Severe familial left ventricular non-compaction cardiomyopathy due to a novel troponin T (TNNT2) mutation

Mark Luedde†, Philipp Ehlermann†, Dieter Weichenhan†, Rainer Will†, Raphael Zeller†, Stefan Rupp2, Andreas Müller3, Henning Steen1, Boris T. Ivandic1, Herbert E. Ulmer3, Michael Kern4, Hugo A. Katus1, and Norbert Frey5*

1Department of Internal Medicine III, University of Heidelberg, 69120 Heidelberg, Germany; 2Department of Pediatric Cardiology, University of Giessen, 35392 Giessen, Germany; 3Department of Pediatric Cardiology, University of Heidelberg, 69120 Heidelberg, Germany; 4Department of Pathology, University of Heidelberg, 69120 Heidelberg, Germany; and 5Department of Cardiology and Angiology, University of Kiel, Schittenhelmstraße 12, 24105 Kiel, Germany

Received 14 December 2009; revised 4 January 2010; accepted 11 January 2010; online publish-ahead-of-print 18 January 2010

Aims Left ventricular non-compaction (LVNC) is caused by mutations in multiple genes. It is still unclear whether LVNC is the primary determinant of cardiomyopathy or rather a secondary phenomenon with intrinsic cardiomyocyte dysfunction being the actual cause of the disease. Here, we describe a family with LVNC due to a novel missense mutation, pE96K, in the cardiac troponin T gene (TNNT2).

Methods and results The novel mutation was identified in the index patient and all affected relatives, but not in 430 healthy control individuals. Mutations in known LVNC-associated genes were excluded. To investigate the pathophysiological implications of the mutation, we generated transgenic mice expressing human wild-type cTNT (hcTNT) or a human troponin T harbouring the pE96K mutation (mut cTNT). Animals were characterized by echocardiography, histology, and gene expression analysis. Mut cTNT mice displayed an impaired left ventricular function and induction of marker genes of heart failure. Remarkably, left ventricular non-compaction was not observed.

Conclusion Familial co-segregation and the cardiomyopathy phenotype of mut cTNT mice strongly support a causal relationship of the pE96K mutation and disease in our index patient. In addition, our data suggest that a non-compaction phenotype is not required for the development of cardiomyopathy in this specific TNNT2 mutation leading to LVNC.

Keywords Cardiomyopathy, left ventricular non-compaction • Transgenic animal model • Troponin T • Mutation

1. Introduction

Left ventricular non-compaction (LVNC) is a cardiac disorder characterized by an excessive trabecular meshwork of deep intertrabecular recesses within the ventricular myocardium. It is often associated with depressed ventricular function and can lead to fatal clinical events such as heart failure, systemic embolism, and ventricular arrhythmias. LVNC can be associated with other congenital heart abnormalities like ventricular septal defect or pulmonary valve stenosis as well as neuromuscular disease. LVNC has a higher prevalence than previously thought, presumably due to improved precision of cardiac imaging, leading to a more accurate diagnosis. It was argued that LVNC is rare in adults and associated with a high mortality and morbidity. Recent reports, however, documented a much better prognosis than previously published. LVNC is often found in familial forms of dilated cardiomyopathy (DCM) and includes both males and females, suggesting an autosomal mode of inheritance. Several genes have been associated with the disease, among them the X-chromosomal G4.5 (TAZ) gene encoding tafazzin. Mutations in G4.5 have also been linked to Barth syndrome, an X-chromosomal-recessive disorder characterized by skeletal myopathy, endocardial fibroelastosis, neutropenia, abnormal mitochondria, and several metabolic disturbances. Other causes of LVNC are mutations in DTNA, LDB3, LMNA, MYH7, ACTC, and

† These authors contributed equally to this work.
* Corresponding author. Tel: +49 431 597 1441; fax: +49 431 597 1470; Email: norbert.frey@uk-sh.de
Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2010. For permissions please email: journals.permissions@oxfordjournals.org.
TNNT2 encoding α-dystrobrevin, LIM domain binding protein 3, lamin A/C, β-myosin heavy chain (MHC), α-cardiac actin, and cardiac troponin T, respectively. The genes LDB3, LMNA, MYH7, and TNNT2 have also been associated with DCM or, in the case of MYH7, α-cardiac actin, and TNNT2, hypertrophic cardiomyopathy (HCM).

LVNC has recently been classified as a distinct genetic cardiomyopathy. However, it is still a matter of debate whether LVNC itself constitutes a specific entity of DCM or may rather be a secondary phenotype due to a maladaptive remodelling process in response to a disease-causing mutation. We describe a family with LVNC which is caused by a novel missense mutation (pE96K) in exon 10 of the cardiac troponin T gene. In order to validate the causal relationship between the mutation and cardiomyopathy, we generated a transgenic mouse model expressing the cTNT mutation found in this family with LVNC.

2. Methods

Please see Supplementary material online for a detailed description of isolation of RNA and cDNA synthesis, cDNA cloning, and site-directed mutagenesis, generation of transgenic mouse lines, quantitative gene expression analysis, protein expression analysis and echocardiography.

In brief, total RNA was prepared from human (explanted) or mouse heart with the Trizol method (Invitrogen, Karlsruhe, Germany). First-strand cDNA was synthesized from total RNA using Superscript III reverse transcriptase. Cloning of a plasmid expressing human TNNT2 gene (accession no. NM_001001430, position 70–936) under the control of a heart-specific α-MHC promoter was performed using the gateway system (Invitrogen). This TNT entry clone was then used to change human TNNT2-codon 96 from GAG to AAG (pPE96K) by site-directed mutagenesis using the QuickChange II-XL kit (Stratagene, Amsterdam, The Netherlands).

2.1 Patients

DCM was diagnosed in the index patient and affected family members according to established criteria. Echocardiography was performed according to national and international standards using a Vivid 7 ultrasound system (GE Healthcare). Left ventricular ejection fraction (LVEF) was estimated according to the Simpson’s rule using a mean value from apical 4- and 2-chamber views. Echocardiographic criteria for left ventricular non-compaction were a ratio of non-compacted to compacted layer (NC/C) >2.0 during end-systole and perfused ventricular recesses as shown by colour Doppler in the absence of other structural abnormalities. Cardiac MRI was performed in supine position at end-expiration employing a 1.5 T whole body superconducting MRI scanner system (ACHIEVA, Philips Medical Systems, Best, The Netherlands) using a five-element cardiac-phased array surface coil with vector ECG-gating. Assessment of resting LV function was determined by cine images using a steady-state free precession sequence (matrix = 160/256, sense-factor = 2, flip angle = 60°, slice thickness/gap = 8/2 mm) in continuous short axes covering the whole left and right ventricle from base to apex as well as 2-, 3-, and 4-chamber views in anatomically correct heart axes. MRI data were transferred to a clinical workstation (Viewforum, Philips Medical Systems). LVEF (EF in %) was generated manually using short axes volumetry by determination of end-diastolic and end-systolic cine images and subsequent delineation of endocardial borders excluding papillary muscles. The diagnostic criterion for the diagnosis of LVNC on the basis of the MRI scan was an NC/C ratio of >2.3 in diastole. The investigation conforms with the principles outlined in the Declaration of Helsinki and was approved by the ethics committee of the Medical Faculty at the University of Heidelberg.

2.2 Mutation screening

Mutation screening in genes LDB3, LMNA, MYH7, MYBPC3, and TNNT2 of the index patient was performed by denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing of amplicons displaying an aberrant DGGE banding pattern as described previously. Mutation screening in the coding exons 3–23 of the DNA gene was done by direct sequencing. Available relatives of the index patient were screened for the presence of the identified mutation by direct sequencing of TNNT2 exon 10.

2.3 Generation of transgenic mouse lines

A fragment harbouring the wild-type (WT) or mutant HTNNT2 coding sequence flanked by the α-MHC promoter and hGH poly(A)+ signal was used for the generation of transgenic founders of mouse strain C57BL/6 according to the standards of the transgenic animal core facility at the University of Heidelberg. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

2.4 Gene expression analyses

Quantitative gene expression analyses were performed by quantitative reverse transcriptase (qRT)-PCR using the Platinum SYBR Green qPCR superMix UDG (Invitrogen).

2.5 Echocardiography

Echocardiography of mice was performed using an ATL HDI 5500 device and a 10 MHz probe as described previously.

2.6 Statistical analyses

All results are depicted as the mean ± standard error of the mean unless stated otherwise. Statistical analyses of the data were performed using one- or two-way ANOVA pursued by Student–Newman–Keuls post hoc tests. P-values <0.05 were considered statistically significant.

3. Results

3.1 Clinical evaluation

A 52-year-old Caucasian female was referred to our hospital due to exercise-related dyspnoea and newly diagnosed impaired left ventricular systolic function. Echocardiography revealed typical signs of LVNC: prominent trabeculations and intertrabecular recesses which were perfused, as shown by colour Doppler imaging. A cardiac MRI examination was performed. This study confirmed that the segments of non-compacted myocardium mainly involved the apical, lateral (Figure 1A), and inferior wall of the left ventricle and showed a two-layered structure with a ratio greater than 2.3 between the non-compacted subendocardial layer and the compacted subepicardial layer at end-diastole (Figure 1A and B). Cardiac catheterization
excluded coronary artery disease, and this patient met the diagnostic criteria for LVNC.\textsuperscript{11,22–24} She received standard drug therapy for congestive heart failure (ACE-inhibitor, diuretics, β-blocker).

As part of our screening programme for familial cardiomyopathy, a detailed family history was obtained. The patient (I-4; Figure 2A) reported that three of her four children died early, \(<1\) year old. One death was related to suspected heart disease, the two others were of unknown cause. In her 5-month-old granddaughter (III-1), recently admitted to our department of paediatric cardiology because of decompensated heart failure, LVNC was diagnosed by echocardiography, which revealed numerous deep trabeculations of the inferior, apical, and lateral wall of the LV, and the two-layered structure of the myocardium typical for LVNC. Rapid disease progression despite optimized medical treatment subsequently necessitated orthotopic heart transplantation at the age of 26 months. Tissue sections of the explanted heart showed typical features of LVNC (Figure 1E and F), including intertrabecular recesses with enhanced fibrosis of the myocardial layer. As a consequence of this striking family history, the asymptomatic son (II-4) of the index patient and father of patient III-1 was examined. Echocardiography and cardiac MRI (Figure 1C and D) of patient II-4 revealed a moderately reduced left ventricular function with non-compacted myocardium and regional hypokinesia, predominantly apical and inferior (clinical data of all three affected patients are outlined in Table 1). A detailed

\begin{figure}[h]
\centering
\begin{tabular}{cc}
\multicolumn{1}{c}{A} & \multicolumn{1}{c}{B} \\
\includegraphics[width=0.4\textwidth]{A.pdf} & \includegraphics[width=0.4\textwidth]{B.pdf} \\
\multicolumn{1}{c}{C} & \multicolumn{1}{c}{D} \\
\includegraphics[width=0.4\textwidth]{C.pdf} & \includegraphics[width=0.4\textwidth]{D.pdf} \\
\multicolumn{1}{c}{E} & \multicolumn{1}{c}{F} \\
\includegraphics[width=0.4\textwidth]{E.pdf} & \includegraphics[width=0.4\textwidth]{F.pdf}
\end{tabular}
\caption{Cardiac imaging and histology of three affected individuals. Cardiac MRI of index patient I-4: (A) short-axis view, end-diastolic; (B) 4-chamber view, end-diastolic. Cardiac MRI of patient II-4: (C) short-axis view, end-diastolic; (D) 4-chamber view, end-diastolic. (E) HE staining and (F) Masson trichrome staining of the explanted heart of patient III-1 at 50× magnification showing characteristic features of LVNC such as deep recesses and fibrosis. LV, left ventricle; RV, right ventricle; LA, left atrium.}
\end{figure}
17-segment MRI analysis of patients I-4 and II-4 is outlined in Table 2, revealing 10 (I-4) or nine (II-4) segments with an NC/C ratio 2.3, consistent with the diagnostic criteria for LVNC by Petersen et al.24

Drug therapy consisting of an ACE-inhibitor and β-blocker was initiated in patient I-4. Currently, none of the patients has received an implantable cardioverter–defibrillator for primary prophylaxis.

3.2 Genetic evaluation

In the course of our mutation screening programme for cardiomyopathy patients,25 the genes LDB3, LMNA, MYH7, MYBPC3 and TNNT2 were analysed, and a novel GAG>AAG (pE96K) missense mutation in TNNT2 (NCBI reference sequence: NM_001001430.1) encoding cardiac troponin T could be identified in patient I-4 (Figure 2B). The part of exon 10 in which the mutation is located is highly conserved among different species (Figure 2C). Subsequent genetic testing of the entire family revealed that the two other affected family members, II-4 and III-1, are also heterozygous carriers of the mutation, while two other family members, mother and grandfather of the young girl, have the WT genotype and are healthy. Two more genes, G4.5 and DTNA, are also known candidates for the non-compaction phenotype. We excluded the G4.5 gene because it causes LVNC in an X-linked recessive mode of inheritance and hence is unlikely to be responsible for the disease in the two female patients. DTNA was analysed by sequencing all coding exons in index patient I-4, revealing no sequence alteration deviating from WT. Therefore, it seems likely that the LVNC phenotype in this family is caused by the novel pE96K missense mutation in cardiac troponin T. When the mother of patient II-1 was pregnant again, a prenatal genetic diagnosis of a biopsy from chorionic villi revealed that the foetus carried the TNNT2 mutation. Soon after birth, the boy showed clinical signs of heart failure as well as a decreased LV function on echocardiography (III-2, Figure 2A).
3.4 Mut cTNT mice reveal cardiomyopathy

While analysis of cardiac morphology and heart to body weight revealed no difference between hcTNT mice, mut cTNT, and WT controls until the age of 30 weeks (data not shown), older mut cTNT mice (>36 weeks) revealed grossly enlarged hearts compared with WT littermates and hcTNT mice. Figure 3C displays representative images of gross morphology of WT, hcTNT, and mut cTNT mice hearts at the age of 40 weeks as well as representative haematoxylin- and eosin-stained sections of these hearts. Moreover, mut cTNT mice revealed an increased heart/body weight ratio of 5.48 mg/g (±0.38) compared with WT littermates (4.2 mg/g ± 0.2; P < 0.05) and hcTNT mice (4.51 mg/g ± 0.24; P < 0.05, Figure 3D). Of note, characteristic histological features of left ventricular non-compaction in humans, such as formation of deep recesses with enhanced fibrosis, as detected in patient III-1 (Figure 1E and F), were not present in mut cTNT hearts, as analysed in HE and Masson trichrom stained sections (see figure in Supplementary material online). As no transgenic mouse died suddenly up to an age of 45 weeks, there was no evidence that the pE96K mutation caused malignant ventricular arrhythmias.

3.5 Mut cTNT mice display decreased cardiac contractile function in vivo

To assess the impact of the pE96K troponin T mutation on cardiac dimensions and function in vivo, we conducted echocardiographic measurements of transgenic and WT mice at the age of 35–45 weeks (n = 15 per group). Figure 4A–C depicts representative M-Mode pictures from WT, hcTNT, and mut cTNT hearts. Mut cTNT mouse hearts displayed decreased fractional shortening (39.7 ± 2.0%) compared with WT (51.7 ± 1.2%, P < 0.001) and hcTNT mice (46.0 ± 2.6%, P < 0.05, Figure 4D). Consistently, the left end-diastolic diameter (normalized to body weight) of mut cTNT hearts was found to be significantly increased (mut cTNT: 0.139 ± 0.011 mm/g; hcTNT: 0.120 ± 0.013 mm/g; P < 0.05; WT: 0.115 ± 0.01 mm/g; P < 0.05, Figure 4E). All echocardiographic parameters are summarized in Supplementary material online, Table S2.

In conclusion, mut cTNT mice develop a DCM-like phenotype at the age of 36–40 weeks comprising a dilated left ventricle with impaired systolic function. The findings in the mut cTNT transgenic mouse further support a causal relationship between the PE96K mutation in the cardiac troponin T molecule and the cardiomyopathic phenotype in patients harbouring this mutation.

3.6 Induction of the foetal gene programme in mut cTNT mice hearts

To test whether the development of the DCM-like phenotype in mut cTNT mice was also accompanied by re-induction of foetal gene expression as frequently observed in failing hearts, mRNA levels of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and β-MHC were analysed by quantitative real-time PCR. These genes were markedly induced in mut cTNT mice compared with WT and hcTNT mice (ANF: +522 ± 15%, P < 0.05; BNP: +635 ± 100%, P < 0.01; β-MHC: 1094 ± 293%, P < 0.05, Figure 5). The induction of these genes indicates the re-activation of a cardiac foetal gene programme consistent with that of a heart failure phenotype.

### Table 2 Cardiac 17-segment MRI analysis of patient I-4 and patient II-4 (end-diastolic)

<table>
<thead>
<tr>
<th>Segment</th>
<th>Non-compact (mm)</th>
<th>Compact (mm)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segment 1, basal anterior</td>
<td>5</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>Segment 2, basal anterosal</td>
<td>8</td>
<td>6</td>
<td>1.3</td>
</tr>
<tr>
<td>Segment 3, basal inferosal</td>
<td>1</td>
<td>7</td>
<td>0.1</td>
</tr>
<tr>
<td>Segment 4, basal inferior</td>
<td>5</td>
<td>6</td>
<td>0.8</td>
</tr>
<tr>
<td>Segment 5, basal inferolateral</td>
<td>13</td>
<td>5</td>
<td>2.6</td>
</tr>
<tr>
<td>Segment 6, basal anterolateral</td>
<td>13</td>
<td>5</td>
<td>2.6</td>
</tr>
<tr>
<td>Segment 7, mid-anterior</td>
<td>10</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>Segment 8, mid-anterosal</td>
<td>6</td>
<td>9</td>
<td>0.7</td>
</tr>
<tr>
<td>Segment 9, mid-inferosal</td>
<td>7</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Segment 10, mid-inferior</td>
<td>13</td>
<td>5</td>
<td>2.6</td>
</tr>
<tr>
<td>Segment 11, mid-inferolateral</td>
<td>12</td>
<td>5</td>
<td>2.4</td>
</tr>
<tr>
<td>Segment 12, mid-inferolateral</td>
<td>15</td>
<td>3</td>
<td>5.0</td>
</tr>
<tr>
<td>Segment 13, apical anterior</td>
<td>11</td>
<td>4</td>
<td>2.8</td>
</tr>
<tr>
<td>Segment 14, apical septal</td>
<td>10</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>Segment 15, apical inferior</td>
<td>10</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>Segment 16, apical lateral</td>
<td>12</td>
<td>4</td>
<td>3.0</td>
</tr>
<tr>
<td>Segment 17, apex</td>
<td>10</td>
<td>3</td>
<td>3.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Segment</th>
<th>Non-compact (mm)</th>
<th>Compact (mm)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segment 1, basal anterior</td>
<td>1</td>
<td>6</td>
<td>0.2</td>
</tr>
<tr>
<td>Segment 2, basal anterosal</td>
<td>1</td>
<td>11</td>
<td>0.1</td>
</tr>
<tr>
<td>Segment 3, basal inferosal</td>
<td>1</td>
<td>7</td>
<td>0.1</td>
</tr>
<tr>
<td>Segment 4, basal inferior</td>
<td>13</td>
<td>7</td>
<td>1.9</td>
</tr>
<tr>
<td>Segment 5, basal inferolateral</td>
<td>17</td>
<td>5</td>
<td>3.4</td>
</tr>
<tr>
<td>Segment 6, basal anterolateral</td>
<td>1</td>
<td>9</td>
<td>0.1</td>
</tr>
<tr>
<td>Segment 7, mid-anterior</td>
<td>8</td>
<td>7</td>
<td>1.1</td>
</tr>
<tr>
<td>Segment 8, mid-inferosal</td>
<td>1</td>
<td>7</td>
<td>0.1</td>
</tr>
<tr>
<td>Segment 9, mid-inferior</td>
<td>1</td>
<td>7</td>
<td>0.1</td>
</tr>
<tr>
<td>Segment 10, mid-inferior</td>
<td>12</td>
<td>4</td>
<td>3.0</td>
</tr>
<tr>
<td>Segment 11, mid-inferolateral</td>
<td>12</td>
<td>4</td>
<td>3.0</td>
</tr>
<tr>
<td>Segment 12, mid-inferolateral</td>
<td>15</td>
<td>5</td>
<td>3.0</td>
</tr>
<tr>
<td>Segment 13, apical anterior</td>
<td>16</td>
<td>5</td>
<td>3.2</td>
</tr>
<tr>
<td>Segment 14, apical septal</td>
<td>17</td>
<td>5</td>
<td>3.4</td>
</tr>
<tr>
<td>Segment 15, apical inferior</td>
<td>15</td>
<td>5</td>
<td>3.0</td>
</tr>
<tr>
<td>Segment 16, apical lateral</td>
<td>14</td>
<td>5</td>
<td>2.8</td>
</tr>
<tr>
<td>Segment 17, apex</td>
<td>12</td>
<td>2</td>
<td>6.0</td>
</tr>
</tbody>
</table>
4. Discussion

We describe a family with highly variable clinical severity of left ventricular non-compaction (LVNC). Rapidly progressive LVNC led to urgent cardiac transplantation in a 26-month-old child (individual III-1), while individual II-4 would not have been diagnosed without family screening which was performed due to the severe disease detected in his mother and daughter. As the molecular cause for cardiomyopathy, we identified the same mutation in all affected individuals, a novel missense mutation in the gene encoding for cardiac troponin T ($\text{TNNT2}$). Mutations in known genes for LVNC, namely the X-linked gene $\text{TAZ}$ as well as the autosomal genes $\text{DTNA}$, $\text{LDB3}$, $\text{LMNA}$, $\text{MYH7}$, and $\text{MYBPC3}$, could be excluded. A recent report by Klaassen et al.$^5$ linked LVNC to a spontaneous de novo mutation in cardiac troponin T (Arg131Trp). We describe a familial LVNC-causing $\text{TNNT2}$ mutation that is inherited, spanning three generations.

Mutations in $\text{TNNT2}$ have so far mainly been described as a cause for HCM and DCM. In patients with DCM, $\text{TNNT2}$ mutations are frequently associated with a severe disease phenotype.$^{18}$ Moreover, many HCM-causing $\text{TNNT2}$ mutations are associated with a high incidence of sudden cardiac death due to malignant arrhythmias.$^{19}$ An
additional disease phenotype, restrictive cardiomyopathy (RCM), has recently also been associated with a spontaneous nonsense mutation in TNNT2.27 Remarkably, this mutation results in deletion of amino acid 96, thus affecting the same codon as the missense mutation in our study. The patient, a 12-month-old girl, also had to undergo urgent cardiac transplantation, supporting the notion that this amino acid residue of the troponin T molecule is critical for sarcomere integrity and contractile function. Specifically, it is known that this region of cTNT binds to tropomyosin, which is essential for the linkage of the troponin complex to the thin filament.28 A recent report demonstrated that the RCM-associated mutation of residue 96 of cardiac troponin T impairs the cooperativity of the thin filament by altering the Ca^{2+} sensitivity of force development and by impairing fibre relaxation.29 Off note, the affected residue is very close to Arg92, which has been termed a mutational hotspot which leads to different clinical phenotypes.30 The functional importance of the segment flanking residue 96 of troponin T is further emphasized by the fact that this region is highly conserved between different species (Figure 2C).

The fact that different TNNT2 mutations lead to a highly variable severity of symptoms and/or different cardiomyopathy phenotypes (LVNC, DCMP, HCM, RCM) suggests the presence of different modifiers that promote the development of a specific phenotype. Disease modifiers might include environmental factors such as exercise,31,32 but also unknown genetic factors. These modifier loci might be situated in sarcomeric genes because it has been demonstrated that the presence of two distinct sarcomeric gene mutations (‘double hit’) is associated with more severe cardiac disease.33 Moreover, genes encoding components of the renin–angiotensin–aldosterone system have been considered as modifier genes of cardiomyopathy.34

**Figure 4** mut cTNT mice show decreased contractility in vivo. (A–C) Representative M-mode echocardiography recordings of WT, hcTNT, and mut cTNT mouse hearts at the age of 35–45 weeks (n=15 each group). (D) Mut cTNT mice revealed a decreased fractional shortening (FS) compared with WT and hcTNT mice, as a measure of cardiac contractility. FS was also slightly reduced in hcTNT mice compared with WT mice. (E) Left ventricular end-diastolic diameter (LVEDD) normalized to body weight (BW) was increased in mut cTNT mouse hearts compared with WT and hcTNT mouse hearts. LVEDD, Left ventricular end-diastolic diameter. *P < 0.05; ‡P < 0.001.

**Figure 5** mut cTNT mouse hearts display the re-induction of a foetal gene programme. ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain. *P < 0.05, †P < 0.01.
4.1 Transgenic mouse model for TNT-(pE96K)-associated cardiomyopathy

To validate the causal relationship between the pE96K mutation and the LVNC phenotype in affected patients as well as to assess the functional impact of the pE96K mutation, we generated a new transgenic mouse model. To accomplish this, a human troponin T molecule harbouring the pE96K mutation (mut cTNT) was expressed under control of the cardiac-specific α-MHC promoter. These mice display a typical DCM phenotype including left ventricular dilatation and decreased contractility as well as re-induction of the foetal gene programme. Thus, we could reproduce important characteristics of the pathological phenotype of patients harbouring the pE96K mutation in mice, further supporting the notion that this troponin T mutation leads to cardiomyopathy in vivo. Interestingly, mice expressing WT human TNT also reveal a slight but significant decrease in contractile function compared with non-transgenic controls. This effect might be due to qualitative differences of human and mouse troponin T which reveal only 92% homology on the amino acid level.

Of note, while mutant TNT mice reproduce the DCM phenotype of our patients, we did not observe a non-compaction phenotype in these animals. Careful histopathological analyses of the transgenic animals excluded increased trabeculations or intertrabecular recesses, which were typical features in our patients. These findings mirror data from transgenic rats harbouring an HCM-causing troponin T mutation that do not show HCM-typical histological characteristics like myocardial disarray or fibrosis, but nevertheless reveal an increased incidence of malignant ventricular arrhythmias. As these results challenge the mandatory connection between fibrosis/cardiomycyte disarray and arrhythmias in HCM, the absence of the LVNC phenotype in our mouse model might indicate the link between the non-compaction phenotype and cardiomyopathy in LVNC. Several explanations for the lack of an obvious LVNC phenotype in our transgenic mice can be considered: (i) Species differences may render mice resistant to a LVNC phenotype. Consistent with this notion, we are unaware of any rodent model (either spontaneously occurring or genetically modified) that reveals LVNC in the adult heart. (ii) A specific ‘permissive’ genetic background may be required for this mutation to also cause a non-compaction phenotype in addition to cardiomyopathy with contractile dysfunction. The importance of the genetic background for the development of a specific phenotype has also been shown in transgenic mouse models for HCM. For example, while the Arg403Gln mutation of the cardiac MHC gene causes a severe form of HCM in humans, the hypertrophic response of transgenic mouse models expressing this mutation varies significantly between different mouse strains, with some mice not revealing cardiac hypertrophy at all. It is conceivable that the induction of the LVNC in humans by the pE96K mutation is dependent on a ‘human-specific’ genetic background; however, the generation of a cardiomyopathy phenotype by the pE96K mutation could be conclusively demonstrated also in mice. (iii) Two independent mutations may cause DCM with contractile dysfunction and LVNC, respectively, e.g. a ‘double hit’ is required. While we cannot formally exclude this possibility, we feel that at least for our family this explanation is unlikely because all carriers of the TNT mutation revealed LVNC. Moreover, several other known candidate genes for LVNC could be excluded.

The development of cardiomyopathy in mut cTNT mice at a relatively old age of 35–40 weeks might seem to challenge the validity of the E96K mutation as a disease model of LVNC as LVNC has been termed a ‘developmental defect’ with non-compaction of the left ventricle already being present at birth. However, it has been demonstrated that the age of onset of symptoms of heart failure in patients with LVNC is highly variable and can occur at any age of 60 years. Thus, the late onset of cardiomyopathy in mut cTNT mice is consistent with the clinical course in many patients and may further corroborate the disconnection between a non-compaction phenotype and the onset of cardiomyopathy.

Although we could demonstrate a causative role of the troponin T mutation for cardiomyopathy in mice, the exact mechanism by which altered troponin T causes LVNC/DCM still needs to be elucidated. Several mouse models point to an important role of troponin T during embryonic development: Targeted disruption of the TNNT2 gene in mice leads to a compromised sarcomere assembly during myofibrillogenesis, causing early embryonic lethality due to a lack of heart beats. Of note, cTNT−/− cardiomyocytes showed regular calcium transients, indicating that cTNT does not affect intracellular calcium handling during embryonic development. Another study confirmed the lethal effect of TNNT2 ablation during embryonic development and additionally demonstrated that ablation of one allele of TNNT2 did not alter cardiac morphology or function, arguing against haploinsufficiency as a disease-causing factor. However, crossbreeding of cTNT+/− mice with transgenic mice overexpressing a DCM causing troponin T (cTNT−/−/TG210) mutation severely aggravated the DCM phenotype compared with cTNT+/−/TG210 animals, pointing to an important role of the ratio of mutant to WT cTNT transcript. Of note, cTNT−/− embryos showed normal embryonic heart looping and no sign of a non-compaction phenotype.

4.2 Is left ventricular non-compaction cardiomyopathy an independent entity?

Remarkably, even in the absence of left ventricular non-compaction, we observed DCM in mutant TNT mice. This finding suggests that the pE96K mutation directly impairs contractile function independent of hypertrabeculation of the LV, and challenges the notion that the non-compaction phenotype is required for the development of cardiomyopathy. Our data rather support a recent hypothesis that questions the causal role of non-compaction in the pathogenesis of cardiomyopathy. This concept considers LVNC a secondary consequence of a genetic alteration, which may be well compensated when cardiac function is otherwise normal, yet may secondarily exacerbate the deleterious effects of a disease-causing mutation on cardiac contractility. Moreover, the diagnostic criteria for LVNC are frequently fulfilled by healthy individuals or patients with HCM and DCM. Although LVNC was included as an independent entity into the 2006 World Health Organization revised classification of cardiomyopathies, it may thus be problematic to consistently delineate LVNC from other cardiomyopathies. Nevertheless, the marked variability of the severity of cardiomyopathy observed in our family as well as others strongly implies that additional modifiers of the phenotype must exist and the extent of non-compaction may therefore play a disease-modifying role.

5. Conclusion

In conclusion, we describe a novel TNNT2 mutation, pE96K, causing LVNC of highly variable severity even within a single family. Moreover,
the phenotype of the first transgenic animal model of an LNVN-associated mutation supports the notion that non-compaction per se is not a prerequisite for the deterioration of contractile function. Thus, the primary defect in LNVN appears to be cardiomyocyte-autonomous, similar to other genetic cardiomyopathies such as HCM and DCM.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Acknowledgements**

The excellent technical assistance of Sandra Manthey, Oliver Mücke, Ulrike Oehl and Jutta Krebs is gratefully acknowledged.

**Conflict of interest**: none declared.

**Funding**

The excellent technical assistance of Sandra Manthey, Oliver Mücke, Ulrike Oehl and Jutta Krebs is gratefully acknowledged.

**References**