

Remodeling Cell Adhesion Releases the Cardiac Potential of Human Pluripotent Stem Cells with Continuous Proliferation and Accelerated Maturation

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Figure S1

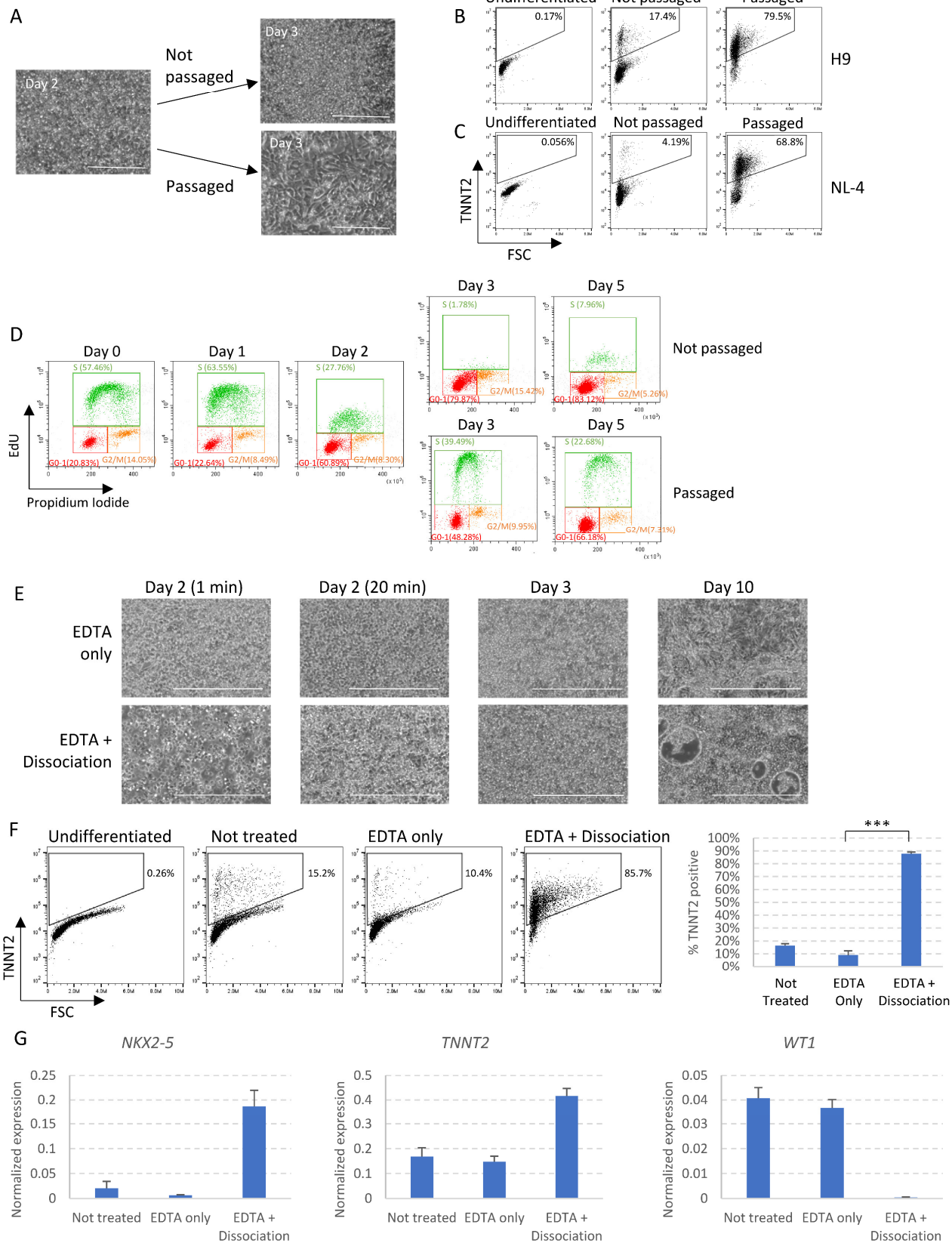


Figure S1. Dissociation of cell-matrix adhesion releases cardiac potential during critical period of cell fate determination. Related to Figure 1.

A) Phase contrast images of Day 3 cells. Scale bar, 400 μ m. B-C) Impact of Cell adhesion remodeling on spontaneous differentiation of mesendoderm progenitors induced from H9 hESC (B) and NL-4 iPSC (C) lines. TNNT2⁺ cells were analyzed by flow cytometry on Day 10 of differentiation. D) Cell cycle analysis using Edu and propidium iodide (PI). Red, G0-1 phase; Orange, G2/M phase; Green, S phase. Data are representative of three independent experiments. E) Comparison of cells treated with EDTA and dissociated / replated on Day 2 (bottom row) versus those treated with EDTA but not dissociated on Day 2 (top row). Phase contrast images were taken right after treatment (1 min) on Day 2, 20 minutes after treatment on Day 2, and on Day 3 and Day 10 of differentiation. Scale bar, 400 μ m. F) Flow cytometry analysis of TNNT2⁺ cells on Day 10 of differentiation, comparing untreated, EDTA-treated and EDTA-treated + dissociated cells. Data presented as mean $\pm$ SD of three biological replicates. ***, $p < 0.001$ (One-Way ANOVA with Dunnett's Multiple Comparison Test). G) qPCR analysis on Day 10 of differentiation, comparing untreated, EDTA-treated and EDTA-treated + dissociated cells. Data are normalized to *GAPDH* and presented as mean $\pm$ SD of three biological replicates.

Figure S2

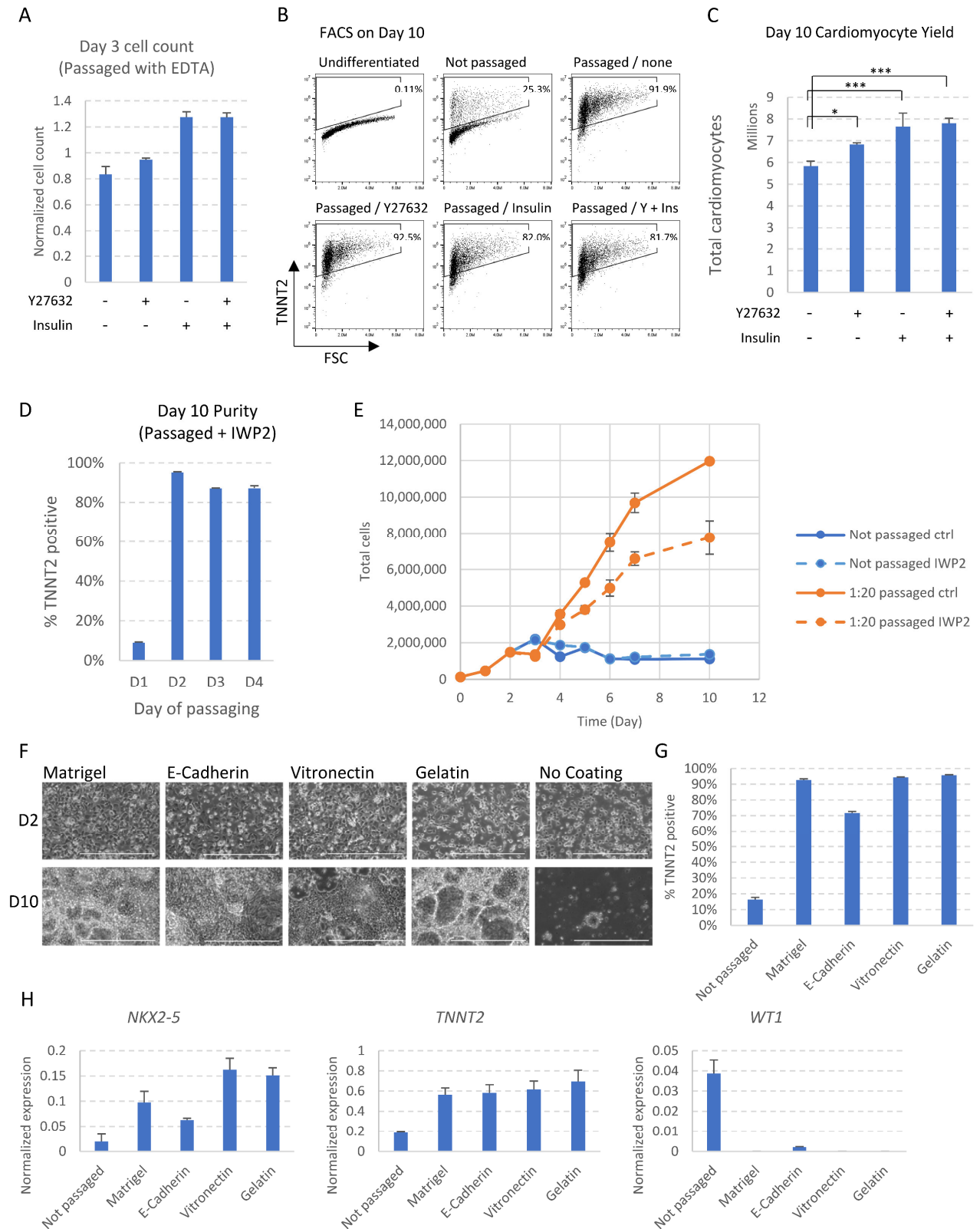


Figure S2. Synergistic signaling modulation maximizes cardiomyocyte production after cell dissociation. Related to Figure 2.

A) Cell count on Day 3, 24 hours after cell dissociation and replating on Day 2 using EDTA method. Cell numbers were normalized to the number of cells seeded on Day 2. Data presented as mean $\pm$ SD of three biological replicates. B-C) FACS analysis of TNNT2⁺ cells on Day 10 of differentiation, showing the impact of ROCK inhibitor Y27632 and insulin. Cells were induced toward cardiomyocytes by dissociation on Day 2 in the presence or absence of Y27632 (5 μ M) or insulin (1 μ g/ml), applied on Day 2. Data presented as mean $\pm$ SD of three biological replicates. *, $p < 0.05$; ***, $p < 0.001$ compared to control (One-Way ANOVA with Dunnett's Multiple Comparison Test). D) FACS analysis of TNNT2⁺ cells on Day 10 of differentiation, comparing cells dissociated and replated on different days in the differentiation process with IWP2 applied Day 2-4. Data presented as mean $\pm$ SD of three biological replicates. E) Cell growth curve through the differentiation process. Cells were passaged on Day 2 at a split ratio of 1:20 and compared to non-passaged cells. IWP2 was applied Day 2-4. Data presented as mean $\pm$ SD of three biological replicates, and numbers represent the total cell count generated from one initial well on a 24-well plate. F) Morphology of cells plated in Matrigel-, E-Cadherin-, vitronectin-, gelatin-coated or uncoated plates after dissociation on Day 2. Phase contrast images were taken on Day 2 (2 hours after dissociation) and Day 10 of differentiation. Scale bar, 400 μ m. G) FACS analysis of TNNT2⁺ cells on Day 10 of differentiation, comparing cells plated on different substrates. Data presented as mean $\pm$ SD of three biological replicates. H) qPCR analysis on Day 10 of differentiation, comparing cells plated on different substrates. Results are normalized to *GAPDH*. Data presented as mean $\pm$ SD of three biological replicates.

Figure S3

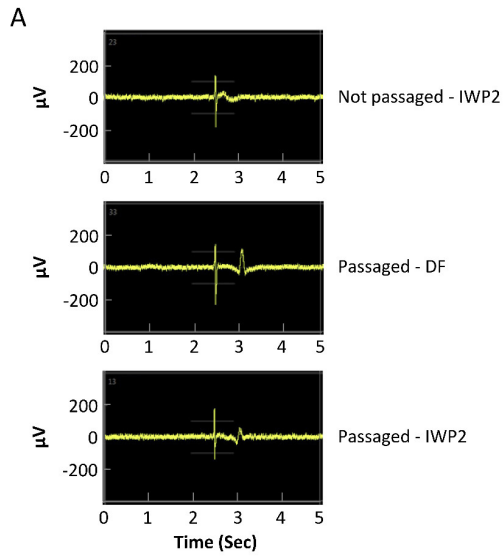


Figure S3. Characterization of passaging-induced cardiomyocytes. Related to Figure 3.

A) Representative electric activity recordings of cardiomyocytes induced by IWP2, passaging, and combined treatment with both IWP2 and passaging, measured on microelectrode array (MEA).

Figure S4

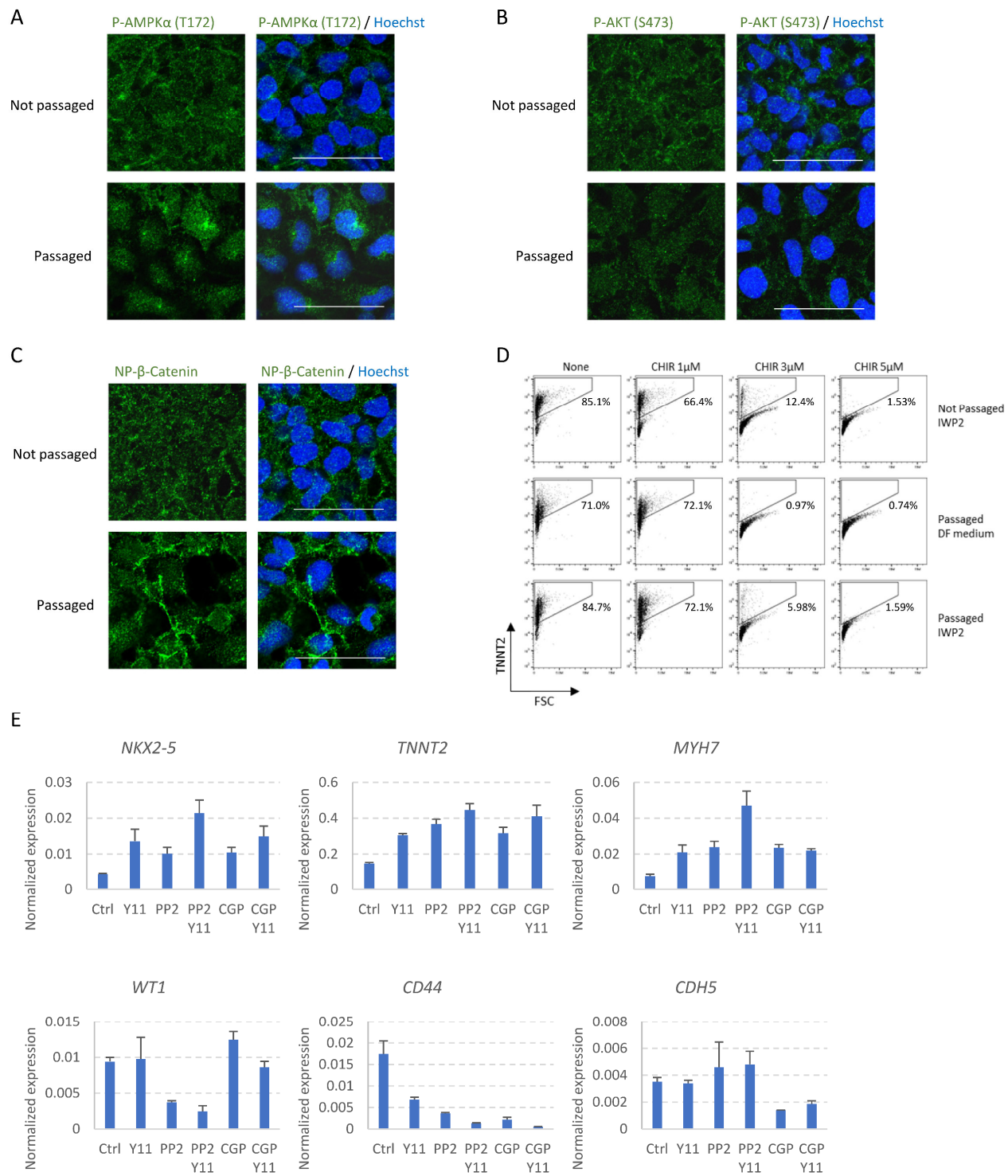


Figure S4. Cell adhesion remodeling regulates AMPK and AKT signaling through the integrin pathway. Related to Figure 4.

A-C) Immunostaining showing the impact of passaging on phosphorylated AMPK α (T172), phosphorylated AKT (S473), and non-phosphorylated (active) β -catenin. Mesendoderm progenitors (derived from H1 cells as shown in Figure 1A) on Day 2 of differentiation were either passaged using EDTA or changed to fresh DF medium without passaging, and fixed for immunostaining 1-2 hours after manipulation (1 hour for P-AMPK, 2 hours for P-AKT and NP- β -catenin). Nuclei were stained with Hoechst (blue). Scale bar, 50 μ m. D) Flow cytometry analysis of TNNT2⁺ cells on Day 10, showing impact of CHIR99021 (applied Day 2-4) on cardiac differentiation induced by IWP2 (applied to non-passaged cells Day 2-4), passaging, or both. E) qPCR analysis on Day 10 of differentiation, showing the impact of SRC inhibitor (PP2, 5 μ M or CGP77675, 5 μ M) and/or FAK inhibitor (Y11, 10 μ M) on cell fate. Treatments were applied on Day 2 for 6 hours, followed by culturing in DF medium with daily medium changes. Data are normalized to *GAPDH* and presented as mean $\pm$ SD of three biological replicates.

Figure S5

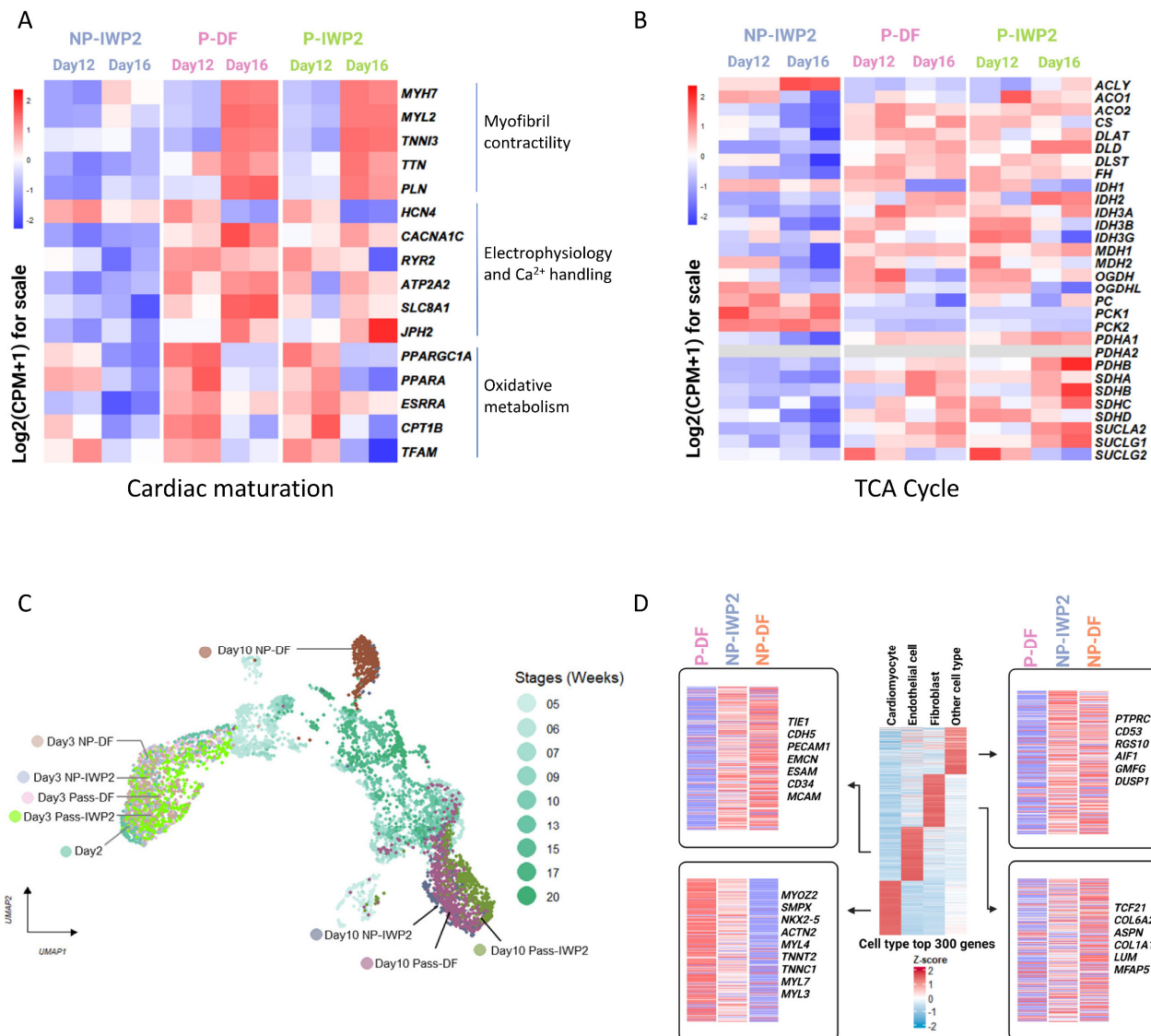


Figure S5. Cell adhesion remodeling accelerates cardiac fate determination and maturation.

Related to Figure 5.

A) Heatmap showing the expression of cardiac maturation marker genes on Day 12 and 16 in cardiomyocytes induced by IWP2 (NP-IWP2), passaging (P-DF), or both (P-IWP2), generated from bulk RNA sequencing data. B) Heatmap showing the expression of TCA cycle genes on Day 12 and 16, generated from bulk RNA sequencing data. C) Uniform Manifold Approximation and Projection (UMAP) plots visualization of human embryonic heart cells (GSE106118) (Cui et al., 2019) by developmental stage, integrated with scRNA-seq data of H1-derived cells. Different shades of green represent gestational weeks in human embryonic heart cell data. D) Middle: Heatmap displaying the Z-score-scaled average expression levels of the top 300 DEGs in human embryonic heart cell types. Left and right: Heatmaps showing the expression of top 300 DEGs from human embryonic heart cells in H1-derived cells generated under different treatments, based on Day 10 bulk RNA-seq results.

Supplemental Video 1. Beating cardiomyocytes induced by passaging. Related to Figure

1. Mesendoderm progenitors were passaged on Day 2 of differentiation, and then cultured in DF medium (DMEM/F12, ascorbic acid, selenite, transferrin, chemically defined lipid, penicillin/streptomycin) without other inducers. Video taken on Day 8.

Supplemental Video 2. Beating cardiomyocytes induced by passaging combined with

IWP2. Related to Figure 2. Mesendoderm progenitors were passaged on Day 2 of differentiation, treated with IWP2 (applied Day 2-4 in DF medium), and then cultured in DF medium. Video taken on Day 8.

Table S1. Sequence of primers used for quantitative PCR

Gene	Forward primer	Reverse primer
<i>NKX2-5</i>	CAAGTGTGCGTCTGCCTTT	CAGCTCTTTCTTTTCGGCTCTA
<i>TNNT2</i>	GGAGGAGTCCAAACCAAAGCC	TCAAAGTCCACTCTCTCTCCATC
<i>MYH7</i>	ACTGCCGAGACCGAGTATG	GCGATCCTTGAGGTTGTAGAGC
<i>MYL7</i>	ACATCATCACCCATGGAGACGAGA	GCAACAGAGTTTATTGAGGTGCCC
<i>MYL2</i>	TGTCCCTACCTTGTCTGTTAGCCA	ATTGGAACATGGCCTCTGGATGGA
<i>WT1</i>	ATAGGCCAGGGCATGTGTATGTGT	AGTTGCCTGGCAGAACTACATCCT
<i>CD44</i>	TGGCACCCGCTATGTCGAG	GTAGCAGGGATTCTGTCTG
<i>CD73</i>	CTCCTCTCAATCATGCCGCT	CATCAATGGGCGACCGGATA
<i>PECAM</i>	TCTATGACCTCGCCCTCCACAAA	GAACGGTGTCTTCAGGTTGGTATTTCA
<i>CDH5</i>	TGGAGAAGTGGCATCAGTCAACAG	TCTACAATCCCTTGCAGTGTGAG
<i>AFP</i>	CAAACCTATTGGCCTGTGGCG	TGGCCAACACCAGGGTTTAC
<i>CDX2</i>	GGAACCTGTGCGAGTGGAT	TCCGTGTACACCACTCGATATT
<i>NANOG</i>	TGTGATTTGTGGGCCTGAAG	AAGTGGGTTGTTTGCCTTTGG
<i>POU5F1</i>	CTTGAATCCCGAATGGAAAGGG	GTGTATATCCCAGGGTGATCCTC

Table S2. qPCR data used to generate heatmaps. Related to Figure 1B. This table shows results of qPCR analysis on Day 10 of differentiation, comparing cells differentiated in static culture (not passaged), cells passaged 1:6 on Day 2, and undifferentiated H1 cells.

Table S3. List of differentially expressed genes (DEGs) from RNA-seq analysis. Related to Figure 3A. This table shows the expression levels of DEGs corresponding to each cluster in the heatmap in Figure 3A.