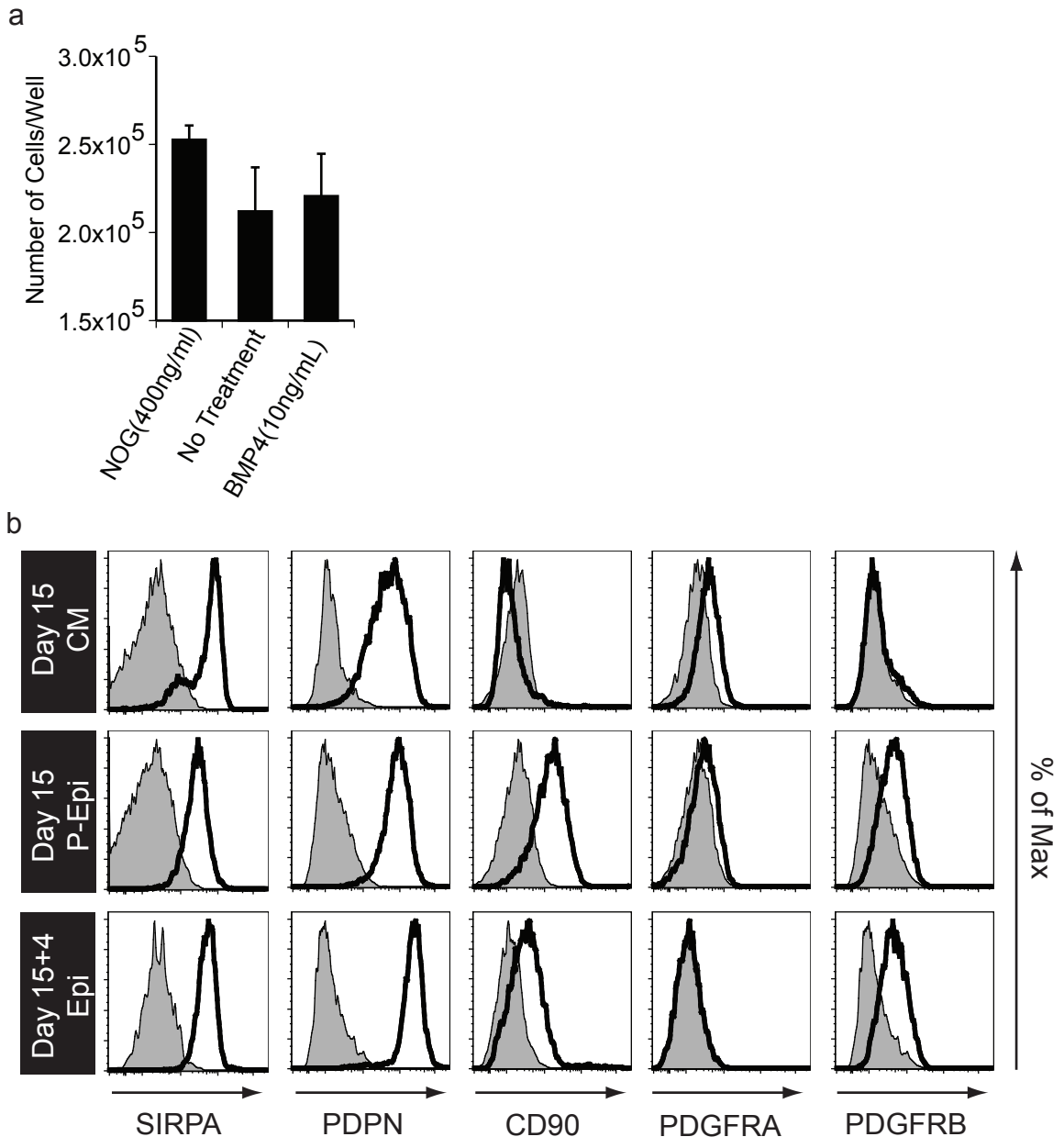
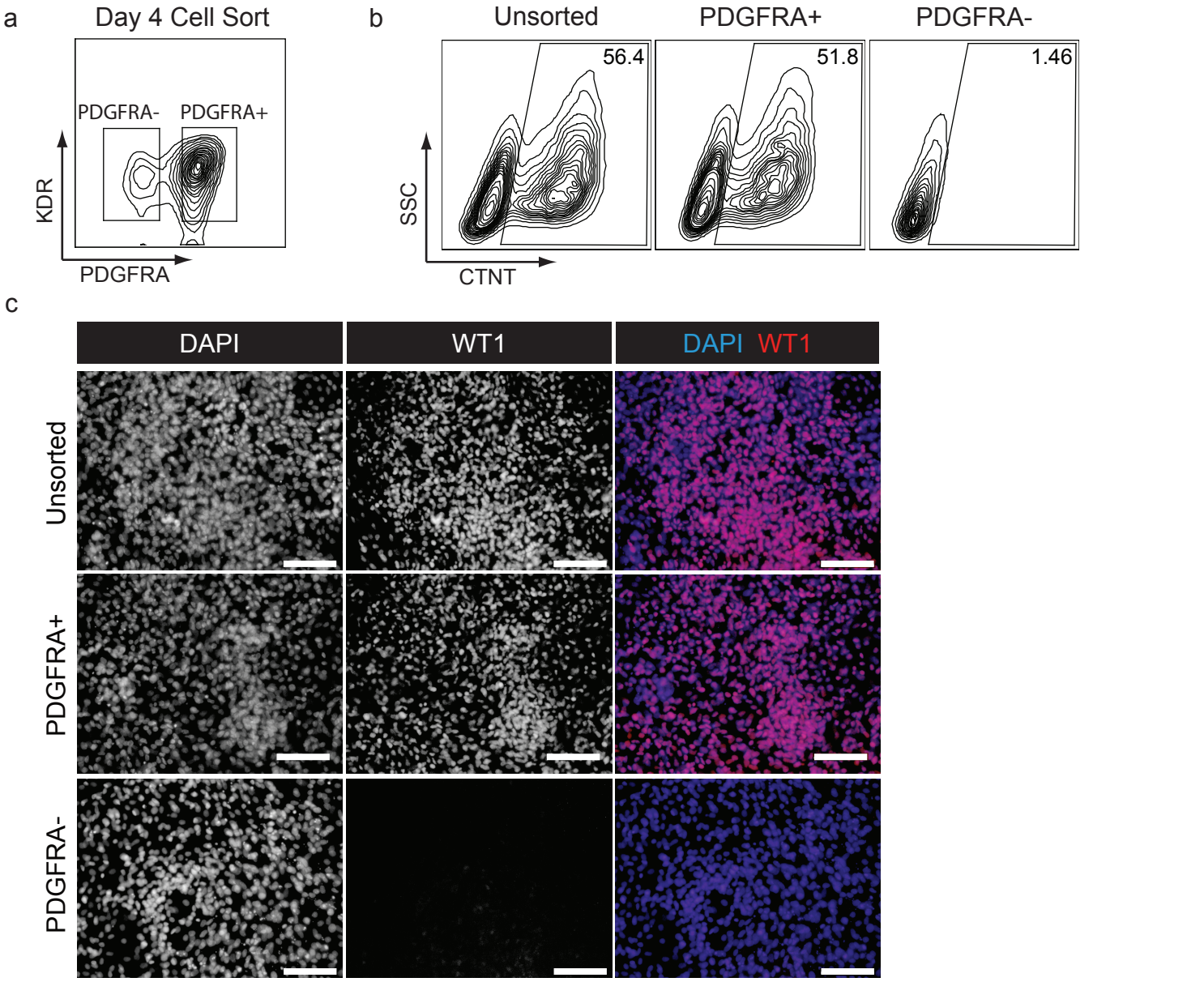
Supplementary Figure 1. Cell quantification and extracellular antigen expression.



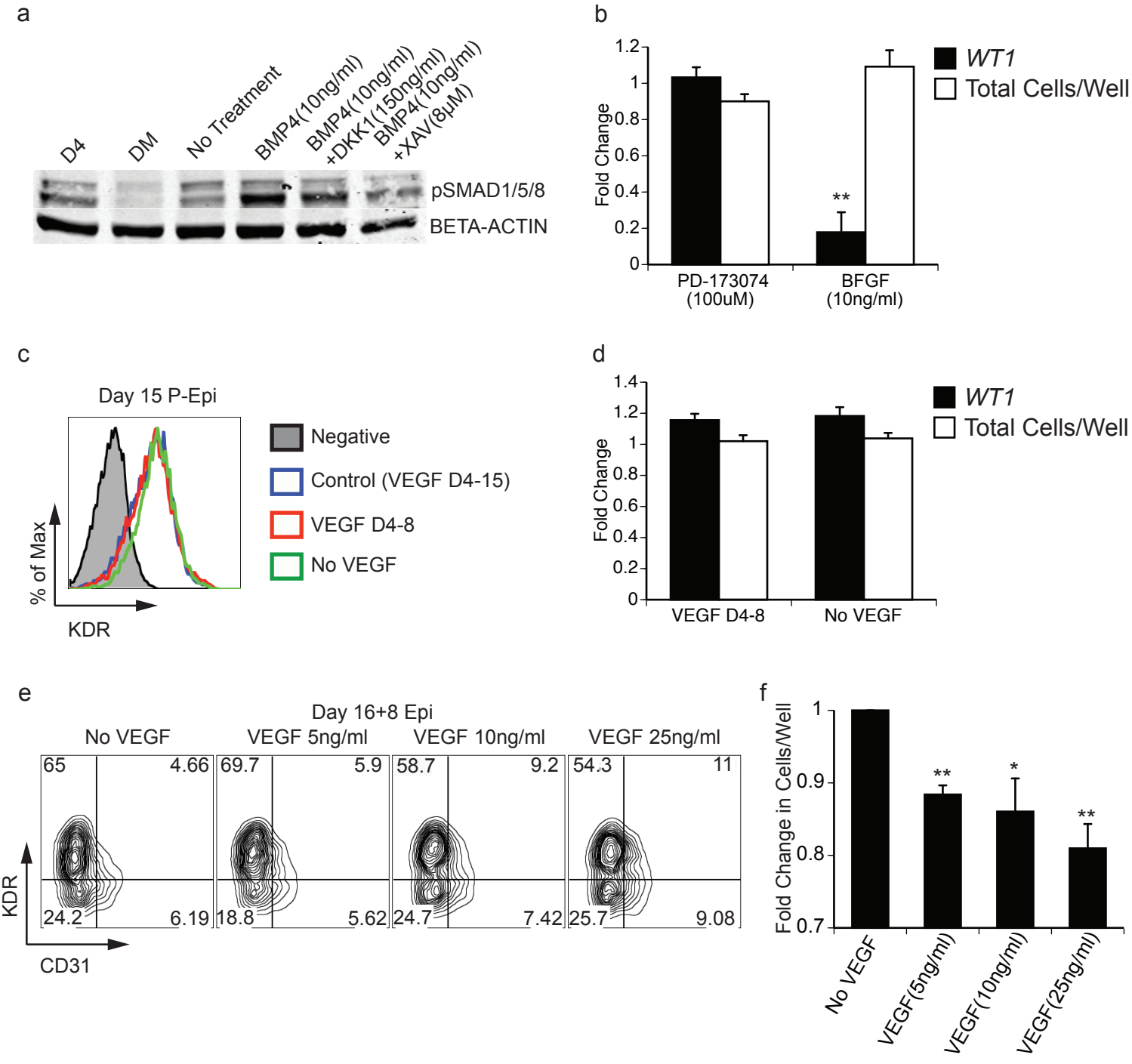
(a) Cell numbers in D15 cultures treated with BMP4 (10ng/ml), NOGGIN (NOG, 400ng/ml) or no treatment (control). Bars represent standard error of the mean of the values from three independent experiments (N=3); No significant differences in cell numbers was detected between the groups based on analysis by Student's T-test.
(b) Flow cytometric analyses of the frequency of cells expressing the indicated markers (black line) in D15 CM, D15 P-Epi and D15+4 Epi populations. In tinted gray area, negative controls.

Supplementary Figure 2. The cardiomyocyte and epicardial lineages are derived from day 4 PDGFRA+ Cells.



(a) PDGFRA+ and PDGFRA- populations were isolated from D4 EBs after which the cell are plated under conditions that support cardiomyocyte or epicardial development.
(b) Flow cytometry for CTNT in D15 cultures from populations plated under cardiomyocyte conditions.
(c) Fluorescent immunostaining for the presence of WT1 positive cells in D15 cultures following BMP4 (10ng/ml) treatment from D4 to D6. DAPI staining shows the cell nuclei. Scale bars represent 100μm.
(d) qRT-PCR-based expression of the epicardial markers *WT1* and *TBX18* in D15 cultures following BMP4 (10ng/ml) treatment from D4 to D6. Values are fold change compared to the unsorted cultures. Bars represent standard error of the mean of the values from three independent experiments (N=3); **P≤0.01 from unsorted cultures as analyzed by Student's T-test.

Supplementary Figure 3. BFGF and VEGF signaling during epicardial specification and maturation.



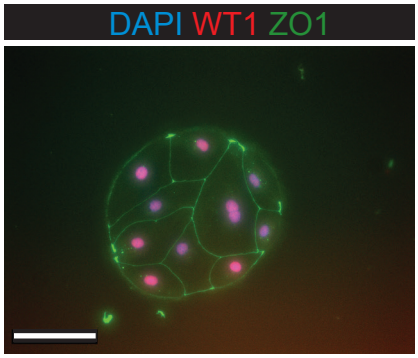
Supplementary Figure 3. BFGF and VEGF signaling during epicardial specification and maturation.

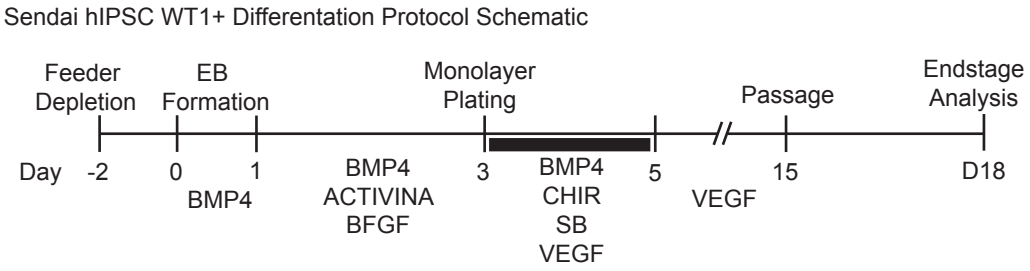
(a) Western blot analyses for pSMAD1/5/8 in D4 populations following treatment with the indicated agonists and antagonists for 30 minutes. BETA-ACTIN is shown as loading control.
(b) WT1 expression as determined by qRT-PCR analyses and total cell numbers per well in D15 populations generated from D4 mesoderm by treatment with BMP4 and CHIR together with either the FGF inhibitor PD-173074 (100u μM) or BFGF (10ng/ml). Values shown are relative to populations generated from mesoderm treated with only BMP4 and CHIR without manipulation of the FGF pathway. Bars represent standard error of the mean of the values from three independent experiments (N=3); **P≤0.01 from unsorted cultures as analyzed by Student's T-test.
(c) Flow cytometric analyses of the frequency of KDR+ cells in D15 P-Epi populations generated from D4 mesoderm treated with BMP4 and CHIR (D4-D6) together with VEGF (5ng/ml) from either D4-D8 or D4-15 (control) or no additional factor (no VEGF).
(d) WT1 expression as determined by qRT-PCR analyses and total cell numbers per well in D15 populations generated from D4 mesoderm by treatment with BMP4 and CHIR (D4-D6) together with either VEGF (D4-D8) or no additional VEGF. Values shown are relative to populations generated from mesoderm treated with only BMP4 and CHIR. Bars represent standard error of the mean of the values from three independent experiments (N=3).
(e) Flow cytometric analyses of the frequency of KDR+ and CD31+ cells in D16+8 Epi populations cultured in the presence of the indicated amounts of VEGF.
(f) Relative number of Epi cells in D16+8 populations following culture in the indicated concentrations of VEGF. Values shown are fold change in total cell relative to untreated (no VEGF) control, which is set at 1. Bars represent standard error of the mean of the values from three independent experiments (N=3); *P≤0.05, **P≤0.01 compared to no treatment control as analyzed by Student's T-test.

Supplementary Figure 4. The generation of epicardial cells from additional hPSC lines.

a

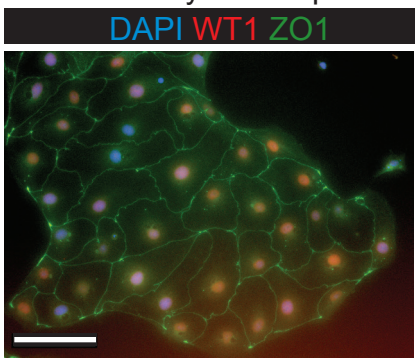
Sendai hiPSC Day 15+3 Epi

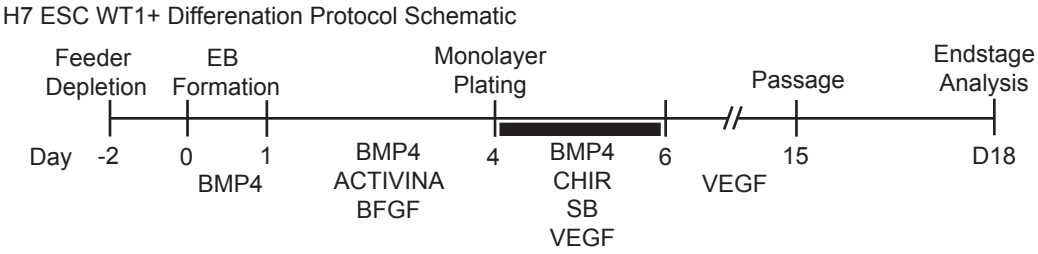




b

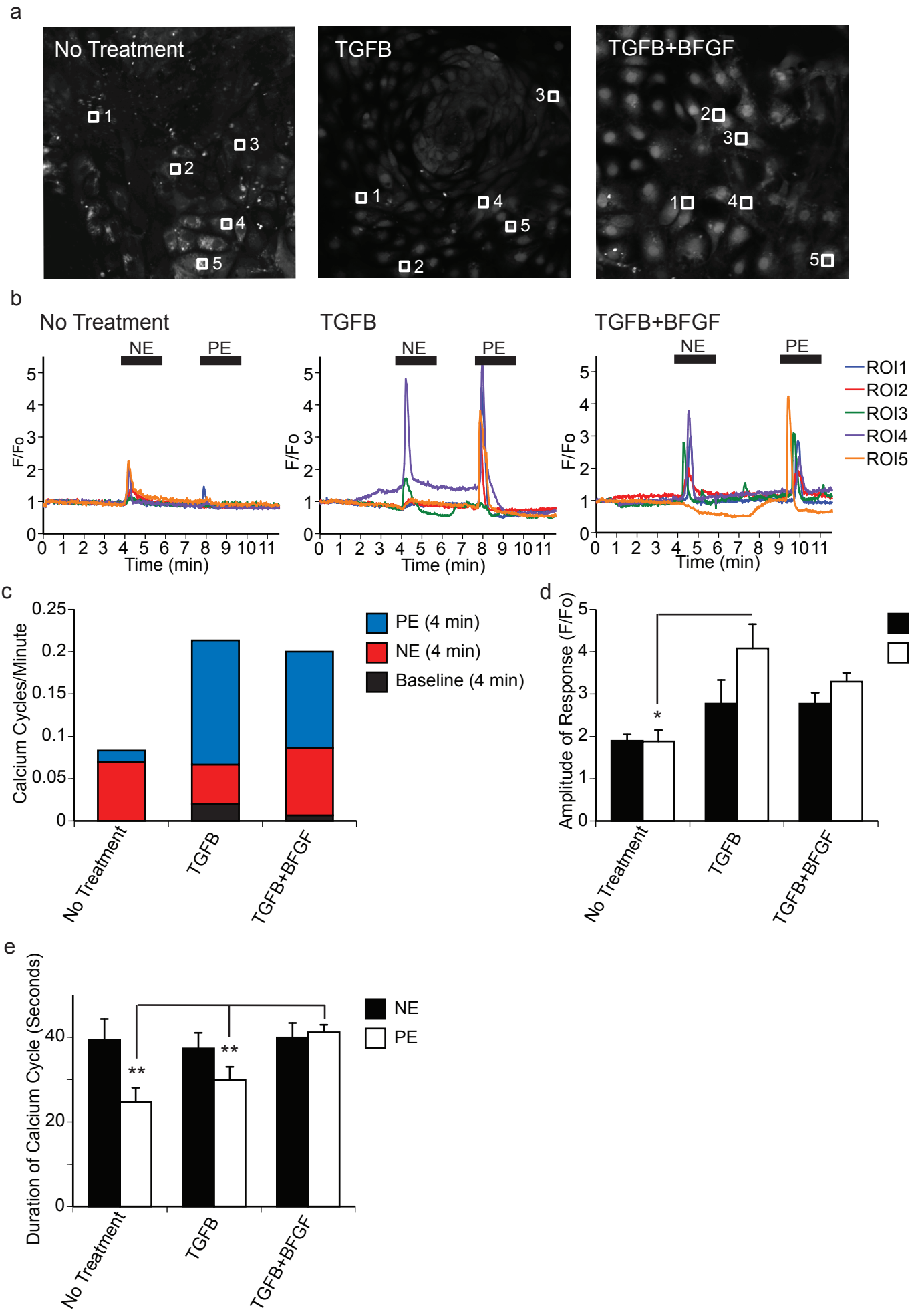
H7 Day 15+3 Epi





(a) Fluorescent immunostaining for the presence of WT1 (red) and ZO1 (green) protein in hiPSC-derived epicardial population. DAPI (blue) staining shows cell nuclei. Scale bars represent 100μm. Scheme indicates timing of manipulations and analysis.
(b) Fluorescent immunostaining showing the presence of WT1 (red) and ZO1 (green) protein in a H7 hESC-derived epicardial population. DAPI (blue) staining shows cell nuclei. Scale bars represent 100μm. Scheme indicates timing of manipulations and analysis.

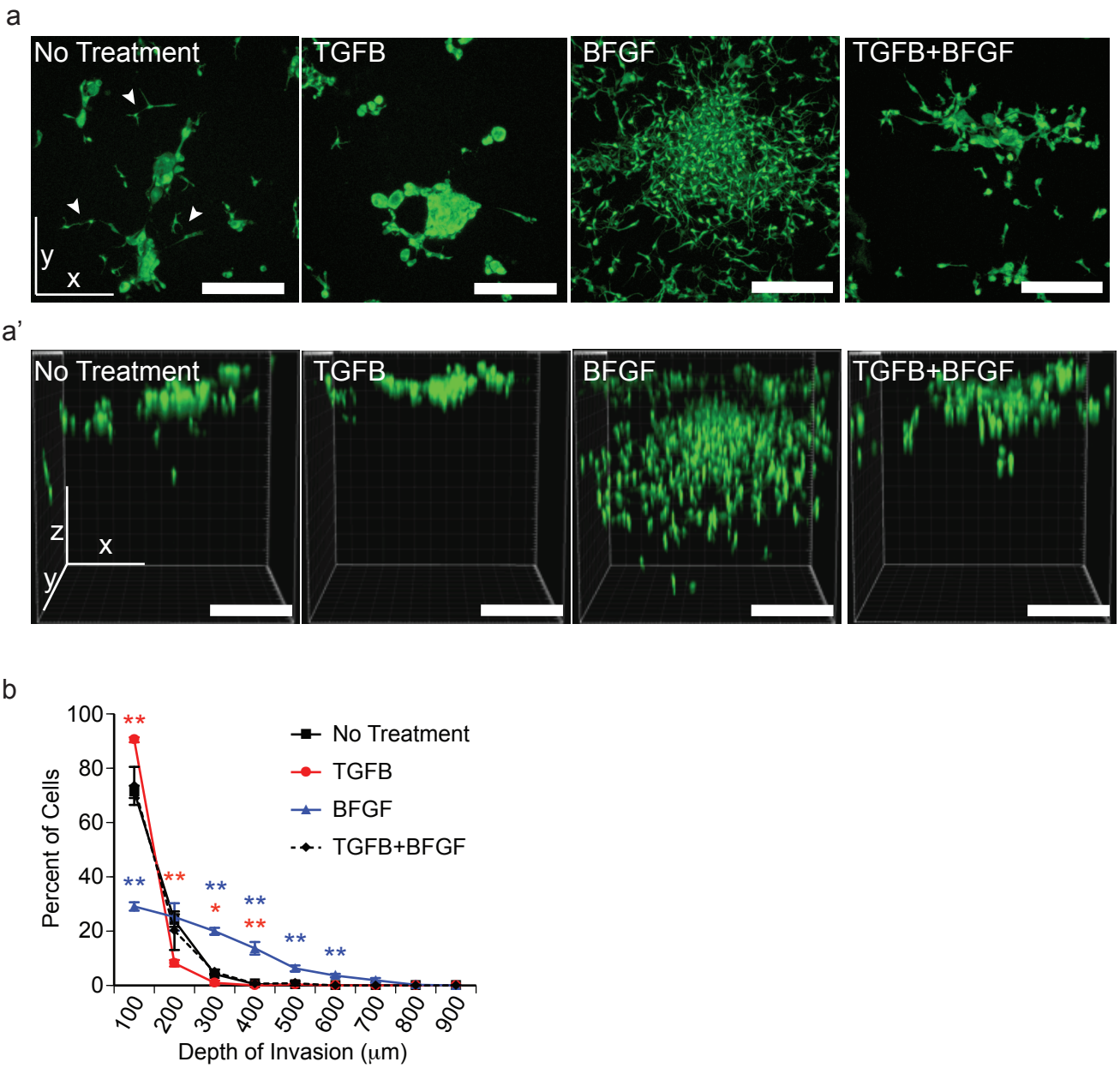
Supplementary Figure 5. Calcium imaging in epicardial-derived populations.



Supplementary Figure 5. Calcium imaging in epicardial-derived populations.

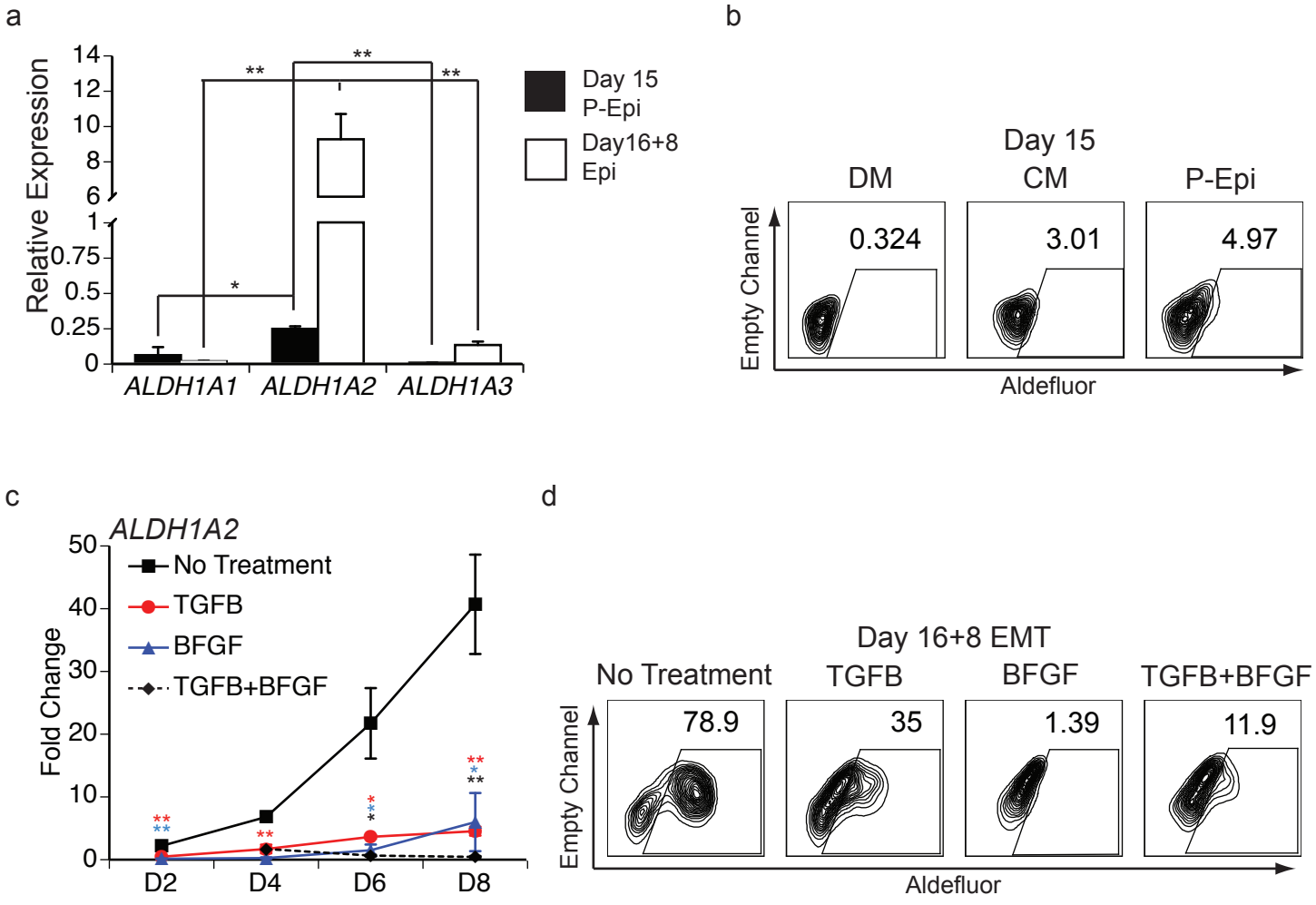
(a) Representative fields of view depict Fluo 4-AM-generated fluorescence signal averaged over the 12-min recording in cultures on D8 following EMT induced with the indicated factors. Numbered boxes correspond to regions of interest (ROI) from where fluorescence was recorded.
(b) Calcium imaging examining the functional response of epicardial-derived SMCs to the agonists norepinephrine (NE) and phenylephrine (PE). ROIs corresponding to cells measure the average fluorescence intensity normalized to baseline (F/Fo). Peaks correspond to active calcium cycles in response to agonists. 20 μM NE was added at 4min, and 15 μM PE was added at 8min, eliciting varied responses between the groups.
(c) The frequency of calcium cycles in actively cycling cells in the conditions as indicated at baseline and after NE and PE addition. Stacked bars represent the contribution to frequency of calcium cycling during baseline recording (blue), and after NE (red) or PE (green) treatment.
(d) The amplitude of calcium transients after NE and PE addition in the EMT induced cultures. No Treatment NE N=6 cells, PE N=4 cells; TGFB NE N=6 cells, PE N=12 cells; TGFB+BFGF NE N=13 cells, PE N=25 cells. *P<0.05 compared by one-way ANOVA with Tukey post hoc test.
(e) The duration of calcium transients after NE and PE addition in the EMT induced cultures. No Treatment NE N=6 cells, PE N=4 cells; TGFB NE N=6 cells, PE N=12 cells; TGFB+BFGF NE N=13 cells, PE N=25 cells. **P<0.01 compared by one-way ANOVA with Tukey post hoc test.

Supplementary Figure 6. Matrigel invasion of epicardial-derived populations.



(a) Representative fields of view in the XY plane (top view) of the matrigel invasion assay on D8 after EMT induction. White arrowheads in the no treatment condition indicate cells displaying EMT morphology. Scale bars represent 100μm.
(a') Representative 3D reconstruction (side view) of the matrigel invasion assay on D8 after EMT induction. Scale bars represent 100μm.
(b) Percent of cells binned within a given matrigel invasion depth on D8 following EMT initiation. Bars represent standard error of the mean of the values from three independent experiments (N=3); *P≤0.05, **P≤0.01 compared to non-treated controls as analyzed by Student's T-test.

Supplementary Figure 7. Epicardial cells up regulate ALDH1A2 expression and display aldehyde dehydrogenase activity following passage.



(a) qRT-PCR-based expression of ALDH1A1, ALDH1A2 and ALDH1A3 in D15 P-Epi cultures and D16+8 post-passage non-treated Epi cultures. Values are relative to the housekeeping gene TBP. Bars represent standard error of the mean of the values from three independent experiments (N=3); *P≤0.05, **P≤0.01 compared to ALDH1A2 expression levels as analyzed by Student's T-test.
(b) Flow cytometry for Aldefluor on representative D15 cultures generated from cells treated specified with DM, BMP4+XAV (cardiomyocytes; CM) or BMP4+CHIR (P-Epi cells).
(c) qRT-PCR-based expression of ALDH1A2 on days (D) 2, 4, 6 and 8 after EMT initiation in Epi cultures. Values are expressed as fold change to experiment-matched pre-passaged D15 P-Epi cultures. Values are relative to the housekeeping gene TBP. Bars represent standard error of the mean of the values from three independent experiments (N=3); *P≤0.05, **P≤0.01 compared to no treatment as analyzed by Student's T-test.
(d) Flow cytometry for Aldefluor on representative Epi-derived populations 8 days after EMT initiation with the indicated treatments.

A.Witty Supplementary Table 1 - qRT-PCR Primer List

Gene	Forward Primer	Reverse Primer
ALDH1A1	TTT GGA AGA TAG GGC CTG CAC TGA	TAA AGA TGC CAC GTG GAG AGC AGT
ALDH1A2	TTT GCC AAG TTC CAT TGT GCC AGG	TGG TGG AGT CAC TGG AAA GCA GAA
ALDH1A3	GGT CTT TGT GGA TTG CAT GTT	GAC TAA GCT CTC TGG GCT ATT G
ANXA8	ACA CGA ATC CAT CCC AAC CGA GAT	AAC ACA GTG TCC TTG GGT CAG GAA
AXIN2	TTG AGC TAG CAG TGC GTTCATGGT	TCT GCA TGT GTC AAT GGT AGG GCA
BNC1	TGG GTT GCC CAT AAC CTG TCA TCT	TAC TTT GCA TTT GTG GAG CTG CCG
CNN1	AAG GAC GCA CTG AGC AAC GCT ATT	ACG CCA CTG TCA CAT CCA CAT AGT
GATA4	CGA ATG ACG GCA TCT GTT TGC CAT	ATT TGG TAT TAG GGA TGC AGG GCG
GATA5	ACC AAG ATT CCC AGT GAA GCA CCT	TCC GTC TAT CCA TGT GGG CAA TGA
GPM6A	TTC CCT ATG CCT CTC TGA TTG CCA	ACA TCC AGT GTG TCT CCA GCA GTT
ISL1	GAA GGT GGA GCT GCA TTG GTT TGA	TAA ACC AGC TAC AGG ACA GGC CAA
KRT19	ATA GTG AGC GGC AGA ATC AGG AGT	AGA GGA CCT TGG AGG CAG ACA AAT
KRT8	CCA TTA AGG ATG CCA ACG CCA AGT	TGA CGT TCA TCA GCT CCT GGT ACT
MYH11	AGA AGC CAG GGA GAA GGA AAC CAA	TGG AGC TGA CCA GGT CTT CCA TTT
NKX2-5	TTT GCA TTC ACT CCT GCG GAG ACC TA	ACT CAT TGC ACG CTG CAT AAT CGC
SMTN	AGC ACC ATG ATG CAA ACC AAG ACC	TCT GCG CCT TCA TCA GCT CTT TCT
SNAI1	TTT CTG GTT CTG TGT CCT CTG CCT	TTC CCA GTG AGT CTG TCA GCC TTT
SNAI2	TTT CTG GGC TGG CCA AAC ATA AGC	ACA CAA GGT AAT GTG TGG GTC CGA
TAGLN	ATG GCC AAC AAG GGT CCT TCC TAT	ATC AGG GCC ACA CTG CAC TAT GAT
TBP	TGA GTT GCT CAT ACC GTG CTG CTA	CCCTCAAACCAACTTGTCAACAGC
TBX18	TTA ACC TTG TCC GTC TGC CTG AGT	GTA ATGGGC TTT GGC CTT TGC ACT
TBX5	ACA AAG TGA AGG TGA CGG GCC TTA	ATC TGT GAT CGT CGG CAG GTA CAA
TCF21	AGG CAG ATC CTG GCT AAC GAC AAA	TCC AGG TAC CAA ACT CCA AGG TCA
TNNT2	TTC ACC AAA GAT CTG CTC CTC GCT	TTA TTA CTG GTG TGG AGT GGG TGT GG
UPK1B	AGT GAC TCT GGA TTT GGT GCT GGA	AAG TCC GTA CCA TCT GAC TTG GCA
WT1	ATA GGC CAG GGC ATG TGTATG TGT	AGT TGC CTG GCA GAA CTA CAT CCT