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Molecular Signatures of Beneficial Class Effects of Statins on Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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Statins prevent cardiac diseases via inhibition of cholesterol biosynthesis and mediation of pleiotropic effects on the cardiovascular system[1]. Statins may also act as anti-hypertrophic and anti-apoptotic agents to prevent cardiomyocyte injury. However, the effects of clinically relevant concentrations of statins (i.e., serum peak concentration (C^{max})) on cardiomyocytes remain largely unknown. In the current study, we investigate the class effects of atorvastatin, lovastatin, simvastatin, and fluvastatin applied at their respective C^{max}, on the transcriptome and functional properties of human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs)[2].

Human iPSCs from two male (Lines #1–2) and female (Lines #3–4) control individuals were differentiated to iPSC-CMs (Figure A) with higher than 80% efficiency, as measured by expression of cardiac troponin (data not shown). Statins mediated no effect on the viability (2 and 7 days) and contractile properties of iPSC-CMs (7 days) when tested in a dose-dependent manner (data not shown). To examine the transcriptional effects of statins, we performed comprehensive RNA sequencing (RNA-seq) analysis of each iPSC-CM line using the C^{max} of each statin, corresponding to the clinical dosage of 40 mg. We focused on

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DISCLOSURES

Dr Wu is a cofounder of Khloris Biosciences but has no competing interests, because the work presented here is completely independent. The other authors report no conflicts.

significantly differentially expressed genes (DEGs) compared to vehicle-treated cells (FDR adjusted p-value <0.05). Heatmap analysis revealed that fluvastatin (F1-F4) mediated the most potent effects on the iPSC-CM transcriptome, followed by atorvastatin (A1-A4), simvastatin (S1-S4), and lovastatin (L1-L4) (Figure B**, upper panel**). Thirty-three common DEGs were significantly affected (FC≥2) by atorvastatin, simvastatin, and fluvastatin (Figure B**, lower panel**). Many of the commonly regulated genes were related to cholesterol biosynthesis, such as methylsterol monooxygenase-1 (*MSMO1*), stearoyl-CoA desaturase (*SCD*), 3-hydroxy-3-methylglutaryl-CoA synthase-1 (*HMGCS1*), low-density lipoprotein receptor (*LDLR*), 24-dehydrocholesterol reductase (*DHCR24*), and 7-dehydrocholesterol reductase (*DHCR7*) (Figure B**, lower panel**). These results suggest that statins induce a molecular signature in a drug-specific manner independent of genetic background.

Additional transcriptomic profiling of three different iPSC-CM batches (Rep 1–3) derived from a single donor (Line #2) was performed to ensure reproducibility of the observed effects on iPSC-CM, independent of technical variability. Comprehensive assessment of these iPSC-CM transcriptomes following drug treatment was performed using the Ion AmpliSeq™ Human Transcriptome Kit. In accordance with the RNA-seq analysis, fluvastatin mediated the most potent effects on iPSC-CM transcriptome, followed by atorvastatin, simvastatin, and lovastatin (Figure C**, upper panel**). Further analysis revealed that fluvastatin, atorvastatin, and simvastatin regulate a common set of 6 DEGs related to cholesterol biosynthesis (*MSMO1*, *SCD*, *LDLR*, *DHCR24*, *HMGCS1*, and *DHCR7*) (Figure C**, lower panel**). This set of commonly regulated genes represents the core molecular signature of the effects of fluvastatin, atorvastatin, and simvastatin in iPSC-CMs. Our results correlate with the clinical efficacy of statins, with the exception of fluvastatin, which may have cardiac specific effects and needs to be further investigated in the future.

The induced protein level of HMGCS1 was confirmed by Western blot analysis in all iPSC-CMs following statin treatment (Figure D). Atorvastatin and fluvastatin mediated the strongest effects on HMGCS1 protein expression, followed by simvastatin and lovastatin. These results are in accordance with both RNA-seq and AmpliSeq analysis, further suggesting all statins regulate the expression of key regulators of the metabolic properties of iPSC-CMs in a drug-specific manner. Subsequently, we performed functional enrichment analysis to uncover the signaling pathways and cellular processes affected by statins in iPSC-CMs. All statins affected signaling pathways/cellular processes that are primarily involved in cholesterol metabolism, secondary alcohol biosynthesis, and sterol biosynthetic pathway, as shown by bubble chart analysis (Figure E). Although the hypertrophic cardiomyopathy (HCM) signaling pathway was not significanlty enriched, various genes were found to be significantly regulated. Significant down-regulation of two HCM pathway genes, *TPM2* and *MYL3*, was observed in iPSC-CMs differentiated from a HCM patient (*MYBPC3* p.Val321Met (c.961G>A))[3] following treatment by atorvastatin, simvastatin, and lovastatin (Figure F). To confirm the anti-hypertrophic effects at the cellular level, we next treated the HCM iPSC-CMs with statins and found that simvastatin and lovastatin significantly reduced the cell size (Figure G). Finally, we also tested the anti-apoptotic effects of statins in two healthy iPSC-CM lines (Line #2–3) following treatment with Doxorubicin (0.1 uM DOX), as previously described[4]. Our data show that simvastatin and

lovastatin significantly reduced the number of DOX-induced apoptosis in iPSC-CMs (Figure H), demostrating pro-survival effects for each of these drugs.

Overall, our study reveals that fluvastatin mediates the strongest effects on the transcriptome of healthy iPSC-CMs. On the other hand, simvastatin and lovastatin exerted stronger anti-hypertrophy effects in HCM iPSC-CMs and pro-survival effects in DOX-treated iPSC-CMs. When applied at physiologically relevant concentrations, statins primarily affect genes related to cholesterol and fatty acid homeostasis, which are in accordance with previous reports implicating long-chain polyunsaturated fatty acids in statin's cardio-protective effects[5]. In addition, statins exert anti-hypertrophic and anti-apoptotic cellular processes in human iPSC-CMs in a drug-specific manner. These effects might be independent from the clinical action of statins on atherosclerosis, although further work is needed to confirm this observation.

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The data supporting the findings of this study are available from the corresponding author upon request. RNA-seq data is publicly available with GEO accession number GSE113546.

J.C. Wu is a co-founder of Khloris Biosciences but has no competing interests, as the work presented here is completely independent.

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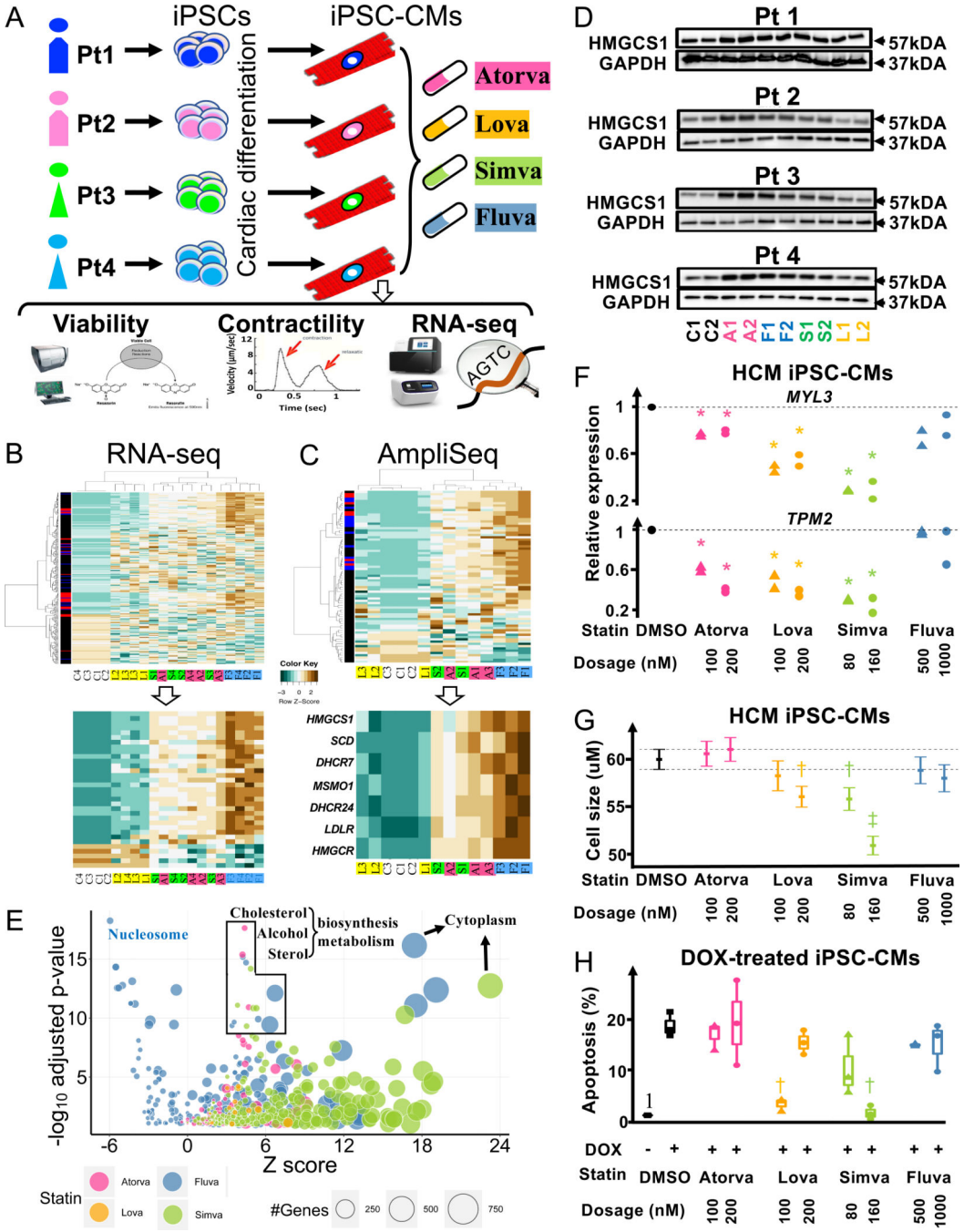

Figure: Statin class effects on the transcriptome and functional properties of iPSC-CMs.

(A) Schematic depiction of study design. **(B) Upper panel:** Heatmap of DEG fold-changes (FCs) from RNA-seq analysis. Red boxes (left side) indicate genes significantly affected by fluvastatin, atorvastatin, and simvastatin, blue indicates genes significantly affected by any two statins, and black indicates genes affected by only one drug. Genes with FDR adjusted p-value < 0.05 under likelihood ratio test were considered as significant. **Lower panel:** Heatmap of FCs of commonly affected DEGs by all drugs. C: control, A: atorvastatin, F: fluvastatin, S: simvastatin, L: lovastatin. 1–4: Lines 1–4. **(C) Upper panel:** Heatmap of

AmpliSeq data showing FCs of DEGs in statin-treated iPSC-CMs from Line #2. **Lower panel:** Heatmap of Ampli-seq data showing FCs of DEGs regulated in common by atorvastatin, fluvastatin, simvastatin, and lovastatin in iPSC-CMs. Genes with FDR adjusted p-value < 0.05 under were considered as significant. C1–3: control, A1–3: atorvastatin, F1–3: fluvastatin, S1–2: simvastatin, L1–3: lovastatin. **(D)** Representative Western blot analysis of HMGCS1 protein expression in iPSC-CMs following statin treatment. **(E)** Bubble chart showing the top signaling pathways and cellular processes with the largest number of affected DEGs, acccording to transcriptomic analysis. **(F)** qRT-PCR quantification of HCM pathway genes in HCM *MYBPC3* p.Val321Met iPSC-CMs treated with statins. **(G)** Cell size analysis of HCM *MYBPC3* p.Val321Met iPSC-CMs following treatment with statins. Error bars indicate 95% confidence intervals and square dots represent mean values. Dash lines show 95% confidence interval in the control group. **(H)** Cell death analysis (apoptosis) of healthy iPSC-CMs (Linese #2 and #3) treated with four statins. *p<0.05, †p<1e-6, ‡p<1e-33 under paired t-test.