Supporting Information

for Adv. Healthcare Mater., DOI: 10.1002/adhm.202100926

CHIR99021 Promotes hiPSC-Derived Cardiomyocyte Proliferation in Engineered 3D Microtissues

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Figure S1. 3D reconstruction of part of an engineered cardiac microtissue. Different views of an engineered cardiac microtissue containing 25 x 10^6 cells/ml at day 7 stained for Nkx2.5. Nuclei were visualized with DAPI.
Figure S2. CHIR99021 stimulation increases the density of hiPSC-cardiomyocytes in collagen I hydrogels containing 25 x 10^6 cells/ml. a) Representative brightfield images of engineered cardiac microtissues at indicated times of stimulation. b) Quantitative analysis of the density of hiPSC-cardiomyocytes in collagen I hydrogels based on the coverage of the tissues with cell colonies (dark areas) (n = 3 in triplicates). c) Quantification of the metabolic activity of tissues at indicated time points measured by CCK-8 assay (n = 3). Data are mean ± SD. *: p < 0.05, **: p < 0.01, ***: p < 0.001. ns: statistically not significant. Scale bars: 1000 µm.
Figure S3. The majority of CHIR99021-stimulated cells are hiPSC-cardiomyocytes. a) Representative projections of confocal images as examples of areas of microtissues at day 7 containing different cardiomyocyte (sarcomeric α-actinin-positive) densities validating Nkx2.5 staining as cardiomyocyte-specific. Nuclei were visualized with DAPI. b) Quantitative analysis of hiPSC-cardiomyocytes (Nkx2.5-positive/DAPI-positive) and non-myocytes (Nkx2.5-negative/DAPI-positive) (n = 3 in duplicates, four individual areas were analyzed for every n). c) Representative example of a projection of confocal images of a part of an engineered cardiac tissue containing 6.25 x 10⁶ cells/ml stained for sarcomeric α-actinin and Nkx2.5 (hiPSC-cardiomyocytes), Ki-67 (proliferation marker), and DNA (DAPI identifying nuclei) at day 7 stimulated with CHIR99021. Note, the majority of Ki-67 positive cells are also positive for Nkx2.5. The arrow highlights one proliferating non-myocyte. d) Representative projections of confocal images as examples of overviews of microtissues at day 7. Yellow boxes: low cardiomyocyte density. Green box: high cardiomyocyte density. Height of Z-stacks: as indicated. Data are mean ± SD. ns: statistically not significant. Scale bars: white: 50 µm; yellow: 500 µm.
Supplementary videos

**Video S1.** Engineered cardiac tissue (12.5 x 10^6 cells/ml) without CHIR99021 at day 10

**Video S2.** Engineered cardiac tissue (12.5 x 10^6 cells/ml) with CHIR99021 at day 10

**Video S3.** Engineered cardiac tissue (25 x 10^6 cells/ml) without CHIR99021 at day 10

**Video S4.** Engineered cardiac tissue (25 x 10^6 cells/ml) with CHIR99021 at day 10

**Video S5.** Calcium flow in tissue (12.5 x 10^6 cells/ml) without CHIR99021 at day 10

**Video S6.** Calcium flow in tissue (12.5 x 10^6 cells/ml) with CHIR99021 at day 10

**Video S7.** Calcium flow in tissue (25 x 10^6 cells/ml) without CHIR99021 at day 10

**Video S8.** Calcium flow in tissue (25 x 10^6 cells/ml) with CHIR99021 at day 10

**Video S9.** Engineered cardiac tissue (12.5 x 10^6 cells/ml) without CHIR99021 at day 35

**Video S10.** Engineered cardiac tissue (12.5 x 10^6 cells/ml) with CHIR99021 at day 35

**Video S11.** Engineered cardiac tissue (25 x 10^6 cells/ml) without CHIR99021 at day 35

**Video S12.** Engineered cardiac tissue (25 x 10^6 cells/ml) with CHIR99021 at day 35

**Video S13.** 150 µl cardiac tissue (12.5 x 10^6 cells/ml) without CHIR99021 at day 10

**Video S14.** 150 µl cardiac tissue (12.5 x 10^6 cells/ml) with CHIR99021 at day 10

**Video S15.** Calcium flow in 150 µl tissue (12.5 x 10^6 cells/ml) without CHIR99021 at day 10

**Video S16.** Calcium flow in 150 µl tissue (12.5 x 10^6 cells/ml) with CHIR99021 at day 10