iPSC-derived cardiomyocytes reveal abnormal TGF-β signalling in left ventricular non-compaction cardiomyopathy

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Left ventricular non-compaction (LVNC) is the third most prevalent cardiomyopathy in children and its pathogenesis has been associated with the developmental defect of the embryonic myocardium. We show that patient-specific induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) generated from LVNC patients carrying a mutation in the cardiac transcription factor TBX20 recapitulate a key aspect of the pathological phenotype at the single-cell level and this was associated with perturbed transforming growth factor beta (TGF-β) signalling. LVNC iPSC-CMs have decreased proliferative capacity due to abnormal activation of TGF-β signalling. TBX20 regulates the expression of TGF-β signalling modifiers including one known to be a genetic cause of LVNC, PRDM16, and genome editing of PRDM16 caused proliferation defects in iPSC-CMs. Inhibition of TGF-β signalling and genome correction of the TBX20 mutation were sufficient to reverse the disease phenotype. Our study demonstrates that iPSC-CMs are a useful tool for the exploration of pathological mechanisms underlying poorly understood cardiomyopathies including LVNC.

Left ventricular non-compaction (LVNC) is increasingly recognized as a cause of cardiomyopathy1,2, especially in children. In a recent study, LVNC accounted for 9.2% of all children with primary cardiomyopathies, and was the third most prevalent form of cardiomyopathy, after dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy2. LVNC is characterized by deep and extensive hypertrabeculation of the left ventricle, and causes heart failure, arrhythmias and thromboembolism.

LVNC has been theorized to result from the arrest of compaction of the developing LV myocardium, as it passes through several distinct evolutionarily conserved steps. Trabeculations in the human embryo emerge after looping of the primitive heart tube at the end of the fourth week of gestation3. Trabecular remodelling begins at 8 weeks with an increase in LV volume compressing the trabeculations, leading to an increase in the thickness of the compacted myocardium. Serial pathologic studies suggest that LVNC arises from impaired/arrested compaction of the myocardium, or abnormalities of vascularization, or in development of the multilayered spiral system3,4. Among these steps, emergence of trabeculations and trabecular remodelling are thought to be the key steps to understanding LVNC. The trabeculation patterns are ventricle-specific, which are generally thicker and the corresponding intertrabecular spaces are larger in the LV than in the right ventricle. When this embryonic pattern persists postnatally, the morphologic appearance strongly resembles the embryonic 'spongiform' myocardium, which was the original nomenclature for this cardiomyopathy.

Like many congenital cardiomyopathies, the genetics of LVNC is complex and the full spectrum of the disorder is still undefined. The mechanisms that lead to LVNC are not well understood, although animal models of LVNC have suggested that abnormal regulation of growth signals, including the transforming growth factor beta (TGF-β)5-8, NOTCH and NRG1/ERBB210,11, may be causative factors. Since most of these animal models harbouring non-compaction-like myocardium showed alterations in cell-cycle regulation in developing cardiomyocytes, it is possible that TGF-β and NOTCH signalling play a role in LVNC.
cardiomyocytes, it is thought that the abnormal proliferation of embryonic cardiomyocytes may be associated with the pathogenesis of LVNC. However, studies have differed on whether this proliferation is increased or decreased. Furthermore, recent human studies have identified mutations in genes that are associated with regulation of cardiomyocyte proliferation. However, it is still unclear which phenotypes in developing cardiomyocytes are actually associated with the pathogenesis seen in humans and investigation of this disease has been challenging due to its complex genetic basis.

To overcome the problems for the investigation of human cardiac cell development with a pathological background of LVNC, we used patient-specific induced pluripotent stem cells (iPSCs). Here we demonstrated the use of human iPSC-derived cardiomyocytes (iPSC-CMs) from patients carrying the TBX20 mutation affected by LVNC as a model to define cell-specific phenotypes and elucidate potential mechanisms of this disease.

RESULTS

TBX20 mutation is a candidate genetic cause of LVNC

To identify potential genetic causes of LVNC, we recruited a family with LVNC including the proband no. 1 (A-III-4), who had undergone heart transplantation for restrictive physiology, two siblings (A-III-2 and A-III-3) with significantly deeper and more extensive trabeculation of the left ventricle (a forme fruste of LVNC referred to clinically as ‘hypertrabeculation’) but with normal systolic function, and the father (A-II-2) with asymptomatic DCM without LVNC (Fig. 1a–c and Supplementary Table 1). Genetic testing by genome-wide exome sequencing revealed a stop-gain mutation in the TBX20 gene (Y317*) in the proband no. 1, two siblings, and father (Fig. 1d). No mutations in maternally transmitted and de novo modifiers known to contribute to cardiomyopathies were detected (Supplementary Table 2). To investigate whether TBX20 mutations are seen in other LVNC patients, we performed genetic testing in an additional 77 LVNC patients and detected another de novo mutation (T262M) from one additional isolated LVNC patient (proband no. 2: B-II-2) (Fig. 1a,d and Supplementary Table 1).

TBX20 is an essential cardiac transcription factor that regulates cardiomyocyte differentiation and proliferation. TBX20 has also been described in conjunction with congenital heart disease and DCM. The TBX20 Y317* mutation resulted in a truncated protein (Fig. 1e). TBX20 Y317* and T262M mutant proteins showed disturbed synergistic activity with other cardiac transcription factors, including NKX2-5, GATA4 and TBX5 (Supplementary Fig. 1a,b). Furthermore, both TBX20 Y317* and T262M mutations were found to impair the negative transcriptional regulatory function of TBX20 compared with the wild type (Supplementary Fig. 1c). These results suggest that LVNC-associated mutations caused disturbance of transcriptional regulation by TBX20.

LVNC iPSC-CMs possess a defect of proliferative capacity

To investigate the pathological mechanism of LVNC, we generated iPSCs using Sendai virus reprogramming from a family with an LVNC history along with three unrelated control volunteers; two clones per patient were established (Supplementary Fig. 1d–f and Supplementary Table 3). We classed all lines into three subgroups: unrelated control, mild DCM (A-II-2) and LVNC (A-III-2, 3, 4). iPSCs were differentiated into cardiomyocytes (iPSC-CMs) followed by glucose deprivation to enrich for iPSC-CMs (Supplementary Fig. 2a). Although LVNC iPSC-CMs were similar to control iPSC-CMs in terms of structural and electrophysiological phenotype as well as sarcomeric gene expression at 2–4 weeks, LVNC iPSC-CMs showed an approximately 50% reduction of the expression of TBX20 downstream target genes compared with control iPSC-CMs (Fig. 1f and Supplementary Table 4). Furthermore, the differentiation efficiency was also halved in LVNC iPSC lines compared with the control prior to glucose deprivation (Fig. 1g). On the other hand, mild DCM iPSC-CMs showed intermediate defects in TBX20 target gene expression and differentiation efficiency between LVNC and control iPSC-CMs. Despite comparable expression of mesodermal markers including MESP1 and brachyury (T) across all lines during cardiac differentiation (Supplementary Fig. 2k), LVNC iPSCs had significantly lower expression of cardiac transcription factors compared with control cell lines especially between day 6 and 9 (Fig. 1h and Supplementary Fig. 3a). These results suggest an impaired induction of cardiac lineage from mesodermal progenitors in LVNC iPSCs.

Since past human and mouse studies have shown possible association between cell-cycle defects in developing cardiomyocytes and pathogenesis of LVNC, we next assessed the proliferative potential in iPSC-CMs. We found that iPSC-CMs were responsive to growth factors and had increased numbers within 3 weeks after induction of differentiation although their growth was temporary (Supplementary Fig. 3b). To assess the proliferation potential in developing iPSC-CMs, the distribution of S-phase cardiomyocytes was validated by EdU incorporation with or without stimulation of serum or growth factors. LVNC iPSC-CMs showed reduced baseline proliferative capacity by ~50% without any stimulation as well as in the presence of serum and growth factors (Fig. 1i,j). On the other hand, undifferentiated iPSCs showed no significant difference in their growth speed (Supplementary Fig. 3c), suggesting that the proliferation defect may be characteristic in differentiated cardiac cells.

Abnormal activation of TGF-β signalling is associated with proliferation defect in LVNC iPSC-CMs

To clarify potential signalling pathways associated with the proliferation defect in LVNC iPSC-CMs, we next performed RNA-sequencing using control, mild DCM, and LVNC iPSC-CMs at 2 weeks. Upstream regulator analysis predicted that activation of TGF-β signalling may be potentially responsible for this differential gene expression between control and LVNC iPSC-CMs (Fig. 2a and Supplementary Fig. 4a and Supplementary Table 5). Most of the TGF-β signalling pathway, especially TGF-β1-associated genes, were upregulated in LVNC and mild DCM iPSC-CMs compared with control iPSC-CMs (Fig. 2a and Supplementary Fig. 4b–c). Previous studies have shown that aberrant TGF-β signalling causes incomplete compaction of myocardium in mice, and that TGF-β signalling inhibits proliferation of embryonic cardiomyocytes via upregulation of cyclin-dependent kinase inhibitors (CKIs) including CDKN1A. Consistent with these studies, LVNC iPSC-CMs showed significant upregulation of TGF-β signalling-related genes and 1.7-fold higher phosphorylation of SMAD2/3 (Fig. 2c and
Figure 1. Characterization of patient-specific LVNC iPSC-CMs carrying a TBX20 mutation. (a) Schematic pedigree of two families with LVNC. The probands are indicated by arrows (A-III-4 and B-II-2). Plus and minus signs indicate the presence and absence of the TBX20 Y317* mutation in the family A and the T262M mutation in the family B, respectively. (b) The LVNC phenotype of the proband no. 1 (A-III-4), two siblings (A-III-2 and A-III-3), and an isolated proband no. 2 (B-II-2) is assessed by echocardiography. LA, left atrium; LV, left ventricle. Scale bars, 1 cm. (c) The proband no. 1's explanted heart (left) and Masson's trichrome staining of the left ventricle (right). Scale bars, 1 cm. (d) Heat map showing mRNA expression of cardiac transcription factors in iPSCs from day 0 to day 16 after induction of cardiac differentiation. The LVNC iPSCs showed a significant decrease in cardiac transcription factors in day 6 and day 9 (red boxes). n = 6 independent experiments. Mean = 0. (e) Immunostaining of nuclear (blue), TNNT2 (red) and EdU (green) in iPSC-CMs at 2 weeks. Scale bar, 100 μm. (f) Percentage of EdU-positive CMs in control, mild DCM and LVNC iPSC-CMs with or without serum (FBS; n = 10, 10 and 30 for CON, mild DCM and LVNC independent experiments respectively at 2 weeks; n = 4 independent experiments per group at 4 and 8 weeks) or with growth factors (IGF1, IGF2, FGF1 or NRG1; n = 4 independent experiments per group at 2, 4 and 8 weeks). CON, unrelated controls. + P < 0.05, ***P < 0.005; NS, not significant in one-way ANOVA followed by Tukey post hoc test. The bar graphs show the mean and error bars represent s.e.m. Statistics source data can be found in Supplementary Table 12. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
Figure 2  Upregulation of TGF-β signalling in the LVNC phenotype.
(a) Upstream regulator analysis of the signalling pathway comparing LVNC and control iPSC-CMs at 2 weeks after induction of cardiac differentiation. (b) Heat map showing upregulation of the TGF-β signalling pathway in LVNC (III-2, 3, 4; mean of four samples) and mild DCM (II-2; mean of two samples) compared with control iPSC-CMs (unrelated controls; mean of four samples). Mean = 0, variance = 1. (c) Western blot of total and phospho-SMAD2/3 in control and patient-specific iPSC-CMs (upper) and densitometry analysis, normalized against α-tubulin (lower). (d) Western blot of CDKN1A protein in control and patient-specific iPSC-CMs (upper) and densitometry analysis, normalized against α-tubulin (lower). (e) Immunostaining of nuclear (blue), alpha-sarcomeric actin (green) and phospho-SMAD2 (red) in the LV of control donor heart tissue versus the explanted heart of proband no. 1. (f) Allele-specific mRNA expression analysis by mRNA-sequencing (upper panel) and digital droplet PCR (lower panel) showed a higher ratio of TBX20 mutant allele expression in LVNC iPSC-CMs compared with mild DCM iPSC-CMs. n = 6 independent experiments. (g) The effect of TGF-β isoform treatments on the percentage of EdU+ cardiomyocytes in control iPSC-CMs with or without growth factors. PBS-treated control; n = 4 independent experiments, TGF-β-treated samples; n = 3 independent experiments. CON, unrelated controls. *P < 0.05, **P < 0.01, ***P < 0.005 in unpaired two-tailed t-test or one-way ANOVA followed by Tukey post hoc test. The bar graphs show the mean and error bars represent s.e.m. Scale bar, 100 μm. Statistics source data can be found in Supplementary Table 12. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

Supplementary Fig. 4d,e), whereas mild DCM iPSC-CMs had less phosphorylation (1.2-fold versus control). LVNC iPSC-CMs also showed a 1.8-fold increased CDKN1A expression compared with control iPSC-CMs (Fig. 2d and Supplementary Fig. 4f,g), suggesting that activation of the TGF-β1-CDKN1A regulatory axis may be responsible for the early cell-cycle exit seen in LVNC iPSC-CMs. Histological analysis of the proband’s explanted LV myocardium revealed higher phosphorylation of SMAD2 in comparison with
control LV tissue, providing further evidence of activated TGF-β signalling as a pathogenesis of LVNC (Fig. 2e). Interestingly, LVNC iPSC-CMs showed significantly higher expression of TGF-β signalling pathway-associated genes compared with mild DCM iPSC-CMs that were associated with milder phenotypic severity (Supplementary Fig. 4b–g).

To understand the difference in disease severity between the father (mild DCM phenotype) and his children (LVNC phenotype), despite carrying the same TBX20 mutation, we next assessed expression of the mutant TBX20 allele by RNA-sequencing and digital droplet PCR. We found that the ratio of mutant allele against wild-type allele expression was higher in LVNC iPSC-CMs than in mild DCM iPSC-CMs (Fig. 2f), suggesting a dosage effect of the TBX20 mutant allele on the ectopic activation level of the TGF-β signalling pathway in patient-specific iPSC-CMs and perhaps explaining the differences in phenotypic severity among affected family members. To confirm that stimulation with TGF-β isoforms could affect the proliferation potential in iPSC-CMs, we treated control iPSC-CMs with TGF-β isoforms with or without serum or growth factors. We found that, like LVNC iPSC-CMs, TGF-β-treated control iPSC-CMs showed a significant decrease of proliferative response to serum, IGF-1, and IGF-2 stimulation at 2 weeks compared with TGF-β-un-treated control iPSC-CMs (Fig. 2g). These results suggest that activation of TGF-β signalling has a negative effect on the proliferative potential of developing cardiomyocytes.

**Ectopic activation of TGF-β signalling causes a cardiomyocyte proliferation defect in vivo**

To assess how abnormal TGF-β1 signalling affects myocardial development in vivo, we next generated cardiac-specific TGF-β1-overexpressing transgenic mice (β1 glo/αMHC-Cre; Supplementary Fig. 5). These double-transgenic mice exhibit embryonic lethality by embryonic day (E) 11.5 due to decreased proliferation in developing cardiomyocytes (Fig. 3a–d and Supplementary Fig. 5c and d and Supplementary Table 6), providing further proof implicating aberrant TGF-β signalling in the embryonic heart with developmental arrest of the compact layer.

Next, to assess the later stages of compact layer development, we crossed β1 glo transgenic mice with NK-TGCK transgenic mice that express GFP-Cre fusion protein under the control of the Nkx2-5 cardiac-specific enhancer/promoter and Tet-Off system to negatively regulate GFP-Cre expression following doxycycline (DOX) exposure (Supplementary Fig. 5e). The αMHC-Cre transgene labelled >90% of cardiomyocytes at E12.5 and the NK-TGCK transgene activated Cre in ~40% of cardiomyocytes without DOX and ~25% of cardiomyocytes with DOX treatment and showed significantly lesser TGFβ1 mRNA expression compared with αMHC-Cre/β1 glo double-transgenic mouse embryos at E10.5 (Supplementary Fig. 5f–h). The double-transgenic embryos carrying both NK-TGCK and β1 glo (β1 glo/NK-TGCK) without DOX treatment showed a significant increase of TGF-β downstream target genes whereas the double-transgenic embryos with DOX treatment showed only a mild increase of these genes at E10.5 (Supplementary Fig. 5i). The β1 glo/NK-TGCK double-transgenic embryos showed embryonic lethality around E12.5 to E13.5. In contrast, when mice were treated with DOX, the embryos could survive normally at E12.5 (Fig. 3e and Supplementary Table 7). Histological analysis showed a significantly thinner compact layer and reduced Ki67 or phospho-histone H3 (PHH3)-positive cardiomyocytes in the compact layer in β1 glo/NK-TGCK double-transgenic embryos without DOX compared with wild-type littermates, although the trabecular layer was well developed in these double-transgenic embryos (Fig. 3f–h and Supplementary Fig. 5j,k). Half of DOX-treated β1 glo/NK-TGCK double-transgenic embryos showed reduced thickness of the LV compact layer, and the proportion of Ki67+ or PHH3+ cardiomyocytes in the compact layer in β1 glo/NK-TGCK embryos was mildly decreased compared with wild-type littermates (Fig. 3f,i and Supplementary Fig. 5j,k).

To assess the fate of TGF-β1-activated CMs in DOX-treated transgenic mice, we next generated triple-transgenic embryos carrying NK-TGCK/β1 glo transgenes with a Cre-dependent tdTomato overexpression system (Ai14)30. We found that the proportion of TGF-β1-activated CMs in LV was significantly decreased in triple-transgenic embryos and neonates during development compared with control (NK-TGCK/Ai14) mice, suggesting reduced proliferation by TGF-β1-expressing CMs (Fig. 4a,b). Furthermore, the hearts of triple-transgenic neonates at postnatal day 3 showed a thicker trabecular layer and a thinner compact layer resulting in a significantly higher non-compaction/compaction (NC/C) ratio in LV myocardium compared with control neonates (Fig. 4c–e). Taken together, these results support the role of abnormal activation of TGF-β signalling to cause developmental arrest of cardiomyocytes in a temporal and dose-dependent manner in vivo.

**Functional disturbance of TBX20 causes abnormal activation of TGF-β signalling**

Next, to assess whether the defective TBX20 transcriptional cascade causes abnormal activation of TGF-β signalling, we analysed the gene expression profile of the Tbx20 knockout mouse31. Interestingly, messenger RNA expression analysis of Tbx20 knockout mouse heart revealed a similar gene expression profile to that of LVNC iPSC-CMs, including upregulation of TGF-β signalling (Fig. 5a and Supplementary Table 8)33. Furthermore, ChIP-sequencing data of Tbx2030 showed conserved TBX20-binding sites in the genes that are associated with TGF-β signalling regulation and modification between human and mouse (Fig. 5b and Supplementary Table 9). This gene expression profile was significantly disturbed in LVNC iPSC-CMs compared with control iPSC-CMs, as well as in the Tbx20 knockout mouse heart compared with the wild type (Fig. 5c,d). We further employed short hairpin RNA (shRNA) against Tbx20 in the H7 human embryonic stem cell (ESC) line (Tbx20KD–H7) (Fig. 6a,b), and found that Tbx20KD–H7 CMs showed less proliferative capacity and increased expression of TGF-β-related genes compared with scramble control lines (Fig. 6c–g). These results confirm that the dysfunction of Tbx20 is associated with the pathological proliferation phenotype of LVNC iPSC-CMs through disturbance of the TGF-β signalling.

**TBX20–PRDM16 axis regulates TGF-β signalling and contributes to cardiomyocyte proliferation**

To better understand the regulatory mechanism of TGF-β signalling by TBX20, we next studied TGF-β modifiers with conserved TBX20-binding sites31 between human and mouse (Supplementary Table 9), and selected the PRDM16 gene as one of the potential...
downstream targets of TBX20. PRDM16, a repressor of TGF-β signalling and a known genetic cause of LVNC and DCM, was significantly downregulated in all LVNC iPSC-CMs, TBX20KD-H7-CMs and the Tbx20 knockout mouse heart (Figs 5d and 6h,i). A previous genetic study of human LVNC with the PRDM16 mutation also revealed that truncation in exon 9 of PRDM16 is associated with the LVNC phenotype. To confirm whether LVNC iPSC-CMs with the PRDM16 mutation showed abnormal activation of TGF-β signalling and a proliferation defect, as seen in LVNC iPSC-CMs with the TBX20 mutation, we next generated a genome-edited iPSC line carrying the frameshift mutation in exon 9 of the PRDM16 gene (PRDM16fs) and induced cardiac differentiation (Fig. 6j). Importantly, PRDM16fs iPSC-CMs showed significantly decreased proliferative response when exposed to growth factors and upregulation of TGF-β downstream

![Image of Figure 3 from Nature Cell Biology](https://example.com/figure3.png)

**Figure 3** Developmental arrest in cardiomyocyte-specific TGF-β1-overexpressing mouse embryo heart. (a) Lateral view of a double-transgenic embryo (β1glo/αMHC-Cre) at embryonic day (E) 10.5 compared with a wild-type littermate (control). The lower panel shows a higher-magnification view of the double-transgenic embryo heart (white box in upper panel). Scale bars, 1 mm. (b) Haematoxylin and eosin (H&E) staining and immunostaining of nuclear (blue), TGF-β1 (red) and TNNI (cyan) in control and β1glo/αMHC-Cre double-transgenic (DTG1) embryo hearts at E10.5. Higher-magnification pictures of the compact layer (indicated by the white box in the upper panels) are shown in the lower lane. Scale bars, 100 μm. (c) Immunostaining for nuclear (blue), Ki67 (red) and αSA (cyan) in coronal sections of control and DTG1 hearts at E10.5. Scale bars, 100 μm. (d) Percentage of Ki67+ cardiomyocytes in the compact layer of control and DTG1 embryo hearts at E10.5. (e) The systemic phenotypes of a partially TGF-β1-overexpressing double-transgenic embryo (β1glo/NK-TGCK) at E12.5 compared with the wild-type littermate (control) with or without doxycycline (DOX) treatment. Scale bars, 5 mm. (f) H&E and immunostaining of nuclear (blue), Ki67 (red) and αSA (cyan) in wild-type (control) and β1glo/NK-TGCK double-transgenic embryo hearts without DOX treatment at E12.5. (g) Dot and box plot of the thickness of the left ventricle (LV) compact layer in control and β1glo/NK-TGCK double-transgenic embryo (DTG2–) hearts without DOX treatment at E12.5. (h) DTG2–embryo hearts showed a significant decrease in the percentage of Ki67+ cardiomyocytes in the compact layer at E12.5. (i) Dot and box plot of the thickness of the left ventricle (LV) compact layer in control and DOX-treated β1glo/NK-TGCK double-transgenic embryo (DTG2+) hearts at E12.5. (j) DTG2+ embryo hearts showed a significant decrease in the percentage of Ki67+ cardiomyocytes in the compact layer at E12.5.

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target genes (Fig. 6k,l). Furthermore, the explanted heart tissue of proband no. 1 showed a significant decrease of PRDM16 expression compared with control donor heart tissue (Fig. 6m), suggesting that the TBX20–PRDM16–TGF-β axis as one of the mechanisms causing LVNC.

**Modification of TGF-β signalling or genetic correction of the TBX20 mutation exacerbate the proliferation defect in LVNC iPSC-CMs**

Finally, we evaluated whether modification of aberrant TGF-β signalling could rescue the proliferation defect in LVNC iPSC-CMs.

Inhibition of TGF-β signalling with TGF-β receptor-1 inhibitors or overexpression of a dominant-negative form of TGF-β receptor-2 increased S-phase cells in both LVNC iPSC-CMs and TBX20KD-H7-CMs compared with untreated cells (Fig. 7a–c and Supplementary Fig. 6). Likewise, knockdown of the downstream effector CDKN1A also improved the distribution of S-phase LVNC iPSC-CMs (Fig. 7df). To validate the effects of the TBX20 Y317F mutation on the phenotype of LVNC iPSC-CMs, we generated TBX20 Y317F mutation-corrected (LVNC-corrected) iPSCs from the proband’s iPSCs (Supplementary Fig. 7ae). LVNC-corrected iPSC-CMs showed increased cardiac differentiation efficiency, expression of

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**Figure 4** Disturbed expansion of embryonic cardiomyocytes and trabecular/compact layer ratio in the left ventricle of the TGF-β1-overexpressing mouse. (a) Immunostaining of nuclear (blue), tdTomato (red) and αSA (green) in coronal sections of control (Ai14/NK-TGCK) and Ai14/NK-TGCK/β1glo triple-transgenic embryo hearts with doxycycline treatment at E10.5 and E15.5. Scale bars, 100 μm. (b) Percentage of tdTomato-positive area per αSA-positive area in the compact layer of control and Ai14/NK-TGCK/β1glo triple-transgenic (TTG) mouse hearts with doxycycline treatment at E10.5 (control, n=5 hearts; triple-transgenic, n=5 hearts), E12.5 (control, n=6 hearts; triple-transgenic, n=5 hearts), E15.5 (control, n=6 hearts; triple-transgenic, n=5 hearts), and postnatal day 3 (P3) (control, n=18 hearts; triple-transgenic, n=8 hearts). (c) Haematoxylin and eosin staining in coronal sections of control (CON; Ai14/NK-TGCK) and Ai14/NK-TGCK/β1glo triple-transgenic (TTG) mouse hearts with doxycycline treatment at postnatal day 3. The lower panels show magnified views of areas indicated by the black box in the upper panels. (d) Thickness of the trabecular and compact layer and total myocardium in control (n=8 hearts) and Ai14/NK-TGCK/β1glo triple-transgenic mouse (n=11 hearts) hearts with doxycycline treatment at P3. (e) Trabecular layer/compact layer (NC/C) ratio in control (n=8 hearts) and Ai14/NK-TGCK/β1glo triple-transgenic mouse (n=11 hearts) hearts with doxycycline treatment at P3. Scale bars, 0.5 mm. *P<0.05, **P<0.01, ***P<0.005 in unpaired two-tailed t-test. The bar graphs show the mean and error bars represent s.e.m. Statistics source data can be found in Supplementary Table 12.
Figure 5 TBX20 regulates the expression of TGF-β signalling modifier genes in developing cardiomyocytes. (a) Venn diagram to show the overlap between genes upregulated (upper) or downregulated (lower) in LVNC iPSC-CMs compared with control iPSC-CMs (q < 0.05 following Benjamini–Hochberg correction), and genes upregulated (upper) or downregulated (lower) in Tbx20 knockout (Tbx20KO) mouse heart compared with the wild type (q < 0.05). The mouse data were obtained from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30943). (b) Predicted number of upregulated (upper) or downregulated (lower) genes with Tbx20-binding sites in Tbx20KO mouse heart and number of genes with a conserved Tbx20-binding site between mouse and human. The ChIP-sequencing data were obtained from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM734426). (c) The heat map of the TGF-β signalling pathway showing upregulation of the TGF-β signalling pathway in Tbx20KO mouse heart compared with wild-type mouse heart. Mean = 0, variance = 1. (d) Significant mRNA expression changes of TBX20 downstream target genes that are involved in the TGF-β signalling pathway as validated by RNA-sequencing. These genes were found to have conserved binding sites in both human (LVNC versus control iPSC-CMs; upper) and mouse (Tbx20KO versus wild-type mouse heart; lower). The bar graphs show the mean and error bars represent s.e.m.

**DISCUSSION**

Previously, both accelerative\(^9,11\) and decelerative proliferation\(^5,7,12\) in embryonic cardiomyocytes have been reported in animal models. In our transgenic mouse model, high overexpression of TGFβ1 in the developing myocardium led to a severe developmental arrest in the compact layer and the severity of this non-compaction phenotype was directly correlated with the levels of TGFβ1 overexpression. These cardiac transcription factors, and restored proliferative capacity compared with non-corrected LVNC iPSC-CMs along with decreased TGF-β signalling activity (Fig. 7g–k). These results suggest that the TBX20 mutation contributes to the pathological phenotype of LVNC iPSC-CMs via disturbance of TGF-β signalling, which is associated with developmental defects of the compact layer during embryogenesis (Supplementary Fig. 7f).
observations suggest that at the early developmental stage of LV compact layer remodelling, proper activation of TGF-β signalling in the embryonic heart is required to ensure normal ventricular development.

Human iPSC-CMs have been used to model familial DCM, familial hypertrophic cardiomyopathy, long QT syndrome, and chemotherapy-induced cardiomyopathy, among others. In this study, we demonstrate that iPSC-CM technology is useful not only for delineating the detailed molecular pathogenesis of congenital developmental defects, but also to clarify genetic causes in such diseases. We successfully found an association between functional disturbance of TBX20 and ectopic activation of TGF-β signalling in...

Figure 6 PRDM16 is a candidate target gene of TBX20 in cardiomyocytes. (a) Validation of TBX20 shRNA knockdown efficiency in lentiviral TBX20 shRNA-transduced H7 ESC-CMs (TBX20KD) compared with scrambled shRNA-transduced H7 ESC-CMs (Scrambled) at 2 weeks. n = 9 independent experiments per group. (b) Validation of pluripotent gene expression profile in TBX20KD and scrambled ESCs by qRT–PCR. (c) Immunostaining of nuclear (blue), TNNT2 (green), and EdU (red) in scrambled and TBX20KD ESC-CMs at 2 weeks. (d) Percentage of EdU-positive CMs in scrambled and TBX20KD ESC-CMs at 2 weeks. (e) Significant decrease of TBX20 downstream target gene (GJA5, TBX5 and MYCN) expression in TBX20KD ESC-CMs compared with scrambled ESC-CMs at 2 weeks. (f,g) Significant increase of TGF-β downstream target gene expression in PRDM16fs iPSC-CMs. (h) Immunostaining of nuclear (blue), alpha-sarcomeric actin (green) and PRDM16 (red) in LVNC iPSC-CMs compared with control iPSC-CMs (h) and in TBX20KD-H7-CMs compared with scrambled H7-CMs (i). (j) CRISPR–Cas9-based frameshift mutation in exon 9 of the PRDM16 gene in control iPSC lines (PRDM16 p.T532fs*8, c.1595delC: highlighted in yellow). wt, wild-type. (k) Percentage of EdU-positive cardiomyocytes in control and PRDM16 frameshift mutation-created iPSC-CMs (PRDM16fs) with or without growth factors. (l) Significant increase of TGF-β downstream target gene expression in PRDM16fs iPSC-CMs. (m) Immunostaining of nuclear (blue), alpha-sarcomeric actin (green) and PRDM16 (red) in LV of donor’s control heart tissue and explanted heart of proband no. 1. CON, unrelated controls.

Table 12.
Figure 7 Rescue of pathological features of LVNC iPSC-CMs. (a,b) Percentage of EdU+ control and LVNC iPSC-CMs (a) or scrambled control and TBX20 knockdown ESC-CMs (b) at 2 weeks after induction of cardiac differentiation with or without treatment of TGF-β receptor-1 inhibitors (SD208 or RepSox) for 2 continuous days. n=7 independent experiments. (c) Adenoviral-mediated overexpression of a dominant-negative form of TGF-β receptor-2 (TGFBRIIDN) significantly restored proliferative potential in LVNC iPSC-CMs compared with the control (adenoviral-mediated GFP overexpression, GFP-Ad). n=8 independent experiments. (d) Knockdown efficiency of CDKN1A protein (upper) and mRNA (lower) in iPSC-CMs by siRNA. n=6 independent experiments. (e) Immunostaining for nuclear (blue), TNNT2 (green) and EdU (red) in control and LVNC iPSC-CMs with CDKN1A or scramble siRNA knockdown. (f) Percentage of EdU+ iPSC-CMs at 2 weeks after induction of cardiac differentiation with CDKN1A or scramble siRNA knockdown. n=6 independent experiments. (g) The efficiency of cardiac differentiation of LVNC and mutation-corrected LVNC iPSC lines before glucose deprivation as validated by FACS for TNNT2. n=5 independent experiments per group. (h) mRNA expression of cardiac transcription factors in differentiating LVNC and mutation-corrected LVNC iPSCs at day 6 and day 9 after induction of cardiac differentiation. LVNC: n=6 independent experiments; LVNC corrected: n=5 independent experiments. (i) Immunostaining for nuclear (blue), TNNT2 (red) and EdU (green) in control, LVNC and TBX20 mutation-corrected (LVNC corrected) iPSC-CMs. (j) Percentage of EdU+ iPSC-CMs at 2 weeks in the control, LVNC and LVNC-corrected group. n=6 independent experiments. (k) Reversible CDKN1A, TGFB1 and PRDM16 mRNA expression abnormality in LVNC-corrected iPSC-CMs compared with LVNC iPSC-CMs. n=6 independent experiments. CON, unrelated controls. Scale bars, 100 μm. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.005; NS, not significant in unpaired two-tailed t-test or one-way ANOVA followed by Tukey post hoc test. The bar graphs show the mean and error bars represent s.e.m. Statistics source data can be found in Supplementary Table 12. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
patient-specific iPSC-CMs as well as perturbed regulation of putative downstream targets of TBX20. We showed that PRDM16 is a possible downstream target gene of TBX20. PRDM16 is a known cause of LVNC in humans and morpholino knockdown of PRDM16 causes a proliferation defect in the developing zebrafish heart. PRDM16 is a repressor of TGF-β signalling via binding of SMAD2/3, and our results revealed ectopic activation of TGF-β signalling in LVNC iPSC-CMs consistent with these previous studies. Although the comprehensive RNA expression data and ChiP-sequencing data showed that TBX20 has a large number of potential downstream target genes associated with TGF-β signalling regulation, our data suggest that the TBX20/PRDM16/TGF-β signalling pathway may be one of the key regulatory cascades for proper development of the compact/trabecular layer.

It is known that patients with LVNC generally show a wide spectrum of clinical manifestations. Some are asymptomatic, and the age at presentation can vary considerably, from as early as the newborn period to as late as older adulthood. This may be explained by varying expression levels of mutant allele-specific mRNA expression between mild DCM and LVNC as shown here. TBX20 is known to interact with other cardiac transcription factors including NKX2-5, GATA4 and TBX5 that are essential for embryonic cardiomyocyte proliferation and ventricular development. As shown here, the disturbance of the interaction between TBX20 and these cardiac transcription factors may negatively impact the expansion of embryonic cardiomyocytes and impair the formation of the multilayered compact myocardium. The differences of allele-specific TBX20 expression and the background expression of these cardiac transcription factors that may also be affected by heterogeneous factors could then impact the various cardiac phenotypes in patients with TBX20 mutations.

In conclusion, iPSC-CMs recapitulate the proliferative defects associated with LVNC at the single-cell level. Importantly, this study presents the compelling evidence of a proliferation defect, a consequence of abnormal activation of TGF-β signalling, as a pathological feature of human LVNC. Whether this defect is common to all cases of LVNC or just a genetic subset remains to be determined. Our data suggest that iPSC-CMs may be useful in therapeutic screening to identify potential interventions for this cardiomyopathy in the future.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

All authors have read and approved the manuscript. K.K., S.-G.O., D.B. and J.C.W. designed research, performed the experiments and wrote the manuscript; F.I., D.B. and J.C.W. recruited the patients; K.K., J.M.C. and S.-G.O. performed and performed experiments on iPSCs/ESC with the help of A.-D.E. and O.J.; K.K., F.J., K.I. and J.C.W. performed sequencing and bioinformatics analysis; S.M.W. generated NK-TGK mouse; K.K., V.T. and I.K. generated genome-corrected iPSC lines; K.K., P.S. and O.J.A. performed and interpreted the patch clamping and calcium imaging data; M.P.S. provided scientific advice; and J.C.W. provided funding and supervised the entire research project.

COMPETING FINANCIAL INTERESTS

J.C.W. is co-founder of Stem Cell Theranostics. The remaining authors declare no competing financial interests.

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METHODS

Derivation of human induced pluripotent stem cells (iPSCs). Fibroblasts or peripheral blood mononuclear cells (PBMCs) were obtained from family members with LVNC with informed consent under protocols approved by the Stanford University Human Subjects Research Institutional Review Board. Human skin punch biopsies were digested with collagenase II and transferred into 6-well culture dish (BD Biosciences). At passage 2 or later, primary fibroblasts were used for reprogramming to iPSCs using the non-integrating Sendai virus-based CytoTune-iPS Reprogramming Kit (Life Technologies). After 24 h and 72 h, the medium was replaced. At day 4, cells were detached using TrypLE (Life Technologies) and replated onto mouse embryonic fibroblast (MEF)-coated 6-well plates in DMEM/Glutamax with 10% FBS and Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor, Y27632 (Selleckchem). From day 5 onwards, cells were cultured in mTeSR medium (STEMCELL Technologies). Colonies were picked after day 20 and transferred to a Matrigel-coated culture dish (BD Biosciences), and subsequently passaged using Accutase (Global Cell Solutions). After passage 10, the culture medium was changed to Essential 8 (Life Technologies) with 2% FBS and 2% FBS 2 days before EdU staining to accelerate the proliferation. Staining was performed using the Click-it EdU Imaging Kit (Life Technologies) according to the manufacturer’s instructions. EdU was incorporated for 24 h before the experiment. Cells were fixed with 4% PFA, permeabilized with 0.5% Triton X-100 in PBS, and stained with Alexa Fluor-conjugated secondary antibody and Hoechst 33342 at day 20. At least more than one hundred cells were counted per sample and more than three independent studies per group were performed. Measurement of the fluorescent signals was performed using NIH-elements BR analysis 4.13.0.64-bit software (Nikon).

Immunocytochemistry. Cells grown on coverslips were fixed using 4% PFA, permeabilized with 0.5% Triton X-100, incubated with primary antibodies and Hoechst 33342, and detected using Alexa Fluor-conjugated secondary antibodies. Primary antibodies used include mouse anti-FLAG M2 (Sigma-Aldrich), rabbit anti-cardiac troponin T (Abcam), mouse anti-cardiac troponin I (Thermo Scientific), or mouse anti-sarcromeric alpha-actinin (Sigma-Aldrich), as published previously. Image acquisition was performed on an Eclipse 80i fluorescence microscope and a confocal microscope (Carl Zeiss, LSM 510 Meta) and ZEN software (Carl Zeiss). Measurement of cell surface area and length was performed using NS-Elements Basic Research 3.0 software (Nikon). A detailed list of antibodies used is shown in Supplementary Table 10.

EdU-based proliferation analysis. iPSC-CMs were cultured in RPMI with B27 supplement and 2% FBS 2 days before EdU staining to accelerate the proliferation. Staining was performed using the Click-it EdU Imaging Kit (Life technologies) according to the manufacturer’s instructions. EdU was incorporated for 24 h before the experiment. Cells were fixed with 4% PFA, permeabilized with 0.5% Triton X-100 in PBS, and stained with Alexa Fluor-conjugated secondary antibody and Hoechst 33342. At least more than one hundred cells were counted per sample and more than three independent studies per group were performed. Measurement of the fluorescent signals was performed using NIH-elements BR analysis 4.13.0.64-bit software (Nikon).

RNA-sequencing. Eight paired-end cDNA libraries (two biological replicates of proband iPSC-CMs, two biological replicates of father iPSC-CMs, one biological replicate of two sibling iPSC-CMs, and one biological replicate of two control iPSC-CMs) were prepared and sequenced. Total RNA was extracted and quantified using the miRNAeasy Kit (Qiagen) according to the manufacturer’s protocol. Ten micrograms of total RNA was used to generate index-tagged paired-end cDNA libraries. Briefly, mRNA were purified by the polyA enrichment procedure using Dynal Oligo(dT) beads (Life Technologies). mRNA fragmentation was performed using RNA Fragmentation Reagents (Life Technologies) to obtain 200–300 bp fragments. cDNA was generated using the SuperScript Double-Stranded cDNA Synthesis Kit (Life technologies). Illumina sequencing adapters were ligated to cDNA using LigaFast (Promega) and PE Adapter Oligo Mix (Illumina). PCR was performed on the adapter-ligated cDNA with 2X Phusion DNA polymerase Master Mix (New England Biolabs). Sequencing was performed with Illuminna’s HiSeq2000 or 2500 platform using paired-end reads at an average length of 100 bp (2 x 100).

RNA-seq data processing and differential expression analysis. Paired-end fastq sequence reads from each sample were assembled against hg19 using the TopHat v2.0.6 (http://tophat.cbcb.umd.edu) with the Illumina-supplied hg19 gene-model annotation file (gtf annotation). The expression level (FPKM, fragments per kilobase of exon per million fragments mapped) was estimated by Cufflinks (http://cufflinks.cbcb.umd.edu/). The Q value is the same as the false discovery rate. Cuffdiff was used to call differentially expressed genes with a false discovery rate less than 0.05. Cuffdiff was run against the UCSC Genomes GTF file from Illumina (http://cole-trapnell-lab.github.io/cufflinks/igenome_table/index.html). Some transcripts that were differentially expressed were not used for filtering and visualization purposes. Only genes with expression values > 1 FPKM in at least one sample were considered for subsequent analysis. Samtools (http://samtools.sourceforge.net) was used to calculate allele-specific expression of the TBX20 gene. We used this software to analyse the differentially expressed genes between controls and LVNC patients. Using IPA Upstream Regulator analysis, the cascade of upstream transcriptional regulators was predicted, and this explained the observed gene expression changes. This upstream regulator analysis is based on prior knowledge of expected effects between transcriptional regulators and their target genes stored in the Ingenuity Knowledge Base. The activation z-score was validated to infer the activation states of predicted transcriptional regulators by the IPA Upstream Regulator analysis. The P value measures whether there is a statistically significant overlap between the data sets and the genes that are regulated by a transcription regulator. The P value calculated using Fisher’s exact test, and significance is generally attributed to P < 0.01. RNA and ChIP-sequencing data of wild-type and Tbx20 knockout mouse heart were obtained from the NCBI GEO database (RNA-sequencing: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30943, ChIP-sequencing: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM374428). All of the RNA sequencing data can be accessed as the GEO reference GSE63161.

Quantifying allele-specific expression using RNA-seq data. Allele-specific expression of the TGFB gene was measured using RNA-Seq data. Alignment was performed using tophat v2.0.6 (http://tophat.cbcb.umd.edu) and processed using
SAMtools v0.1.2, which produces site-specific allele frequencies using overlapping reads (read pileup). Allele-specific expression was calculated by determining whether or not each overlapping read at mutant site fitted the reference or the mutant allele. These summed counts represented our measures of relative allelic abundance at that site. Any deviation from equal allelic abundance was reflected as allelic imbalance.

Quantitative RT-PCR. Total RNA was prepared using the RNAeasy plus kit (Qiagen). cDNA was synthesized by the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a StepOne Real-Time PCR System (Life Technologies) using the TaqMan Universal PCR Master Mix (Life technologies) according to the manufacturer’s protocol. Relative quantification was normalized against GAPDH. More than three respective sets of experiments were performed.

Digital droplet PCR for the detection and quantification of TBX20 wild-type and mutant allele-specific mRNA. Total RNA of 2-week-old LNCV and mild DCM IPS-CMs was extracted and prepared using the RNAeasy plus kit (Qiagen). cDNA was synthesized by the High Capacity cDNA Reverse Transcription Kit (Life Technologies). The quantification of allele-specific mRNA expression was analysed using the QX100 Droplet Digital PCR system (Bio-Rad). The Y317* mutation was detected with an FAM probe: 5'-FAM-CCATCTCGTACTGAGGAGGAG- Black Hole Quencher-3 and replaced the forward primer (5'-TCGTTGTTGGGAAAGCTGAA-3') and the reverse primer (5'-TGACTCTTCATTCCCCAACAG-3'), 20× mutant-specific FAM probes, 20× wild-type-specific HEX probe, 5 ng cDNA template, and the mixture was adjusted with PCR-grade water to a final volume of 20 µl. The thermal cycling conditions were as follows: enzyme activation, 95 °C for 10 min (1 cycle); denaturation, 94°C for 30 s (40 cycles); annealing/extension, 61 °C for 1 min (40 cycles); and hold 98 °C for 10 min (1 cycle). The ddPCR data were analysed with QuantaSoft analysis software (Bio-Rad), and the quantification of either the deletion or the insertion allele was presented as the number of copies per microlitre of PCR mixture.

Western blotting. Following SDS-PAGE, proteins were transferred to 0.45 µm nitrocellulose membranes (Bio-Rad) using a mini Bio-Rad Mini PROTEAN 3 Cell system in NuPAGE transfer buffer (Life Technologies). The membrane was then blocked in Membrane Blocking Solution (Life technologies) and incubated with primary antibody overnight at 4 °C. Blots were incubated with the appropriate secondary antibodies for 1 h at room temperature and visualized using the ECL Western Blotting Analysis System (GE Healthcare). Primary antibodies used were biotinylated SMAD2/3 (Cell Signaling), phospho-SMAD2/3 (Cell Signaling), CDKN1A (Cell Signaling), TBX20 (Sigma-Aldrich), and HRP-conjugated α-tubulin (Cell Signaling). A detailed list of the antibodies used is shown in Supplementary Table 10.

Ca2+ imaging. Briefly, 30-day-old iPS-CMs were dissociated with TrypLE and reseeded on Matrigel-coated 22-mm round coverslips. The Fluo-4 Direct Calcium Assay kit was used (Life Technologies) as per the manufacturer’s instructions. Fluo-4 loading solution was incubated with the cells at 37 °C for 30 min and fluorescence was measured at 495 ± 20 nm excitation and 515 ± 20 nm emission. Videos were taken of iPS-CMs spontaneously beating or electrically field-stimulated at 1 and 2 Hz, 10 V cm-1, and 10 ms biphasic pulse width. Measurements were taken on an AxioObserver Z1 inverted microscope (Carl Zeiss) equipped with a Lambda DG-4 300 W xenon light source (Sutter Instruments), an ORCA-ER CCD (charge-coupled device) camera (Hamamatsu), and AxioVision 4.7 software (Zeiss). All experiments were conducted at 37 °C with normal culture medium (RPMI-1640 with B27 supplement). The mean Ca2+ signals of ten paced beats were analysed with Image-J software. More than three sets of experiments were performed.

Patch clamp. Whole-cell action potentials (APs) were recorded with the use of a standard patch clamp technique, as previously described. Briefly, cultured iPS-CMs were plated on No. 18 coverglass coverslips (Warner Instruments) coated with Matrigel, placed in a RC-26C recording chamber (Warner Instruments), and mounted onto the stage of an inverted microscope (Nikon). The chamber was continuously perfused with warmed (35–37 °C) extracellular solution (pH 7.4) of the following composition: (mM) 150 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 1.0 Na pyruvate, 15 HEPES, and 15 glucose. Glass micropipettes were fabricated from standard wall borosilicate glass capillary tubes (Sutter BF 100-50-10, Sutter Instruments) using a programmable puller (P-97; Sutter Instruments) and filled with the following intracellular solution (pH 7.2): 120 KCl, 1.0 MgCl2, 10 HEPES, 10 EGTA, and 3 Mg-ATP. A single beating cardiomyocyte was selected and APs were recorded in whole-cell current clamp mode using an EPC-10 patch-clamp amplifier (HEKA). Data were acquired using patchMaster software (HEKA) and digitized at 1.0 kHz. The following criteria are used for classifying observed APs into ventricular-, atrial- and nodal-like iPS-CMs. For ventricular-like, the criteria were a negative maximum diastolic membrane potential (<−50 mV), a rapid AP upstroke, a long plateau phase, AP amplitude >90 mV, and AP duration at 50% repolarization/AP duration at 50% repolarization (APD90/APD50) < 1.4. For atrial-like, the criteria were an absence of a prominent plateau phase, a negative diastolic membrane potential (<−50 mV), and APD90/APD50 > 1.7. For nodal-like, the criteria were a more positive MDP, a slower AP upstroke, a prominent phase 4 depolarization, and APD90/APD50 between 1.4 and 1.7.

Transgenic mice. oMHC-Cre mice, mTORC1 mTORC1, and NK-TGCK mice were previously described. The tdTomato reporter mice (A114) and transgenic mice that conditionally express active TGF-β1 following genomic recombination by Cre recombinase (β1glo) were purchased from the Jackson Laboratory. All transgenic mice were backcrossed with C57BL6 background more than five times. oMHC-Cre mice and NK-TGCK mice were crossed with mTORC1 reporter transgenic mice to observe the Cre activity in embryonic heart. oMHC-Cre mice and NK-TGCK mice were also crossed with β1glo mice to obtain cardiac-specific TGF-β1-overexpression (oMHC-Cre/β1glo or NK-TGCK/β1glo) double-transgenic embryos. Doxycycline (2 mg ml−1 plus 50 mg ml−1 sucrose) was administered orally in the drinking water and replaced every 4 days. Pregnant female mice were treated with doxycycline from embryonic day 0.5 to 7.0. For the generation of the NK-TGCK/β1glo double-transgenic adult female mice, pregnant female mice were treated with doxycycline from embryonic day 6.5 to 12.5 to inactivate the NK-TGCK transgene during pregnancy. NK-TGCK/β1glo double-transgenic female mice were crossed with A114 male mice to obtain triple-transgenic embryos and neonates. All experimental procedures were approved by the ethics committee of Stanford University and were in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health).

Immunohistochemistry. Mouse embryos and neonates were collected, fixed in 4% PFA, embedded in OCT, and frozen in dry ice/hexane. Frozen sections were blocked with 5% goat serum, stained with mouse anti-TNNIT (Thermo Scientific), mouse anti-sarcoplasmic alpha-actinin (Sigma-Aldrich), rabbit anti-TNNT2 (Santa Cruz), rabbit anti-TGF-β1 (Abcam), rabbit anti-Ki67 (Thermo Scientific), rabbit anti-α-smooth muscle actin (Abcam), mouse anti-sarcomeric alpha-actinin (Sigma-Aldrich) and rabbit anti-smooth muscle actin (Abcam). Tissue sections were rehydrated and autoclaved with citrate buffer, pH 6.0 (Sigma-Aldrich) or HEPES buffer (Sigma-Aldrich) or sodium citrate, pH 6.0 (Sigma-Aldrich) or HEPES buffer (Sigma-Aldrich) and visualized using Alexa Fluor-conjugated secondary antibodies (Life Technologies). Image acquisition was performed on a Zeiss LSM510 Meta inverted confocal microscope (Carl Zeiss) or an Eclipse 80i fluorescence microscope (Nikon). Transgenes were detected by PCR from yolk sac DNA of embryos or tail DNA of neonatal pups. For human heart tissues, formalin-fixed paraffin-embedded tissue sections were rehydrated and autoclaved with citrate buffer, pH 6.0 (Sigma-Aldrich) and stained with rabbit anti-phospho-Smad2 (EMD Millipore), rabbit anti-smooth muscle actin (Abcam), mouse anti-sarcomeric alpha-actinin (Sigma-Aldrich) and rabbit anti-smooth muscle actin (Abcam). Tissue sections were rehydrated and autoclaved with citrate buffer, pH 6.0 (Sigma-Aldrich) or sodium citrate, pH 6.0 (Sigma-Aldrich) or HEPES buffer (Sigma-Aldrich) and visualized using Alexa Fluor-conjugated secondary antibodies (Life Technologies). Image acquisition was performed on an Eclipse 80i fluorescence microscope (Nikon). A detailed list of the antibodies used is shown in Supplementary Table 10.

Exome sequencing in case-parent trio. Exome sequencing was performed on patient and both parents using the Agilent SureSelectXT Human All Exon V4 (50 Mb) (Agilent Technologies). Briefly, 3 µg of genomic DNA was sheared in 130 µl of low-TE buffer to a peak size of 150–200 bp using Covaris E220, and then purified with AmpPure XP beads to remove fragments less than 100 bp. The purified DNA fragments were then subjected to the Agilent SureSelect Library preparation kit, ILM, to be end-repaired, A-tailed, and ligated to indexing-specific paired-end adapter. The adapter-ligated libraries were amplified for five cycles using HercuII (Agilent Technologies). Amplified pre-capture libraries (750 ng) were concentrated in 3 µl and hybridized to the target specific baits (SureSelectXT Human All Exon V4; Agilent Technologies) according to the manufacturer’s recommendations. Hybridized material was captured using streptavidin-coated beads (Invitrogen) and amplified for 10 cycles. Captured libraries were pooled in pairs and paired-end sequenced on one lane of the Illumina HiSeq 2000 at the Stanford Center for Genomics and Personalized Medicine.

Exome-sequencing data analysis. For each exome, raw reads in FASTQ format were aligned to hg19 using the Burrows–Wheeler aligner (BWA, http://bio-bwa.sourceforge.net) to produce a BAM (binary alignment/map) file. For case and each parent, approximately 12 Gb high-quality mappable data were obtained with an average read coverage of 178×, and 90% of bases in the captured region covered more than 49×. SGBenomics BWA-GATK- and Ingenuity Variant Calling programs version 2.0.20130604 were used to identify potentially pathogenic variants associated with LNCV. An initial variant data set (in variant call format, VCF) was...
METHODS

Transcription activator-like effector nuclease (TALEN)-mediated genome editing. TALEN pair vectors were designed and constructed using the rapid TALEN assembly system as previously described45. Five-hundred-base-pair fragments of wild-type TBX20 exon 7 and adjacent intronic sequences were synthesized as GeneArt String DNA fragments (Life Technologies) to make left and right homologous arms, and cloned into PB-MV1Puro-TK vector (Transposagen), as previously described45. Four silent mutations in homologous arms were inserted to create an artificial TTAa site and to avoid re-cleavage of the genomic sequence. Both TALEN pair and targeting vectors were delivered into LVNC iPSCs by nucleofection using P3 Primary Cell 4D-Nucleofector X Kit (Lonza). Afterwards, cells with correct targeting vector integration were selected by puromycin (Life Technologies) and genotyped. To excise the selection cassette, the transient expression of piggyBac transposase was performed by nuclease of excision-only piggyBac transposase plasmid, PBx (Transposagen). After negative selection using ganciclovir (Sigma-Aldrich), the established clones were genotyped by PCR and bidirectional direct sequencing.

Statistics and reproducibility. The experiments were not randomized and no statistical method was used to predetermine sample size. No inclusion/exclusion criteria were applied to the animal study. The investigators were not blinded to allocation during experiments and outcome assessment and replicate experiments were performed on the basis of the severity and variability of phenotypes obtained. Data were expressed as mean ± s.e.m. Immunoblots show representative of at least two independent experiments. All other experiments are the average of at least two independent assays, and for cell number calculation in immunostaining assays, at least 100 cells per sample were counted for each independent experiment. To confirm the reproducibility, in vitro experiments were performed by two independent operators. Statistical analyses were performed using SPSS statistics 21 software (IBM). An unpaired two-tailed Student’s t-test was used to calculate significant differences between two groups. Multiple comparison correction analysis was performed using ANOVA followed by Tukey’s post hoc HSD test. A P value of <0.05 was considered statistically significant. For RNA-sequencing, raw P values were adjusted for multiple testing with the Benjamin–Hochberg procedure. Genes with an adjusted P value of 0.05 or less were termed as differentially expressed genes. The investigators were not blinded to allocation during experiments and outcome assessment. Source data are provided in Supplementary Table 12.

Data availability. Primary RNA-seq data sets have been deposited in the Gene Expression Omnibus ( GEO) under accession code GSE63161. The Exome-sequencing processed data can be accessed on https://www.ncbi.nlm.nih.gov/VariantInfo/ Jahanbani2016 and raw data can be accessed as the SRA reference SRP080041. Source data for Figs 1–7 and Supplementary Figs 1–5 have been provided in Supplementary Table 12. All other data supporting the findings of this study are available from the corresponding author on request.

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