for the ligand of AT₁ and AT₂ receptors was also determined in the heart of both genotypes and ages. Similar to expression pattern of its receptor, expression of angiotensin II gene was slightly higher in heart of 18-day-old Hspa4⁻/⁻ and 4-fold higher in heart of 3-month-old Hspa4⁻/⁻ mice than in heart of control littermates (Fig.3.26 C). The expression of endothelin (ET-1) coding for ligand of ETₐ and ETₐ also showed the same pattern (Fig.3.26 F). These results suggest that the Hspa4 deficiency alters the activity of angiotensin-endothelin pathway. To study whether the upregulation of these genes coding for ligands and receptors of angiotensin-endothelin pathway of 3-month-old Hspa4⁻/⁻ mice is accompanied by increase of the activity of Calcineurin-NFAT signaling pathway, quantitative real-time PCR assays were performed to determine the mRNA levels of several genes that are involved in this signaling. The studied genes include natriuretic peptide receptor A (Npr1), calmodulin 1 (Calm), protein kinase C (PKC), myocyte-specific enhancer factor 2C (Mef2C), GATA4 binding protein 4 and histone deacetylase 7A (Hdac7a). As shown in figure 3.27, except for Calm, mRNA levels of all other genes were slightly higher in heart of 18-day-old Hspa4⁻/⁻ mice than in control mice. In heart of 3-month-old mice, expression levels of all these genes were more than 2-fold higher in Hspa4⁻/⁻ mice compared to control mice. These results suggest that the expression of genes involved in calcineurin/NFAT signaling is altered in heart of Hspa4⁻/⁻ mice.
Results

Fig. 3. 26. Upregulation of genes that are involved in angiotensin-endothelin system in heart of Hspa4-mutants and controls. Quantitative real-time PCR analysis of mRNA levels for angiotensin II type 1 (AT₁) (A) and type 2 (AT₂) (B) receptors, angiotensin II (AngII) (C), endothelin A (ETₐ) (D) and B (ET₉) (E) receptors and endothelin (ET-1)(F) wild type (WT) and mutant (KO) mice was determined at 18-day- and 3-month-old. Expression levels were normalised to the expression of endogenous control (Sdha). 2-3 biological replicates were performed for each stage and genotype.
In calcineurin/NFAT signaling, calcineurin dephosphorylates and activates the NFAT transcription factors. The activated NFAT protein promotes together with other transcription factors such as Mef2C and GATA4 the expression of several hypertrophic induced genes. To determine whether calcineurin/NFAT pathway is highly activated in heart of $Hspa4^{-/-}$ mice, we determined the expression of the NFAT-target gene, cardiac-specific transcript modulatory calcineurin interacting protein 1.4 (MCIP1.4). The promoter of MCIP1.4 gene contains specific-acting element for NFAT proteins. Expression analysis revealed that the MCIP1.4 transcript level was 15-fold higher in heart of 3-month-old $Hspa4^{-/-}$ mice than in control mice. These results demonstrate that Calcineurin/NFAT signaling pathway is highly activated in heart of $Hspa4^{-/-}$ mice (Fig. 3.28). These results further suggest that elevated calcineurin/NFAT activity can modulate the cardiac hypertrophy of $Hspa4^{-/-}$ mice.

### 3.2.6.7 Microarray analysis of cardiac gene expression

To investigate the pathways regulating the development of cardiac hypertrophy in Hspa4-deficient mice, microarray analysis was used as a screening tool to indicate genes with altered expression in the $Hspa4$-null hearts at 25 days compared with that of wild type mice. We selected 25-day-old $Hspa4^{-/-}$ and control mice because at that age expression of marker genes for cardiac hypertrophy and fibrosis is slightly increased in heart of $Hspa4^{-/-}$ mice (sections 3.2.6.4 and 3.2.6.5). RNA from these wild-type and $Hspa4^{-/-}$ mice, respectively, was prepared and labeled coda was hybridized to GeneChip® Mouse Gene 1.0 ST Arrays. The specificity of results obtained by microarray analysis was firstly confirmed by studying downregulation of $Hspa4$ gene (4-fold decrease) and upregulation of hypertrophic markers such as $Anf$, $Bnf$, $Myh7$ and $Acta1$, which were found to be upregulated in $Hspa4^{-/-}$ heart by real time PCR described in section 3.2.6.4. There were 98 genes with statistically significant altered expression of at least $+/-1.5$ fold with P values of $< 0.05$, of which 42 were upregulated and 56 downregulated in $Hspa4^{-/-}$ heart compared to corresponding wild-type (Table 3.1). Several genes were selected and classified according to their function in the development of cardiac hypertrophy (Table 3.2).
Fig 3.27. Expression levels of Npr1 (A) calmodulin 1 (B), PKC (C), Mef2C (D), GATA4 (E) and Hdac 7a (F) in heart of Hspa4<sup>+/−</sup> and control mice at 18 days and 3 months of age. Expression levels were normalized to the expression of endogenous control (Sdha). 2-3 biological replicates were used for each stage and genotype.
Fig. 3.28. Overexpression of MCIP1.4 in heart of Hspa4-deficient mice. Values represent relative levels of MCIP1.4 in heart of 25-day- and 3 month-old animals. mRNA expression levels in each sample were normalized to the expression of Sdha. 2-3 biological replicates were used for each stage and genotype.
Results

Table 3.1 Microarray analysis showing genes altered in \textit{Hspa4}^-- as compare to wild-type.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Function</th>
<th>log2FC KOvsWT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nppa (Anf)</td>
<td>natriuretic peptide precursor type A</td>
<td>Hormone activity, regulation of blood vessel size</td>
<td>1.79</td>
</tr>
<tr>
<td>Kcne1</td>
<td>potassium voltage-gated channel, Isk-related subfamily, member 1</td>
<td>Potassium ion transport, epithelial cell Maturation</td>
<td>1.02</td>
</tr>
<tr>
<td>Acta1</td>
<td>actin, alpha 1, skeletal muscle</td>
<td>striated muscle thin filament, skeletal muscle fiber development</td>
<td>1.01</td>
</tr>
<tr>
<td>BC023105</td>
<td>cDNA sequence BC023105</td>
<td>unknown</td>
<td>0.90</td>
</tr>
<tr>
<td>Myl9</td>
<td>myosin, light polypeptide 9, regulatory</td>
<td>Calcium ion binding, motor activity</td>
<td>0.80</td>
</tr>
<tr>
<td>Dpysl3</td>
<td>dihydropyrimidinase-like 3</td>
<td>Hydrolase activity, SH3 domain binding</td>
<td>0.76</td>
</tr>
<tr>
<td>Gnao1</td>
<td>guanine nucleotide binding protein, alpha O</td>
<td>GTP binding, GTPase activity, signal transduction</td>
<td>0.75</td>
</tr>
<tr>
<td>Leng8</td>
<td>leukocyte receptor cluster (LRC) member 8</td>
<td>Protein binding</td>
<td>0.72</td>
</tr>
<tr>
<td>Fhl1</td>
<td>four and a half LIM domains 1</td>
<td>metal ion binding</td>
<td>0.72</td>
</tr>
<tr>
<td>Rasd2</td>
<td>RASD family, member 2</td>
<td>nucleotide binding, GTP binding</td>
<td>0.69</td>
</tr>
<tr>
<td>Adh1</td>
<td>alcohol dehydrogenase 1 (class 1)</td>
<td>NAD or NADH binding, oxidoreductase activity, zinc ion binding</td>
<td>0.66</td>
</tr>
<tr>
<td>Igfbp3</td>
<td>insulin-like growth factor binding protein 3</td>
<td>regulation of cell growth, insulin-like growth factor binding, Negative regulation of protein amino acid phosphorylation</td>
<td>0.64</td>
</tr>
<tr>
<td>Rps26</td>
<td>ribosomal protein S26</td>
<td>structural constituent of ribosome</td>
<td>0.63</td>
</tr>
<tr>
<td>Timp4</td>
<td>tissue inhibitor of metalloproteinase 4</td>
<td>Metal ion binding, metalloendopeptidase inhibitor activity</td>
<td>0.62</td>
</tr>
<tr>
<td>Casq1</td>
<td>calsequestrin 1</td>
<td>Calcium ion binding, regulation of muscle contraction</td>
<td>0.61</td>
</tr>
<tr>
<td>Pfn1</td>
<td>profilin 1</td>
<td>actin binding, regulation of actin polymerization or depolymerization</td>
<td>0.61</td>
</tr>
</tbody>
</table>
## Results

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Function</th>
<th>log2FC KOvsWT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myh7</td>
<td>myosin, heavy polypeptide 7, cardiac muscle, beta</td>
<td>calmodulin binding, striated muscle thick filament, striated muscle contraction, myosin complex</td>
<td>0.58</td>
</tr>
<tr>
<td>Irx4</td>
<td>Iroquois related homeobox 4 (Drosophila)</td>
<td>Regulation of transcription, heart development, regulation of transcription</td>
<td>0.57</td>
</tr>
<tr>
<td>Pitpnm2</td>
<td>phosphatidylinositol transfer protein, membrane-associated 2</td>
<td>calcium ion binding, phospholipid binding</td>
<td>0.56</td>
</tr>
<tr>
<td>Nrbp2</td>
<td>nuclear receptor binding protein 2</td>
<td>protein kinase binding, ATP binding, protein amino acid phosphorylation</td>
<td>0.55</td>
</tr>
<tr>
<td>Rnf207</td>
<td>ring finger protein 207</td>
<td>protein binding, intracellular, zinc ion binding, metal ion binding</td>
<td>0.55</td>
</tr>
<tr>
<td>9030624G23Rik</td>
<td>RIKEN cDNA 9030624G23 gene</td>
<td>Unknown</td>
<td>0.54</td>
</tr>
<tr>
<td>Zyx</td>
<td>zyxin</td>
<td>protein binding, cell-cell adherens junction, cell adhesion</td>
<td>0.54</td>
</tr>
<tr>
<td>Eif4ebp1</td>
<td>eukaryotic translation initiation factor 4E binding protein 1</td>
<td>translation initiation factor activity regulation of translation, regulation of translational initiation</td>
<td>0.53</td>
</tr>
<tr>
<td>Hspg2</td>
<td>perlecan (heparan sulfate proteoglycan 2)</td>
<td>protein binding, cell adhesion</td>
<td>0.52</td>
</tr>
<tr>
<td>Gata6</td>
<td>GATA binding protein 6</td>
<td>transcription regulator activity, metal ion binding</td>
<td>0.52</td>
</tr>
<tr>
<td>Rmrp</td>
<td>RNA component of mitochondrial RNAase P</td>
<td>Molecular function</td>
<td>0.51</td>
</tr>
<tr>
<td>Nppb (Bnf)</td>
<td>natriuretic peptide precursor type B</td>
<td>regulation of blood vessel size</td>
<td>0.50</td>
</tr>
<tr>
<td>Stk17b</td>
<td>serine/threonine kinase 17b (apoptosis-inducing)</td>
<td>protein amino acid phosphorylation, apoptosis, kinase activity, transferase activity</td>
<td>-0.50</td>
</tr>
<tr>
<td>Zfp72</td>
<td>zinc finger protein 72</td>
<td>zinc ion binding, metal ion binding</td>
<td>-0.50</td>
</tr>
<tr>
<td>Mrc1</td>
<td>mannose receptor, C type 1</td>
<td>receptor activity, calcium ion binding, integral to membrane</td>
<td>-0.50</td>
</tr>
<tr>
<td>Mpdz</td>
<td>multiple PDZ domain protein</td>
<td>protein binding, tight junction, cell adhesion</td>
<td>-0.51</td>
</tr>
<tr>
<td>Kras</td>
<td>v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
<td>nucleotide binding, GTPase activity, GTPase activity, protein binding, GTP binding</td>
<td>-0.51</td>
</tr>
<tr>
<td>Ctsc</td>
<td>cathepsin C</td>
<td>cysteine-type endopeptidase activity, lysosome, proteolysis</td>
<td>-0.51</td>
</tr>
<tr>
<td>Mrgprh</td>
<td>MAS-related GPR, member H</td>
<td>signal transducer activity</td>
<td>-0.52</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td>Function</td>
<td>log2FC KOvsWT</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>B3galt2</td>
<td>UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2</td>
<td>protein amino acid glycosylation integral to membrane, manganese ion binding</td>
<td>-0.52</td>
</tr>
<tr>
<td>Pkia</td>
<td>protein kinase inhibitor, alpha</td>
<td>negative regulation of transcription from RNA polymerase II promoter, protein kinase inhibitor activity</td>
<td>-0.52</td>
</tr>
<tr>
<td>Tm4sf5</td>
<td>transmembrane 4 superfamily member 5</td>
<td>integral to membrane</td>
<td>-0.52</td>
</tr>
<tr>
<td>Lrtm1</td>
<td>leucine-rich repeats and transmembrane domains 1</td>
<td>protein binding, membrane, integral to membrane</td>
<td>-0.53</td>
</tr>
<tr>
<td>Kcnd2</td>
<td>potassium voltage-gated channel, Shal-related family, member 2</td>
<td>potassium channel activity</td>
<td>-0.54</td>
</tr>
<tr>
<td>3110057O12Rik</td>
<td>RIKEN cDNA 3110057O12 gene</td>
<td>extracellular region</td>
<td>-0.54</td>
</tr>
<tr>
<td>C7</td>
<td>complement component 7</td>
<td>complement component 7</td>
<td>-0.55</td>
</tr>
<tr>
<td>Gpm6a</td>
<td>glycoprotein m6a</td>
<td>membrane, integral to membrane</td>
<td>-0.55</td>
</tr>
<tr>
<td>P4ha1</td>
<td>procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide</td>
<td>metal ion binding oxidation reduction</td>
<td>-0.56</td>
</tr>
<tr>
<td>Mrpl50</td>
<td>mitochondrial ribosomal protein L50</td>
<td>mitochondrion, ribosome, ribonucleoprotein complex</td>
<td>-0.56</td>
</tr>
<tr>
<td>Dcun1d1</td>
<td>DCN1, defective in cullin neddylation 1, domain containing 1 (S. cerevisiae)</td>
<td>unknown</td>
<td>-0.56</td>
</tr>
<tr>
<td>Dab2</td>
<td>disabled homolog 2 (Drosophila)</td>
<td>involved in differentiation cell morphogenesis and utero embryonic development</td>
<td>-0.57</td>
</tr>
<tr>
<td>Alkbh8</td>
<td>alkB, alkylation repair homolog 8 (E. coli)</td>
<td>oxidoreductase activity, transferase activity</td>
<td>-0.59</td>
</tr>
<tr>
<td>ENSMUSG00000049380</td>
<td>predicted gene, ENSMUSG00000049380</td>
<td>unknown</td>
<td>-0.59</td>
</tr>
<tr>
<td>Zmpste24</td>
<td>zinc metalloproteinase, STE24 homolog (S. cerevisiae)</td>
<td>metalloendopeptidase activity, proteolysis, nuclear envelope organization, zinc ion binding</td>
<td>-0.59</td>
</tr>
<tr>
<td>Scn4a</td>
<td>sodium channel, voltage-gated, type IV, alpha</td>
<td>Sodium ion transport, muscle contraction,</td>
<td>-0.61</td>
</tr>
<tr>
<td>Myl1</td>
<td>myosin, light polypeptide 1</td>
<td>motor activity, calcium ion binding, myosin complex</td>
<td>-0.61</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td>Function</td>
<td>log2FC KOvsWT</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>OTTMUSG00000003605</td>
<td>predicted gene, OTTMUSG00000003605</td>
<td>unknown</td>
<td>-0.63</td>
</tr>
<tr>
<td>Ptp4a1</td>
<td>protein tyrosine phosphatase 4a1</td>
<td>p protein amino acid dephosphorylation &amp; cell cycle</td>
<td>-0.65</td>
</tr>
<tr>
<td>AW061290</td>
<td>expressed sequence AW061290</td>
<td>membrane, integral to membrane</td>
<td>-0.65</td>
</tr>
<tr>
<td>Angpt1</td>
<td>angiopoietin 1</td>
<td>angiogenesis, receptor binding, signal transduction, cell differentiation</td>
<td>-0.66</td>
</tr>
<tr>
<td>6430514L14Rik</td>
<td>RIKEN cDNA 6430514L14 gene</td>
<td>unknown</td>
<td>-0.66</td>
</tr>
<tr>
<td>Tbc1d8b</td>
<td>TBC1 domain family, member 8B</td>
<td>GTPase activator activity &amp; calcium ion binding intracellular</td>
<td>-0.68</td>
</tr>
<tr>
<td>Fkm</td>
<td>fukutin</td>
<td>integral to membrane, transferase activity</td>
<td>-0.71</td>
</tr>
<tr>
<td>Hcn1</td>
<td>hyperpolarization-activated, cyclic nucleotide-gated K+ 1</td>
<td>potassium channel activity, sodium channel activity, ion transport</td>
<td>-0.71</td>
</tr>
<tr>
<td>100039652</td>
<td>predicted gene, 100039652</td>
<td>unknown</td>
<td>-0.72</td>
</tr>
<tr>
<td>Upk1b</td>
<td>uroplakin 1B</td>
<td>membrane, integral to membrane</td>
<td>-0.73</td>
</tr>
<tr>
<td>EG433229</td>
<td>predicted gene, EG433229</td>
<td>unknown</td>
<td>-0.76</td>
</tr>
<tr>
<td>Chordc1</td>
<td>cysteine and histidine-rich domain (CHORD)-containing, zinc-binding protein 1</td>
<td>calcium ion binding</td>
<td>-0.77</td>
</tr>
<tr>
<td>EG434373</td>
<td>predicted gene, EG434373</td>
<td>unknown</td>
<td>-0.77</td>
</tr>
<tr>
<td>Hsp1</td>
<td>heat shock 105kDa/110kDa protein 1</td>
<td>response to stress, chaperone cofactor-dependent protein folding</td>
<td>-0.77</td>
</tr>
<tr>
<td>Mme</td>
<td>membrane metallo endopeptidase</td>
<td>proteolysis, peptidase activity, metallopeptidase activity, zinc ion binding</td>
<td>-0.85</td>
</tr>
<tr>
<td>C3</td>
<td>complement component 3</td>
<td>positive regulation of type IIa, hypersensitivity, immune response</td>
<td>-0.94</td>
</tr>
<tr>
<td>Lgi1</td>
<td>leucine-rich repeat LGI family, member 1</td>
<td>protein binding, extracellular region</td>
<td>-1.09</td>
</tr>
<tr>
<td>Maob</td>
<td>monoamine oxidase B</td>
<td>mitochondrial, amine oxidase activity, oxidoreductase activity</td>
<td>-1.11</td>
</tr>
<tr>
<td>EG628276</td>
<td>predicted gene, EG628276</td>
<td>unknown</td>
<td>-1.51</td>
</tr>
<tr>
<td>Hspa4</td>
<td>heat shock protein 4</td>
<td></td>
<td>-2.01</td>
</tr>
</tbody>
</table>
### Results

#### Table 3.2 Selected genes which their expression was altered in \textit{Hspa4} mutant hearts.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>log2FC KOVsWT</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>A- Hypertrophic markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anf</td>
<td>natriuretic peptide precursor type A</td>
<td>1.79</td>
<td></td>
</tr>
<tr>
<td>Acta1</td>
<td>actin, alpha 1, skeletal muscle</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>Myh7</td>
<td>myosin, heavy polypeptide 7, cardiac muscle, beta</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Bnf</td>
<td>natriuretic peptide precursor type B</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>B- Hypertrophy related genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fktn</td>
<td>fukutin</td>
<td>-0.71</td>
<td>-Mutation of Fktn cause hypertrophic cardiomyopathy and muscular dystrophy</td>
</tr>
<tr>
<td>Gata6</td>
<td>GATA binding protein 6</td>
<td>0.52</td>
<td>-GATA factors are sufficient regulators of cardiomyocyte hypertrophy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Overexpression in transgenic mice induce hypertrophy</td>
</tr>
<tr>
<td>Igfbp3</td>
<td>insulin-like growth factor binding protein 3</td>
<td>0.64</td>
<td>Mice overexpressed of hIgfbp3- has organomegaly</td>
</tr>
<tr>
<td></td>
<td><strong>C- Ion transport</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcne1</td>
<td>potassium voltage-gated channel, Isk-related subfamily, member 1</td>
<td>1.02</td>
<td>- Involved in potassium channel</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Overexpression of KCNE1 in transgenic mice resulted in a cardiac phenotype similar to that in the thyroid hormone receptor-alpha1 (TRalpha1)-deficient mice, including a lower heart rate and prolonged QT time</td>
</tr>
<tr>
<td>Kcnd2</td>
<td>potassium voltage-gated channel, Shal-related</td>
<td>-0.54</td>
<td>- Involved in potassium channel</td>
</tr>
</tbody>
</table>
### Results

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Expression</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scn4a</strong></td>
<td>sodium channel, voltage-gated, type IV, alpha</td>
<td>-0.61</td>
<td>Involved in sodium channel</td>
</tr>
<tr>
<td><strong>Hcn1</strong></td>
<td>hyperpolarization-activated, cyclic nucleotide-gated K+ 1</td>
<td>-0.7</td>
<td>It contribute to spontaneous rhythmic activity in both heart and brain</td>
</tr>
<tr>
<td><strong>Irx4</strong></td>
<td>Iroquois related homeobox 4 (Drosophila)</td>
<td>0.57</td>
<td>- controls cardiac potassium channel Kv4.2 gene transcription</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Adult Irx4 KO mice developed cardiac hypertrophy</td>
</tr>
<tr>
<td><strong>Maob</strong></td>
<td>monoamine oxidase B</td>
<td>-1.11</td>
<td>Neutralized oxygen in mitochondria, deletion of Mao caused hypertrophy</td>
</tr>
<tr>
<td><strong>Gnao1</strong></td>
<td>guanine nucleotide binding protein, alpha O</td>
<td>0.75</td>
<td>Is critical target of oxidative stress</td>
</tr>
<tr>
<td><strong>Ptp4a1</strong></td>
<td>protein tyrosine phosphatase 4a1</td>
<td>-0.65</td>
<td>-It stimulates cell growth (stimulates progression from G1 into S phase during mitosis)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-It is modulated under oxidative stress</td>
</tr>
<tr>
<td><strong>Mme (NEP)</strong></td>
<td>membrane metallo endopeptidase</td>
<td>-0.85</td>
<td>-Hypoxia resulted in a significant decrease mme</td>
</tr>
</tbody>
</table>

#### D- Oxidative stress

#### E- synaptic junction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Expression</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lgi1</strong></td>
<td>leucine-rich repeat LGI family, member 1</td>
<td>-0.109</td>
<td>- Lgi1(-/-) causes abnormal synaptic transmission and epilepsy</td>
</tr>
</tbody>
</table>
Results

The differentially expressed genes include natriuretic peptide precursor type A \((Anf)\), natriuretic peptide precursor type B \((Bnf)\), actin, alpha 1, skeletal muscle \((Acta1)\) and myosin, heavy polypeptide 7, cardiac muscle, beta \((Myh7)\) and genes involved in ion channel signaling, potassium voltage-gated channel, Isk-related subfamily, member 1 \((Kcne1)\), potassium voltage-gated channel, Shal-related family, member 2 \((Kcnd2)\), sodium channel, voltage-gated, type IV, alpha \((Scn4a)\), hyperpolarization-activated, cyclic nucleotide-gated K+ 1 \((Hcn1)\) and Iroquois related homeobox 4 \((Irx4)\). In addition, differential expression of genes, which are involved in protection of cells against oxidative stress, were also observed. These genes are guanine nucleotide binding protein, alpha O \((Gnao1)\), protein tyrosine phosphatase 4a1 \((Ptp4a1)\) and membrane metalloendopeptidase \((Mme)\). We have also studied the expression of the monoamine oxidase B \((Maob)\), which induces the oxidative stress in cardiomyocyte by oxidation of monoamine to hydrogen peroxide in the mitochondria \((\text{Naoi et al.}, 2006)\). The \(Maob\) is downregulated in \(Hspa4^{-/-}\) heart by microarray analysis.

3.2.6.8 Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was used to confirm the expression levels of selected genes shown to be differentially expressed by microarray analysis. cDNAs of heart were prepared from 25- day- and 3-month-old mice and used to determine the expression of \(Kcne1\), \(Kcnd2\), \(Scn4a\), \(Hcn1\), \(Irx4\), \(Maob\), \(Mme\), \(Gnao1\), \(Ptp4a1\), GATA6, Fktn, Igfbp3, Lgi1 by quantitative real-time PCR. In 18-day- and 3-month-old \(Hspa4\)-null heart, expression of \(Kcne1\) and \(Irx4\) transcripts that control K+ channel was upregulated in \(Hspa4\)-null ventricle compared with control (Fig.3. 29A, B). Whereas the expression of \(Kcnd2\), another gene encoding for Potassium voltage-gated channel subfamily D member 2, was significantly downregulated in mutant heart (Fig.3.29C). The expression of \(Hcn1\), encoding pacemaker channel protein, was significantly reduced in \(Hspa4\)-mice in comparison to wild-type (Fig.3.29D). Expression of \(Scna4\) was also downregulated in \(Hspa4^{-/-}\) heart compared with control at both ages (Fig.3. 29E).
Results

Fig. 3.29. Expression levels of Kcne1 (A), Irx4 (B), Kcnd2 (C), Hcn1 (D) and Scn4a (E) in hearts of wild-type and Hspa4−/− mice at age of 25 days and 3 months. Quantitative real time PCRs were carried out in triplicate and in 3 mice per group at different postnatal stages. All values obtained were normalized to Sdha.
Maob is involved in the metabolism of serotonin and catecholamines that generates hydrogen peroxide (H₂O₂). Defects in this pathway lead to an increase of serotonin and catecholamines, which leads to an increase of blood pressure, and in consequence to cardiomyopathy. In 25-day- and 3-month-old Hsp4a-null heart, the expression level of Maob was significantly reduced compared with wild-type at both ages (Fig. 3.30A). The downregulation of maob may be one of the causes for development of hypertrophy in Hspa4-deficiency. Expression of Gnao1 is known to be induced by reactive oxygen species (Nishida, et al., 2000). Levels of Gnao1 mRNA were significantly increased in heart of 25-day- and 3-month-old mice mutant compared with wild-type (Fig. 3.30B). The Mme is a proteolytic enzyme responsible for degradation of Anf. Quantitative real time PCR showed that gene expression of Mme is significantly reduced in Hspa4⁻/⁻ heart compared with wild type (Fig. 3.30C). The zinc finger-containing transcription factors GATA4 and GATA6 are important regulators of basal and inducible gene expression in cardiac and smooth muscle cell types. Overexpression of either GATA4 or GATA6 is sufficient to induce cardiomyocyte hypertrophy characterized by enhanced sarcomeric organization (Liang, et al., 2001). In Hspa4⁻/⁻ heart, GATA6 was significantly increased in comparison with wild-type (Fig. 3.30D). Treatment of cardiac myocyte cultures with Igfbp-3 demonstrated an increase in Anf expression level (Henson et al., 2000). A significant increase of Igfbp-3 transcript was observed in heart of Hspa4⁻/⁻ mice at 25 days and 3-months of age compared with wild type (Fig. 3.30E). Mutation of Fktn cause hypertrophic cardiomyopathy and muscular dystrophy (Arimura, et al., 2009). Analysis of the expression of Fktn showed that expression of this gene is relatively low in 25-day-old wild-type and mutant heart, but its expression level at 3-month-old heart did not significantly differ between WT and KO mice (Fig. 3.30F). Leucine-rich, glioma inactivated 1 (Lgi1) regulates voltage-gated potassium channels assembly of Kcna1, Kcna4 and Kcnab1 and positively regulates synaptic transmission mediated by AMPA-type glutamate receptors. At day 18 and at 3-months, expression levels of Lgi1 sharply decreased in mutant heart compared with wild type (Fig. 3.30G).
Results

**Fig. 3.30** Relative levels of Maob (A), Gnao1 (B) Mme (C) GATA6 (D), Igfbp3 (E) Fktn (F) and Lgi1 (G) mRNA expression in heart of Hspa4<sup>−/−</sup> mice at age of 25 days and 3 months. Expression levels were normalised to the expression of endogenous control (Sdha). The results are representations of 3 independent experiments, each carried out in triplicate.
Results

3.2.7 Expression analysis of HSPA4L and HSPH1 in Hspa4-deficiency

Two other members of HSP110 family (HSPA4L and HSPH1) have been reported to be localized in cytoplasm as HSPA4. We examined the expression of both genes in Hspa4\(^{-/-}\) heart at protein level, because Hspa4-deficiency may alter the expression levels of both proteins. Total protein extracts were isolated from heart of 3-month-old wild-type and Hspa4\(^{-/-}\) mice, separated on PAGE-SDS gel and blotted on nitrocellulose filters. Blots were probed with HSPA4, HSPA4L and HSPH1 antibodies. No change in expression levels of HSPA4L and HSPH1 proteins were detected in the heart of Hspa4-deficient mice (Fig. 3.31). These results suggest that the loss of Hspa4 expression in Hspa4\(^{-/-}\) heart is not compensated for by a detectable increase of the expression of Hspa4l and Hsp1.

\[
\begin{array}{c|c|c}
\text{Heart} & +/+ & -/- \\
\hline
94-kDa & \text{HSPA4} & \text{HSPA4L} \\
96-kDa & \text{HSPH1} & \alpha\text{-tubulin}
\end{array}
\]

Fig. 3.31. Expression of HSPA4L and HSPH1 in heart of Hspa4\(^{-/-}\) mice. Western blots with protein extracts from heart of 3-month-old Hspa4\(^{+/+}\) and Hspa4\(^{-/-}\) mice were probed with anti-HSP4A , HSPA4L and HSPH1 antibodies. The membranes were stripped and subsequently probed with \(\alpha\text{-tubulin}\) antibody.
3.2.8 The effect of aging and oxidative stress on expression of HSPA4

At least 11 families of HSPs have been described. Most of them are expressed constitutively or strongly induced by stress conditions such as heat, osmotic or oxidative stress. Expression of other HSPs is induced at certain developmental stages (Bodega et al., 2002). It is also known that biochemical stresses resulting from hypertension, hypoxia and other forms of myocytes injury induce cardiac hypertrophy. Biochemical stresses affect the folding of proteins and cause a progressive loss of cardiomyocytes. Therefore, increased expression of HSP proteins prevents accumulation of misfolded proteins in cells. We have determined whether the expression of HSPA4 is increased in response to different stresses. Firstly, we determined the HSPA4 expression in wild-type heart at different ages. It is known that old hearts are exposed to more stresses than young hearts. Total cellular proteins were isolated from 3-, 6-, 9-, 12-month-old wild-type heart, electrophoresed on SDS-PAGE and transferred onto a nitrocellulose membrane. The Western blot was probed with HSPA4 antibody. Thereafter, the membrane was stripped and subsequently probed with HSP4L antibody. As shown in figure 3.32, the expression of HSPA4 is significantly increased in hearts with advancing age. In contrast, the expression level of HSP4L was not changed with increasing age.

These results lead us to suggest that the upregulation of Hspa4 in aging heart is linked with accumulation of stress such as oxidative stress. To prove the validity of this hypothesis, the expression of Hspa4 in heart was studied after paraquat injection, which induces oxidative stress. 3-month-old- wild type mice (n=3) were intraperitoneally injected with paraquat (50 mg/kg body weight). Control mice (n=3) were injected with PBS. Heart tissues were isolated from the mice 7 hrs after injection. Firstly, we checked whether the paraquat treatment induces oxidative stress in heart by analysis of the expression of oxidative stress marker superoxide dismutase [Cu-Zn] (SOD1) and Gnao1 (G protein binding) by quantitative real time PCR. The results demonstrate that the expression levels of SOD1 and Gnao1 are increased in heart of paraquat- injected mice compared to untreated mice (Fig. 3.33A, B). This result suggests that paraquat induces the oxidative stress in heart. However, there were no significant differences in the expression levels of Hspa4 between heart of paraquat-treated and control mice (Fig. 3.33C). These results suggest that Hspa4 is not induced by oxidative stress.
Fig. 3.32. Increased expression of HSPA4 with aging. Western blot of HSPA4 expression in 3-, 6-, 9-, 12 wild-type heart. The membrane was stripped and subsequently probed with anti HSP4L antibody (B).
Fig. 3.33. Effect of oxidative stress on expression of Hspa4 in heart. Expression of heart SOD1 (A), Gnao1 (B), Hspa4(C) genes in 3-month-old wild-type mice after paraquat (PQ) injection measured by real-time PCR. Expression of these genes normalized by Sdha and compared with control (C) without stress.
4. Discussion

4.1 Overview of results of this study

Analysis of Hspa4 mutant mice in hybrid C57BL/6J x 129/Sv genetic background demonstrated that male infertility is the most apparent phenotype. Number of spermatozoa and their motility in epididymis of infertile Hspa4-null mice were found to be drastically reduced, suggesting that spermatogenesis is disrupted (Held, 2008). To identify the spermatogenic stage at which spermatogenesis is affected by Hspa4 deficiency, testicular sections from different postnatal days were histologically and immunohistologically analysed. The results of these studies revealed that Hspa4-deficiency resulted in partial arrest of the first wave of spermatogenesis at the late stage of prophase I. To determine whether the observed loss of germ cells is a result of enhanced apoptosis in Hspa4-null mice, Tunnel assay was performed. The results of these analyses indicated that germ cells at meiotic stages appear to be the most affected cells in Hspa4-deficient testis. Furthermore, the down-regulation of transcription levels of genes known to be expressed in spermatocytes at late stages of prophase I and post-meiotic spermatids let us suggest that spermatogenesis is arrested at late stages of meiotic prophase I. These results provide evidence that HSPA4 is required for normal spermatogenesis.

In contrast to Hspa4 deficiency in the hybrid C57BL/6Jx129/Sv genetic background, most of Hspa4-deficient mice with the inbred 129/Sv genetic background display growth retardation and die between the third and fourth week after birth. To identify whether the cause of growth retardation is due to defect in glucose homeostasis, we determined the serum glucose levels during postnatal development. The results showed that a reduction of blood glucose level is only manifested in Hspa4−/− animals at age between 22 to 28 days. However, hypoglycemia found in fasted Hspa4−/− mice is not due to impaired gluconeogenesis. To address the question whether their growth retardation is a result of malabsorption of lipids in the intestine, expression analyses of apolipoprotein components of chylomicrons (ApoB and ApoAIV) were performed by RNA analysis. The ApoB gene is upregulated in intestine and liver of Hspa4−/− mice at postnatal days 20 and 25, while the level of ApoAIV mRNA is higher in intestine of 20- and 25-day-old Hspa4−/− mice. The upregulation of both apolipoprotein genes let us to suggest that the proteins in Hspa4−/− intestine are unstable. The degradation of both proteins may lead to feedback regulation and upregulation of ApoB and
ApoIV genes. However, Western blot analysis did not show a significant reduction of APOB proteins in Hspa4−/− intestine (Held, 2008). These results suggest that the absorption of lipid in Hspa4−/− intestine is not affected. We have then determined whether the growth retardation can be due to a defect in growth hormone (GH) signaling. However, normal expression of two GH-responsive genes in liver of Hspa4−/− mice excludes the impairment of the GH-signaling in Hspa4-deficient mice.

Development of kyphosis in Hspa4−/− deficient mice of advanced age leads us to examine whether these mice suffer from skeletal muscle myopathy. Microscopic examination of different skeletal muscles was carried out on 4-month-old wild-type and Hspa4−/− mice. Tibialis anterior, Vastus intermedius and Soleus muscles of Hspa4−/− mice were found to display myopathic changes. An abundant pathology was found in paraspinal muscle of 18-month-old mice. These results suggest that myopathy of the paraspinal muscle is a cause of kyphose development in aging Hspa4−/− mice. Furthermore, skeletal muscle myopathy was also shown in 13- and 19-day-old Soleus muscle of Hspa4−/− mice. This indicates that growth retardation in Hspa4−/− mice is a result of skeletal muscle myopathy.

To determine whether skeletal muscle myopathy is also realized in heart as hypertrophic cardiomyopathy, hearts from 12-month-old wild-type and Hspa4-deficient mice in the inbred 129/Sv genetic background were subjected to histological analysis. These histological analyses revealed the development of hypertrophic cardiomyopathy and fibrosis in Hspa4−/− mice. Further histological analyses revealed that cardiac hypertrophy and fibrosis were first observed in hearts of 2-month-old Hspa4−/− mice. Two-dimensional directed M-mode echocardiograms were performed to further confirm the cardiac hypertrophy phenotype. Significant hypertrophy with increase of interventricular septal thickness, left ventricle posterior wall thickness during diastole compared with control littersmates was found. Furthermore, the calculated ratio of wall thickness to heart radius at diastole (H/R) was significantly increased in mutant mice, suggesting a concentric pattern of enlargement. Echocardiographic measurement in Hspa4-null mice at 17-days of age showed a lower degree of hypertrophy but with a significant increase in septal wall thickness compared with wild-type controls. Ultrastructure analysis revealed myofibrillar disarray and disassembly, abnormal nuclei and increased collagen accumulation (fibrosis) in heart of Hspa4−/− mice. To identify when the development of cardiac hypertrophy and fibrosis starts in Hspa4−/− heart, we studied expression of marker genes for hypertrophy and fibrosis in hearts from different
Discussion

postnatal stages by Northern blot and real time PCR analyses. These results indicated that the development of cardiac hypertrophy and fibrosis in \textit{Hspa4}\textsuperscript{-null} mice starts in early postnatal life. We suggest that the early postnatal lethality in \textit{Hspa4}\textsuperscript{-/-} mice in inbred 129/Sv genetic background could be due to the progression of skeletal and cardiac muscle myopathy.

In order to identify the signal pathways that mediate cardiac hypertrophy in \textit{Hspa4}\textsuperscript{-/-} mice, we determined the expression of some genes and proteins, which are involved in Gp130/STAT3, MAPK and calcineurin-NFAT signaling pathways, in heart of wild-type and \textit{Hspa4}\textsuperscript{-/-} mice. The results of these analyses suggest that STAT3 and MAPK signaling pathways are not responsible for the development of cardiac hypertrophy in \textit{Hspa4}\textsuperscript{-null} mice. However, the increased activity of calcineurin/NFAT in \textit{Hspa4}\textsuperscript{-/-} heart can be responsible for modulation of the cardiac hypertrophy.

Microarray analysis was used as a screening tool to identify altered expression in the \textit{Hspa4}-deficient hearts at 25 days compared with that of wild-type mice. 98 genes were identified as differentially expressed. Several genes were selected and classified according to their function in the development of cardiac hypertrophy (Table 3.2). To confirm the results of microarray analysis, quantitative real-time PCR was performed. We confirmed that genes involved in ion channel signaling (\textit{Kcne1}, \textit{Kcnd2}, \textit{Scn4a}, \textit{Hcn1} and \textit{Irx4}), in protection of cells against oxidative stress (\textit{Gnao1}, \textit{Ptp4a1} and \textit{Mme1}) and in inducing oxidative stress (\textit{Maob}) are differentially expressed in heart of \textit{Hspa4}\textsuperscript{-/-} mice as compared to wild-type mice.

Expression analysis of HSPA4L and HSPH1 proteins in \textit{Hspa4}\textsuperscript{-/-} heart was done in order to show whether the expression of these members of HSP110 family are altered in \textit{Hspa4} deficiency. The loss of expression of \textit{Hspa4} in \textit{Hspa4}\textsuperscript{-/-} heart is not compensated for by a detectable increase in the expression of \textit{Hspa4l} and \textit{HspH1}. It is known that older hearts are exposed to more stress than young hearts. To investigate whether the expression of HSPA4 protein increases with aging, we determined the HSPA4 expression in wild-type heart at different ages. The data showed that the expression level of HSPA4 is significantly increased in hearts with advanced age. This result leads us to examine the expression of \textit{Hspa4} under condition of oxidative stress. Results of this experiment suggest that expression level of \textit{Hspa4} is not induced by oxidative stress.
4.2 The role of HSPA4 for germ cell development

Expression of HSPA4 is widespread in different tissues (Yasuda et al., 1995; Kaneko et al., 1997; Xue et al., 1998). However, the HSPA4 expression is highly enriched in male and female germ cells of prenatal gonads. Expression of HSPA4 in male gonocytes is gradually decreased after their migration to basement layers of seminiferous tubules and differentiation to spermatogonia (Held, 2008). The preferential expression leads us to study the specific role of HSPA4 in germ cell development. Analyses of Hspa4-deficient mice revealed that all Hspa4-null mice of the hybrid 129Sv X C57Bl/6J background were born at Mendelian ratio and were apparently normal, although expression of HSPA4 can be detected in all tissues of wild-type mice. Male infertility was the most apparent phenotype for Hspa4-deficient mice. Male infertility is histologically characterised by a decreased number of postmeiotic germ cells, and an increased number of meiotic and postmeiotic cells undergoing apoptosis. Hspa4 mutants display an arrest of the first wave of spermatogenesis in juvenile testes by postnatal day 15 when the most advanced germ cells in the testes remain at the late pachytene spermatocyte stage. These results indicate that Hspa4 deficiency impair the development of most germ cells in late prophase I.

Numerous proteins that are required for the development of male germ cells through meiotic and post-meiotic stages are mostly translated in pachytene spermatocytes (Mons et al., 1999). Failure of molecular chaperones to direct correct folding of newly synthesized proteins in pachytene spermatocytes might lead to accumulation of misfolded and damaged proteins, which would trigger spermatocytes to release meiotic division and initiate apoptosis. Based on the high similarity of HSP110 family members, we expected that the molecular chaperones, which also include the NEF members of HSP110 family, would be abnormal or partially affected in Hspa4-/- mice.

The relatively leaky phenotype of Hspa4-deficient mice led us to suggest that other members of HSP110 family can partially compensate for the loss of HSPA4 function. HSPA4l and HSPH1 were possible candidates, since both proteins are widely expressed and localized in the cytoplasm like HSPA4. Therefore, compensation for the absence of HSPA4 by HSPA4L and HSPH1 would be the cause that Hspa4-/- mice are viable and display normal development except for disruption of spermatogenesis. We tested this possibility by Western blot analysis. However, we did not detect a significant up-regulation of HSP4AL or HSPH1 expression in Hspa4-/- testes.

Impaired male fertility was also reported for Hspa4l-deficient mice (Held et al., 2006). Thus, approximately 42% of Hspa4l-/- male mice suffered from fertility defects. Whereas the
spermatogenic cells, the number of mature sperm in the epididymis and sperm motility was drastically reduced. The reduction of the sperm count was due to the elimination of a significant number of developing germ cells via apoptosis. Partial penetrance and similarity of male infertility phenotype in both Hspa4 and Hspa4l knockout mice prompted us to generate HSPA4 and HSPA4L double knockout mice. Analyses of double knockout mice revealed that the Hspa4+/Hspa4l-/- mice are dying immediately after birth (Held, 2008). Immunohistological analysis demonstrated that the number of gonocytes in Hspa4-/-/Hspa4l-/- testes of E18.5 was not significantly reduced compared to wild-type littermates (data not shown).

The expression of some HSP proteins is inducible by environmental stress, but expression of others can be either constitutive or developmentally regulated (Dix, 1997). The HSPA4L is highly expressed in spermatogenic cells, from late pachytene spermatocytes to postmeiotic spermatids. No HSPA4L could be detected in germ cells of prenatal testis and ovary (Held et al., 2006; unpublished results). In contrast, HSPA4 and HSPH1 are ubiquitously expressed proteins and become relatively enriched in gonocytes after colonization of gonads by primordial germ cells (Held, 2008). The enrichment of both proteins in germ stem cells suggests their significant role for male and female germ cells. The results showed that gonocytes are not affected in Hspa4-deficient mice suggesting a redundant function of both proteins in germ cell development. To our knowledge, there is no report describing abnormal spermatogenesis in Hsph1-deficient mice. In one study, Hsph1-null mice were described as normally fertile (Nakamura et al., 2008). The generation of HSPA4 and HSPH1 double knockout mice will help us to study the role of both proteins in development of germ stem cells in male and female gonads.

Several reports which used microarray analysis to identify preferentially expressed genes in different stem cells revealed that the Hspa4 is highly expressed in embryonic and different tissue-specific stem cells and its expression is down-regulated in their differentiated counterparts (Ramalho-Santos et al., 2002; Bhattacharya et al., 2004). Hspa4 was one of 216 enriched genes, which were found to be expressed at high levels in embryonic, neural and hematopoietic stem cells. Our results demonstrating high expression of HSPA4 in gonocytes further confirm the requirement of HSPA4 function for development of germ stem cells. Although the physiological role of molecular chaperones for self-renewal of stem cells is not known, it is believed that molecular chaperones may protect stem cells from aging due to oxidative stress (Ramalho-Santos et al., 2002). Caenorhabditis elegans that have an extended life-span have elevated levels of...
molecular chaperones and enzymes that process oxidative free radicals and appear to be resistant to environmental stress (Finkel and Holbrook, 2000).

A high incidence of male infertility was found among Hspa4-null mice in F2 generation, which contains a high level of inter-individual genetic variability. The decline in incidence of infertility phenotype in subsequent generations would point to a selection bias against that genotype. Therefore, partial penetrance of male infertility among Hspa4-null mice may be due to segregation of genetic modifier on the mixed genetic background. Analyses of different mice models revealed the consequence of background-related differences in male infertility phenotypes. Mice deficient for Tnp2, mitochondria solute carrier protein (MSCP), POU domain class 5 transcription factor 2 (Sperm-1), Hspa4l and insulin-like 6 (Insl6) showed the highest incidence of male infertility in F2 generation and in inbred genetic background (Bitgood et al., 1996; Pearse et al., 1997; Yu et al., 2000; Adham et al., 2001; Nayernia et al., 2002; Burnicka-Turek et al., 2009).

Several reports demonstrated the essential role of other heat shock proteins in the progress of spermatogenesis. Male Hsp70-2 knockout mice are infertile, whereas females are fertile. Like spermatogenic arrest in Hspa4-deficient mice, spermatogenesis of Hsp70-2-/- is arrested in prophase I of meiotic division. The number of apoptotic spermatocytes was slightly increased in Hsp70-2-mutant mice on postnatal day 15 and increased significantly at P17 (Dix et al., 1997; Mori et al., 1997). According to phenotype similarity between Hspa4- and Hsp70-2-deficient mice, we suggest that HSPA4 might be a cochaperone of HSP70-2 and the chaperone containing HSP70-2 and HSPA4 has an essential role in repair and clearance of misfolded proteins during meiotic phase of spermatogenesis. To prove this hypothesis, we have to check in the future whether HSPA4 interacts with HSP70-2.

BAT3 is a member of the Bag family that acts as a cochaperone for the heat shock protein HSP70 and is involved in various developmental processes, cellular stress and viability (Corduan et al., 2009). Most Bat3-deficient male germ cells die at meiotic prophase I and Bat3-/- testes contain increased number of apoptotic germ cells. Interestingly, the testis specific Hsp70-2 protein was undetectable in Bat3-deficient germ cells even though Hsp70-2 transcript levels were normal. Further experiments revealed that Bat3 interacts with Hsp70-2 and this interaction protects Hsp70-2 from degradation (Sasaki et al., 2008). In yeast, HSP110 proteins are a nucleotide exchange factor for HSP70 proteins. On the basis of the similarity in phenotype
between knockout mice for *Hspa4*, *Hsp70-2* and *Bat3*, we suggest that HSPA4, BAT3 and HSP70-2 cooperate to regulate diverse chaperone-assisted processes in male germ cells at pachytene stage. Adepletion of one of these proteins could be a cause for accumulation of damaged proteins and a signal to induce cell death.

In a current study immunostaining with an antibody against synaptonemal complex protein 3 (SYCP3) showed that the formation of synaptonemal complexes in the *Hspa4*<sup>-/-</sup> meiotic cells was indistinguishable from that in wild-type. In contrast, a higher number of unsynapsed or partially synapsed chromosomes was observed in pachytene spermatocytes of *Bat3*-deficient testis. Immunostaining with anti-phosphorylated histone H2AX antibody, which recognizes DNA double strand breaks, revealed that multiple γ-H2AX–positive foci were observed on paired and unpaired *Bat3*<sup>-/-</sup> chromosomes (Sasaki *et al*., 2008). These data indicate that multiple synaptic abnormalities occur in *Bat3*-deficient pachytene spermatocytes. HSP70-2 has been found to associate with the lateral element of the synaptonemal complex (SC) (Allen *et al*., 1996). Although SC assembled in early pachytene stage of *Hsp70-2*<sup>-/-</sup> mice, later studies showed that SC was fragmented at late pachytene stage. Immunostaining of wild-type pachytene spermatocytes with anti-HSPA4 antibody revealed that HSPA4 protein is not associated with SC. These data suggest that HSPA4 is not involved in the regulation of chromosome dynamics during meiosis.

The expression of heat shock *Hsc70t* gene is restricted to post-meiotic spermatids (Zakeri and Wolgemuth, 1987; Maekawa *et al*., 1989; Matsumoto and Fujimoto, 1990). *Hsc70t*<sup>-/-</sup> male mice have normal fertility and histological studies indicated that germ cell development during the late postmeiotic phase was not affected. However, ATP levels are low in the sperm of *Hsc70t*-deficient testis and their ability to produce ATP appears to be compromised. This suggests that HSC70t possesses unique chaperone capabilities that are required by postmeiotic germ cells for the assembly and function of protein complexes involved in energy production (Eddy, 2002).

### 4.3 Potential role of *Hspa4* in regulation of cell cycle

Arrest of spermatogenesis during meiosis lead us to suggest that *Hspa4* has a role in control of cell cycle during meiotic division. Among the most well established heat shock effects on cell cycle kinetics are the transient arrests of cells at the G1/S or G2/M border (Kühl and Rensing, 2000). During cell cycle, chaperones play an important stabilizing role. They associate with cell cycle or signal proteins in order to translocate them to their targets, to keep them in a required conformational state, or to eliminate degraded or mutated forms (Sato and Torigoe,
It has been reported that HSPA4 regulates cell cycle by competing the transcription factor ZONAB for binding to the SH3 domain of ZO-1. As a result of a dissociation of ZONAB/ZO-1, ZONAB is translocated to the nucleus where it regulates G1/S phase progression (Fig. 4.1).

**Fig. 4.1.** Potential function of HSPA4 as a regulator of cell cycle. HSPA4 compete with ZONAB for binding to the SH3 domain of ZO-1. At low level of HSPA4 or during binding of HSPA4 with HSP70 chaperone, ZO-1 binds to ZONAB. This interaction accumulates ZONAB in cytoplasm and arrest the cell cycle at G1 phase. At high level of HSPA4 or when the process of folding and correct the misfolded protein successfully completed during the G1 phase, HSPA4 releases the HSP70 chaperone and then binds to ZO-1 resulting in dissociation of ZONAB from ZO-1. Subsequently, ZONAB interacts then with CDK4 and ZONAB-CDK4 translocates from cytoplasm to nucleus. In nucleus, ZONAB-CDK4 regulates G1/S phase transition by transcriptional regulation of cyclin D1 and the initiation of DNA replication by transcriptional regulation of replication factors, such as PCNA (modified from Sourisseau et al., 2006; Tsapara et al., 2006).
ZONAB affects cell cycle progression by two distinct mechanisms. Firstly, it regulates the nuclear accumulation of the cell division kinase CDK4 via a direct interaction. Second, it regulates expression of genes encoding cell cycle regulators such as PCNA and cyclin D1 (Balda et al., 2003; Sourisseau et al., 2006). These data lead us to suggest that during binding of HSPA4 with HSP70 chaperone and their function to correct the misfolding proteins and folding of new synthesized proteins. ZO-1 binds to and keeps ZONAB inactive in the cytosol. Thus, inhibition of nuclear accumulation of ZONAB by its binding with ZO-1 reduces the nuclear pool of CDK4. A consequence of that is the inhibition of G1/S phase progression. When the process of folding and correction of misfolded proteins is successfully completed, HSPA4 translocates to plasma membrane and binds to ZO-1. Then, ZONAB interacts with CDK4 and this complex ZONAB - CDK4 codistributed to the nucleus, where they promoted G1/S phase transition. We expected that percentage of cells in S phase would be decreased in Hspa4-mutant testis as a result of cytosol accumulation of ZONAB and missfolded proteins. The arrest of cell cycle at G1 phase might be triggered the spermatocytes to initiate apoptosis.

4.4 The cause of growth retardation in Hspa4-deficient mice

In several mouse models, growth retardation in early postnatal development has been shown to be a result of defect in growth hormone signaling. Growth hormone (GH), acting through its receptor (GHR), is essential for somatic growth, development and maintaining metabolic homeostasis. Growth hormone receptor-deficient (GHR−/−) mice exhibit proportional growth retardation and drastically diminished insulin-like growth factor- (IGF-1) levels (Robertson et al., 2006). Insulin-like growth factor-I (Igf-1)-deficient animals have severe growth deficiency, organ hypoplasia and diminished survival (Baker et al., 1993; Liu et al., 1993; Powell-Braxton et al., 1993; Liu et al., 1998; Wang et al., 1999). In addition, hepatocyte nuclear factor 1α (HNF-1α) is required for postnatal growth and development in mice. Growth retardation in Hnf-1α null mice was found to be due to downregulation of growth hormone -responsive genes that are crucial for growth and development (Lee et al., 1998). Therefore, HNF-1α -related growth retardation results from a defect in the GH signaling (Lin et al., 2008). To determine whether the growth retardation of Hspa4−/− mice is due to defect in GH-signaling, we analyzed the expression of two GH- responsive genes, Amyloid P component (APCS) and solute carrier organic anion transporter family, member 1b2 ( SLCO1B2) , in liver of wild-type and Hspa4−/− animals. The results suggested that GH-signaling is not altered in Hspa4−/− mice.
Other mouse models reveal that growth retardation is due to malabsorption of lipids in the intestine. Fat malabsorption is one of several causes, which are contributing to growth retardation in glial cell line-derived neurotrophic factor family receptor α2 (Gfra2)-null mice (Rossi et al., 2003). During fat absorption in the intestine, luminal digestion products of dietary fats are absorbed across the microvillus border of the enterocyte. Apolipoprotein B (apoB) and apo AIV are known to have a particularly critical role in the assembly of the triglyceride-rich lipoproteins. (Young, 1990; Havel and Kane, 1995; Kane and Havel, 1995). ApoA-IV may physically interact with ApoB in the secretory pathway to modulate the process of triglyceride-rich lipoprotein assembly and secretion (Gallagher et al., 2004). The chylomicron-deficient mice lack all apoB expression in the intestine, which prevents the assembly and secretion of TG-rich lipoproteins from the intestine. These mice accumulate massive amounts of cytosolic fat droplets within the absorptive enterocytes of the intestine and manifest severe intestinal fat malabsorption and retarded growth (Young et al., 1995). To address the question whether growth retardation in Hspa4-deficient mice is due to lipid malabsorption, we determined the expression levels of ApoB and ApoAIV in liver and intestine of Hspa4+/− and wild-type. This expression analysis indicated that ApoB mRNA level is upregulated in intestine and liver of Hspa4+/− mice, while ApoAIV is higher in intestine of Hspa4+/− mice compared with wild-type animals. However, Held (2008) found in Western-blot analysis that the ApoB levels markedly reduced in Hspa4+/− intestine. It has been reported that yeast HSP110, SSE1p, associates with and stabilizes ApoB (Hrizo et al., 2007). These results suggest that HSPA4 might stabilize ApoB and overexpression of ApoB gene in Hspa4+/− mice is due to feedback regulation of ApoB gene in consequence of ApoB degradation.

4.5 Role of Hspa4 in skeletal muscle development

Mammalian skeletal muscle consists of multinucleated myofibers which are formed during development by fusion of mononucleated muscle progenitors, some of which remain associated to adult myofibers as satellite cells, which is a specific type of stem cells (Ciciliot and Schiaffino, 2010). Adult skeletal muscle fibers are terminally differentiated. Muscle growth and regeneration are accomplished by proliferation of satellite cells. In the unperturbed state, satellite cells remain in a nonproliferative, quiescent state. However, in response to stimuli such as myotrauma, satellite cells present around the damaged muscle become activated, proliferate, and
resulting myoblasts express myogenic markers (Fig. 4.2). Ultimately, these myoblasts fuse to existing muscle fibers or fuse together to form new myofibers during regeneration of damaged skeletal muscle (Bischoff et al., 1994; Schultz, 1996).

Several knockout mouse models display growth retardation, which is a result of skeletal muscle myopathy. Analysis of these mouse models revealed that the skeletal muscle myopathy is either due to severe muscle degradation, impaired muscle regeneration or both. Severe muscle degradation was the main cause for the postnatal growth retardation of knockout mice for Bag3, serum response factor (SRF) and mammalian target of rapamycin (mTOR), respectively. Similar to skeletal muscle myopathy observed in Hspa4-deficient mice, Bag3−/− mice cease to gain weight after postnatal day 12 and die. Skeletal muscle of Bag3−/− animals showed a marked variation in myofiber size, with evidence of atrophic fibers characterized by non-inflammatory myofibrillar degradation with apoptotic features (Homma et al., 2006). Few centrally located nuclei were found in Bag3−/− myofibers, suggesting absence of a regenerative response. In contrast, myofibers with centrally located nuclei can be detected in Hspa4−/− mice suggesting a regenerative response in Hspa4−/− mice following muscle degradation. However, we have not determined in this work whether the growth retardation of the Hspa4−/− is due to severe muscle degradation or impairment in the process of muscle regeneration. The mTOR knockout mouse displays severe skeletal muscle myopathy, displaying features of muscle dystrophy and metabolic myopathy leading to decreased growth rate, which starts at 4 weeks of age. By the age 13 week, mutant mTOR mice start to develop spinal deformity (kyphosis), a sign of muscle weakness, and most mutants eventually die between 22 and 28 weeks of age (Risson et al., 2009).

Impaired regeneration capacity is the cause for growth retardation in a number of gene knockout mouse models such for melanoma antigen, family D, 1 (Maged1), tetradecanoyl phorbol acetate-induced sequence 7 gene (Tis7) and for forkhead/winged helix transcription factor (Foxk1/Mnf). Maged1 deficiency results in defective cell cycle exit and impaired myotube maturation (Nguyen et al., 2010). While disruption of the Tis7 gene delayed muscle regeneration (Vadivelu et al., 2004). Foxk1−/− mice display growth retardation and a severe impairment in skeletal muscle regeneration following injury. Such impairment in muscle regeneration in Foxk1−/− skeletal muscle is due to a reduction in number of satellite cells and arrest cell cycle progression at G0/G1 phase (Hawke et al., 2003). It is possible according to the hypothesis (Fig 4.1) that the competition between the HSPA4 and ZONAB to bind with ZO-1 may be regulated
Discussion

the transition of cell cycle at G1/S phase of proliferated satellite cells and the HSPA4 depletion results in a G1/S arrest in significant number of proliferated satellite cells. Other possibility that the Hspa4-deficiency delays the withdrawal of myoblasts from the cell cycle to differentiation. ZO-1 is a tight junction adaptar protein that regulates gene expression and junction assembly. Aijaz et al. (2007) have reported that the interaction of HSP4A with ZO-1 is also important for junction formation and epithelial morphogenesis. Thus, depletion of HSPA4 in Madin-Darby canine kidney epithelial cells (MDCK) retards junction assembly and inhibits epithelial morphogenesis. On the basis on these data, we propose that Hspa4 deficiency may impair the fusion of myogenic cells during skeletal muscle regeneration and formation of new myofibers.

4.6 Development of cardiac hypertrophy in Hspa4-deficient mice.

Cardiomyocytes are terminally differentiated cells and lose their ability to proliferate soon after birth. Thereafter, cardiomyocytes grow in cell size without cell division to adapt to a demand for an increased workload. In a number of pathological conditions (e.g., hypertension, valvular disease, myocardial infarction, and cardiomyopathy) that impose overwork on the heart, postnatal cardiomyocytes undergo cardiac hypertrophy. Although cardiac hypertrophy is initially compensatory for an increased workload, prolongation of this process leads to congestive heart failure, arrhythmia, and sudden death (Levy et al., 1990; Lorell and Carabello, 2000). Cardiac myocyte hypertrophy is associated with changes in gene expression including increased expression of immediate early genes (e.g., c-jun, c-fos and egr1), heat shock protein genes (such as Hsp70 and CryAB) and re-expression of fetal genes for atrial natriuretic factor (Anf), brain natriuretic peptide (Bnf), β-myosin heavy chain 7 (Myh7) and skeletal α actin 1 (Acta1). High expression of these fetal genes in heart has been used as indices of hypertrophy (Hoshijima and Chien, 2002; Dorn et al., 2003; Kumarapeli et al., 2008). Our RNA analysis revealed that expression levels of hypertrophic markers Anf, Myh7 and Acta1 are increased in Hspa4-null heart at postnatal day 15 compared with control littermates. Thereafter, a significant increase in expression levels of hypertrophic markers was observed through the postnatal developmental stage of Hspa4-deficient hearts. These results revealed that cardiac hypertrophy develops in the heart of young Hspa4-deficient mice.
Fig. 4.2 Satellite cell response to myotrauma. Skeletal muscle trauma or injury may be minor - (e.g., resistance training) or may be more extensive (e.g., toxin injection, Duchenne muscular dystrophy). In response to an injury, satellite cells become activated and proliferate. Some of the satellite cells will reestablish a quiescent satellite cell pool through a process of self-renewal. Satellite cells will migrate to the damaged region and, depending on the severity of the injury, fuse to the existing myofiber or align and fuse to produce a new myofiber. In the regenerated myofiber, the newly fused satellite cell nuclei will initially be centralized but will later migrate to assume a more peripheral location (Hawke and Garry, 2001).
Discussion

There are different stress pathways that modulate cardiac hypertrophy, such as P13k/GSK-3, dependent MAPK/ERK, IL6/gp130/STAT3 and calcineurin/NFAT signaling pathway (Ruwhof and van der Laarse, 2000; Frey and Olsen, 2003; Baines and Molkentin, 2005; Hill and Olson, 2008). To determine the molecular pathway which is responsible for the development of cardiac hypertrophy in Hspa4-null mice, we determined the activities of signaling pathways in heart of Hspa4-deficient mice.

4.6.1 MAPK/ERK signaling pathway

Mitogen-activated protein kinase (MAPK) signaling pathways consist of a sequence of successively acting kinases that ultimately result in the dual phosphorylation and activation of terminal kinases such as p38, c-Jun N-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERKs) (Widmann et al., 1999). MAPK pathways provide an important link between external stimuli and the nucleus via phosphorylation and regulation of multiple transcription factors. The MAPK signaling cascade is initiated in cardiac myocytes by G protein–coupled receptors (angiotensin II, endothelin-1, and adrenergic receptors) and cardiotrophin-1 (gp130 receptor), and by stress stimuli (Sugden and Clerk, 1998). Once activated, p38, JNKs and ERKs each phosphorylate a wide array of intracellular targets that includes numerous transcription factors resulting in the reprogramming of cardiac gene expression as part of the hypertrophic program (Bueno and Molkentin, 2002) (Fig. 4.3). The ERK family members, ERK1 (p42) and ERK2 (p44), are directly phosphorylated by two MAPK kinases, MEK1 and MEK2 (Garrington and Johnson, 1999). Transgenic overexpression of MEK1, that activates ERK1/2, results in considerable cardiac hypertrophy (Bueno et al., 2000). Glennon et al., (1996) demonstrated that antisense oligodeoxynucleotides against the ERK isoforms p42 and p44 inhibited the morphological changes of hypertrophy in cardiomyocytes exposed to phenylephrine. MAPK/ERK signaling was found to mediate cardiac hypertrophy in several mouse models (Table 4.1). Deletion of Smad4, which is the central intracellular mediator of TGFβ signaling, resulted in cardiac hypertrophy and fibrosis. Phosphorylated extracellular signal-regulated kinase (ERK) 1/2 and mitogen-activated protein kinase-ERK (MEK) 1 were increased in the Smad4 mutants (Wang et al., 2005). To investigate whether the activation of MAPK signaling is responsible for development of cardiac hypertrophy in Hspa4-null mice, we analyzed the expression of ERK 1/2 and phospho-ERK1/2. Our study showed that MAPK/ERK signaling is not activated in heart of
Table 4.1. Activated signal pathway in different mouse models for cardiac hypertrophy

<table>
<thead>
<tr>
<th>Knockout mice</th>
<th>Activated signaling pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natriuretic peptides receptor1 (Npr1&lt;sup&gt;-/-&lt;/sup&gt;)</td>
<td>calcineurin–NFAT (Ellmers et al., 2007)</td>
</tr>
<tr>
<td>α B-crystallin (CryAB)/Hspb2 CryAB&lt;sup&gt;−/−&lt;/sup&gt;/Hspb2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>calcineurin-NFAT pathway (Kumarapeli et al., 2008)</td>
</tr>
<tr>
<td>cardiac myosin binding protein C (cMyBP-C&lt;sup&gt;−/−&lt;/sup&gt;)</td>
<td>MAPK pathway including JNK, p38-MAPK but not ERK pathway (Eijssen et al., 2008)</td>
</tr>
<tr>
<td>Heat shock protein 70 (Hsp70&lt;sup&gt;−/−&lt;/sup&gt;)</td>
<td>JNK, p38-MAPK, and Raf-1/ERK. (Kim et al., 2006)</td>
</tr>
<tr>
<td>Forkhead box transcription factor,O3 (Foxo3&lt;sup&gt;−/−&lt;/sup&gt;)</td>
<td>Calcineurin/NFAT signaling (Ni et al., 2006)</td>
</tr>
<tr>
<td>Neurofibromatosis type 1 (Nf1&lt;sup&gt;-/-&lt;/sup&gt;)</td>
<td>Ras-Erk pathway (Xu et al., 2009)</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase kinase (Mkk4&lt;sup&gt;−/−&lt;/sup&gt;)</td>
<td>JAK-calcineurin-NFAT signaling pathway (Liu et al., 2009)</td>
</tr>
<tr>
<td>Ras associated with diabetes GTPase (rad&lt;sup&gt;−/−&lt;/sup&gt;)</td>
<td>CaMKII pathway (Chang et al., 2007)</td>
</tr>
<tr>
<td>Profilin 1 (Pfn1 -TG)</td>
<td>JNK and ERK pathways (Moustafa-Bayoumi et al., 2007)</td>
</tr>
<tr>
<td>Adiponectin (Adipoq&lt;sup&gt;−/−&lt;/sup&gt;)</td>
<td>ERK signaling pathway (Shibata et al., 2004)</td>
</tr>
<tr>
<td>The Insulin-like Growth Factor 1 Receptor (IGF1R-TG)</td>
<td>PI3K pathway and the mitogen-activated protein kinase (MAPK) pathway (McMullen et al., 2004)</td>
</tr>
<tr>
<td>mothers against decapentaplegic homolog 4 (Smad4&lt;sup&gt;−/−&lt;/sup&gt;)</td>
<td>MEK1–ERK1/2 signaling (Wang et al., 2005)</td>
</tr>
</tbody>
</table>
Discussion

| angiotensin converting enzyme 2 (Ace2<sup>-/-</sup>) | AT1-ERK1/2, JNK1/2 and p38 pathways (Oudit et al., 2007) |

### 4.6.2 IL-6-gp130-STAT3 signalling pathway

Activation of the JAK/STAT pathway by overload was found to be mediated by glycoprotein 130 receptor (gp130), and at least cadiotrophin-1(CT-1) and IL-6 were involved in activation of this pathway (Pan et al., 1997). Binding of ligands (i.e. member of interleukin (IL)-6 family such as IL-6 and CT-1) to gp130 receptor, leads to formation of dimers of gp130 and activated JAKs (Kishimoto et al., 1994). As presented in figure 4.4, STAT factors bind to certain phosphotyrosines of gp130, leading to phosphorylation by JAK. Phosphorylated STAT factors form homo- and hetero- dimers, and translocate to the nucleus, where they induce gene expression (Kamimura et al., 2003). It has been found that induction of gp130-dependent signaling leads to activation of both MAPK and JAK/STAT pathways (Hoshijima and Chien., 2002). Specifically, STAT3 is translocated to the nucleus in response to gp130 activation, which results in the induction of genes involved in hypertrophy and survival pathways (Yamauchi-Takahara and Kishimoto, 2000). Overexpression of STAT3 in transgenic mice is sufficient to induce cardiomyocyte hypertrophy in vitro (Kunisada et al., 1998) and in vivo (Kunisada et al., 2000). Eijssen et al. (2008) reported that the lack of the cMyBP-C protein resulted in cardiac hypertrophy, which is mediated by activation of the gp130/STAT3 and MAPK signaling pathways. We examined whether STAT3 activation is involved in cardiac hypertrophy in Hspa4<sup>-/-</sup> mutant mice. Protein analysis revealed that the levels of phosphorylated STAT3 in Hspa4<sup>-/-</sup> hearts are not significantly different from that in wild-type. This data suggest that gp130/STAT3 pathway is not involved in development of cardiac hypertrophy of Hspa4<sup>-/-</sup> mice.

### 4.6.3 Calcineurin/NFAT signaling pathway

Neuroendocrine factors such as angiotensin II (Ang II) and endothelin-1 (ET-1) can stimulate G protein-coupled receptors (GPCRs). GPRCRs are typically coupled to G proteins, which lead to elevation of interacellular Ca<sup>2+</sup>. An increase in cytoplasmic Ca<sup>2+</sup> binds to calmodulin and activates calcineurin, which can dephosphorylate NFATs (nuclear factors of activated T-cells) (Fig. 4.5). Dephosphorylated NFATs migrate into the nucleus and promotes gene expression.
Fig. 4.3 Schematic representation of the activation of MAPK pathway. Activation of specific MAPKs involves highly regulated and modulated cascades of phosphorylation events mediated by sequential and concerted activation of upstream kinases. ERKs are phosphorylated by members of the MEK family; JNK/SAPKs and p38 MAPKs are phosphorylated by SEKs and MKKs.
Fig. 4.4. Binding of ligands to their cytokine receptors, such as cardiotrophin-1 (CT-1) leads to phosphorylation and activation of receptor-JAK complex with subsequent recruitment of STATs. Activated STATs migrate into the nucleus, and bind to promoters of targeted genes and stimulate gene transcription (Ruwhof and van der Laarse, 2000).

Genetic manipulation of signaling pathways in mice and biochemical analyses have shown that calcium/calmodulin (Ca\textsuperscript{2+}/Calm)-dependent signaling plays a pivotal role in pathological cardiac hypertrophy (Wilkins et al., 2004). NFAT and GATA4 cooperatively activate transcription of hypertrophic gene program, including Anf and Bnf genes. Anf and Bnf may create negative-feedback loop that suppress calcineurin activity (Tokudome et. al, 2005). Deletion of several genes such as Npr1, CryAB/HspB2, Foxo3 and Mkk in mice leads to activate calcineurin/NFAT signaling and induces hypertrophic responses (summarized in table 4.1) (Ni et al., 2006; Ellmers et al., 2007; Kumarapeli et al., 2008; Liu et al., 2009). In our current study we have determined whether the inactivation of the Hspa4 gene stimulates the activity of calcineurin-NFAT signaling pathway. Expression analysis revealed that calcineurin/NFAT pathway is significantly activated in Hspa4\textsuperscript{-/-} heart.
Discussion

**Fig 4.5** Calcineurin/NFAT signaling pathway. Activation of G protein-coupled or mechanical stretch receptors leads to an elevation of intracellular Ca2+ and activation of the calmodulin-regulated phosphatase, calcineurin. Calcineurin activation causes nuclear localization of NFAT transcription factors by direct dephosphorylation. Calcineurin also directly activates nuclear MEF2 factors. These factors, along with GATA-4 and other partners cooperatively activate transcription of the hypertrophic gene programme (Wilkins and Molkentin, 2002).

Endothelin 1 (ET-1), a potent vasoconstrictor peptide expressed by endothelium, is also upregulated in the heart in response to a variety of stresses (Shohet et al., 2004). ET-1 activates two G-protein-coupled receptors, endothelin A (ET_A) and endothelin B (ET_B), with approximately equal affinity (Arai et al., 1990, Sakurai et al., 1990). The binding of ET-1 to both ET_A and ET_B receptors in cardiomyocytes results in activation of G protein signaling and increased intracellular calcium (Beyer, et al., 1995). ET-1 and its receptors mediate stress-induced remodeling in the mammalian heart. In vitro experiments show that ET-1-mediated activation of either ET_A or ET_B receptors on cardiomyocytes results in cellular hypertrophy (Ito et al., 1993a; Cullen et al., 2001). ET-1 acts as a local factor involved in cardiac and craniofacial development, as well as in the regulation of cardiac contractility and hypertrophy (Kedzierski and Yanagisawa 2001). It has been reported that ET-1 stimulates the accumulation of Hsp 27 (Kawamura et al. 1999) and Hsp70 (Pan et al., 2004). In our present study, ET-1, ET_A and ET_B
expression levels were markedly increased in heart of *Hspa4*-null mice at 3 months. We suggest that HSPA4 protect cells against deleterious stimuli after induction of ET-1 in response to stress.

Calmodulin1 (CALM) is a small cytoplasmic Ca\(^{2+}\) binding protein that regulates numerous cellular activities. CALM may exert its action through a direct interaction with its target proteins or indirectly by regulating the activity of Ca\(^{2+}\)/CALM-dependent protein kinase and CALM stimulated protein phosphatase (calcineurin) (Maier and Bers, 2002; Saimi and Kung, 2002). Calm transgenic (*Calm*-TG) mice develop marked cardiac hypertrophy and exhibit upregulation of calcineurin, atrial natriuretic factor (ANF) and β-myosin heavy chain gene expression in the heart during the first 2 weeks after birth (Obata *et al*., 2005). In the present study calmodulin 1 expression level was found to be upregulated in heart of 3-month-old *Hspa4*\(^{-/-}\) mice. We suggest that calmodulin, which is a central mediator of several hypertrophic-signalling pathways, mediates the cardiac hypertrophy that occurs in response to the lack of *Hspa4*.

The activity of calcineurin is influenced by cofactors known as modulatory calcineurin-interacting proteins (MCIPs) or calcipressins (Rothermel *et al*., 2003). MCIP1 is upregulated by calcineurin signaling and has been proposed to function in a negative feedback loop to modulate calcineurin activity (Vega *et al*., 2003). The MCIP1.4 isoform has been shown to be tightly controlled by an alternative promoter containing 15 cis-acting elements of transcription factor NFAT cis-acting elements in the intron located upstream of MCIP1 exon 4 (Yang *et al*., 2000). MCIP1.4 expression can serve as a functional surrogate for calcineurin activity (van Rooij *et al*., 2004). Because MCIP1.4 is a target gene for activated NFAT transcription, MCIP1.4 expression is a better marker for calcineurin activity than calcineurin protein level (Ni *et al*., 2006). Calsarcin-1 overexpression in transgenic mice prevents AngII-induced cardiomyocyte hypertrophy via inhibition of calcineurin signaling, which results in downregulation level of MCIP1.4 (Frank *et al*., 2007). In addition, mice lacking calsarcin-1 are sensitized to calcineurin signaling and show accelerated cardiomyopathy in response to pathological biomechanical stress (Frey *et al*., 2004). Foxo proteins decrease calcineurin phosphatase activity and repress both basal and hypertrophic agonist-induced expression of MCIP1.4. Furthermore, hearts from Foxo3-null mice exhibit increased MCIP1.4 abundance and a hypertrophic phenotype (Ni *et al*., 2006). We have found that expression levels of MCIP1.4 is increased 15-fold in heart of 3-month-old *Hspa4*\(^{-/-}\) mice compared to wild type mice. These results indicate that activation of calcineurin-NFAT signaling is responsible for development of cardiac hypertrophy in *Hspa4*-deficient
mice.

Activated NFAT transcription factor induces in collaboration with transcription factor Mef and GATA4 the expression of hypertrophic genes such Anf, Bnf and Myh7 (Akazawa and Komuro, 2003). In current study, RNA analysis revealed the significant increase in expression of Mef2 and GATA4 in the heart of Hspa4-/- mice.

Histone deacetylases (HDACs) regulate chromatin remodeling. Phosphorylation of class II HDACs are regulated by various signal-transduction pathways. For example prohypertrophic signals modify HDAC kinases such as protein kinase D (PKD), PKC and Calm Kinase, which phosphorylate HDACs, resulting in their subsequent nuclear export. Histone deacetylases (HDACs) have been shown to associate with and repress MEF2 activation (Miska et al., 1999; Lu et al., 2000a, b; Youn et al., 2000). In hypertrophic heart of Calm kinase IV transgenic mice, MEF2 activation was suggested to occur through phosphorylation and dissociation of class II HDACs from MEF2 (Passier et al., 2000; Zhang et al., 2007). Ellmers et al. (2007) showed that Hdac 7a expression was increased in hypertrophic heart of Npr1-/- males. Here the expression level of Hdac 7a was increased in RNA of Hspa4-null hearts at 18 days and 3 months. This finding let us suggest that calmodulin and its kinase contribute to cardiac hypertrophy in Hspa4-null hearts through nuclear export of HDAC7 by phosphorylation and MEF2 transcription factors activation. Finally figure 4.6 gives our proposal for a pathway that modulates the cardiac hypertrophy of Hspa4-/- mice.

### 4.6.4 Cardiac fibrosis in Hspa4-/- mice

Cardiac fibrosis is a classical feature of hypertrophy and is characterized by the expansion of the extracellular matrix due to the accumulation of collagen, particularly collagen types I and III (Manabe et al. 2002). TGF-β stimulates fibroblast growth, enhances collagen synthesis, and suppresses collagen degradation (Mehta and Attramadal, 2007). TGF-β is an important mediator of cardiac hypertrophy induced by Ang II (Williams, 2001; Schultz et al., 2002). It plays an important role in myofibroblast differentiation during wound healing and fibrocontractive diseases by regulating the expression of alpha-SM actin in these cells (Desmoulière et al., 1993). In our study, collagen 1, collagen II and TGF-β expression levels were markedly increased in 3-month Hspa4-/- animals, but were not apparent in the heart of 18 day-old mice.
Fig. 4.6 Schematic representation of our proposal signaling pathways contributing to cardiac hypertrophy in Hspa4-null mice. Hypertrophic stimuli acting via the α subunit of guanine nucleotide-binding proteins after binding of G protein-coupled receptors (GPCRs), recruit PLCβ to the membrane, where it hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP2), releasing inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to receptors in the sarcoplasmic reticulum (SR), releasing calcium. The increase in cytosolic [Ca2+] together with calmodulin activates the protein phosphatase calcineurin. Calcineurin dephosphorylates several residues in the amino-terminal region of the transcription factor NFAT, allowing it to translocate to the nucleus and activate transcription of hypertrophic response genes. Calm regulates myogenesis and prevents formation of MEF2/HDAC complexes by inducing phosphorylation and nuclear export of HDACs. MCIP1.4 proteins actually facilitate Calcineurin activity for specific target proteins by serving as targeting subunits.
Discussion

The late increase in the expression of collagen I, collagen III and TGF-β in 3-month-old $Hspa4^{-/-}$ mice indicates that in this mouse model of cardiac disease, hypertrophy occurs in early postnatal life, whilst the fibrotic response occurs later.

4.6.5 Role of heat shock in heart protection

Hypertrophic growth of cardiomyocytes is a result of different physiological and pathological stresses. Heat shock proteins were originally discovered as proteins, whose expression is induced by heat shock. Subsequent experiments have revealed that HSPs expression is also induced by different cellular stresses, including mechanical ischemia/hypoxia and neural/hormonal. Therefore, this generic stress response molecule such HSP may also have a highly specific role in regulating cardiac hypertrophy under pathological stimulation (Vondriska and Wang, 2008). A number of studies have shown that HSPs in the heart have a protective effect against severe stress such as mechanical stress or pressure overload (Morimoto, 1993). These results have been obtained by analysing the effects of overexpression of an individual HSP in cultured cardiac cells and in the heart of transgenic animals. Kumarapeli, et al. (2008) showed that $Cryab/HspB2$ deficiency activates the NFAT/calceneurin signaling and induces cardiac hypertrophic responses at the unstressed or minimal stress conditions and exacerbates cardiac malfunction on pressure overload. In contrast, Cryab overexpression significantly attenuates pressure-overloaded. Overexpression of NFAT-targeted gene MCIP1.4 was observed in $Cryab/Hspb2$-deficient heart. Kim et al. (2006) studied the role of $Hsp70$ in maintaining cardiac contractility and calcium handling by investigation of $Hsp70$-knockout mice. We have shown that the depletion of HSPA4 causes cardiac hypertrophy, which suggests that HSPA4 confers cardioprotection against stimulated or metabolic stress. To confirm the cardioprotective effect of HSPA4, we have to determine which stress induces the expression of HSPA4 in heart. Generation of $Hspa4$ transgenic mice, in which HSPA4 is specifically overexpressed in cardiomyocytes, will help us to determine the protective effect of $Hspa4$ against different stresses that induces cardiac hypertrophy.

4.6.6 Expression profiles of $Hspa4^{-/-}$ heart

With the emergence of the DNA microarray technique, it is possible to have a more comprehensive characterization of the hypertrophic response at the global gene expression than in traditional single-gene studies (Liew and Dzau 2004, Liew 2005). Microarray analysis was
Discussion

Discussion performed to identify gene expression profiles and expand the knowledge of pathways regulating the development of cardiac hypertrophy in Hspa4-null mice. Results of DNA microarray analysis identified 98 differentially expressed genes that were upregulated or downregulated in Hspa4-null heart at least 1.5 fold with P values of < 0.05. Differentially expressed genes related to oxidative stress, ion channel signaling and synaptic junction were selected for further study (Table 3.2).

Result of DNA microarray showed a wide spectrum of changes in gene expression, which may be responsible for development of cardiac fibrosis and hypertrophy of Hspa4-null mice. A key finding of the microarray analysis was that expression of hypertrophic genes Anf, Bnf, Acta1 and Myh7 was upregulated. These results confirm our hypothesis suggesting that pathologic hypertrophy of Hspa4-/- heart is accompanied by reactivation of fetal gene programs. DNA microarray studies of other genetic animal models of hypertrophy revealed that activation of Anf and/or Bnf seems to be the most predominant change at the gene expression level (Aronow et al. 2001; Fischer, et al., 2005; Mirotsou et al. 2006).

Monoamine oxidases (MAOs) are mitochondrial flavoenzymes, which catalyze oxidative deamination of catecholamines (CAs) and biogenic amine such as serotonin. During this process, they generate hydrogen peroxide (H$_2$O$_2$), which can potentially source of oxidative stress in the heart. MAOs exist in two forms, MAOA and MAOB (Shih et al., 1999). Both enzymes catalyze the oxidative deamination of monoamine neurotransmitters such as serotonin, norepinephrine and phenylethylamine (Youdim and Bakhle, 2006; Bortolato et al., 2008). Maoa-knockout mice have elevated brain levels of serotonin, norepinephrine and to a lesser extent, dopamine (Cases et al., 1995), whereas only 2-phenylethylamine levels are increased in Maob-Knockout mice (Youdim et al., 2006). Maoa-KO mice displayed exaggerated ventricular hypertrophy which is due to increase serotonin levels (Lairez et al., 2009). Cardiomyocyte receptor that couples to Gq stimulates cardiomyocyte hypertrophy, the most important receptor of which are the 1-adrenergic receptors for norepinephrine and phenylephrine (Molkentin and Dorn 2001). Mice that are unable to synthesize norepinephrine, because of targeted disruption of the dopamine ß-hydroxylase gene, exhibit less cardiac hypertrophy and preserved ventricular function after surgical constriction of the transverse aorta (Esposito et al., 2002). In contrast, depletion of MAOA was associated with an increase in whole blood serotonin and exacerbates left ventricle thickening and fibrosis (Lairez et al., 2009). The present results showing reduced Maob expression in heart of 25-day and 3-month-old Hspa4-null mice suggest that the reduction of
Discussion

*Maob* in *Hspa4*-mutant mice increases the catecholamine stress and blood pressure. Such increase of catecholamine stress and blood stress induces cardiac hypertrophy via receptor 1-adrenergic receptors that initiate G-protein signaling cascade.

The main receptors involved in the regulation of heart muscle contraction are prototypical G protein-coupled receptors in response to neurohumoral induction (Rockman *et al*., 2002). They activate heterotrimeric G proteins that are comprised of α-, β-, and γ-subunits (Offermanns, 2003). The activation of a G protein is accomplished by the dissociation of its α subunit from the γβ dimer, both of which in turn modulate target effectors (Zhu and Birnbaumer, 1996). Guanine nucleotide binding protein also known as alpha O (Gnao1/GαO) protein belongs to the G-alpha family. Targeted deletion of both isoforms of Ga_o in mice lead to altered potassium and calcium channel regulation in neuronal cells and neurological abnormalities (Valenzuela *et al*., 1997; Tanaka *et al*., 1999; Greif *et al*., 2000; Kamp and Hell, 2000). Nishida *et al*. (2000) demonstrated that hydrogen peroxide (H2O2) induced *Gnao1* expression in rat neonatal cardiomyocytes. The α-Adrenoceptors mediate the contractile response in rat aorta by coupling to both binding of Gq protein and the GNAO1 protein (Gurdal *et al*., 1997). A constitutively active form of GNAO* (Ga_o*) exerts positive effect on cardiac Ca2-cycling and contractile function in ventricular myocytes of Gnao* transgenic mice (Zhu *et al*., 2008). The present results show a significant increase of Gnao1 expression in heart of 25-day- and 3-month-old *Hspa4*-null mice. We suggest that increase of GNAO1 activates phospholipase C, resulting in production of inositol trisphosphate and release of intracellular calcium after binding of norepinephrine to α-adrenoceptor (AR) in *Hspa4*-knockout. This results support the involvement of this pathway in the development of cardiac hypertrophy.

Insulin-like growth factor-binding protein3 (*Igfbp3*) is one of six members of protein family that bind with insulin-like growth factor1 (IGF1). These IGFBPs are capable to increase the half-life of IGF1 in the circulation and are able to either potentiate the cell specific effects of IGF1 or act as inhibitors to block its action by preventing delivery to responding cell types (Ito *et al*., 1993b). Murphy *et al*. (1995) reported that expression of human IGFBP3 in transgenic mice causes organomegaly (heart, liver and spleen). Henson *et al*. (2000) demonstrated that *Igfbp3* expression was significantly increased in heart of rodent models for heart hypertrophy. Increased IGFBP-3 protein was shown to induce transcription of atrial natriuretic factor (*Anf*) and β-myosin heavy chain (β-Mhc/Myh7). Moreover, the hypertrophy in these rodent models is independent of
ANGII, cardiotrophin-1 and IGF-1. In this study, Igfbp-3 transcript was markedly increased in heart of Hspa4−/− mice at 25 days and 3-months of age. Prolongation of the repolarization time has often been observed before fibrosis or clinical signs of heart failure in animal model become evident. Cardiac hypertrophy is characterized by electrical remodeling with increased risk of arrhythmogenicity. This electrical remodeling process is at least partially determined by a reprogramming of cardiac gene expression and the reactivation of ‘fetal’ cardiac genes. Among the differentially expressed genes in the Hspa4-null heart are genes encoding ion channel proteins. The hyperpolarization-activated cyclic nucleotide-gated channel (HCN) gene family, Ca2+, K+ and Na+ channels playing an important role in the electrical remodeling process (Boixel et al., 2006). Four genes that encode HCN channels have been identified: Hcn1, Hcn2, Hcn3, and Hcn4. HCN channels carry an inward current, the depolarizing Na/K current It, that underlies cardiac pacemaker activity (Jongbloed et al., 2008). KCNE1 encodes the potassium ion channel β subunit that co-assembles with KCNQ1 to produce a channel that mediates the slowly activating delayed rectifier potassium current, IKs (Barhanin et al., 1996; Sanguinetti et al., 1996). Loss of Kcne1 function prolongs ventricular action potentials in humans and in Kcne1−/− mice and display unexpectedly shortened atrial action potentials and prolonged electrocardiographic QT intervals (Temple et al., 2005). Leucine-rich glioma inactivated gene 1 (Lgi1) regulates voltage-gated potassium channels assembly of Kcna1, Kcna4 and Kcnab1 and positively regulates synaptic transmission mediated by AMPA-type glutamate receptors (Schulte et al., 2006; Diani et al., 2008; Sagane, 2008). The expression of the Hcn2, Hcn4, as well as Kcne1 and Kcne2 genes in cultured ventricular cell, which are isolated from acute myocardial infarction rat hearts, underwent dynamic expression changes, reaching peak levels at 1 or 2 weeks post-acute myocardial infarction. The increased expression may be related to ventricular arrhythmogenesis after acute myocardial infarction (Xia et al., 2010). In current study, we showed that expression of Kcne1 transcripts in heart of Hsp4a-null mutant is upregulated. In contrast, the expression levels of Kcnd2, Hcn1 and Lgi1 are significantly decreased in Hspa4-mutant heart compared with wild type. We propose that electrical remodeling is associated with ventricular hypertrophy in Hspa4-null.
5. Summary

The aims of this study were to determine the underlying causes of male infertility, growth retardation and cardiac hypertrophy in Hspa4−/− mice.

In order to identify the spermatogenic stage, at which spermatogenesis is affected by Hspas4 deficiency in mice with hybrid C57BL/6J x 129/Sv genetic background, we performed histological and immunological analysis. Analysis of germ cell development during juvenile life of Hspa4−/− mice showed an arrest of first wave of spermatogenesis in late stage of prophase I. RNA analysis showed a marked reduction in expression of late meiotic and postmeiotic-specific marker genes, whereas expression of early meiotic-specific genes was unaffected in the Hspa4−/− testes. These results suggest that HSPA4 is required for the regulation of diverse chaperone processes and cell cycle during germ cell progression.

The second aim of this study was concentrated to study the cause of growth retardation in Hspa4−/− mice with inbred 129/Sv genetic background. To investigate whether growth retardation is due to skeletal muscle myopathy, histological analyses of different skeletal muscles were carried out. Histological analyses revealed that Tibialis anterior, Vastus intermedius, Soleus and paraspinal muscles of Hspa4−/− adult mice displayed myopathic changes. Moreover, myopathic change was already present at an early postnatal stage of Hspa4−/− mice development. This suggested that skeletal muscle myopathy is the cause of growth retardation. Furthermore, these results suggest that HSPA4 has protective effect that confers the protection of skeletal muscle against mechanical or metabolic stress, which induce skeletal muscle degradation.

To determine whether skeletal muscle myopathy is also realized in heart as hypertrophic cardiomyopathy, histological molecular analyses and two-dimensional directed M-mode echocardiograms were performed. Histological analyses revealed the development of cardiac hypertrophy and fibrosis in heart of Hspa4-deficient mice. Ultrastructure analysis revealed myofibrillar disarray and disassembly in cardiomyocytes and increased collagen accumulation (fibrosis) in heart of Hspa4-deficient mice. Echocardiographic measurements further confirm the cardiac hypertrophy phenotype in Hspa4-null mice. To identify when the development of cardiac hypertrophy and fibrosis starts in Hspa4−/− hearts, we studied expression of hypertrophic and fibrotic marker genes in heart from different postnatal stages by Northern blot and real time PCR.
analyses. These results indicate that the development of cardiac hypertrophy and fibrosis in Hspa4-null mice starts in early postnatal life and let us to suggest that the early postnatal lethality in Hspa4−/− mice in an inbred 129/SV genetic background can be contributed to the progression of skeletal muscle myopathy and hypertrophic cardiomyopathy. In contrast to skeletal muscle and heart phenotype in Hspa4−/− mice with inbred background, histological analysis and expression analysis of hypertrophic genes showed that cardiac hypertrophy is not developed in Hspa4−/− mice with hybrid 129/Sv x C57BL genetic background.

In order to identify the signal pathways that mediate cardiac hypertrophy in Hspa4−/− mice, we determined the expression of some genes and proteins, which are involved in Gp130/STAT3, MAPK and calcineurin-NFAT signalings, in heart of wild-type and Hspa4−/− mice. Results of these analyses suggest that STAT3 and MAPK signaling pathways are not responsible for the development of cardiac hypertrophy in Hspa4-null mice. On the contrary, increased activity of calcineurin/NFAT in Hspa4−/− heart seems to modulate the cardiac hypertrophy.

Microarray analysis was used as a screening tool to identify altered gene expression in Hspa4-deficient heart at 25 days compared with that of wild-type mice. 98 genes were identified as differentially expressed. Several genes were selected and classified according to their function in the development of cardiac hypertrophy. To confirm the results of microarray analysis, quantitative real-time PCR was performed. We confirmed that genes involved in ion channel signaling (Kcne1, Kcnd2, Scn4a, Hcn1 and Irx4), in protection of cells against oxidative stress (Gnao1, Ptp4a1 and Mme1) and those inducing oxidative stress (Maob), are differentially expressed in heart of Hspa4−/− mice. The altered expression of these genes may be involved in the development of cardiac hypertrophy in Hspa4−/− mice.
6. References


References


References


References


References


References


Vega R.B., Rothermel, B.A., Weinheimer, C.J., Kovacs, A., Naseem, R.H., Bassel-Duby, R.,
Williams, R.S., and Olson, E.N. 2003. Dual roles of modulatory calcineurin-interacting

Res.* 103:1194.

between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families.*Biochem.* 47:7001–7011.

disruption of Smad4 in cardiomyocytes results in cardiac hypertrophy and heart failure. *Circ


Wilkins B. J., Molkentin J. D. (2002) Calcineurin and cardiac hypertrophy: Where have we

R., Molkentin, J. D. (2004). Calcineurin/NFAT coupling participates in pathological, but

Cardiol.* 87: 10C-17C.
References


References


Acknowledgements

There are many people to whom I wish to thank and who helped me, taught and encouraged me along the way.

I would like to express my gratitude to Prof. Dr. med. Dr. W. Engel for the chance he gave me, to make PhD study in the Institute of Human Genetics. I would like to warmly thank for his support, encouragement, excellent scientific supervision, valuable scientific discussions, and supervision and for always being ready to lend a helping hand in all situation. It has been a pleasure working in such an inspiring and friendly atmosphere as he has created at the Institute of Human Genetics.

I would like to express my gratitude and my sincere to Prof. Dr. I. M. Adham, for his valuable efforts, kind assistance, and encouragement through the course of this work and for all valuable things learned from him. Thank you for provided me with excellent scientific guidance, instructive ideas and theoretical discussion over the period of my PhD study. Thank you for the long hours at your office for learning, for your patience with my language mistakes and for all the corrections and revisions.

I sincerely thank PD Dr. S. Hoyer-Fender for being my co-referee. I also extend my sincere thank you to Prof Dr. J. Wienands, Prof Dr. E. A. Wimmer, Prof Dr. W. Wuttke and Prof. Dr. A. Mansouri for being my dissertation examiners.

I would like to thank all my institute colleagues and co-worker for helpful during my stay. I would like to appreciate the current and former members of our group: Maiada, Ola, Ilona, Lili, Belal, Shuai, Karina, Ozii, Oggie, Gonjee, Chimgee, Chiranjeevi, Katy, Krishna, Sandra, Sandra, Saskia, Janine, Thanks for spending so much time with me in and outside the lab. I really enjoyed it!
I would like also thank S. Meyer and S. Lührig for kindness, support for excellent assistance.

I am grateful to secretaries Mrs. P. Albers and Mrs. A. Winkles at the Institute of Human Genetics for excellent administrative assistance and for kind.

Most especially, thank my friends Maiada for her kindness, support, care, friendship, thank you for all things (support, helping inside and outside the lab). I would also thank my friends Heba, Basma for support and helping while I was ill.

I would like to thank Krishna (for friendship, support and discussion), Grzmil. Dr. Pawel and Zibat, Dr. Arne (for helping and support)

Finally I would like to thank my husband for his support, making me smile, help and love.
Curriculum vitae

Personal details:
Name: Amal Z. A.-Z. Barakat
Address: Am Vogelsang 1, 37075 Göttingen
Telephone: 017663066808
E-mail: amalbarakat2001@yahoo.co.uk
Date of Birth: 30.05.1975
Place of Birth: Cairo, Egypt
Nationality: Egypt
Sex: Female
Marital Status: Married

Educational background:
Since November 2006: PhD study, Institute of Human Genetics, Georg-August University, Goettingen
1999-2002 Scholarships for master's degree student in National Research Center, Egypt
2001- 2004: Master thesis, Biochemistry Department, Faculty of Science, Ain Shams University, Egypt
Title of thesis: “Purification and characterization of lipase from Cucurbitaceae“
1994- 1997: Study of Biology at the Ain Shams, University, Cairo, Egypt
1986- 1994: Primary and Secondary School (Cairo, Egypt)

Work Experience:
1998-1999 work in the lab of the hospital in Egypt
2002-2004 Research Assistant in National Research Center, Egypt
Since 2004 Assistant Researcher in National Research Center, Egypt

Publications: