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Exercise Reveals Proline Dehydrogenase as a Potential Target in Heart Failure

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ABSTRACT

The benefits of physical activity in cardiovascular diseases have long been appreciated. However, the molecular mechanisms that trigger and sustain the cardiac benefits of exercise are poorly understood, and it is anticipated that unveiling these mechanisms will identify novel therapeutic targets. In search of these mechanisms we took advantage of unbiased RNA-sequencing (RNA-seq) technology to discover cardiac gene targets whose expression is disrupted in heart failure (HF) and rescued by exercise in a rat model. Upon exhaustive validation in a separate rat cohort (qPCR) and human datasets, we shortlisted 16 targets for a cell-based screening, aiming to evaluate whether targeted disruption of these genes with silencing RNA would affect the abundance of a CVD biomarker (BNP, B-type natriuretic peptide) in human cardiomyocytes. Overall, these experiments showed that Proline Dehydrogenase (PRODH) expression is reduced in human failing hearts, rescued by exercise in a rat model of HF, and its targeted knockdown increases BNP expression in human cardiomyocytes. On the other hand, overexpression of PRODH increases the abundance of metabolism-related gene transcripts, and PRODH appears to be crucial to sustain normal mitochondrial function and maintenance of ATP levels in human cardiomyocytes in a hypoxic environment, as well as for redox homeostasis in both normoxic and hypoxic conditions. Altogether our findings show that PRODH is a novel molecular target of exercise in failing hearts and highlight its role in cardiomyocyte physiology, thereby proposing PRODH as a potential experimental target for gene therapy in HF.

Contents

Abbreviations and acronyms: CV, Cardiovascular; CVD, cardiovascular disease; RNA-seq, RNA sequencing; qPCR, quantitative real-time Polymerase Chain Reaction; PRODH, Proline Dehydrogenase; BNP, B-type natriuretic peptide; AMPK, AMP-activated protein kinase; HF, heart failure; MI, myocardial infarction; GSEA, Gene Set Enrichment Analysis; iPSC, induced-pluripotent stem cell-derived cardiomyocytes; OCR, oxygen consumption rate; PA, physical activity; ROS, reactive oxygen species; LV, left ventricle or ventricular; GEO, Gene Expression Omnibus; siRNA, silencing RNA; GFP, Green-Fluorescent Protein.

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Cardiovascular (CV) diseases (CVDs) are by far the main cause of death worldwide.[1] The Global Burden of Disease Study shows that the burden of ischemic heart disease increased by ~21% from 2007 to 2017, making it the leading cause of years of life lost.[2] Unfortunately, current drug therapies fall short at delivering optimal clinical benefits. Antagonists of neurohumoral factors, inhibitors of beta-adrenergic receptors and diuretics are the most widely used medical treatment options.[3,4] Although these approaches provide a general positive effect in cardiac function, the progression towards heart failure is not stopped, and potential side-effects are constantly debated.[4] Therefore, the field calls for novel therapeutic targets.

Regular physical activity delivers powerful benefits to CVD patients and is currently one of the most effective interventions for reducing CVD burden.[5,6] Several clinical studies have shown that exercise relieves pathological cardiac remodelling, improves left ventricular (LV) function, and increases functional capacity and quality of life even in heart failure (HF) patients.[7,8] Larger clinical trials suggest that these effects may translate into lower morbidity and mortality rates.[9] Although it is now clear that the failing heart displays remarkable plasticity in response to exercise, the cellular and molecular mechanisms that trigger, propagate and maintain these benefits are poorly understood. Previous studies reported adaptations induced by exercise at the cellular level,[10,11] and although they provided an initial characterization, most of the findings were associative and unable to distinguish cause from consequence. In addition, the molecular signals recruited by exercise in the failing heart have not yet been catalogued with high-coverage and unbiased technologies. Despite this knowledge gap, the scientific community anticipates that identification and understanding of molecular mechanisms recruited by exercise will shed new light on novel pharmacological and therapeutic targets.[12]

In face of these challenges, we took advantage of RNA sequencing (RNA-seq) technology and set out to discover novel molecular mechanisms induced/mobilized by physical activity (PA) in the failing heart. A pronounced effect of exercise was validated in a subset of genes, evidencing a shortlist selected for manipulation in human cardiomyocytes (derived from stem cells). Further experiments showed that Proline Dehydrogenase (PRODH) expression is reduced in HF and rescued by exercise. Finally, by manipulating PRODH expression we show that it contributes to sustain mitochondrial function and redox homeostasis in cardiomyocytes.

Methods

Rat model of HF

Three-month-old female Sprague–Dawley with ad libitum access to water and rodent chow diet were used. The study was approved by the Norwegian Animal Research Authority (FOTS ID 5829). For induction of post-myocardial infarction (MI) HF, left coronary artery ligation was performed during thoracotomy under anesthesia provided by 3% isoflurane mixed with 30% O_2/70% N_2O, whereas Buprenorphine (0.05 mg Temgesic; Reckitt and Colman, Hull, UK) was given subcutaneously immediately and 8 h after the thoracotomy, as previously described.[13,14] Sham-operated animals were used as controls. Echocardiography was performed four weeks post-surgery, and MI-operated animals with signs of HF (poor systolic and diastolic function, LV dilation, and low exercise capacity) were included in the study. Investigators involved in animal examinations were blinded to group allocation during examinations. Animals matching the inclusion criteria were randomly allocated to an exercise (HF-Exercise) or sedentary (HF-Sedentary) protocol, and the SHAM group (also sedentary) was used as control. Intensity-controlled exercise training was performed 5 days per week for 8 weeks, on motorized treadmills (25° incline) equipped with an electrical grid at the end of the lane (Columbus Instruments, USA). The training sessions had 90 min duration at 60% of maximum running speed. Intensity was adjusted based on running tests to exhaustion. Following a 15 min warm-up, band speed was increased by 1.8 m/min every 2 min until exhaustion, which was achieved when the animals were not able to run further despite receiving electrical stimuli, as we previously described.[15] The validation cohort was comprised of HF rat samples from the University of Split School of Medicine, Laboratory for Cellular Physiology, with physiological data previously reported by Kraljevic et al.[14]

Echocardiography

Echocardiography was performed under 2.5% isoflurane anesthesia, four weeks after surgery and repeated at the end of the protocol, when exercise training was concluded. Cardiac function was assessed using echocardiography including M-Mode assessments (short axis view) and pulsed-waved Doppler measurements (Vevo 2100 high-resolution ultrasound, VisualSonics, Canada). An apical four-chamber view was used to identify the mitral valve and the Doppler flow spectra recorded at the center of the left atrioventricular orifice. Early and late diastolic transmitral peak flow velocity (E and A velocities) were quantified and E/A ratio calculated. LV mass was estimated from M-Mode measurements and contractile function was assessed by LV fractional shortening and ejection fraction.

RNA library preparation, RNA sequencing and hierarchical clustering analysis

At the end of the protocol rats were sacrificed by heart removal under deep anesthesia (5% isoflurane). Hearts were cleaned from

blood, weighted and the viable (non-infarcted) region of the LV was immediately frozen in liquid nitrogen for posterior RNA isolation. RNA was isolated with the miRNEasy spin column kit, following manufacturers protocol (Qiagen, Norway). The protocol isolates total and small RNAs. Amount and purity (A260/A230 and A260/280 > 2.0) of isolated RNA were measured by spectrophotometry (Nanodrop, Thermo Fisher Scientific, Norway), while RNA integrity (RNA integrity number > 8) was assessed using Agilent RNA 6000 Nano Kit on a 2100 Bioanalyzer instrument (Agilent Technologies, USA). All 24 samples passed RNA quality control. cDNA libraries were prepared with the TruSeq Stranded mRNA kit (Illumina, USA) following Illumina's protocol. Libraries were quantitated by qPCR and validated using Agilent High Sensitivity DNA Kit on a Bioanalyzer. Libraries were normalized to 22 pM and subjected to cluster and paired-end sequencing was performed on a HiSeq2500 instrument (Illumina, USA), according to the manufacturer's instructions. RNA-seq alignment was performed with the STAR software.[16] Differences in gene expression were determined by a Benjamini and Hochberg's False Discovery Rate (FDR) of 5% or less. RPKM (Reads Per Kilobase Million) values for all samples are shown in the Supplementary Material. RPKM values were used to perform hierarchical clustering analysis, an algorithm that groups samples based on how close they are to one another. The result is a tree structure, referred to as dendrogram. Hierarchical clustering analysis was performed with GenePattern software.[17] RNA-seq was performed at the Genomics Core Facility (GCF), Norwegian University of Science and Technology (NTNU). GCF is funded by the Faculty of Medicine and Health Sciences at NTNU and Central Norway Regional Health Authority.

Human datasets

We obtained microarray and RNA-seq data from datasets reporting gene expression from human cardiac biopsies, collected from HF patients and non-failing donors. The microarray dataset (GEO dataset ID GSE57345, reported in[18]) consisting of left ventricle samples from 313 adult individuals: 136 non-failing, 95 ischemic HF and 82 non-ischemic HF. The RNA-seq dataset (GEO dataset ID GSE46224[19]) reported data from 24 individuals: 8 non-failing, 8 ischemic HF and 8 non-ischemic HF. Information about the patients/donors can be found in the original publications.[18,19] We analysed the datasets to obtain the expression patterns of our genes of interest, as described in detail under the Results section. Differential gene expression in the microarray dataset GSE57345 was analysed with GEO2R, a bioinformatics tool from NCBI (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html) that allows us to compare groups of samples from the GEO database. We verified that information provided by the data submitter was sufficient to identify the group allocation, and performed differential gene expression analysis comparing non-failing vs. ischemic HF samples, as well as non-failing vs. non-ischemic HF. A FDR below 0.05 (5%) was considered significant. The RNA-seq dataset GSE46224 was analysed using the RPKM values deposited by the submitters at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46224. The entire analyses of both datasets are shown in the Supplementary Material.

Real-time quantitative Polymerase Chain Reaction (RT-qPCR)

Reverse transcription (RT) of 4.5 μL of isolated RNA was performed using the High-Capacity RNA-to-cDNA™ Kit (#4387406, Thermo Fisher Scientific, Norway) following the manufacturer's recommendations. After RT, samples were diluted with nuclease-free water to a final concentration of 5 ng/μL. Gene expression was analysed by real-time qPCR and primers/probes (TaqMan Gene Expression Assays, Thermo Fisher Scientific, Norway; product information shown in Supplementary Table 8). The reactions were run on a CFX96 System (BioRad) following cycling conditions recommended by the supplier. Gene expression was calculated by the ΔCt method and normalized to the housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) or ACTB (Actin Beta).

Mitochondrial fractionation and western blotting

To verify if PRODH was located in cardiac mitochondria, we isolated mitochondria from rat left ventricle using the Mitochondria Isolation Kit for Tissue (product #89801, Thermo Fisher Scientific, Norway), following the supplier's protocol. 200 mg of tissue was used for each preparation. The resulting mitochondrial pellet was lysed in RIPA buffer containing protease and phosphatase inhibitors (1% volume). Protein content was quantified with the BCA protein assay (Thermo Fisher Scientific, Norway). Samples were prepared for western blotting by adding LDS buffer (4× Bolt™ LDS Sample Buffer, Thermo Fisher Scientific, Norway) and dithiothreitol (DTT, 50 mM final), followed by heating at 70 °C. Proteins were separated by electrophoresis in Tris-Glycine gels (Novex 10% Tris-Glycine Mini Gels, Thermo Fisher Scientific, Norway) and later transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk or 5% bovine serum albumin (BSA) diluted in Tris-buffered saline solution containing 1% Tween 20 (TBST) and incubated overnight with PRODH (PA5-72540, Thermo Fisher Scientific, Norway), VDAC (ab14734, Abcam, UK) or GAPDH (MA5-15738, Thermo Fisher Scientific, Norway) antibodies (1000× dilution). Membranes were washed with TBST solution and incubated for 2 h with fluorescent secondary antibodies (IRDye® 800CW and IRDye® 680RD, LICOR Biosciences, UK), followed by a final TBST wash and imaging with the LICOR Odyssey scanner. GAPDH was used as cytosolic loading control and VDAC as mitochondrial marker.

Human iPSC-derived cardiomyocytes, siRNA screening and adenovirus experiments

Human cardiomyocytes derived from induced-pluripotent stem cells (iPSC, iCell Cardiomyocytes) were obtained from Cellular Dynamics International (CDI, USA). iPSC cardiomyocytes were cultured according to the user guide provided by CDI. Cells were seeded on fibronectin-coated cultures plates (15,000 cells/well in 96-well plates for siRNA and mitochondrial function experiments, 90,000 cells/well on 24-well plates for RT-qPCR). 48 h after seeding, cells were washed and Plating Medium replaced with Maintenance Medium (both supplied with the cells, with CDI's proprietary formula). Cells were washed with Maintenance medium and fresh Maintenance medium was added every 48 h afterwards. For the siRNA screening, we obtained a Silencer Select siRNA library (Thermo Fisher Scientific, Norway) containing two independent human siRNAs for each of our gene targets, as well as a control non-targeting siRNA. Product information for siRNAs is shown in Supplementary Table 9. Four days after cell seeding siRNAs (3.5 nM) were individually added to cells in a complex with 0.5% Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Norway), following the reagent's data sheet. Two days later, medium was changed and cells were kept for another 24 h before RNA extraction and RT-qPCR using TaqMan® Gene Expression Cells-to-CT™ Kit (Thermo Fisher Scientific, Norway). BNP (NPPB gene) mRNA levels were quantified and analysed using the same RT-qPCR protocol described above. GAPDH and ACTB were used as control housekeeping genes. For the hypoxia experiments, cells were kept in culture for 10 days and siRNA and/or adenovirus are added on day 6 and day 8, respectively (siRNA on day 6, Ad virus on day 8). A normoxic group was kept for the same duration to allow appropriate comparisons. In hypoxia experiments, Maintenance Medium was replaced with hypoxia assay medium on day 6. The composition of hypoxia assay medium was 93% DMEM (no Glucose, no Glutamine, no Phenol Red), 5 mM Creatine, 2.75 mM D-(+) Glucose, 1× GlutaMax, 10 mM HEPES, 2 mM L-carnitine, 1× non-essential Amino Acids (#11140050, Thermo Fisher Scientific), 1 mM Sodium Pyruvate, 5 mM Taurine and 1× Linoleic Acid-Oleic (#L9655, Sigma-Aldrich, Norway). Ad-GFP and Ad-PRODH

were obtained from Vector Biolabs (USA) and were used at 100 MOI (multiplicity of infection).

Mitochondrial function

We used the Agilent Seahorse (XF96) instrument to assess oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in iPSC cardiomyocytes seeded onto fibronectin-coated XF96 Cell Culture Microplates (#102416-100, Agilent Technologies, USA) by following the Mito Stress Test protocol from Agilent Technologies. Cells were kept in culture as described above, until the day of the experiment. On the day of the experiment cells were washed and culture medium was replaced with XF Assay Medium (#102365-100, Agilent Technologies, USA) and kept at 37 °C with atmospheric CO_2 levels (i.e., no additional CO_2) for 2 h. After incubation, the assay started by instrument calibration and equilibration with the assay medium, followed by three cycles of OCR and ECAR recording (basal respiration) alternated with mixing. After this point oligomycin (1 μM, ATP synthase inhibitor) was injected into the wells, resulting in a decrease in OCR, where proton leak could be measured. After three cycles of recording and mixing, carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP, 0.5 μM) was added to uncouple oxidative phosphorylation from the electron transport system and shows the maximal uncoupled respiration rate. Lastly, the mitochondrial electron transport chain was inhibited completely by the addition of the complex I-specific inhibitor rotenone (2 μM). The data also allowed us to calculate the spare respiratory capacity (FCCP stimulated OCR minus basal OCR), which corresponds to the ability of cells to respond to an increase in energy demand.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA)[20] is a well-accepted bioinformatics tool to compare broad gene expression patterns, and was used to detect coordinated differences in expression induced by Ad-GFP vs. Ad-PRODH in iPSC cardiomyocytes. Gene sets are available from Molecular Signatures DataBase (www.broad.mit.edu/gsea/.msigdb/msigdb_index.html). GSEA calculates an enrichment score (ES) that reflects the degree to which a gene set is overrepresented at the extremes (top or bottom) of the entire ranked list of transcripts — where genes are ranked according to the expression difference between Ad-GFP and Ad-PRODH. The ES is calculated by walking down the list, increasing a running-sum statistic when it encounters a gene that is in the gene set and decreasing it when it encounters genes that are not. The magnitude of the increment depends on the correlation of the gene with the phenotype. The software then estimates the statistical significance (nominal p value) of the ES by using an empirical phenotype-based permutation test procedure that preserves the complex correlation structure of the gene expression data. The nominal p value of the observed ES is then calculated relative to this null distribution. Finally, when an entire database of gene sets is evaluated, GSEA adjusts the estimated significance level to account for multiple hypothesis testing. GSEA first normalizes the ES for each gene set to account for the size of the set, yielding a normalized enrichment score (NES). It then controls the proportion of false positives by calculating the false discovery rate (FDR) corresponding to each NES. The FDR is the estimated probability that a set with a given NES represents a false positive finding; it is computed by comparing the tails of the observed and null distributions for the NES. The "H: hallmark gene sets" were used as reference in our GSEA analysis.

Reactive oxygen species (ROS) measurements

Total ROS and superoxide levels were quantified with the fluorescence-based ROS/Superoxide Detection Assay Kit (#ab139476, Abcam, UK), following the supplier's protocol.

Statistical analysis

Echocardiography and physiological parameters were analysed by repeated measures analysis of variance (ANOVA) followed by Fisher LSD post hoc test. RT-qPCR, mitochondrial function and ROS levels were analysed by one-way ANOVA followed by Fisher LSD post hoc test (three or more groups) or by unpaired Student's t-test (two groups). GraphPad Prism 8.0 was used for statistical analysis and figure preparation.

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Results

Exercise reprograms gene patterns in failing hearts

In order to shed new light on mechanisms recruited by exercise in the failing heart, we used a rat model of HF induced by MI; these MI rats displaying signs of HF at week four after surgery (Table 1, "Pre" values) were included in the study and subjected to a sedentary

Table 1
Physiological parameters and echocardiographic variables of animals included in RNA-seq analysis.

		SHAM (n = 8)	HF-SEDENTARY (n = 8)	HF-EXERCISE (n = 8)
VO_{2peak}, mL/kg/min	Pre	90.8 ± 2.0	78.3 ± 4.8*	78.73 ± 3.7*
	Post	89.0 ± 1.8	76.2 ± 4.1*	88.32 ± 2.8#,&
Max running speed, m/min	Pre	28.7 ± 1.0	22.50 ± 1.7*	23.75 ± 1.6*
	Post	28.0 ± 0.8	24.00 ± 1.1	34.50 ± 1.2*,#,&
Body weight (BW), g	Pre	274.4 ± 10.0	269.6 ± 4.8	264.7 ± 3.0
	Post	299.7 ± 8.8	298.0 ± 5.6	288.5 ± 5.7
MV A, mm/s	Pre	480.2 ± 54.3	355.9 ± 74.3*	306.0 ± 58.7*
	Post	486.5 ± 31.7	375.1 ± 80.5*	312.7 ± 68.8*
MV E, mm/s	Pre	701.9 ± 45.47	1176.1 ± 92.6*	1104.5 ± 64.9*
	Post	724.0 ± 44.48	1157.1 ± 90.8*	1081.0 ± 62.5*
MV E/A ratio	Pre	1.72 ± 0.35	4.80 ± 1.19*	5.10 ± 1.30*
	Post	1.51 ± 0.13	4.11 ± 0.85*	4.81 ± 1.03*
LA/BW, mg/g	Pre	0.013 ± 0.001	0.021 ± 0.002*	0.018 ± 0.002*
	Post	0.012 ± 0.001	0.019 ± 0.002*	0.017 ± 0.002*
LVIDd, mm	Pre	6.63 ± 0.24	8.69 ± 0.35*	9.06 ± 0.32*
	Post	6.88 ± 0.22	9.56 ± 0.38*,#	9.46 ± 0.40*
LVIDs, mm	Pre	3.81 ± 0.19	7.57 ± 0.45*	7.73 ± 0.32*
	Post	3.45 ± 0.25	8.36 ± 0.48*,#	7.59 ± 0.60*,&
LVPWd, mm	Pre	2.01 ± 0.18	2.17 ± 0.22	1.83 ± 0.16
	Post	1.86 ± 0.12	1.58 ± 0.11#	2.15 ± 0.14&
LVPWs, mm	Pre	2.70 ± 0.14	2.47 ± 0.27	2.57 ± 0.18
	Post	2.96 ± 0.09	1.93 ± 0.25*,#	2.99 ± 0.23&
LV mass/BW, mg/g	Pre	3.15 ± 0.23	4.27 ± 0.28	3.88 ± 0.31
	Post	3.24 ± 0.12	4.08 ± 0.42*	5.80 ± 0.53*,#
EF, %	Pre	72.02 ± 2.39	26.91 ± 4.22*	28.91 ± 4.52*
	Post	79.08 ± 3.47	25.60 ± 5.10*	39.49 ± 5.82*,#,&
FS, %	Pre	42.53 ± 2.12	13.32 ± 2.24*	14.51 ± 2.42*
	Post	48.74 ± 3.38	12.83 ± 2.71*	20.81 ± 3.44*,#,&

Animals were assessed before (Pre) and after (Post) the exercise (or sedentary) protocol. *Abbreviations*: VO_{2peak}: peak oxygen uptake. MV E: Mitral valve peak inflow in early diastole. MV A: Mitral valve peak inflow in late diastole. LA: left atrium weight. LVIDd: left ventricle internal diameter in diastole. LVIDs: left ventricle internal diameter in systole. LVPWd: left ventricle posterior wall in diastole. LVPWs: left ventricle posterior wall in systole. EF: left ventricle ejection fraction. FS: left ventricle fractional shortening. Data were analysed by repeated measures analysis of variance (ANOVA) followed by Fisher LSD post hoc test.

* Indicates $p < 0.05$ vs. SHAM at the same timepoint (Pre or Post).

\# Indicates $p < 0.05$ vs. "Pre" values from the same group.

& Indicates $p < 0.05$ vs. HF-SEDENTARY at the same timepoint.

protocol (HF-Sedentary) or regular exercise training (HF-Exercise). A sham-operated group (SHAM) was used as control (healthy hearts). Significant benefits in cardiac function, structure and exercise capacity were observed in the HF-Exercise group at the end of the protocol (Table 1). LV shortening and ejection fraction increased significantly after exercise training, while the progression of LV dilation (LV internal diameters) and posterior wall thinning observed in HF-Sedentary was prevented in the HF-Exercise group. Peak oxygen uptake and maximum running speed also increased significantly. In fact, HF-Exercise reached greater maximum speed than SHAM. These results reproduce previous findings[13,21] and show that the exercise training intervention was very efficient.

We performed deep sequencing (~100 million reads per sample) of messenger RNA isolated from cardiac samples (viable left ventricle) from all animals (n = 8 in each group), to identify cardiac genes with altered expression in HF and after the exercise intervention. The dataset shows that expression of 932 genes were significantly affected by HF, with a false discovery rate (FDR) below 0.05 (Fig. 1A and Supplementary Table 1). This finding was expected and reproduces the results on well-known and established HF biomarkers such as ANP (Nppa gene, +2149%), BNP (Nppb gene, +403%), MYH7 (+289%) and many others. These data show that the cardiac gene program of our sedentary HF animals resemble that of human HF and serve as a relevant model for the purpose of this study. We found that only 174 genes differed in abundance (FDR < 0.05) between our control group and exercised HF animals (Fig. 1B).

More importantly, we report for the first time the transcriptome landscape of sedentary vs. exercised HF rats and identify 47 genes with significantly different mRNA abundance between the groups (Fig. 1C), among which 36 have gene symbols annotation in the ENSEMBL genome database. Hierarchical clustering analysis, an algorithm that groups similar samples into clusters based on broad similarities in gene expression, was able to distinguish all sedentary HF animals from SHAM, while most exercised HF rats clustered together with SHAM (Fig. 1D). This suggests that exercise reprograms the failing heart at the transcriptome level, towards a healthier profile. In addition, some genes were partially rescued to normal (SHAM) levels in exercised failing hearts (Supplementary Table 2). We sought to validate these findings for a total of 50 genes with gene symbol annotation, using RT-qPCR in an independent cohort of animals. Quantification of all 50 genes is shown in Supplementary Table 3 and demonstrates that the expression pattern of most genes was validated. Therefore, our RNA-seq experiment and PCR validation revealed the set of cardiac genes modulated or rescued by exercise in HF rats. However, the role of most of these genes in cardiac physiology and pathology remains largely unknown.

Some of the validated genes were found in a previous RNA-seq study[22] to be enriched in fibroblasts rather than cardiomyocytes (separated by centrifugal fractionation), or have poor evolutionary conservation between rodents and humans. These genes were discarded from further analysis, since our primary focus was on evolutionary conserved cardiomyocyte targets. By applying these criteria, we obtained a shortlist of 16 validated genes (Fig. 1E). Among the 16 prioritized targets, 13 displayed reduced expression in HF, and recovered expression in the exercised HF group, while the other three genes showed the opposite pattern (increased in HF and decreased by exercise) (Fig. 1D). To avoid an arbitrary selection of gene targets for further studies, we performed two additional steps of validation in order to prioritize targets of greater biological relevance: (1) we assessed how HF affected our

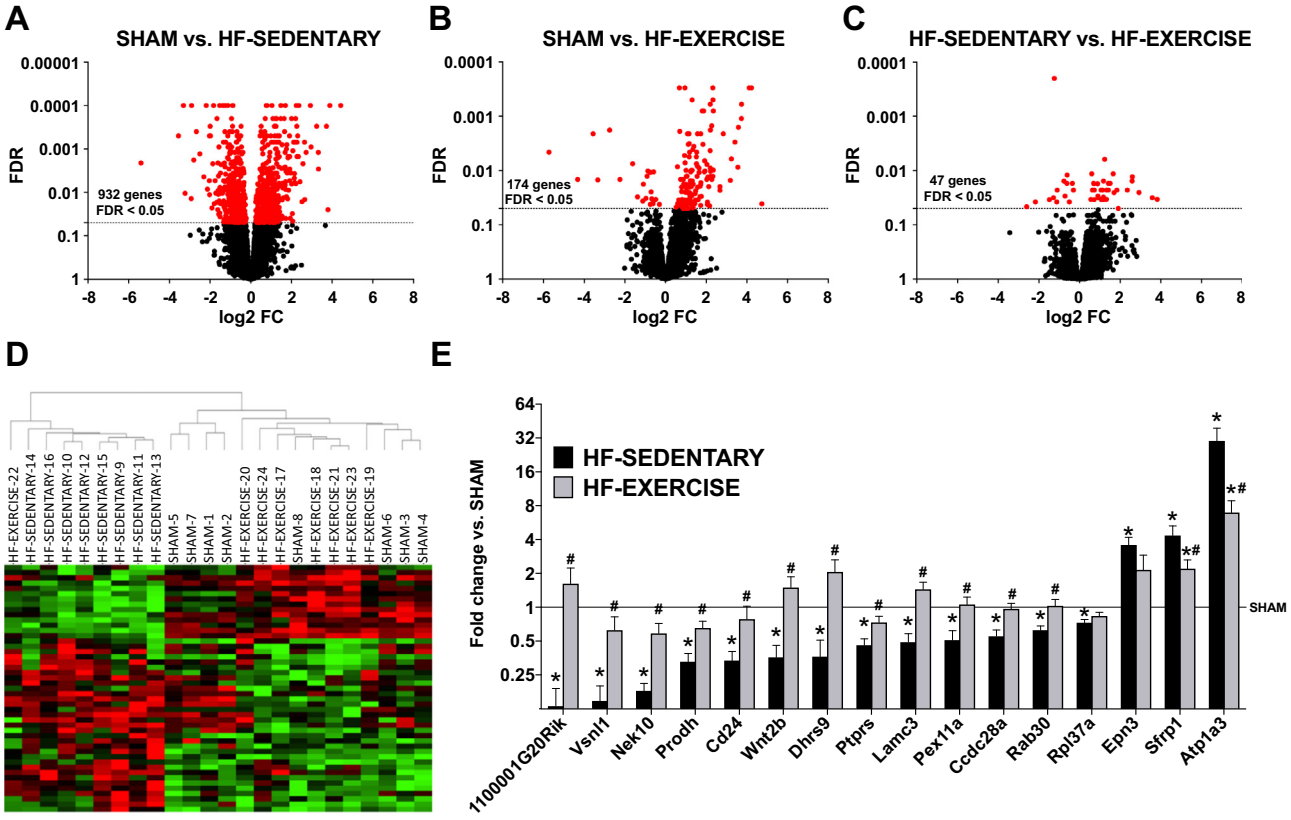


Fig. 1. RNA-seq analysis and RT-qPCR validation. Volcano plots display Benjamini and Hochberg's false discovery rate (FDR) and fold-change in gene expression (log2) between SHAM vs. HF-SEDENTARY (A), SHAM vs. HF-EXERCISE (B) and HF-SEDENTARY vs. HF-EXERCISE (C). Panel D illustrates the hierarchical clustering analysis based on the 47 genes differentially expressed between HF-SEDENTARY vs. HF-EXERCISE, and shows that HF-EXERCISE samples clustered closer to SHAM than HF-SEDENTARY. Panel E shows the RT-qPCR validation (n = 6–9/group) of shortlisted genes. * indicates $p < 0.05$ vs. SHAM. # indicates $p < 0.05$ vs. HF-SEDENTARY. Statistical analysis of RT-qPCR data was performed by one-way ANOVA followed by Fisher LSD post hoc test.

target genes in *human* hearts; (2) we performed a cell-based siRNA screening, targeting each of our target genes and assessing a relevant CVD biomarker in human cardiomyocytes. These results are described below.

PRODH expression is markedly disrupted in human HF

We verified the expression of all 16 targets in human failing hearts, reasoning that only those displaying the same expression pattern in humans as we observed in sedentary rats, would be relevant for further studies. For these analyses, we took advantage of two public datasets (see Methods section) that reported transcriptome analysis of human failing hearts in comparison to healthy donors. Briefly, the first was a microarray dataset (GEO dataset ID GSE57345[18]) consisting of LV samples from 313 adult individuals: 136 non-failing, 95 ischemic HF and 82 non-ischemic HF. The second (GEO dataset ID GSE46224[19]) was a RNA-seq dataset from 24 individuals: 8 non-failing, 8 ischemic HF and 8 non-ischemic HF. Fold-change and statistical analysis for all genes in our analyses of the datasets are shown in Supplementary Table 4. Fig. 2A and B summarize the data on the human homologues of our 16 shortlisted genes, extracted from the human HF datasets. Although many of the genes demonstrated reproducible findings in the human samples, the most pronounced effect was clearly observed in PRODH (>70% reduction in the human RNA-seq dataset), being reproduced in both datasets and both HF etiologies.

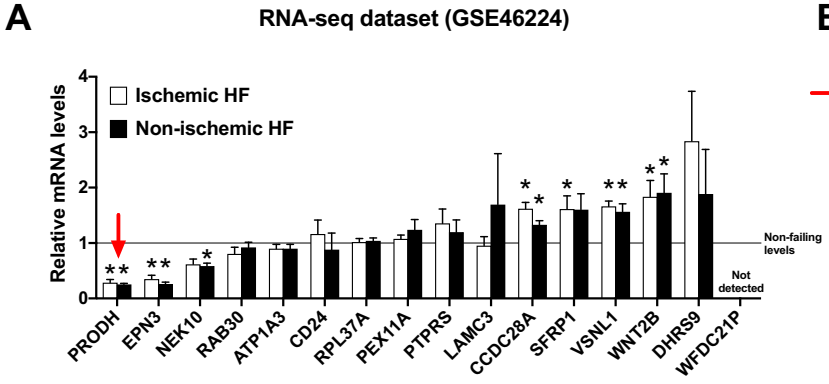
A cell-based siRNAS screening identifies PRODH as a potential target

We obtained small interfering RNAs (siRNAs, i.e. silencing RNA) targeting the human homologues of the shortlisted genes. WFDC21P, the human homologue of rat's 1100001G20Rik, was excluded because its expression was undetectable in human iPSC cardiomyocytes and in cardiac biopsies (Fig. 2A and B). We then performed a cell-based screening, by silencing each of the 15 genes of interest in human iPSC cardiomyocytes and measuring the expression of BNP as a readout. BNP is produced exclusively in cardiac cells and responds to changes in cardiomyocyte health, being used as one of the most widely accepted CVD and HF biomarkers.[23] Therefore, we reasoned that if any of our target genes had an important role in cardiac pathology, silencing their expression would affect BNP in human cardiomyocytes. siRNAs were able to efficiently knock down the genes of interest, with an average silencing of 74% (Supplementary Table 5). Our data show that three siRNAs (targeting PRODH, VSNL1 and ATP1A3) increased BNP expression 72 h after siRNA exposure (Fig. 2C). The most significant effect was found for PRODH siRNA, which resulted in more than 2-fold increase in BNP.

Until here our findings highlight PRODH as the most promising target among the 16 shortlisted genes. In summary, cardiac PRODH expression is reduced in human HF (Supplementary Table 2 and Fig. 2A), rescued by exercise in HF rats at gene and protein levels (Figs. 1E and 2D), and its targeted silencing leads to increased BNP expression in human iPSC cardiomyocytes (Fig. 2C). For these reasons, PRODH was selected for further studies.

PRODH overexpression affects genes related to mitochondrial oxidative phosphorylation

There are no studies describing a role for PRODH in the cardiovascular system. To continue elucidating how PRODH may influence cardiac biology, we infected iPSC cardiomyocytes with adenovirus expressing PRODH (Ad-PRODH) or GFP (Green Fluorescent Protein, Ad-GFP) as a control, to verify how PRODH overexpression would affect the cellular transcriptome (Supplementary Table 6). We performed RNA-seq and



B Microarray dataset (GSE57345)

Gene	Non-failing vs. Ischemic HF FDR	P-value	logFC	Non-failing vs. Non-ischemic HF FDR	P-value	logFC
PRODH	0,00101	0,00021	-0,25	0,00000	0,00000	-0,38
EPN3	0,00000	0,00000	-0,28	0,00000	0,00000	-0,33
RPL37A	0,00757	0,00198	-0,09	0,01490	0,00431	-0,08
PEX11A	0,11900	0,05200	-0,08	0,05020	0,01790	-0,09
LAMC3	0,15500	0,07290	-0,05	0,73700	0,60200	-0,01
WNT2B	0,57300	0,42000	-0,03	0,00579	0,00146	-0,13
NEK10	0,65100	0,50700	0,05	0,64400	0,49000	0,05
DHRS9	0,20400	0,10400	0,07	0,00973	0,00264	0,14
PTPRS	0,12400	0,05480	0,13	0,65200	0,49900	0,04
RAB30	0,00104	0,00021	0,20	0,00082	0,00016	0,21
CCDC28A	0,00000	0,00000	0,30	0,00000	0,00000	0,23
ATP1A3	0,00546	0,00137	0,31	0,00012	0,00002	0,37
VSNL1	0,00019	0,00003	0,38	0,00087	0,00017	0,33
CD24	0,00026	0,00005	0,68	0,01030	0,00283	0,53
SFRP1	0,00000	0,00000	1,06	0,00000	0,00000	1,00
WFDC21P	Not detected			Not detected		

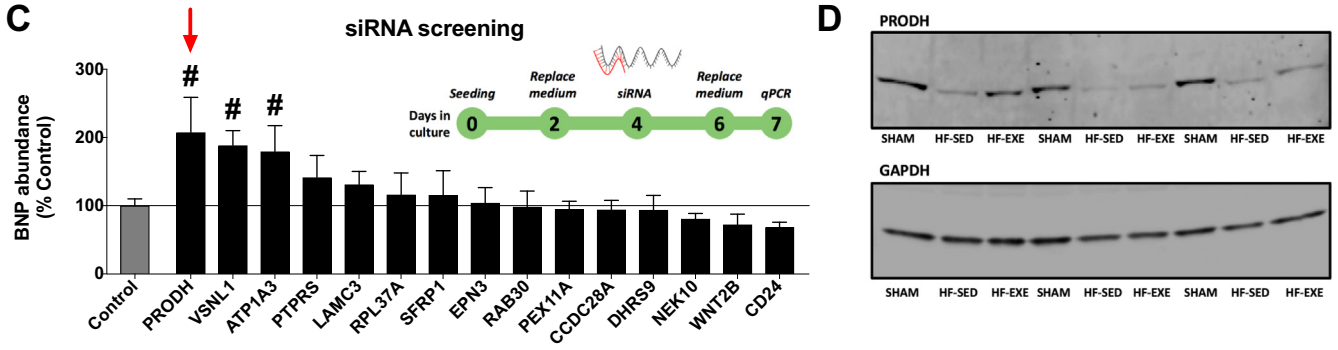


Fig. 2. Human HF datasets and siRNA screening. Expression levels of our genes of interest were verified in public datasets generated from human HF vs. non-failing cardiac samples, analysed by RNA-seq in GEO dataset GSE46224 (A, * indicates $p < 0.05$ vs. non-failing group) or cDNA microarrays in GEO dataset GSE57345 (B). Panel C shows the results of the siRNA screening in human iPSC-human cardiomyocytes. BNP results were analysed by one-way ANOVA followed by Fisher LSD post hoc test. # indicates $p < 0.05$ vs. Control siRNA (panel C). Panel D shows PRODH and GAPDH (loading control) western blotting images from nine rat cardiac samples used in our RNA-seq analysis. *Abbreviations*: HF-SED = HF-SEDENTARY group. HF-EXE = HF-EXERCISE group.

Gene Set Enrichment Analysis (GSEA)[20] to detect significant and concordant differences between the biological states (Ad-PRODH vs. Ad-GFP). GSEA showed that "oxidative phosphorylation" was the gene set most overrepresented among the genes with increased expression in the Ad-PRODH group, and "fatty acid metabolism" was also very significantly enriched (Fig. 3A and Supplementary Table 7). Some of the metabolic genes that resulted in this overrepresentation are shown in Fig. 3B. In addition, PRODH is a mitochondrial protein (Fig. 3C), further suggesting a role in mitochondrial energy metabolism.

PRODH affects mitochondrial function in hypoxic cardiomyocytes

Since PRODH is a mitochondrial protein and PRODH overexpression seems to affect expression of other metabolism-related genes, we questioned what would be the effect of PRODH disruption on mitochondrial function. Therefore, mitochondrial function was assessed by measuring oxygen consumption rate (OCR) in intact iPSC cardiomyocytes. In normoxic conditions (20.95% oxygen), PRODH knockdown did not alter mitochondrial oxygen consumption or cardiomyocyte ATP levels (Fig. 4A). On the other hand, pronounced effects were observed upon PRODH manipulation (by siRNA-PRODH or Ad-PRODH) in cardiomyocytes subjected to 36 h hypoxia (1% oxygen) in medium with reduced nutrient supply (Fig. 4B). The hypoxia protocol was intended to mimic the oxygen-deprived environment of ischemic failing hearts. Basal respiration and maximum OCR were reduced by 31% and 30% (both with $p < 0.05$) (Fig. 4C), respectively, in hypoxic cardiomyocytes receiving siRNA-PRODH. Spare respiratory capacity, a normalized metric less sensitive to variations in cell number, was also 41% lower in cardiomyocytes receiving siRNA-PRODH/Ad-GFP than those receiving control siRNA (Fig. 4C, $p = 0.06$). Importantly, rescuing PRODH expression with adenovirus (siRNA-PRODH/Ad-PRODH) partially recovered maximum OCR and spare respiratory capacity (Fig. 4C, i.e. maximum and spare OCR in siRNA-PRODH/Ad-PRODH were not significantly different from siRNA-control/Ad-GFP, the control group). The highest values for basal, maximum and spare respiratory capacity were observed in cardiomyocytes expressing the highest PRODH levels (siRNA-control/Ad-PRODH). Finally, we quantified intracellular ATP levels with a luciferase assay, showing a 55% reduction of ATP abundance in hypoxic cardiomyocytes upon PRODH knockdown (Fig. 4D). In summary, these findings show that reduced PRODH levels are detrimental to cardiomyocytes exposed to a low-nutrient, hypoxic environment.

Reactive oxygen species

Mitochondria are also a major site of ROS production and play a central role in cardiomyocyte redox control.[24] In addition, several studies have implicated ROS and disrupted redox balance in HF.[25–27] For these reasons, we also quantified total ROS and superoxide levels in cardiomyocytes after PRODH manipulation. In normoxic conditions, PRODH knockdown led to increased levels of total ROS, in an effect that was completely blocked in cells receiving Ad-PRODH to rescue PRODH expression (Fig. 4E). In fact, Ad-PRODH reduced ROS levels below that of control cardiomyocytes (siRNA-control/Ad-GFP)

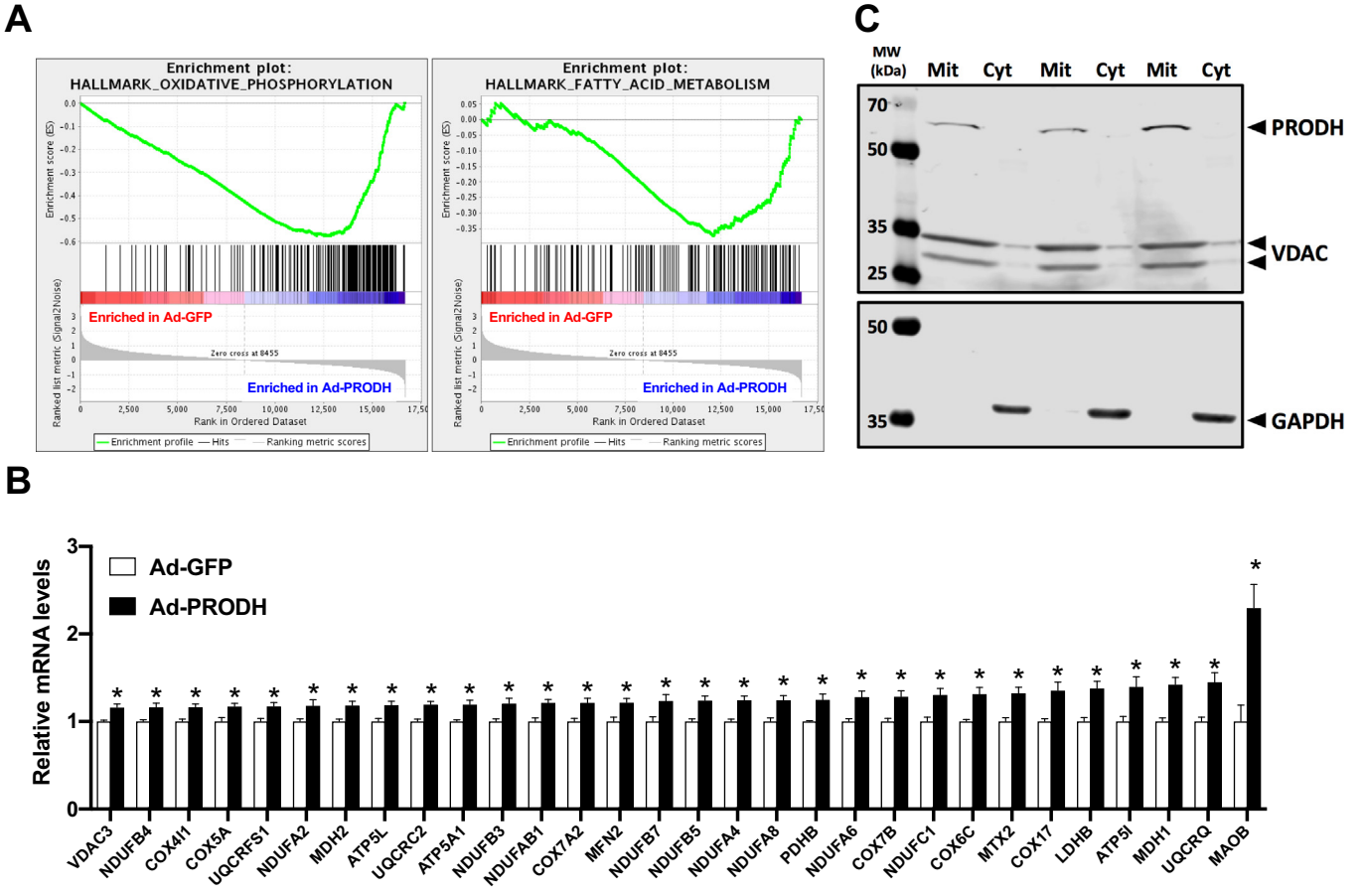


Fig. 3. RNA-seq results from iPSC cardiomyocytes infected with Ad-GFP vs. Ad-PRODH. (A) Graphical representation of gene set enrichment after GSEA (upper portion), the location of the "oxidative phosphorylation" and "fatty acid metabolism" genes in the ranked set of all genes (middle portion), and the distribution of the rank metric score across all genes present in the expression dataset (bottom portion). (B) Expression levels of 30 genes from the "oxidative phosphorylation" gene set. The dataset top list is shown in Supplementary Table 6. (C) Western blotting demonstrating that cardiac PRODH is localized in the mitochondria. VDAC is used as a mitochondrial marker and GAPDH as cytosolic control. *Abbreviations*: MW = molecular weight marker. Cyt = Cytosol sample. Mit = Mitochondria sample. * indicates a FDR < 0.05 (from RNA-seq analysis).

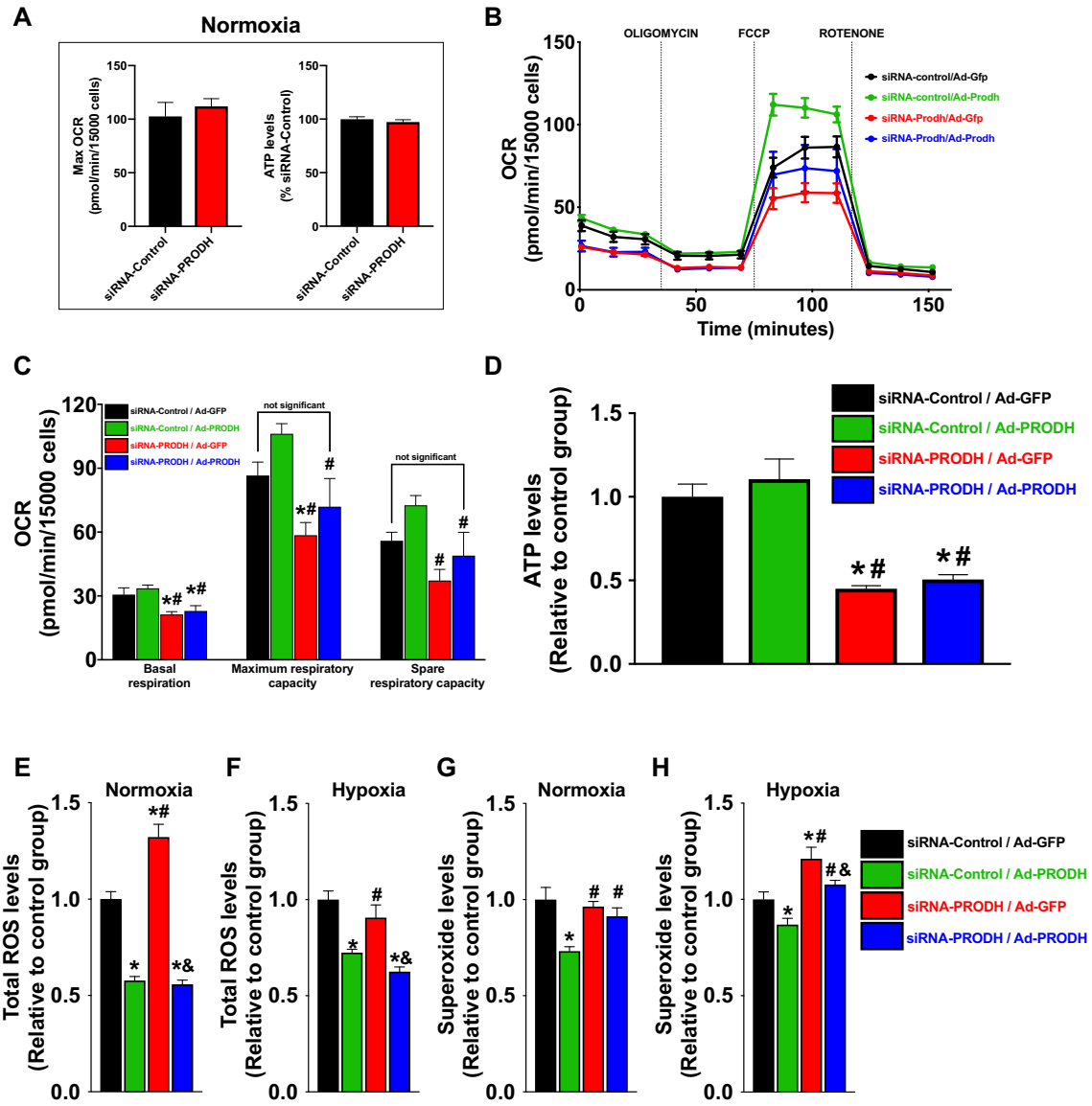


Fig. 4. Mitochondrial function and ROS levels. (A) Maximum OCR (oxygen consumption rate), and ATP levels in iPSC cardiomyocytes receiving control or PRODH siRNA, in a normoxic (20.95% oxygen) environment. (B) Graphical representation of a mitochondrial function experiment in iPSC cardiomyocytes receiving control or PRODH siRNA and/or adenovirus, using the Agilent Seahorse instrument. (C) Quantification of mitochondrial oxygen consumption after PRODH manipulation in hypoxic conditions. (D) Intracellular ATP levels of iPSC cardiomyocytes after PRODH manipulation in hypoxic conditions. (E) Total ROS levels in iPSC cardiomyocytes in normoxic and (F) hypoxic conditions, after PRODH manipulation. (G) Superoxide levels in iPSC cardiomyocytes in normoxic and (H) hypoxic conditions, after PRODH manipulation. * indicates $p < 0.05$ vs. siRNA-Control/Ad-GFP group. # indicates $p < 0.05$ vs. siRNA-control/Ad-PRODH group. & indicates $p < 0.05$ vs. siRNA-PRODH/Ad-GFP group. Data analysis was performed by one-way ANOVA followed by Fisher LSD post hoc test.

(Fig. 4E). In hypoxic conditions, PRODH overexpression also reduced total ROS levels, in the presence or absence of siRNA-PRODH (Fig. 4F). Moreover, superoxide levels were decreased by Ad-PRODH in normoxic conditions (Fig. 4G), while in hypoxia the effects of PRODH knockdown in increasing superoxide levels were also prevented by Ad-PRODH (Fig. 4H). These results provide evidence that PRODH expression contributes to maintain a healthy redox homeostasis in human iPSC-derived cardiomyocytes, in both normoxic and hypoxic conditions.

Discussion

The benefits of exercise in HF are among the most extraordinary medical phenomena. Although the physiological effects of PA have been greatly described and well understood, the knowledge on underlying molecular mechanisms barely scratches the surface. In this study, we advance this body of knowledge and bring into light PRODH, whose expression is negatively affected by HF (in rats and humans) and positively recovered by exercise in a relevant animal model. We found that PRODH depletion has detrimental effects on human cardiomyocytes (derived from iPSC), particularly in regard to mitochondrial function. Part of these effects could be rescued by an adenovirus expressing PRODH, suggesting that preservation of PRODH (as we observed in exercised failing hearts) levels in hypoxic cardiomyocytes may be a potential therapeutic goal.

Previous studies on molecular mechanisms of exercise have reported a few intracellular players (genes or proteins) displaying associations with the effects of PA in the heart. Early studies suggested that the Pi3k/Akt signalling axis is required for physiological, but not pathological, cardiac hypertrophy in mice.[28–31] It was later shown that Pi3k gene therapy may have therapeutic effects in diseased hearts.[32,33] Aware of these findings and taking advantage of our transcriptome (RNA-seq) data, we checked if genes related to the Pi3k/Akt cascade (Akt isoforms, Pi3k isoforms, mTor and Gsk3 genes) were modulated in exercised failing hearts and found no major changes (Supplementary Table 1). Therefore, our findings

do not seem to be related to Pi3k/Akt signalling. Another study reported that C/EBPβ, a transcription factor pinpointed from a qPCR screen, is down-regulated by PA and is an independent mechanism underlying exercise-induced cardiac hypertrophy in healthy mice.[34] The study showed that C/EBPβ represses cardiomyocyte growth and proliferation in the adult mammalian heart, and reduction in C/EBPβ by exercise is a crucial signal for physiological cardiac hypertrophy. C/EBPβ seems to stimulate proliferation of adult cardiomyocytes in vivo, thereby holding potential for future therapies targeting cardiac regeneration.[34] These studies provided an initial characterization of causative mechanisms underlying the benefits of exercise, but a more comprehensive understanding is needed. In this sense, our RNA-seq findings are the first to catalogue the effects of exercise in the failing heart, in an unbiased manner and at transcriptome-wide scale.

By using exercise as the discovery platform, we were able to pinpoint PRODH as a potential novel target. The relevance of PRODH for human HF is suggested by data from different patient cohorts (from the public datasets), where we found reduced PRODH levels in comparison to healthy donors' biopsies. In human iPSC-derived cardiomyocytes, PRODH silencing led to accumulation of the HF biomarker BNP, reduced the maximum capacity of cellular respiration and ATP levels. The results on BNP are most likely due to an overall stress state induced by PRODH knockdown, and not a direct regulation because PRODH has no transcription factor activity or proteolytic function.

PRODH is an enzyme that catalyses the initial process of proline degradation,[35] and, when cellular nutrients and oxygen are limited, proline metabolism becomes increasingly important.[36] The conversion of proline to Δ1-pyrroline-5-carboxylate (P5C), which is catalysed by PRODH, generates electrons that are donated to mitochondrial electron transport through flavine adenine dinucleotide (FAD) to generate ATP.[35] These findings from previous studies provide a plausible explanation for the results we observed in mitochondria from hypoxic cardiomyocytes after PRODH silencing. Phang et al.[37] also showed that PRODH expression contributes to maintain ATP levels in cancer cell lines under nutrient stress. This result was later shown to be dependent on AMP-activated protein kinase (AMPK), a sensor of intracellular nutrient stress[38] with an important role in CV biology.[39] Therefore, the importance of proline metabolism and PRODH has been recognized in cancer biology,[40,41] but largely overlooked in the cardiovascular field. We should also recognize limitations in our study. Although we employed a strategy to narrow the list down to a group of potential gene targets, we cannot ignore the fact that the cardiac benefits of exercise are too complex to be explained by changes in expression of one, or very few, genes. It is reasonable to suppose that it is a combination of multiple signals, later expanded to signal cascades within the cardiomyocyte and neighbouring cells (e.g. cardiac fibroblasts), that eventually leads to the full array of benefits observed in an exercised heart. Our data also contribute to this understanding, as we report here the RNA-seq dataset of differentially expressed genes, which can be further explored towards realizing whether a combined manipulation of multiple genes may enhance cardiomyocyte physiology and function. In addition, iPSC-derived cardiomyocytes are not entirely identical to adult human cardiomyocytes, however, gene expression patterns[42] and drug screening[43] studies have revealed striking similarities in molecular profiles[42] and response to pharmacological stimuli.[43] These findings provide sound justification for the use of iPSC cardiomyocytes in our study.

Altogether, our findings suggest the role of PRODH and proline metabolism in HF may be greater than previously thought. We conclude that PRODH is an important molecular target of exercise in HF, thereby encouraging further studies employing PRODH as an experimental target for gene therapy.

Statement of conflict of interest

None of the authors have any conflicts of interests with regard to this publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pcad.2019.03.002.

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