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Lovastatin improves endothelial dysfunction and cellular crosstalk in LMNA-related dilated cardiomyopathy

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Abstract

Mutations in *LMNA*, the gene that encodes lamin A and C, causes LMNA-related dilated cardiomyopathy (DCM), or cardiolaminopathy. LMNA is expressed in endothelial cells (ECs), however, little is known about the EC-specific phenotype of LMNA-related DCM. Here we studied a family affected by DCM due to a frameshift variant in *LMNA*. Human induced pluripotent stem cell (iPSC)-derived ECs were generated from patients with LMNA-related DCM and phenotypically characterized. Patients with LMNA-related DCM exhibited clinical endothelial dysfunction, and their iPSC-ECs showed decreased functionality as seen by impaired angiogenesis and nitric oxide (NO) production. Moreover, genome-edited isogenic iPSC lines recapitulated the EC disease phenotype in which LMNA-corrected iPSC-ECs showed restoration of EC function. Simultaneous profiling of chromatin accessibility and gene expression dynamics by combining Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) and RNA-seq as well as loss-of-function studies identified Krüppel-like Factor 2 (KLF2) as a potential transcription factor responsible for the EC dysfunction. Gain-of-function studies showed that treatment of LMNA iPSC-ECs with KLF2 agonists, including lovastatin, rescued the EC dysfunction. Patients with LMNA-related DCM treated with lovastatin showed improvements in clinical endothelial dysfunction as indicated by increased reactive hyperemia index. Furthermore, iPSC-derived cardiomyocytes (iPSC-CMs) from patients exhibiting the DCM phenotype showed improvement in cardiomyocyte function when co-cultured with iPSC-ECs and lovastatin. These results suggest impaired crosstalk between ECs and CMs can contribute to the pathogenesis of LMNA-related DCM, and statin may be an effective therapy for vascular dysfunction in patients with cardiolaminopathy.

One-sentence summary

Patient-specific iPSCs model endothelial dysfunction in lamin A and C-related dilated cardiomyopathy and identify lovastatin as a therapy.

INTRODUCTION

Dilated cardiomyopathy (DCM) is characterized by cardiac ventricular enlargement and dysfunction (1) that lead to increased morbidity despite treatment, making it a leading cause for heart transplantation (2, 3). To date, variants in more than 40 genes have been implicated in familial DCM, including genes that encode sarcomeric, cytoskeletal, nuclear, and plasma membrane proteins (4, 5). Among them, variants in the gene that encodes the nuclear envelope proteins lamin A and C (*LMNA*) are amongst the most common (6). Patients who have what is usually known as cardiolaminopathies present with a severe form of the disease, which is often associated with conduction abnormalities, ventricular tachyarrhythmias, progressive heart failure, and sudden cardiac death (SCD) (7). In addition to these cardiac defects, myocardial fibrosis has been identified as a key feature in the hearts of carriers with *LMNA* mutations that exhibit arrhythmias or conduction abnormalities (8). However, the molecular mechanisms that underlie cardiolaminopathy remain elusive, and it is unknown why mutations in this ubiquitously expressed gene have such a disproportionately negative effect on the heart.

Lamins are expressed in all differentiated cells, and in contrast to other genes that cause DCM, *LMNA* variants do not directly affect sarcomeres but still trigger cellular dysfunction (6). Thus, it has been suggested that cardiolaminopathy can arise not only from abnormally functioning lamins in cardiomyocytes (CMs), but also from defects in non-myocytes. Indeed, dermal fibroblasts from patients with cardiolaminopathy exhibit nuclear envelope abnormalities (9, 10), and *LMNA* mutations that cause lipodystrophy or progeria show endothelial cell (EC)-dependent vascular dysfunction leading to premature atherosclerosis (11–13). Therefore, abnormalities in fibroblasts and ECs make it

increasingly clear that dysfunction of non-myocytes in patients with cardiolaminopathy may contribute to progressive heart failure. Moreover, there is evidence to suggest that EC dysfunction can accelerate the progression of myopathy (14).

Although LMNA is abundantly expressed in ECs, and mutations in *LMNA* are known to induce EC dysfunction (13, 15), little is known about the EC-specific phenotype of LMNA-related DCM. To understand this relationship between *LMNA* mutation and EC dysfunction, we hypothesized that induced pluripotent stem cells (iPSC)-derived ECs from a family cohort who harbor the mutation can recapitulate key aspects of the disease phenotype, and thereby provide important insights into the underlying disease mechanisms of cardiolaminopathy.

RESULTS

Patients with *LMNA* mutation exhibit clinical endothelial dysfunction

We studied a large family cohort spanning four generations (Fig. 1A) and carrying a mutation on *LMNA* causing DCM (16). Eight of the recruited family members (Pt. 1, Pt. 2, Pt. 3, Pt. 4, Pt. 5, Pt. 6, Pt. 7, and Pt. 8) harbored a variant that included a heterozygous insertion of a guanine between nucleotides 348 and 349, causing a frameshift mutation at codon 117 (fig. S1A) (16, 17). As a consequence, multiple carriers presented with early-onset atrial fibrillation (AF) and progressive atrioventricular block (AVB) that was followed by DCM and sudden cardiac death (SCD). Indeed, electrophysiological studies on iPSC-derived cardiomyocytes (iPSC-CMs) from these carriers showed aberrant calcium homeostasis as a cause for arrhythmias, with the activation of platelet-derived growth factor (PDGF) as a major contributor to the pathogenesis of LMNA-related DCM (17). However, as lamins are expressed in all differentiated cells, including endothelial cells, and mutations in *LMNA* can induce EC dysfunction, we hypothesized that EC dysfunction could also contribute to the development of DCM in these affected individuals.

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Fig. 1.

Patients carrying an *LMNA* mutation exhibit clinical and molecular EC dysfunction.

(A) Pedigree of family that carry the *LMNA* mutation (Pt. 1, Pt. 2, Pt. 3, Pt. 4, Pt. 5, Pt. 6, Pt. 7, and Pt. 8) and healthy controls (HC1 and HC2). **(B)** Representative images of raw EndoPAT data showing reactive hyperemia index (RHI) from HC1 (left panel) and LMNA Pt. 2 (right panel). Bar graph shows quantification of RHI from HC1 and LMNA Pt. 2. **(C)** Schematic workflow of the experimental design. iPSCs from healthy control and LMNA patients were differentiated to iPSC-ECs for further characterization. **(D)** Schematic of protocol for differentiating iPSCs to ECs. The figure outlines the time course and sequential treatments of growth factors and small molecules. **(E)** Representative brightfield images of healthy control iPSC-ECs and LMNA iPSC-ECs showing typical "cobblestone" monolayer. **(F)** Quantitative PCR data (top panel) and immunoblot (bottom panel) shows *LMNA* expression in healthy control and LMNA iPSC-ECs at the mRNA and protein levels, respectively. Data represented as relative fold-change to patient iPSCs. **(G)** Quantitative PCR data show *eNOS* expression in healthy control and LMNA iPSC-ECs. Data represented as relative fold-change to undifferentiated iPSCs. **(H)** Representative brightfield images of capillary-like networks formed by healthy control and LMNA iPSC-ECs. Right panel shows quantification of the number of tubes. **(I)** Quantification of NO production by healthy control and LMNA iPSC-ECs in response to acetylcholine (Ach) or Ca^{2+} ionophore A23187. (**J**) Quantification of LDL-uptake by healthy control and LMNA iPSC-ECs. All data represented as mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$. Statistical analyses were performed using Student's t-test or one-way ANOVA corrected with Bonferroni method. Scale bar: 50 μm.

To determine whether the carriers exhibited clinical endothelial dysfunction, we first assessed the ability of the patient's endothelium to induce vessel wall relaxation when subjected to reactive hyperemia (18). For this, we used an EndoPAT device to measure any changes in the pulse volume amplitude after reactive hyperemia (19), and calculated a reactive hyperemia index (RHI). Readings above 1.67 are considered normal endothelial function. In accordance with our hypothesis, patients carrying the *LMNA* mutation showed a significant ($P < 0.05$) decrease in their RHI, consistent with endothelial dysfunction, when compared to healthy controls (Fig. 1B, fig. S1, B to G). Importantly, three of these recruited carriers showed reduced RHI even at a young age (Pt. 7 and Pt. 8) or in the absence of other disease confounders known to affect EC function such as atherosclerosis or hypertension (Pt. 6, Pt. 7, and Pt. 8), suggesting an independent effect of the LMNA mutation on clinical EC function (fig. S1, E to G). A decrease in the RHI of a hypertensive patient with known vascular complications confirmed the specificity of the EndoPAT device (fig. S1H). Taken together, these results suggest that carriers of the *LMNA* mutation exhibit clinical endothelial dysfunction in addition to their cardiac abnormalities.

iPSC-ECs from patients with LMNA mutation show impaired phenotype

We used iPSC lines from healthy controls (HC1 and HC2) and patients carrying *LMNA* mutation (Pt. 1 to Pt. 7) that were expanded and confirmed for pluripotency (Fig. 1C, fig. S2A) (17). Using our chemically defined protocol (20), these iPSCs were differentiated to iPSC-ECs as a monolayer (Fig. 1, D and E, fig. S2B) that showed typical cobblestone features and expressed endothelial markers, including *CD31* and *eNOS*. Consistent with our previous results (21), both the healthy control and *LMNA* mutant iPSC-ECs represented a heterogenous population expressing markers from all the three

subtypes of ECs (arterial, venous, and lymphatic). However, as shown in fig. S3A, both groups of iPSC-ECs showed higher expression of arterial (*NRP1*, *EFNB2*, and *NOTCH1*), and lower expression of venous (*EPHB4*, *NR2F2*, and *NOTCH4*) and lymphatic (*PROX1* and *PDPN*) markers, confirming that our differentiated iPSC-ECs represent the arterial subtype and that the phenotypic studies were predominantly conducted on the arterial subfraction of these iPSC-ECs. As expected, *LMNA* mutant iPSC-ECs exhibited significantly ($P < 0.05$) reduced expression of Lamin A/C when compared to healthy controls (Fig. 1F, fig. S3B). Importantly, this reduction in *LMNA* expression had no impact on the differentiation and proliferation potential of these iPSC-ECs (fig. S3, C and D).

Next, we evaluated the phenotypic characteristics of the generated iPSC-ECs from both healthy controls and patients with *LMNA* mutation. As compared to controls, LMNA iPSC-ECs showed significantly ($P < 0.05$) lower expression of *eNOS* and *CD31* (Fig. 1G, fig. S4A). Similarly, when assessing their function, we observed that LMNA iPSC-ECs exhibited a decreased capacity to form networks of tubular structures (Fig. 1H, fig. S4B), a decreased capacity to generate nitric oxide (NO) when stimulated with acetylcholine (Ach) or Ca^{2+} ionophore A23187 (Fig. 1I, fig. S4C), and a decreased capacity to incorporate acetylated LDL (Fig. 1J, fig. S4D) when compared to healthy control iPSC-ECs, all hallmarks of EC dysfunction. In contrast, both healthy control and LMNA iPSC-ECs showed a similar uptake of oxidized LDL (ox-LDL) (fig. S4E), which is internalized in ECs via its receptor LOX-1 and is often considered one of the first steps towards development of atherosclerosis (22). This finding gave us reason to speculate that ox-LDL uptake by ECs in LMNA patients might have only a minimal contribution to the observed EC dysfunction. Indeed, the observed EC dysfunction from our LMNA patients was similar (fig. S4F), and clinical and molecular characterization confirmed that even LMNA patients who were young and without signs of atherosclerosis (table S1) exhibited EC dysfunction. Moreover, this supports our hypothesis that the observed EC dysfunction in LMNA patients is primarily due to the mutation affecting downstream signaling pathways in ECs.

To investigate whether this EC dysfunction truly reflects the EC status in patients, we isolated vessel ECs (VECs) from one of the branches of the circumflex artery from Pt. 2 (obtained during cardiac surgery), and blood endothelial progenitor cells (BECs) (23) from Pt. 3 (fig. S5A). Consistent with our previous findings, both Pt. 2 VECs and Pt. 3 BECs showed downregulation of *CD31* and *eNOS* (fig. S5B) and a decrease in their functional properties, including angiogenic potential (fig. S5C), capacity to produce NO (fig. S5D), and incorporation of Ac-LDL (Fig. S5E), when compared to healthy control ECs (commercially obtained human cardiac microvascular endothelial cells). To further confirm that the EC dysfunction observed in our family cohort is consistent with other LMNA mutations, we also generated iPSC-ECs from another family carrying a different LMNA variant (p.Arg133Gln; c.398G>A) (fig. S5F). Indeed, LMNA iPSC-ECs showed similar phenotypes, including decreased expression of EC markers (fig. S5G), decreased potential to form tubes (fig. S5H), and decreased NO production (fig. S5I). Taken together, these data show that LMNA mutation and EC dysfunction are correlated in both patients and patient-derived iPSC-ECs.

Genome-edited isogenic iPSC-ECs recapitulate disease phenotype

To investigate the correlation between LMNA mutation and EC dysfunction, we next used two isogenic iPSC lines made via TALEN-based genome-editing (17). In the first line, the frameshift-inducing LMNA mutation was corrected (LMNA-WT, wild-type); in the second line, the LMNA frameshift allele was generated in a healthy control line without EC dysfunction (Control-MT, mutant) (fig. S6A). The pluripotent potential was confirmed (fig. S6B) and assessment for off-target effects did not show any variants or indels in our genome-edited isogenic iPSC lines (fig. S6, C to F). Next, we differentiated these isogenic iPSCs into iPSC-ECs to assess their ability to recapitulate the disease phenotype (Fig. 2A). Strikingly, the EC dysfunction observed in LMNA iPSC-ECs was reversed in the genome-edited LMNA-WT iPSC-ECs as evident by the rescue of EC marker expression (Fig. 2B), tube

formation (Fig. 2C), and NO production (Fig. 2D). By contrast, insertion of the LMNA-mutation into healthy control iPSC-ECs (Control-MT) that previously had exhibited normal function now induced dysfunction, linking this LMNA mutation to endothelial dysfunction.

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Fig. 2.

Genome-edited isogenic LMNA iPSC-ECs recapitulate disease phenotype.

(A) Brightfield images of iPSC-ECs from both parental lines (LMNA and healthy control) and genome-edited isogenic lines (LMNA-WT and Control-MT) show typical "cobblestone" appearance. **(B)** Quantitative PCR data show *eNOS* and *CD31* expression in parental (LMNA and healthy control) and genome-edited (LMNA-WT and Control-MT) iPSC-ECs. Data represented as relative fold-change to undifferentiated iPSCs. **(C)** Representative images of capillary-like networks formed by parental (LMNA and healthy control) and genome-edited (LMNA-WT and Control-MT) iPSC-ECs. Right panel shows quantification of the number of tubes. **(D)** Quantification of NO production by parental (LMNA and healthy control) and genome-edited (LMNA-WT and Control-MT) iPSC-ECs in response to acetylcholine (Ach) or Ca^{2+} ionophore A23187. All data represented as mean ± SEM, $n = 3$, $^*P < 0.05$. Statistical analyses were performed using Student's t-test or one-way ANOVA corrected with Bonferroni method. Scale bar: 50 μm.

Transcriptional profiling of *LMNA* mutant iPSC-ECs reveals downregulation of key genes

To elucidate the molecular mechanisms involved in the EC dysfunction, we next performed RNA sequencing (RNA-seq) on healthy control and LMNA iPSC-ECs as well as their isogenic genome-edited lines [denoted as "Control-MT" (mutated) and "LMNA-WT" (corrected), respectively]. A direct comparison of total RNA expression between LMNA iPSC-ECs and isogenic controls revealed a total of 6,766 differentially expressed genes, of which 2,925 were upregulated and 3,841 downregulated (Fig. 3A, fig. S7, A and B). Enrichment analysis of differentially expressed genes showed many dysregulated pathways in LMNA iPSC-ECs compared to isogenic control LMNA-WT iPSC-ECs (Fig. 3B). Further analysis identified a subset of genes that were significantly downregulated in LMNA iPSC-ECs when compared to LMNA-WT. As expected, EC-specific genes such as *PECAM-1* and *CDH5*, and genes responsible for proliferation and angiogenesis such as *netrin-1*, *CD9*, and *ECSCR*, were downregulated in LMNA iPSC-ECs. Interestingly, Kruppel-like factor 2 (KLF2), a transcription factor induced by laminar shear stress, was found to be downregulated in LMNA iPSC-ECs. This is particularly important because *LMNA* has been shown to play a role in mechanotransduction signaling (24). Indeed, our validation studies showed that *KLF2* was downregulated in LMNA iPSC-ECs both at the mRNA and protein levels (Fig. 3C).

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Fig. 3.

Transcriptional profiling of LMNA iPSC-ECs implicates KLF2 as an important regulator in EC dysfunction.

(A) Hierarchical clustering of RNA-seq data from LMNA and LMNA-WT (corrected) iPSC-ECs. **(B)** Enrichment analysis of differentially expressed genes identified by RNA-seq in LMNA and LMNA-WT iPSC-ECs. **(C)** Quantitative PCR data (left panel) and immunoblot (right panel) show *KLF2* expression in healthy control and LMNA iPSC-ECs at the mRNA and protein levels, respectively. **(D)** Normalized ATAC-seq signal across transcription start sites (TSS) in LMNA and LMNA-WT (corrected) iPSC-ECs shown as averaged plots (above) and heatmap image (below). **(E)** Enrichment analysis of ATAC-seq data from LMNA and LMNA-WT iPSC-ECs. **(F)** ChIP analysis to assess H3K4me3 and H3K27me3 of the KLF2 promoters in healthy control and LMNA iPSC-ECs. **(G)** Representative brightfield images of capillary-like networks formed by scramble and KLF2-KD in both healthy control and LMNA-WT iPSC-ECs. Right panel shows quantification of the number of tubes. **(H)** Quantification of NO production by scramble and KLF2-KD in both healthy control and LMNA-WT iPSC-ECs in response to acetylcholine (Ach) or Ca^{2+} ionophore A23187. **(I)** Quantification of LDL-uptake by scramble and KLF2-KD iPSC-ECs in both healthy control and LMNA-WT iPSC-ECs. All data represented as mean ± SEM, $n = 3$, $^{*}P < 0.05$. Statistical analyses were performed using Student's t-test or one-way ANOVA corrected with Bonferroni method. Scale bar: 50 μm.

KLF2 deficiency is responsible for endothelial dysfunction in LMNA patients

LMNA is known to interact with genomic DNA and thereby can modulate local gene expression by interacting with particular sites on promoters (25). Moreover, it has been shown that *LMNA* deficiency can lead to its dissociation from promoters and alter repressive and permissive histone modifications (26). To assess this, we performed Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) to detect differences in signals across transcription start sites (TSS) in LMNA iPSC-ECs when compared to isogenic control LMNA-WT (corrected) iPSC-ECs. Importantly, by overlapping this with our RNA-seq data, we found that genes in LMNA iPSC-ECs exhibited a higher open chromatin positioning on their promoter regions when compared to LMNA-WT iPSC-ECs (Fig. 3, D and E, fig. S7C), thereby demonstrating a strong correlation between *LMNA* expression and chromatin accessibility. Moreover, these data suggest that decreased *LMNA* expression in disease patients could result in abnormal open chromatin state that might interfere with normal gene expression. Next, to determine the effects of LMNA mutation on KLF2 promoters, we performed chromatin immunoprecipitation followed by PCR analysis (ChIP-PCR) to detect trimethylation of histone H3 at lysine 4 (H3K4me3), which would mark transcriptionally active genes, or histone H3 at lysine 27 (H3K27me3), associated with transcriptionally silenced genes. As expected, we observed a significant ($P < 0.05$) increase of H3K4me3 in the promoter regions of KLF2 in healthy control iPSC-ECs when compared to LMNA iPSC-ECs (Fig. 3F). Similarly, we found a concomitant increase of H3K27me3 in the promoter regions of KLF2 in LMNA iPSC-ECs when compared to healthy control iPSC-ECs. These epigenetic modifications confirmed that the LMNA-mutation decreases *KLF2* expression.

To further validate the role of KLF2, we knocked down the *KLF2* expression in healthy control and LMNA-WT (corrected) as well as in LMNA and Control-MT (mutated) iPSC-ECs using short hairpin RNA (shRNA) (fig. S7, D and E). Knockdown (KD) of *KLF2* in healthy control and LMNA-WT

iPSC-ECs not only reduced the expression of *CD31* and *eNOS* (fig. S7F), but also impaired the function of iPSC-ECs by decreasing their ability to form tubular structures (Fig. 3G), capacity to produce NO (Fig. 3H), and capacity to incorporate Ac-LDL (Fig. 3I).

Shear stress fails to induce *KLF2* expression in LMNA iPSC-ECs

Although the endothelium is a diaphanous film of tissue, this delicate monolayer acts as a signal transduction interface for mechanical forces generated by blood flow (27). The transmission of these hemodynamic forces ultimately leads to alterations in gene expression, thereby regulating vascular tone. KLF2 is a mechanotransduction intermediary, and its expression is upregulated by shear stress (28). Moreover, KLF2 can regulate *eNOS* expression and enzymatic activity, thereby acting as a molecular switch for NO production (fig. S8A) (29, 30). This led us to hypothesize that mutations in LMNA can downregulate *KLF2* in ECs even in the presence of shear stress, leading to lower *eNOS* expression and decreased NO production, thereby inducing EC dysfunction (fig. S8B). To test this hypothesis, we exposed iPSC-ECs to well-defined laminar flow with a shear stress of ~15 dynes/cm^2. Consistent with previous findings showing that KLF2 is essential for EC alignment (31), healthy control and LMNA-WT (corrected) iPSC-ECs were aligned in the direction of flow, whereas LMNA and Control-MT (mutated) iPSC-ECs remained non-aligned (Fig. 4A). Importantly, LMNA and Control-MT iPSC-ECs failed to induce *KLF2* and *eNOS* expression in response to shear stress (Fig. 4B). Similarly, functional assays on LMNA and Control-MT iPSC-ECs showed impaired tube formation and NO production after exposure to flow (Fig. 4, C and D).

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Fig. 4.

Shear stress fails to induce *KLF2* expression in LMNA iPSC-ECs.

(A) Representative brightfield images of parental (LMNA and healthy control) and genome-edited (LMNA-WT and Control-MT) iPSC-ECs subjected to shear stress. Arrow represents the direction of the flow. **(B)** Quantitative PCR data show *KLF2* and *eNOS* expression in parental (LMNA and healthy control) and genome-edited (LMNA-WT and Control-MT) iPSC-ECs after being subjected to shear stress. **(C)** Representative brightfield images of capillary-like networks formed by parental (LMNA and healthy control) and genome-edited (LMNA-WT and Control-MT) iPSC-ECs after exposure to shear stress. Bar graph showing quantification of the number of tubes (right panel). **(D)** Quantification of NO production by parental (LMNA and healthy control) and genome-edited (LMNA-WT and Control-MT) iPSC-ECs after shear stress. **(E)** Representative brightfield images of scramble and KLF2-KD in both healthy control and LMNA-WT (corrected) iPSC-ECs when subjected to shear stress. Arrow represents the direction of the flow. **(F)** Quantitative PCR data show *KLF2* and *eNOS* expression in healthy control and LMNA-WT iPSC-ECs when KLF2 is knocked down and subjected to shear stress in comparison to scramble controls. **(G)** Representative brightfield images of capillary-like networks formed by healthy control and LMNA-WT iPSC-ECs when KLF2 is knocked down in comparison to scramble controls. Right panel shows quantification of the number of tubes. **(H)** Quantification of NO production by healthy control and LMNA-WT iPSC-ECs after shear stress when KLF2 is knocked down in comparison to scramble controls. All data represented as mean ± SEM, $n = 3$, $^*P < 0.05$. Statistical analyses were performed using Student's t-test or one-way ANOVA corrected with Bonferroni method. Scale bar: 50 μm.

Next, we exposed the KLF2-KD iPSC-ECs from healthy control and LMNA-WT (corrected) as well as LMNA and Control-MT (mutated) iPSC-ECs to shear stress. As *KLF2* is already downregulated in LMNA and Control-MT iPSC-ECs, further knockdown of *KLF2* did not show any significant ($P < 0.05$) differences in EC alignment, tube formation, and NO production (fig. S8, C to E) when compared to scramble-treated cells. On the contrary, knockdown of *KLF2* in healthy control and LMNA-WT iPSC-ECs resulted in misalignment of ECs (Fig. 4E), downregulation of *KLF2* and *eNOS* (Fig. 4F), decreased tube formation (Fig. 4G), and lower NO production (Fig. 4H) when compared to scramble-treated cells. Collectively, these results suggest that the LMNA mutation impairs KLF2-mediated EC response to shear stress, thus explaining the clinically observed endothelial dysfunction.

Lovastatin improves LMNA-related endothelial dysfunction by induction of KLF2

Human iPSC-derived cells provide a unique platform to screen for compounds that have superior human-specific drug responsiveness. With KLF2 as the main target, we next searched a publicly available database (ChemBank) for small molecules that can regulate KLF2 (fig. S9A). Of the 16 we identified (fig. S9B), six belonged to the statin family, consistent with previous studies showing statin-dependent induction of KLF2 in ECs (32). Screening of these small molecules for *KLF2* expression revealed three compounds (lovastatin, mevastatin, and simvastatin) that showed a dose-dependent increase in *KLF2* expression in LMNA and Control-MT (mutated) iPSC-ECs (fig. S9C). Because lovastatin showed the most robust increase (≈6-fold) in *KLF2* expression, we used lovastatin for all subsequent experiments.

We first assessed whether lovastatin can improve EC dysfunction in LMNA and Control-MT iPSC-ECs. For this, iPSC-ECs were treated with 1 μM lovastatin in flow-mediated conditions (Fig. 5A), followed by genotypic and phenotypic characterization. Under flow conditions, lovastatin not only increased *KLF2* and *eNOS* expression (Fig. 5B), but also improved the EC function as evidenced by increased tube formation and increased NO production (Fig. 5, C and D). After lovastatin treatment, both LMNA and Control-MT (mutated) iPSC-ECs showed alignment with the direction of the flow (Fig. 5B), indicating functional rescue. By contrast, both healthy control and LMNA-WT (corrected) iPSC-ECs showed no significant differences in their alignment (fig. S10A), gene expression of *KLF2* and *eNOS* (fig. S10B), or their functional characteristics (fig. S10, C and D) when treated with lovastatin.

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Fig. 5.

Lovastatin improves EC function in LMNA iPSC-ECs.

(A) Representative brightfield images of LMNA and Control-MT iPSC-ECs when subjected to shear stress in the presence of lovastatin. Arrow represents the direction of the flow. **(B)** Quantitative PCR data show *KLF2* and *eNOS* expression in LMNA and Control-MT iPSC-ECs when subjected to shear stress in the presence of lovastatin. **(C)** Representative brightfield images of capillary-like networks formed by LMNA and Control-MT iPSC-ECs when subjected to shear stress in the presence of lovastatin. Right panel shows quantification of the number of tubes. **(D)** Quantification of NO production by LMNA and Control-MT iPSC-ECs after shear stress in the presence of lovastatin. All data represented as mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{****}P < 0.0001$. Statistical analyses were performed using student's t-test or one-way ANOVA corrected with Bonferroni method. Scale bar: 50 μm.

Because statins are known to generally improve EC function in disease conditions (33), we next sought to determine whether the observed effects of lovastatin were specific to *LMNA* deficiency. For this, we compared the effects of lovastatin on healthy control and LMNA iPSC-ECs to atorvastatin, a widely used statin with known beneficial effects on the endothelium. As expected, both lovastatin and atorvastatin had no effect on EC function in healthy control iPSC-ECs as evident by the absence of changes in the number of capillary-like networks (fig. S10E). By contrast, lovastatin treatment of LMNA iPSC-ECs showed a significant ($P < 0.05$) increase in the number of capillary-like networks when compared to atorvastatin treatment. Importantly, the functional data showed that atorvastatin, despite being a very potent statin for reducing cholesterol, exhibited a more blunted response in LMNA iPSC-ECs when compared to lovastatin, suggesting that the observed effects of lovastatin are specific to *LMNA* haploinsufficiency seen in these LMNA patients. Taken together, these results suggest that by increasing *KLF2* expression, lovastatin improves EC function in LMNA iPSC-ECs by enhancing *eNOS* expression and NO production.

Lovastatin improves endothelial function in cardiolaminopathy patients

We next investigated whether lovastatin can improve clinical EC function *in vivo*. We recruited two members of the family affected by *LMNA* mutation (Pt. 2 and Pt. 3) to start a daily oral regimen of 40 mg lovastatin. After lovastatin treatment, clinical endothelial function was assessed for both LMNA Pt. 2 and LMNA Pt. 3 at 6 months and again at 18 months for Pt. 3. EndoPAT data from both Pt. 2 (Fig. 6A) and Pt. 3 (Fig. 6B) showed significant ($P < 0.05$) improvements in their RHI (Fig. 6C). Importantly, long-term treatment of Pt. 3 with lovastatin showed further improvement in the RHI (Fig. 6D) indicative of continued improvement in the patient's clinical endothelial function. As we already had baseline EC functional data from Pt. 2 and Pt. 3 (fig. S5, A to E), we next determined whether long-term lovastatin treatment improved EC function. At the 6-month treatment point, LMNA Pt. 2 underwent a heart transplant, giving us the opportunity to collect and isolate coronary vessel endothelial cells (VECs). Similarly, we collected blood endothelial cells (BECs) from LMNA Pt. 3 to evaluate their functional characteristics following long-term lovastatin treatment. In addition to increasing *KLF2* and *eNOS* expression in Pt. 2 VECs and Pt. 3 BECs (Fig. S11, A and B), long-term lovastatin treatment also improved EC function in both patients. Their primary ECs showed an improvement in forming tubes and networks (Fig. 6, E and F), suggesting that long-term lovastatin treatment can improve EC function in cardiolaminopathy patients.

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Fig. 6.

Lovastatin improves EC dysfunction in patients with cardiolaminopathy.

(A-B) Representative images of raw EndoPAT data showing reactive hyperemia index (RHI) from LMNA Pt. 2 **(A)** and Pt. 3 **(B)** after 6 months of oral lovastatin treatment. Upper panels show EndoPAT data before lovastatin treatment. Lower panels show EndoPAT data after lovastatin treatment. **(C)** Bar graph shows quantification of RHI from LMNA Pt. 2 and Pt. 3 before and after 6 months lovastatin treatment. **(D)** Representative image of raw EndoPAT data showing RHI from LMNA Pt. 3 after 18 months of oral lovastatin treatment. **(E)** Representative brightfield images of capillary-like networks formed by Pt. 2 vessel ECs (VECs) isolated before (left) and after (right) 6 months of lovastatin treatment. Far right panel shows quantification of the number of tubes. **(F)** Representative brightfield images of capillary-like networks formed by Pt. 3 blood ECs (BECs) before (left) and after (right) 6 months of lovastatin treatment. Far right panel shows quantification of the number of tubes. **(G)** Line graph of percent relaxation in Pt. 2 LADs at 3 and 10 μM acetylcholine when treated with 1 μM lovastatin. All data represented as mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$. Significance of effects of lovastatin treatment in LMNA patient LADs was determined by 2-way ANOVA, followed by a Bonferroni post-test, $n = 3$, (three separate segments of LAD artery from LMNA Pt. 2 and from healthy control heart), $^{*}P < 0.05$. Scale bar: 50 μm.

Next, we evaluated the *ex vivo* effects of lovastatin on coronary arteries obtained from LMNA Pt. 2. For this, the left anterior descending (LAD) artery from Pt. 2 was harvested during heart transplantation and vascular reactivity assessed using a wire myograph that can measure the force generated by vascular smooth muscle cells (SMCs) in response to NO produced by ECs (34). LAD artery collected from a healthy heart of a rejected donor (42 yo female) served as a control. First, we confirmed that the isometric measurements of contraction between vessels form control and Pt. 2 were identical in response to agonists or antagonists (fig. S11, C and D). Next, we measured the vascular relaxation in the presence or absence of lovastatin when stimulated with acetylcholine (fig. S11E). As expected, the percent relaxation of the vessel from Pt. 2 was lower than that of the control (Fig. 6G). When the LAD artery from Pt. 2 was pre-incubated with lovastatin, the percent relaxation showed a significant ($P < 0.05$) improvement (Fig. 6G), suggesting that lovastatin-induced *KLF2* expression improves vascular reactivity in cardiolaminopathy.

Lovastatin improves cardiomyocyte function when co-cultured with ECs

Patients with cardiolaminopathy present with symptomatic conduction system diseases such as arrhythmias or DCM, including heart failure. Indeed, these clinical features have been recapitulated using mouse models that showed impaired contractility in isolated cardiomyocytes and cardiac pathology reflective of DCM (35). Similarly, iPSC-CMs from patients with *LMNA* mutation also recapitulated the disease phenotype bearing the hallmarks of DCM (17, 36, 37). In the healthy heart, due to their close proximity to the myocyte, capillary ECs are known to play an important role in cardiac development and function (38). Moreover, in an adult heart, the endothelium can regulate the cardiac output and rhythm by releasing NO both under normal or stress conditions (39). Despite these studies, little is known about the underlying mechanisms, especially the role of ECs in cardiac diastolic dysfunction, another hallmark of DCM. Because our patients with *LMNA* mutation exhibited diastolic dysfunction as evident by a decrease in their tissue Doppler relaxation velocity (Fig. 7A, fig. S12A),

we next investigated the crosstalk between iPSC-CMs and iPSC-ECs. We hypothesized that lovastatin treatment, by improving EC function in patients with *LMNA* mutation, can indirectly improve CM function when co-cultured together.

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Fig. 7.

Lovastatin improves functional phenotype of LMNA iPSC-CMs when co-cultured with LMNA iPSC-ECs.

(A) Bar graph showing tissue Doppler velocity (lateral E' and medial E') in healthy control and LMNA patients. **(B)** Schematic workflow of the experimental design. iPSC-CMs and iPSC-ECs from healthy control and LMNA patients were co-cultured in the presence or absence of 1 μM lovastatin. iPSC-CMs alone or treated with lovastatin were used as controls. **(C)** Quantification of the contractile properties of iPSC-CMs using video microscopy-based motion vector analysis. Bar graphs show relaxation velocity in LMNA iPSC-CMs when co-cultured with LMNA iPSC-ECs and treated with 1 μM lovastatin for 1-week. **(D)** Quantification of diastolic Ca^{2+} imaging parameter in LMNA iPSC-CMs when co-cultured with LMNA iPSC-ECs and treated with 1 μM lovastatin for 1-week. **(E)** Hierarchical clustering of RNA-seq data in co-cultured LMNA iPSC-ECs after lovastatin treatment. **(F)** Enrichment analysis of RNA-seq data show GO terms in co-cultured LMNA iPSC-ECs after lovastatin treatment. **(G)** Hierarchical clustering of RNA-seq data in co-cultured LMNA iPSC-CMs after lovastatin treatment. **(H)** Enrichment analysis of RNA-seq data show GO terms in co-cultured LMNA iPSC-CMs after lovastatin treatment. **(I)** Immunoblot showing eNOS expression and phosphorylation in LMNA iPSC-ECs when co-cultured with LMNA iPSC-CMs and treated with 1 μM lovastatin for 1-week. GAPDH was used as loading control. Data represented from three biological replicates of healthy control (HC1) and of LMNA Pt. 2. **(J)** Quantification of NO production by LMNA iPSC-ECs when co-cultured with LMNA iPSC-CMs and treated with 1 μM lovastatin for 1-week. All data represented as mean ± SEM, $n = 3$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Statistical analyses were performed using one-way ANOVA corrected with Bonferroni method or one-way multivariate analysis of variance (MANOVA).

To test this, we co-cultured iPSC-CMs and iPSC-ECs from healthy control and patients with *LMNA* mutation in the presence or absence of lovastatin. iPSC-CMs alone or treated with lovastatin served as control groups (Fig. 7B). To mimic the normal heart in which ECs outnumber CMs, we seeded iPSC-CMs with iPSC-ECs at a ratio of 1:3, followed by lovastatin treatment of 1 μM for 1-week (fig. S12B). First, we assessed the contractile properties of iPSC-CMs using high-speed video microscopy with motion vector analysis (40). Measurement of spontaneous beating rate showed no differences in either group following lovastatin treatment (fig. S12C). However, LMNA iPSC-CMs exhibited a significant ($P < 0.05$) decrease in their relaxation velocity when compared to healthy control iPSC-CMs (Fig. 7C). This impaired relaxation was observed even when LMNA iPSC-CMs were treated with lovastatin alone or co-cultured with LMNA iPSC-ECs without lovastatin. Only when the co-cultures of iPSC-CMs and iPSC-ECs were treated with lovastatin did LMNA iPSC-CMs show improvement in their relaxation velocity. Consistent with our previous findings (41), LMNA iPSC-CMs also showed impaired contractility when compared to healthy controls (fig. S12D).

We next assessed the Ca^{2+} handling properties of iPSC-CMs in these co-cultures by measuring Ca^{2+} transient amplitude and kinetics by fluorescent Ca^{2+} imaging. As expected, LMNA iPSC-CMs exhibited increased arrhythmic Ca^{2+} transients when cultured alone or with LMNA iPSC-ECs (fig.

S12, E and F); however, lovastatin treatment on these co-cultures showed a decrease in arrhythmic Ca^{2+} transients. Importantly, lovastatin treatment decreased the diastolic Ca^{2+} (Fig. 7D) and time constant (fig. S12G) and increased the systolic Ca^{2+} amplitude in LMNA iPSC-CMs (fig. S12H), suggesting that lovastatin treatment reduced cytosolic diastolic Ca^{2+} by increasing Ca^{2+} uptake and thereby improved diastolic dysfunction and precursors to arrhythmia.

To support our functional data showing that lovastatin treatment of LMNA iPSC-ECs can improve the function of LMNA iPSC-CMs when cultured together, we next sought to determine the underlying mechanisms driving this crosstalk. To that end, we captured the dynamic changes in the global gene expression of the transcriptomic landscape in co-cultures of iPSC-ECs and iPSC-CMs when treated with lovastatin. iPSC-CMs and iPSC-ECs from both healthy control and patients with *LMNA* mutation were co-cultured and treated with lovastatin for 1-week. Following treatments, iPSC-ECs were separated from iPSC-CMs using magnetic-activated cell sorting (MACS) with CD31 antibody, and both separated cell types were immediately processed for RNA-seq (fig. S13A). Consistent with our previous RNA-seq data of iPSC-EC monocultures, co-cultured iPSC-ECs from patients with *LMNA* mutation exhibited downregulation of EC-specific genes such as *PECAM-1*, *CDH5*, and *ECSCR* (Fig. 7E, fig. S13B). Similarly, *KLF2* and *eNOS* were also downregulated in co-cultured LMNA iPSC-ECs when compared to healthy control. By contrast, the same co-cultured iPSC-ECs when treated with lovastatin for 1-week showed upregulation of EC-specific genes including *KLF2* and *eNOS* (Fig. 7E, fig. S13C). Furthermore, enrichment analysis of our RNA-seq data from co-cultured iPSC-ECs revealed significantly altered Gene Ontology (GO) terms in LMNA iPSC-ECs following lovastatin treatment when compared to vehicle-treated co-cultures, suggesting a global upregulating effect of lovastatin on EC genes (Fig. 7F).

We next analyzed the gene expression profile of the co-cultured iPSC-CMs to establish the effects of lovastatin on healthy control versus LMNA cells. As expected, LMNA iPSC-CMs exhibited an impaired cardiac gene expression profile when compared to healthy control (Fig. 7G, fig. S13D). However, when treated with lovastatin, co-cultured LMNA iPSC-CMs showed an upregulation in genes responsible for cardiac mechanics, including those for cardiac contractility (*MYH6/7*, *ACTC1*, *TNNI3*), Ca^{2+} handling (*CASQ2*, *PLN*), and metabolism (*KLF15*, *PPARα*) (Fig. 7G, fig. S13E). Indeed, enrichment analysis of RNA-seq data from co-cultured LMNA iPSC-CMs showed altered GO terms including cardiac muscle contraction and heart process after lovastatin treatment (Fig. 7H). Importantly, this upregulation in cardiac gene expression was not evident in co-cultured healthy control iPSC-CMs when treated with lovastatin, suggesting that the observed improvement in iPSC-CM function in patients with *LMNA* mutation was due to upregulation of genes responsible for cardiac mechanics. These data suggest that improvement in the cardiac contractile apparatus was due to lovastatin-induced improvement in LMNA iPSC-ECs. The absence of cardiac marker such as *MYH6* in iPSC-ECs and EC marker such as *eNOS* in iPSC-CMs sorted from co-cultures confirmed that the separation of iPSC-ECs and iPSC-CMs from these co-cultures via MACS was clean (fig. S13F).

Lovastatin improves crosstalk between LMNA iPSC-CMs and iPSC-ECs

To further validate this influence of iPSC-ECs on iPSC-CMs, we evaluated the heterocellular signaling by co-culturing healthy control and LMNA cells together. For this, healthy control iPSC-ECs were co-cultured with LMNA iPSC-CMs (fig. S14A) and LMNA iPSC-ECs were co-cultured with healthy control iPSC-CMs (fig. S14B). We focused on measuring the contractile properties of iPSC-CMs and found that, in line with our hypothesis, co-cultured healthy control iPSC-ECs improved the contractile properties of LMNA iPSC-CMs whether or not lovastatin was present (fig. S14, C to E), suggesting that healthy iPSC-ECs with normal *KLF2* and *eNOS* expression have a significant ($P < 0.05$) impact on LMNA iPSC-CM contractility. Interestingly, when co-cultured with LMNA iPSC-ECs, healthy control iPSC-CMs showed no further improvement in their contractile properties when compared to control

iPSC-CMs exposed only to lovastatin (fig. S14, C to E). Taken together, these results demonstrate that the impaired contractile function of LMNA iPSC-CMs can be restored when co-cultured with healthy control iPSC-ECs or lovastatin-treated LMNA iPSC-ECs, thereby suggesting that a critical crosstalk exists between the iPSC-CMs and iPSC-ECs.

To further understand the molecular players involved in this crosstalk between iPSC-ECs and iPSC-CMs, we evaluated the expression and activity of *eNOS* in healthy control and LMNA co-cultures. Because *eNOS* expression is attributed to NO production by the ECs, which thereby exerts positive inotropic and lusitropic effects on the cardiac muscle (38), we hypothesized that downregulation of *KLF2* in LMNA iPSC-ECs will impair *eNOS* expression and thus lead to an attenuated NO production by the ECs. Indeed, lovastatin is widely accepted to upregulate *eNOS* expression via many pathways, including increased expression of *KLF2* (42). Once upregulated, eNOS undergoes post-translational modification, thereby mediating an increase in NO bioavailability in the ECs. Based on this, we evaluated both the expression and phosphorylation of eNOS as well as NO production in the iPSC-ECs of the co-cultures. Consistent with our qPCR data, eNOS protein expression was impaired in LMNA iPSC-ECs when compared to healthy controls (Fig. 7I). However, when treated with lovastatin for 1-week, co-cultured LMNA iPSC-ECs showed an increase in eNOS expression when compared to healthy controls. Importantly, lovastatin treatment induced phosphorylation of eNOS at serine residue 1177 in LMNA iPSC-ECs, which is known to increase eNOS enzymatic activity and NO production. In our co-cultures, we correlated the lovastatin-induced eNOS activation in LMNA iPSC-ECs to NO production. As expected, the increase in eNOS expression in LMNA iPSC-ECs after lovastatin treatment was correlated with the increase in the NO production (Fig. 7J), suggesting that the impaired iPSC-CM contractility in patients with *LMNA* mutation can, at least in part, be attributed to the attenuated NO production.

DISCUSSION

Patients with *LMNA* mutation suffer from a wide range of diseases, including DCM, muscular dystrophy, and progeria, among others (43). Collectively referred to as laminopathies, almost all of these diseases present with vascular dysfunction (44, 45). Despite this evidence, little is known about the role of the endothelium in these diseases, especially in LMNA-related DCM. In this study, we assessed the endothelial function in a large family with an autosomal-dominant cardiolaminopathy. The frameshift mutation in this family resulted in clinical phenotype characterized by early onset atrial fibrillation, progressive atrioventricular block, DCM, and sudden cardiac death (16, 17). Measurement of their vascular RHI and assessment of their iPSC-ECs established that these patients with *LMNA* mutation suffer from endothelial dysfunction. Further characterization of genome-edited isogenic iPSC-ECs confirmed our finding of a direct link between LMNA mutation and endothelial dysfunction. This approach enabled us to study endothelial dysfunction in LMNA patients without the need to recruit additional patients (46), while removing the confounding genetic variability that could occur when comparing one patient to another.

Lamins are expressed in all differentiated somatic cells and are considered important regulators of gene expression due to their interplay with signaling pathways, transcription, and chromatic organization (47, 48). Moreover, lamins can directly interact with transcription factors to regulate gene expression by interacting with transcriptional complexes. Indeed, our simultaneous profiling of chromatin accessibility and gene expression dynamics by combining ATAC-seq with RNA-seq showed downregulation of one such transcription factor, KLF2, in LMNA iPSC-ECs, and further knockdown studies validated the importance of KLF2 in LMNA-induced EC dysfunction. KLF2, a member of the Kruppel-like family, is regulated by biomechanical flow in ECs (28, 49) and in turn can regulate downstream genes such as *eNOS*, a gene primarily responsible for NO production (49). An emerging function of nuclear lamins is to detect “outside-in” signaling such as shear stress, and to react by

remodeling the cytoskeleton and extracellular matrix (50). In other words, *LMNA* genes behave as a "mechanostat" to external forces, allowing the cells to adapt to the environment (24). Knowing that by virtue of their location ECs are excellent mechanotransducers, we modeled EC dysfunction under physiological laminar flow. Our data showed that even under normal shear stress, LMNA iPSC-ECs exhibited EC dysfunction, which further validates our hypothesis that KLF2 is a key mediator for EC dysfunction in patients with *LMNA* mutation.

Although LMNA-associated DCM accounts for about 6% of all familial cases, targeted therapeutic strategies to prevent its onset and progression remain elusive. Knowing that KLF2 is an important regulator in LMNA-related EC dysfunction, we screened for compound libraries that target KLF2-related signaling pathways. Our initial screen revealed three statins (lovastatin, mevastatin, and simvastatin) that improved EC function in LMNA iPSC-ECs, consistent with other studies that showed a similar improvement in EC function (32, 51), suggesting an additional non-lipid lowering beneficial effect of these statins. Our data provide clear evidence that upregulating KLF2 via lovastatin can help restore EC function *in vitro*. To establish whether lovastatin can improve clinical EC function *in vivo*, we tested the effects of lovastatin on two of our recruited human subjects. By as early as six months after initiating treatment and lasting up to 18 months, daily regimen of lovastatin improved the RHI in patients with cardiolaminopathy, which is indicative of an intrinsic improvement in EC function.

Mortality in patients with cardiolaminopathy is usually associated with major cardiovascular complications including atrioventricular block, ventricular tachyarrhythmia, and DCM. In addition to these cardiac defects, LMNA mutations can also affect the non-myocyte cells in the heart, including fibroblasts and endothelial cells. Indeed, it has been observed that around half of all cardiolaminopathy patients will develop myocardial fibrosis, which can be either interstitial or gross, and has been identified as a causative factor in the development of left ventricular dysfunction (52, 53). Similarly, another predominant pathology that is often seen in laminopathy patients is atherosclerosis (54). As EC dysfunction is a precursor to atherosclerosis with reduced NO bioavailability (55, 56), there is substantial evidence to suggest that EC dysfunction can accelerate the progression of myopathy (57, 58). With the knowledge that endocardial vasculature is a key regulator of myocardial integrity, contractile performance, and rhythmicity via secretion of signaling factors such as NO (38, 59), we tested whether improving EC function in LMNA iPSC-ECs can improve cardiomyocyte function for these patients. Our data provide clear evidence that lovastatin treatment of LMNA iPSC-ECs improved function of LMNA iPSC-CMs when cultured together by upregulating genes in the LMNA iPSC-CMs that are responsible for cardiac mechanics, including those for cardiac contractility, Ca^{2+} handling, and metabolism.

In contrast, oral lovastatin treatment in patients with *LMNA* mutation failed to show an improvement in their ejection fraction, suggesting that improving EC function in these patients cannot reverse the cardiac pathology. This failure of lovastatin to reverse the cardiac dysfunction *in vivo* may be partly due to the onset of myocardial fibrosis in these patients with cardiolaminopathy. Although lovastatin improved the EC function in our patients with LMNA mutation *in vivo*, it may not be able to change the amount of myocardial fibrosis. Moreover, EC dysfunction has been shown to contribute to the development of myocardial fibrosis in DCM (8), as ECs in disease conditions can secrete inflammatory cytokines such as tumor necrosis factor-α, interleukin-1, and interleukin-6 that possess pro-fibrotic properties and thus play important roles in cardiac remodeling and heart failure (60). Based on this, we speculate that by improving EC function at an earlier stage, before patients develop cardiac symptoms, it may be possible to delay the onset of cardiolaminopathy.

In summary, we used iPSC technology to understand the disease mechanisms underlying cardiolaminopathy, identifying a key gene responsible for endothelial dysfunction in patients with LMNA-related DCM. We discovered that a subset of statins can ameliorate this endothelial dysfunction by restoring the downregulated *KLF2* expression *in vitro* as well as improving vascular RHI *in vivo*.

Similarly, when co-cultured with iPSC-ECs, iPSC-CMs from patients with LMNA mutation showed improvement in function when treated with lovastatin (fig. S15). Taken together, our results provide mechanistic insights into the pathological processes of LMNA-related DCM, allowing us to conduct a "clinical trial in-a-dish" to identify and validate a potentially effective drug for improving endothelial dysfunction in patients with LMNA-related DCM.

MATERIALS AND METHODS

Study design

To investigate the role of the endothelium in LMNA-related DCM, a large family cohort, spanning four generations and carrying a mutation in *LMNA*, was recruited at Stanford University as previously described (17). Patients' clinical endothelial function was assessed by digital plethysmography using the EndoPAT2000 system (Itamar Medical Ltd) and a detail clinical history was recorded. Blood draws or skin biopsies were performed to generate iPSCs using Institutional Review Board (IRB)-approved protocol. Human heart tissues were procured by the Human Biorepository Tissue Research Bank under IRB-approved protocol. All personal information was de-identified in accordance to relevant HIPAA regulations and tissues were collected with informed patient consent. Recruited subjects were assigned to healthy control or disease groups based on the presence of the *LMNA* mutation. Measurements of endothelial function from both healthy controls and patients with *LMNA* mutation were not blinded. A minimum of $n = 3$ biological replicates were conducted for each experiment.

Genetic phenotype and clinical history

Sequencing of the *LMNA* gene revealed that eight of the recruited family members (Pt. 1, Pt. 2, Pt. 3, Pt. 4, Pt. 5, Pt. 6, Pt. 7, and Pt. 8) harbored a mutation that included a heterozygous insertion of a guanine between nucleotides 348 and 349, causing a frameshift mutation at codon 117 (K117fs). As a consequence, multiple carriers presented with atrial fibrillation (AF), atrioventricular block (AVB), ventricular tachycardia (VT), and DCM, suggesting a phenotype characterized by early-onset AF leading to DCM. Despite an aggressive medical regimen, the three older carriers suffered from severe cardiovascular problems, and a closer examination revealed an abnormal vascular phenotype (table S1). Similarly, another patient carrying a different LMNA mutation (p.Arg133Gln; c.398G>A) was also recruited for our study.

EndoPAT assessment

Clinical endothelial function was assessed by digital plethysmography using the non-invasive EndoPAT2000 system (Itamar Medical Ltd., Caesarea, Israel). More details on conducting the EndoPAT are described in the Supplemental Materials. Endothelial function was presented in the form of reactive hyperemia index (RHI), which was calculated by the post-to-pre-occlusion peripheral arterial tone (PAT) signal ratio in the occluded side after normalizing to the control arm and further corrected for baseline vascular tone. RHI > 1.67 indicates normal endothelial function, and RHI ≤ 1.67 indicates abnormal endothelial function.

iPSC-EC differentiation

Patient-specific iPSCs were generated using the OSKM CytoTune-iPS 2.0 Sendai Reprogramming Kit viral particle factors (Life Technologies) as described previously (17). The iPSCs used for this study were at passages 22–25. iPSCs were cultured as described above until reaching 80% confluence. The medium was switched to RPMI-B27 without insulin (Life Technologies) with 6 μM CHIR99021 for 2 days, and then changed to 6 μM CHIR99021 for another 2 days. During the differentiation process, from day 4 to day 12, the medium was changed to EGM2 (Lonza) supplemented with 50 ng/ml VEGF

(Peprotech), 20 ng/ml BMP4, and 20 ng/ml FGF2 (Peprotech). By day 12, cells were dissociated using TyrpLE for 5 min and sorted using CD144-conjugated magnetic microbeads (Miltenyi Biotec) according to the manufacturer's instructions. CD144-positive cells were seeded on 0.2% gelatin-coated plates and maintained in EGM2 medium supplemented with 10 μM TGFβ inhibitor (SB431542) (Selleckchem). After passage 2, iPSC-ECs were cultured in normal EGM2, without SB431542. The iPSC-ECs used for this study were all at passage 3.

Statistical analysis

Data were analyzed using Prism (GraphPad) or Excel and reported as mean ± SEM unless otherwise specified. Comparisons were measured via the one-way analysis of variance (ANOVA) or one-way multivariate analysis of variance (MANOVA) test for more than two groups, or via an unpaired two-tailed Student's t-test for two groups to assess the significant differences. $P < 0.05$ indicated as significant, $P > 0.05$ indicated as not significant.

Supplementary Material

Supplemental Materials

Fig. S1. Characterization of LMNA-mutation family.

Fig. S2. Generation of iPSCs and differentiation of iPSC-ECs.

Fig. S3. Characterization of iPSC-ECs from healthy control and LMNA patients.

Fig. S4. Characterization of iPSC-ECs from additional healthy control and LMNA patients.

Fig. S5. Characterization of primary ECs isolated from LMNA patients.

Fig. S6. Generation and characterization of genome-edited iPSCs.

Fig. S7. Transcriptional characterization of iPSC-ECs.

Fig. S8. Characterization of iPSC-ECs under shear stress.

Fig. S9. Screening of small molecules that increase *KLF2* expression in LMNA iPSC-ECs.

Fig. S10. Lovastatin improves EC function in LMNA iPSC-ECs.

Fig. S11. Lovastatin improves EC function in cardiolaminopathy patients.

Fig. S12. Lovastatin improves LMNA iPSC-CM phenotype when co-cultured with LMNA iPSC-ECs.

Fig. S13. Lovastatin upregulates genes in LMNA iPSC-CMs responsible for cardiac mechanics when co-cultured with LMNA iPSC-ECs.

Fig. S14. Characterization of LMNA iPSC-CMs in inverse co-cultures.

Fig. S15. Summary figure of modeling endothelial dysfunction in LMNA-related DCM using patient-specific iPSC-ECs.

Table S1. Demographic and clinical characteristics of healthy control and LMNA patients at baseline.

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Footnotes

Competing interests: J.C.W. is a co-founder of Khloris Biosciences; the work presented was performed independently.

Data availability: All data associated with this study are present in the paper or the Supplementary Materials. Data are available from the NCBI BioProject database under accession number: PRJNA533629.

Overline: CARDIOMYOPATHY

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