Supplemental Materials and Methods

Histology, immunohistochemistry and immunocytochemistry

For EPDC labeling, background fluorescence was quenched with 3% H2O2 in methanol at 4°C overnight. All samples were permeabilized in 0.2% Triton X-100 in PBS (PBT) for 5 min (cells), 0.1% PBT for 10 min (cryosections), or antigen retrieval buffer (DAKO) for 10 min in a pressure-cooker (paraffin sections). Non-specific binding was blocked by incubation with 5-10% serum in PBS or Antibody diluent (DAKO) for 30 min at RT. Source of serum was selected based on host of secondary antibody: goat (Vector Laboratories) or donkey (Jackson Immuno Research). Primary antibodies listed in Supplemental Table S3 were diluted in either PBS (cells), 5-10% serum in 0.1% PBT (cryosections), or Antibody Diluent (DAKO, paraffin sections) and added to slides at 4°C overnight. Slides were then incubated with fluorescent-conjugated secondary antibodies (1:200) for 1 hr at RT: donkey (Jackson Immuno Research) anti-goat 488, anti-rabbit Cy3, or anti-rat 405, and goat (Life Technologies) anti-chicken 488, anti-mouse 488 or 594, anti-rabbit 488 or 594, or anti-rat 647. Slides were either mounted with TOPRO-3 Iodide (Life Technologies) or mounting media with DAPI (Vector Laboratories).

Cell culture

For MRTF-A overexpression experiments in EMCs, cells were serum-starved for 24 hrs then transduced with adenovirus expressing HA-MRTF-A or βgal (~4.0 x 10^4 pfu/ml) for an additional 24 hrs in serum-free media. To determine MRTF antibody specificity, COS-7 cells were transfected with 1 μg of empty vector or plasmid expressing Flag-MRTF-A or Flag-MRTF-B using Lipofectamine 2000 (Invitrogen) per manufacturer's instructions and processed for protein isolation.

RNA isolation and analysis

Total RNA was isolated from cell cultures and tissue samples using Trizol reagent (Life Technologies) and cDNA was generated using iScript cDNA Synthesis Kit (Bio-Rad) following manufacturer's protocol. To increase yield from epicardial explants, 5-10 ng of glycogen (Life Technologies) was added prior to RNA precipitation. Gene expression was examined by standard RT-PCR methods or qPCR using iQ SYBR Green Supermix (Bio-Rad) and the CFX Connect Real-Time PCR Detection System (Bio-Rad). Relative mRNA levels were normalized to *Gapdh* and calculated using the $2^{-\Delta\Delta CT}$ method.

Supplemental References

Austin, A.F., Compton, L.A., Love, J.D., Brown, C.B., and Barnett, J.V. (2008). Primary and immortalized mouse epicardial cells undergo differentiation in response to TGFbeta. *Developmental dynamics : an official publication of the American Association of Anatomists* **237**, 366-376.

Wada, A.M., Smith, T.K., Osler, M.E., Reese, D.E., and Bader, D.M. (2003). Epicardial/Mesothelial cell line retains vasculogenic potential of embryonic epicardium. *Circ Res* **92**, 525-531.

Supplemental Figures

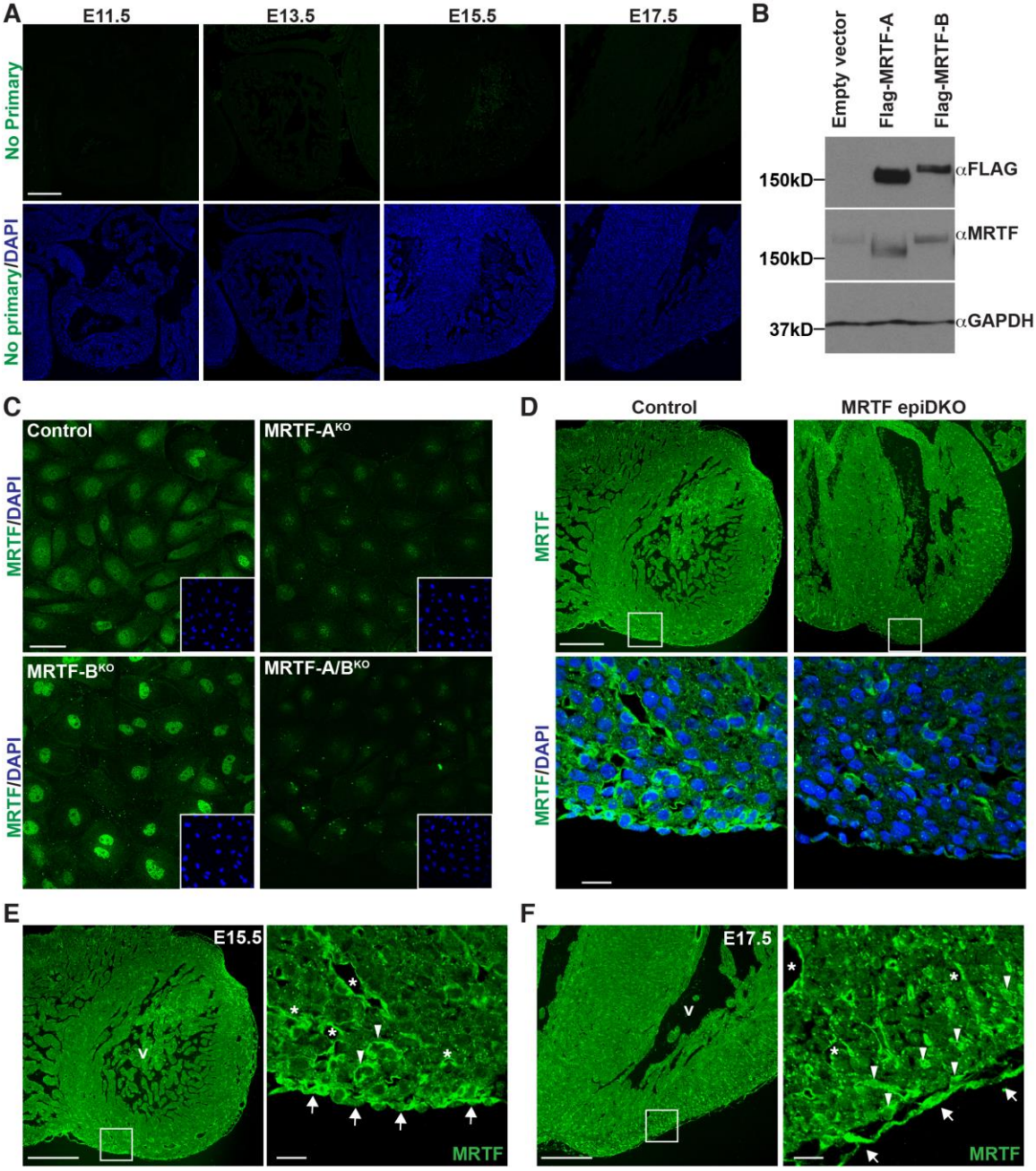

Fig. S1. Anti-MRTF antibody recognizes MRTF-A and MRTF-B

(A) No primary antibody control immunofluorescence performed in parallel to MRTF immunostaining. All images were captured under the same settings as those presented in Fig. S1E, F. Scale bar: 250 μm. **(B)** Western blot analysis of COS-7

cells transfected with Flag-tagged constructs reveals MRTF antibody detects both MRTF-A and MRTF-B. Empty vector control lane demonstrates detection of endogenous MRTF-B. **(C)** MRTF immunostaining of primary EPDCs isolated from the following embryos and cultured in 10% FBS to elicit nuclear accumulation: Control (*Wt1*$^{+/+}$; *Mrtfa*$^{+/+}$; *Mrtfb*$^{/fl}$); MRTF-A^{KO} (*Wt1*$^{+/+}$; *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$); MRTF-B^{KO} (*Wt1*$^{CreERT2/+}$; *Mrtfa*$^{+/+}$; *Mrtfb*$^{fl/fl}$); and MRTF-A/B^{KO} (*Wt1*$^{CreERT2/+}$; *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$). Embryos were isolated from pregnant dams gavaged with 4-hydroxytamoxifen at E9.5 and E10.5. Scale bar: 50 μm. **(D)** E15.5 hearts were collected from wildtype C57BL/6 (Control) or MRTF^{epiDKO} embryos. Global deletion of MRTF-A and epicardial deletion of MRTF–B leads to attenuation of MRTF protein levels, particularly in the epicardium and sub-epicardium as demonstrated by immunostaining with an anti-MRTF antibody (green). White box in upper panel (10X magnification, scale bar: 250 μm) identifies area shown in lower panel (80X magnification, scale bar: 20 μm). **(E, F)** Expression of MRTFs in E15.5 (E) and E17.5 (F) hearts. Arrows mark epicardium, arrowheads mark interstitial cells, and asterisks mark vessels. White box in left panel (10X magnification, scale bar: 250 μm) identifies area shown in right panel (80X magnification, scale bar: 20 μm). v, ventricle.

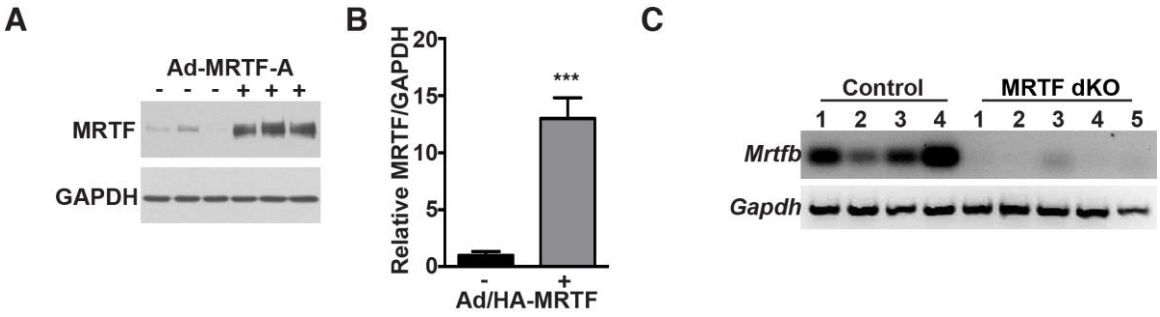

Fig. S2. Modulation of MRTF expression levels

(**A**) Western blot analysis for endogenous MRTF protein in EMCs transduced with adenoviruses expressing HA-epitope tagged MRTF-A (Ad/MRTF-A) or βgal (Control). Control lanes demonstrate detection of endogenous MRTF. **(B)** Quantification of (A). (**C**) EPDCs isolated from *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$ embryos were transduced with Ad/βgal (Control) or Ad/Cre (MRTF dKO). RT-PCR confirms efficient Cre-dependent *Mrtfb* deletion in 4-5 independent samples.

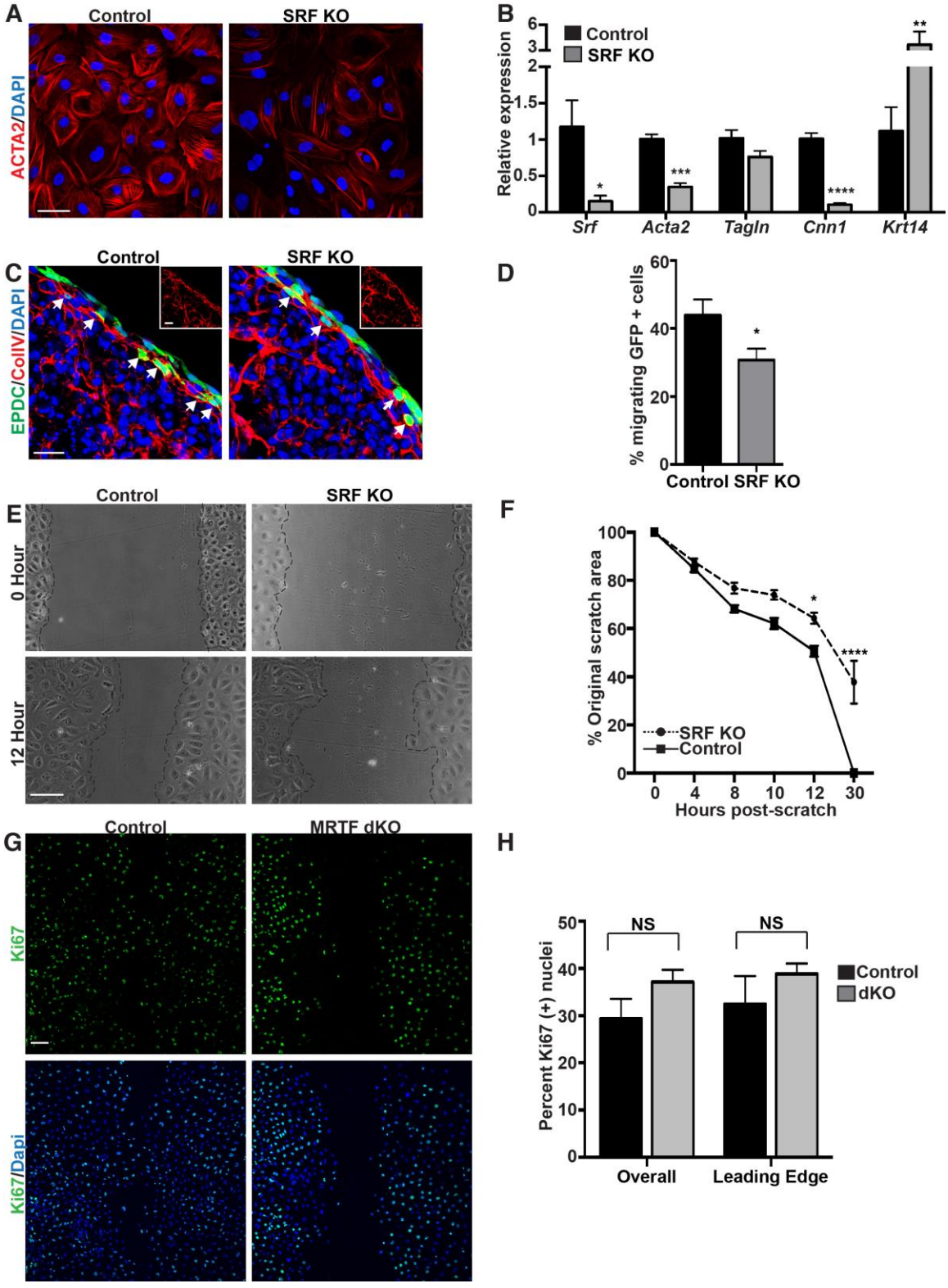

Fig. S3. SRF/MRTFs are essential for epicardial cell migration

(A, B) ACTA2 immunostaining (A) and qPCR analysis (B) of EPDCs isolated from *Srf*$^{fl/fl}$ embryos and transduced with Ad/βgal (Control) or Ad/Cre (SRF KO). n = 7

(Control) and $n = 9$ (SRF KO). **(C, D)** Hearts from E12.5 $Srf^{fl/fl}$ embryos explanted and co-transduced with Ad/GFP to label the epicardium and Ad/βgal (Control) Ad/Cre (SRF KO). (C) Immunostaining for GFP (green) and ColIV (red) reveals reduced migration of SRF KO EPDCs (arrows) across the basement membrane. Migration was quantified and presented as a percentage of all GFP positive cells (D). (**E, F**) Scratch assay on EPDCs isolated from $Srf^{fl/fl}$ embryos transduced with Ad/βgal (Control) or Ad/Cre (SRF KO). Images were captured at the indicated time points (E) and migration was quantified as a percent of original scratch area (F). Statistical differences in (F) were determined using a two-way ANOVA and Tukey post-hoc test. ($n = 4$ for each condition). (**G, H**) Immunostaining for Ki-67 on EPDCs isolated from $Mrtfa^{-/-}$; $Mrtfb^{fl/fl}$ embryos and transduced with control or Cre adenoviruses. Images were captured 16 hours after scratch and quantified as percent Ki-67 positive nuclei at the leading edge of migration or throughout the explant (H). Data are presented as the mean±s.e.m. ($n = 5$ (Control), and $n = 7$ (dKO), data represent at least two independent experiments). NS, not significant using a two-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$. Scale bars: 25 μm (A, C) or 100 μm (E, G).

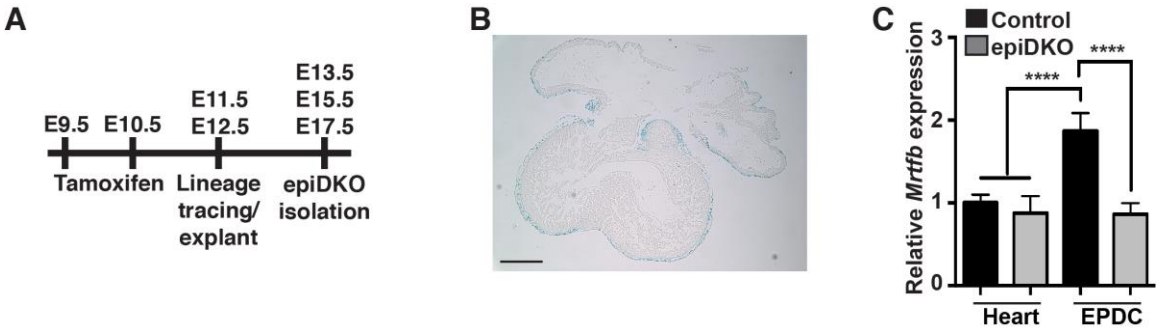

Fig. S4. Epicardium-specific recombination with WT1^{CreERT2}

(A) Experimental timeline for generating and analyzing MRTF^{epiDKO} embryos. **(B)** β-gal expression in E12.5 *Wt1*$^{Cre/ERT2}$;*Rosa*^{lacZ} heart. **(C)** qPCR analysis confirms *Mrtfb* deletion is restricted to the epicardium of E11.5 MRTF^{epiDKO} hearts. [$n = 3$ (Control) and $n = 5$ (MRTF^{epiDKO})]. **** $P < 0.0001$.

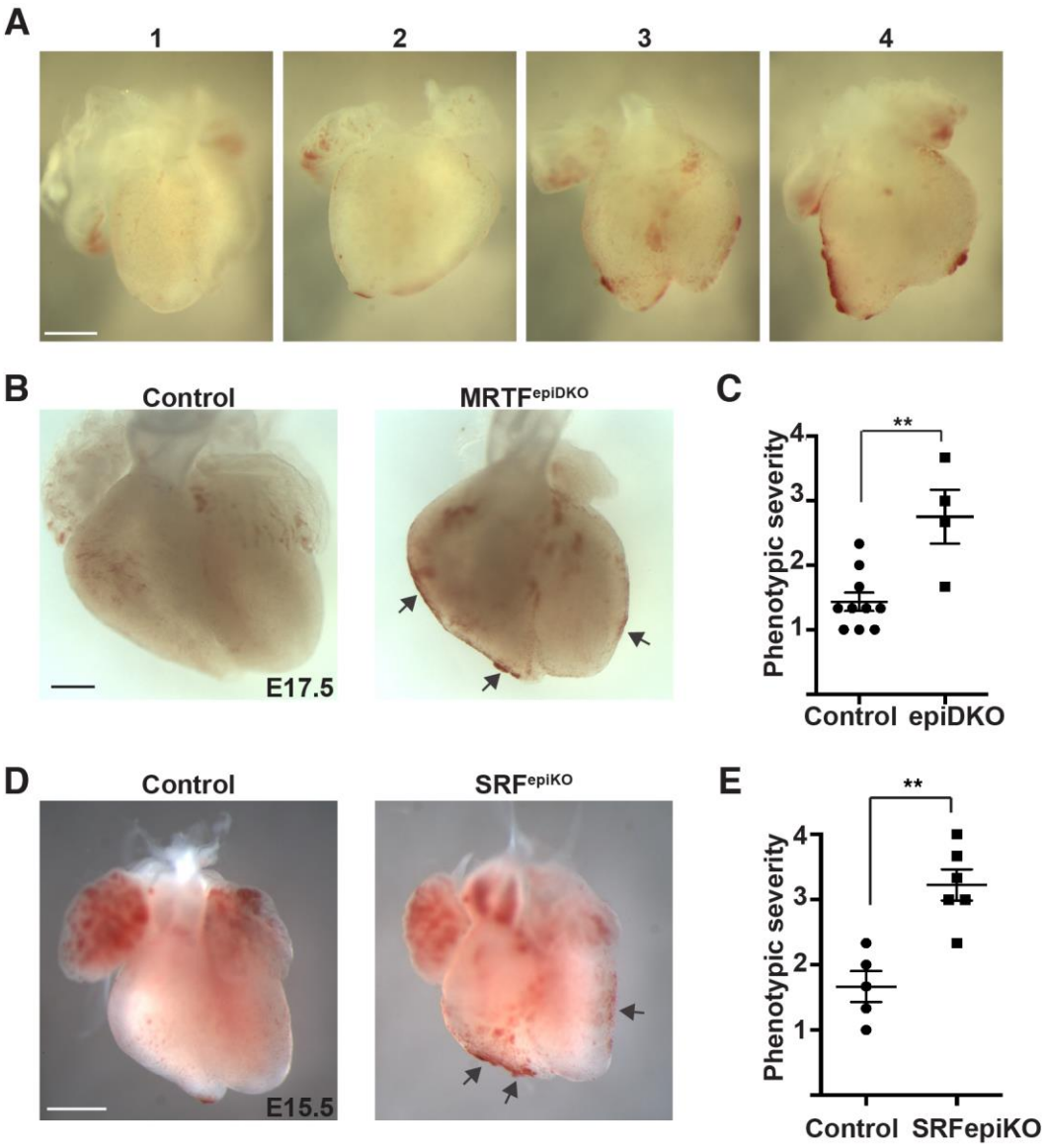

Fig. S5. Sub-epicardial hemorrhage phenotype in MRTF^{epiDKO} and SRF^{epiKO}

(A) Range of sub-epicardial hemorrhaging displayed by E15.5 MRTF^{epiDKO} hearts, "4" being the most severe. This phenotypic scale was used by three blinded reviewers to score hearts. (**B**) Gross morphology of E17.5 MRTF^{epiDKO} hearts display sub-epicardial hemorrhaging, indicated by arrows. **(C)** Hemorrhage severity scored at E17.5. [n = 11 (Control), and n = 4 (MRTF^{epiDKO})]. (**D**) Gross morphology of E15.5-E16.5 hearts isolated from *Wt1*$^{CreBAC/+}$;*Srf*$^{fl/fl}$ (SRF^{epiKO}) and *Wt1*$^{+/+}$;*Srf*$^{fl/fl}$ (Control)

embryos demonstrating a sub-epicardial hemorrhage phenotype in SRF^{epiKO}. (**E**). Quantification of hemorrhage phenotype. All data are presented as the mean ± s.e.m. (n = 5 (Control), and n = 6 (SRF^{epiKO}), data represent the combined results of three independent litters). $^{**}P < 0.01$ using a Mann Whitney test. Scale bars: 500 μm.

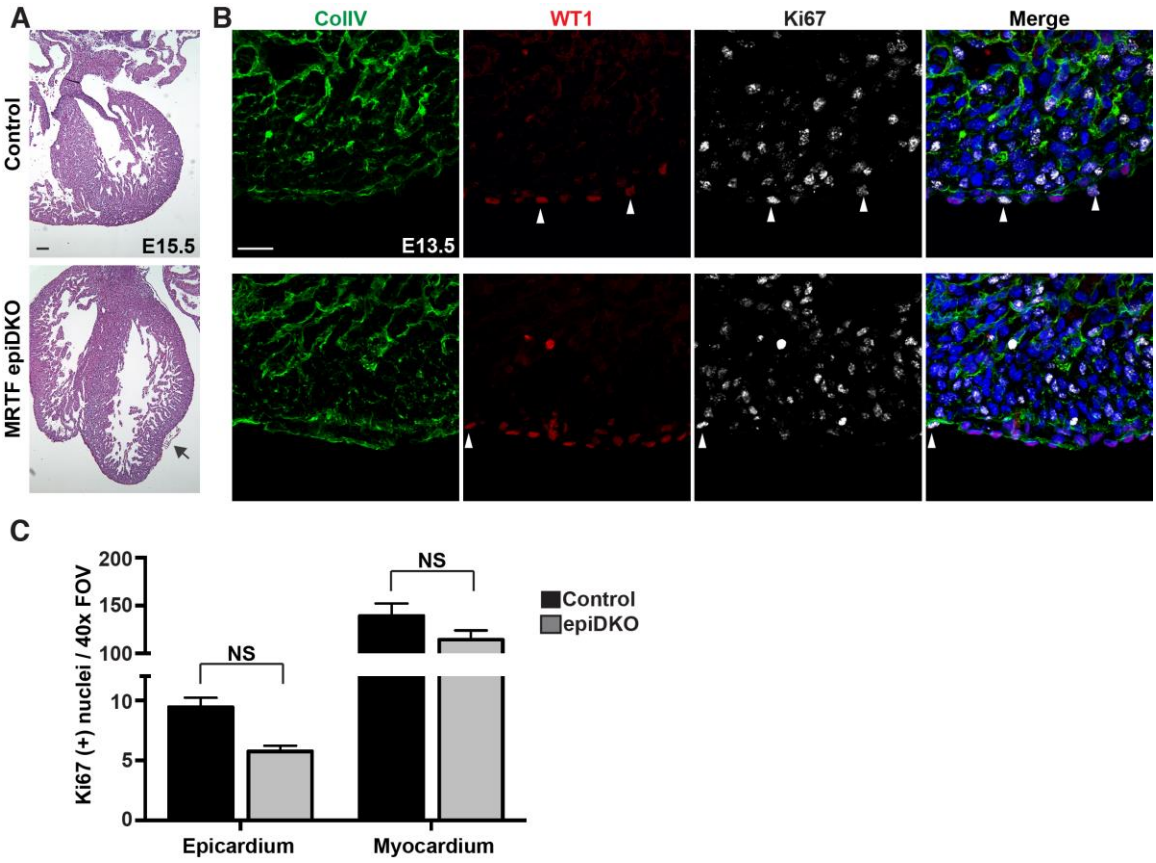

Fig. S6. Epicardial adherence and proliferation defects in MRTF^{epiDKO}

(**A**) H&E stain depicting epicardial dissociation (arrow) from the underlying myocardium in E15.5 MRTF^{epiDKO} hearts. (**B**) Immunostaining for ColIV (green), WT1 (red), and Ki-67 (white) reveals cell proliferation at E13.5. Merged image includes Dapi staining of nuclei. (**C**) Ki-67 positive nuclei per 40X field of view were quantified within the epicardium and myocardium. Data are presented as the mean ± s.e.m. (n = 4 (Control) and n = 8 (MRTF^{epiDKO}), data represent the combined results of two independent litters). NS, not significant using a two-way ANOVA. Scale bars: 100 μm (A) or 25 μm (B).

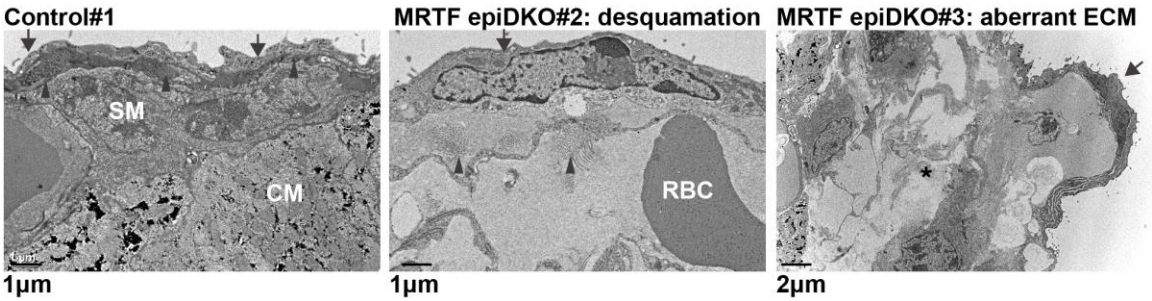

Fig. S7. Ultrastructural analysis of epicardium

TEM of E15.5 control and MRTF^{epiDKO} hearts. Ultrastructure reveals an organized epithelial layer (arrows) and closely associated basal lamina (arrowheads) in the control heart. MRTF^{epiDKO} hearts display desquamation from the subjacent myocardium, epicardial detachment, and an accumulation of disorganized ECM (asterisk) underlying the epicardium. [n = 2 (Control) and n = 3 (MRTF^{epiDKO})]. CM, cardiac myocyte; RBC, red blood cell; SM, sub-epicardial mesenchymal cell.

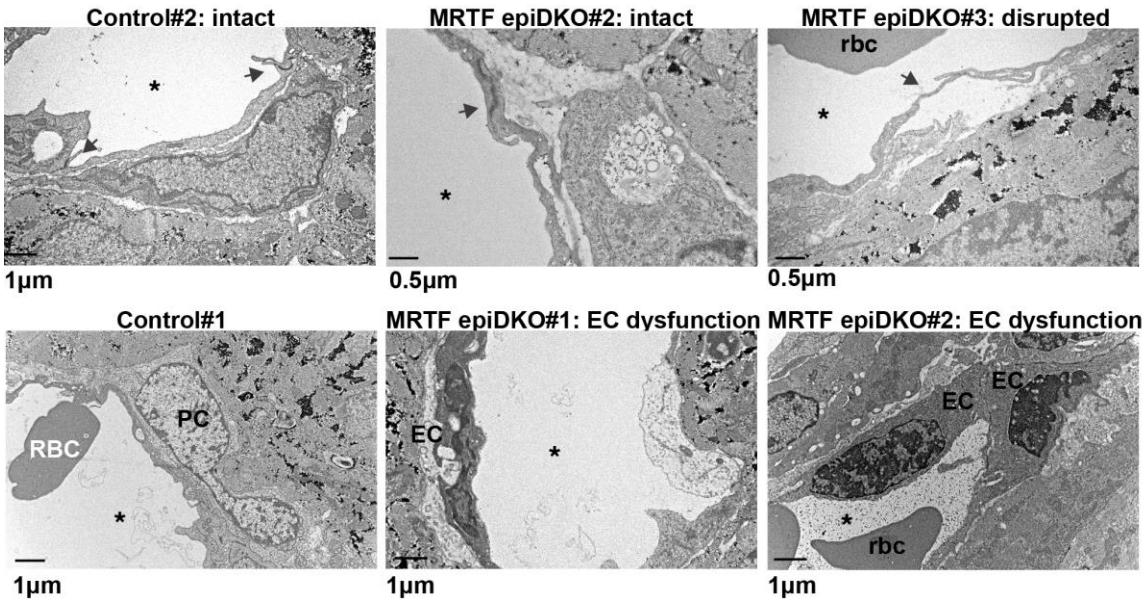

Fig. S8. Ultrastructural analysis of sub-epicardial microvessels

TEM of E15.5 control and MRTF^{epiDKO} hearts. Control hearts display intact vessels with electron dense tight junctions (arrows) between endothelial cells. Some MRTF^{epiDKO} vessels also have intact tight junctions, whereas others display disrupted junctional integrity and occasional EC dysfunction with electron dense chromatin. [n = 2 (Control) and n = 3 (MRTF^{epiDKO})]. EC, endothelial cell; PC, pericyte; RBC, red blood cell; asterick denotes lumen.

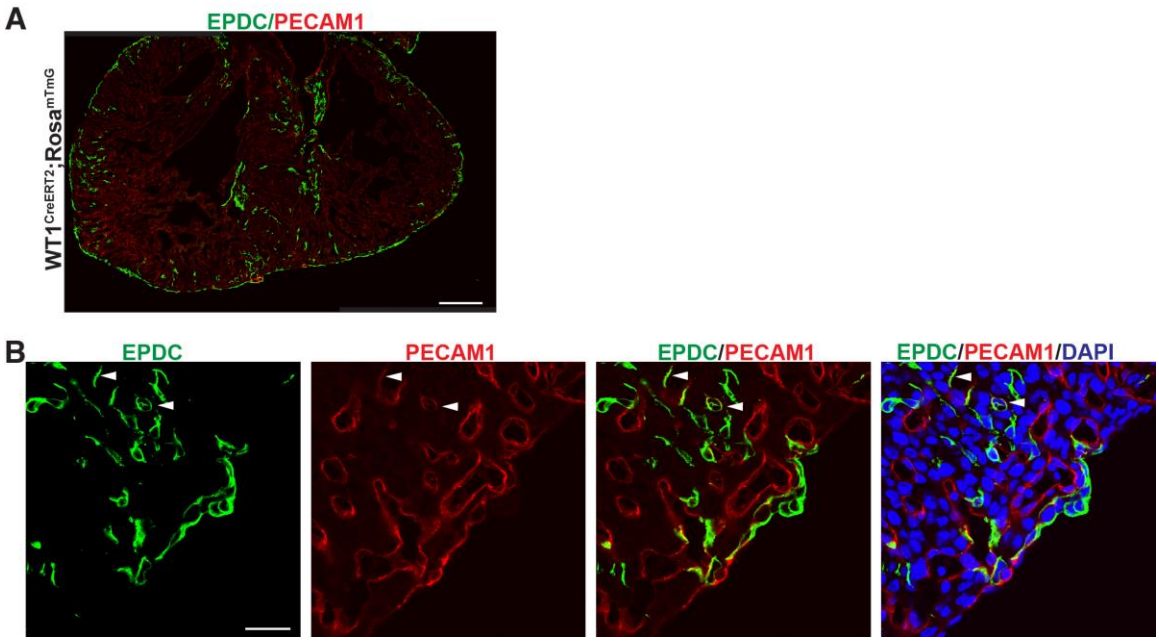

Fig. S9. WT^{CreERT2}; Rosa^{mTmG} lineage tracing

(A) Detection of the epicardium-derived lineage in *Wt1*^{CreERT2};*Rosa*^{mTmG} hearts at E15.5 with an anti-GFP antibody (green) following tamoxifen administration at E9.5 and E10.5. GFP positive cells are present in the epicardium and descendants of the epicardium in the sub-epicardium, compact myocardium and interventricular septum. Co-staining for PECAM1 (red) identifies ECs. Image is a composite of two separate 10X images. Scale bar: 200 μm. **(B)** 80X magnification of a representative heart demonstrates EPDC in close apposition with, but generally distinct from, PECAM1-positive ECs. Arrowheads denote two ECs that co-stain with GFP. Scale bars: 50 μm (A) and 25 μm (B).

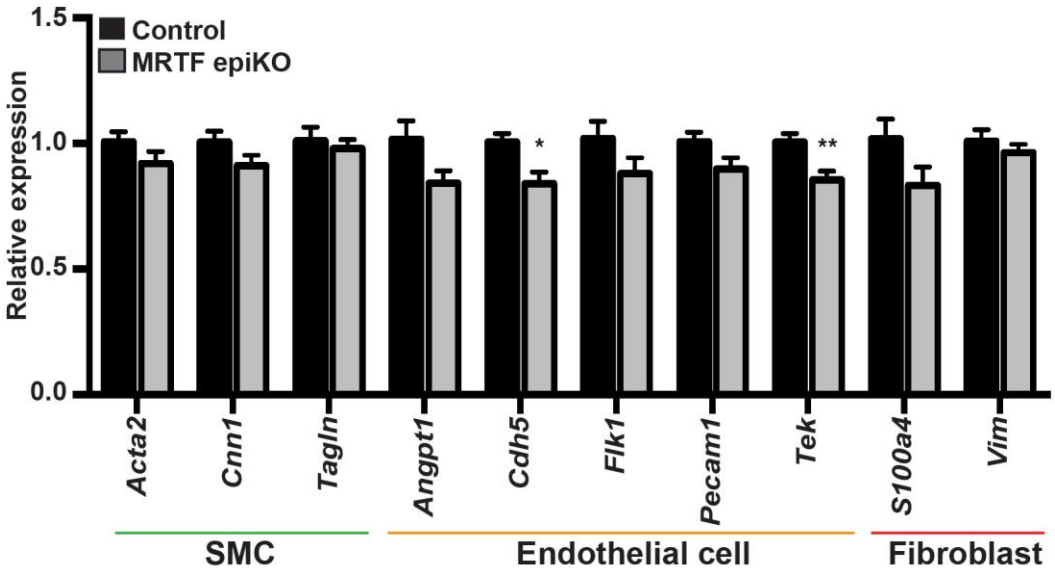

Fig. S10. Gene expression analysis in E15.5 MRTF^{epiDKO} hearts

qPCR analysis of SMC, endothelial cell and fibroblast markers in whole ventricles isolated from control and MRTF^{epiDKO} embryos at E15.5. Data are presented as the mean ± s.e.m. (n = 9 (Control) and n = 14 (epiDKO), data represent the combined results of three independent litters). * P <0.05; ** P < 0.01.

Supplemental Tables

Table S1. Progeny from MRTF^{epiDKO} intercrosses

Embryos were isolated at the indicated times from *Wt1*$^{+/+}$; *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$ dams crossed to *Wt1*$^{CreERT2/+}$; *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$ sires following tamoxifen administration of the dam at E9.5 and E10.5.

Age	*Wt1*$^{CreERT2/+}$; *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$	*Wt1*$^{+/+}$; *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$	Total
E11.5	19 (49%)	20 (51%)	39
E13.5	37 (60%)	25 (40%)	62
E15.5	48 (61%)	31 (39%)	79
E17.5	10 (34%)$^{\Psi}$	19 (66%)$^{\delta}$	29

Data are presented as number (percentage).
Observed frequencies were not significantly different from the expected frequencies by Chi-square analysis.
$^{\Psi}$one or $^{\delta}$two additional resorbing embryos were recovered.

Table S2. Progeny from MRTF^{BACdKO} intercrosses

Survival of pups was monitored at the indicated times from *Wt1*$^{+/+}$; *Mrtfa*$^{+/-}$; *Mrtfb*$^{fl/fl}$ dams crossed to *Wt1*$^{CreBAC/+}$; *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$ sires.

Age	***Wt1*$^{CreBAC/+}$; *Mrtfa*$^{-/-}$; *Mrtfb*$^{fl/fl}$**	***Wt1*$^{CreBAC/+}$; *Mrtfa*$^{+/-}$ *Mrtfb*$^{fl/fl}$**	***Wt1*$^{+/+}$ *Mrtfa*$^{-/-}$ *Mrtfb*$^{fl/fl}$**	***Wt1*$^{+/+}$ *Mrtfa*$^{+/-}$ *Mrtfb*$^{fl/fl}$**	**Total**
P0	16 (19%)	25 (29%)	18 (21%)	26 (31%)	85
P1	12 (15%)$^{\Psi}$	25 (31%)	18 (22%)	26 (32%)	81
P2	11 (13%)$^{\delta}$	25 (32%)	18 (23%)	25 (32%)$^{\delta}$	79
P3	11 (13%)	25 (32%)	18 (23%)	25 (32%)	79
P4	11 (14%)	24 (31%)$^{\delta}$	18 (24%)	24 (31%)$^{\delta}$	77
P51	11 (14%)	24 (32%)	18 (24%)	23 (30%) $^{\delta}$	76

Data are presented as number (percentage).
Observed frequencies were not significantly different from the expected frequencies by Chi-square analysis.
P=0.0113 by Logrank test of survival proportions.
$^{\Psi}$four or $^{\delta}$one pups were recovered dead.

Table S3. Antibodies used for immunostaining

Information regarding antibodies used for immunostaining, including antigen, source, host, dilution, and staining method.

Antigen	Source	Host	Dilution	Protocol
ACTA2	Sigma (Clone 1A4, C6198)	Mouse	1:200	Cell culture
COLIV	Millipore (AB756P)	Rabbit	1:200	Cryosection
CSPG4	Millipore (AB5320)	Rabbit	1:150	Cryosection
GFP	AbCam (ab13970)	Chicken	1:400	Cryosection
HA	Cell Signaling (3724)	Rabbit	1:200	Cell culture
Ki-67	DAKO (clone TEC-3, M7249)	Rat	1:300	Cell culture
			1:1,000	Paraffin
MRTF	Kind gift from Guido Posern	Rabbit	1:100	Paraffin
			1:200	Cell culture
PECAM1	BD Pharmingen (550274)	Rat	1:200	Cryosection
PDGFRβ	R&D Systems (AF1042)	Goat	1:200	Cryosection
TnnT2	Thermo Fisher (MS-295)	Mouse	1:100	Paraffin
VCL	AbCam (ab11194)	Mouse	1:150	Cell Culture
WT1	Thermo Fisher (MS-1837)	Mouse	1:100	Paraffin
ZO1	Invitrogen (402200)	Rabbit	1:200	Cell culture

Table S4. Primers used in RT-PCR studies

Sequence of forward and reverse primers and PCR conditions used in standard and quantitative RT-PCR reactions.

Gene	Forward	Reverse
Acta2	GTTCAGTGGTGCCTCTGTCA	ACTGGGACGACATGGAAAAG
Angpt1	CATCAGCTCAATCCTCAGC	GGGGGAGGTTGGACAGTAA
Cdh5	CAGCAACTTCACCCTCATAAAC	TCCCGATTAAACTGCCCATAC
Cnn1	GAAGGTCAATGAGTCAACTCAGAA	CCATACTTGGTAATGGCTTTGA
Col1a2	AGCAGGTCCTTGGAAACCTT	AAGGAGTTTCATCTGGCCCT
Col3a1	TAGGACTGACCAAGGTGGCT	GGAACCTGGTTTCTTCTCACC
Cspg4	TCTTACCTTGGCCTTGTTGG	ATGTGGAGAACTGGAGCAGC
Flk1	TCTGTGGTTCTGCGTGGAGA	GTATCATTTCCAACCACCCT
Fn1	AGACCTGGGAAAAGCCCTACCAA	ACTGAAGCAGGTTTCCTCGGTTGT
Gapdh†	CGTGCCGCCTGGAGAAAC	TGGGAGTTGCTGTTGAAGTCG
Krt14	ATCGAGGACCTGAAGAGCAA	TCGATCTGCAGGAGGACATT
Mrtfa	ACGAGGCGGTTACCATCAC	GCAGACAGAGACAGGAGCAC
Mrtfb†	TCCCGTGCTCCCTACAA	CGGTGTTTGTCGTTTGGATTC
Myh7	GTGGCTCCGAGAAAGGAAG	GAGCCTTGGATTCTCAAACG
Mylk	AAAAACCGTCTGGACTGCAC	TCACAGCATTGCCCGTTTTC
Myocd	AAGGTCCATTCCAACTGCTC	CCATCTCTACTGCTGTCATCC
Nkx2-5	GACGTAGCCTGGTGTCTCG	GTGTGGAATCCGTCGAAAGT
Pdgfrβ	GGGAGACACTGGGGAATACTTTTG	TGAACAGGTCCTCGGAGTCCATAG
Pecam1	TGGTTGTCATTGGAGTGGTC	TTCTCGCTGTTGGAGTTCAG
Rock1	AAGCTTTTGTTGGCAATCAGC	AACTTTCCTGCAAGCTTTTATCCA
S100a4	CTTCCTCTCTCTTGGTCTGGTC	TTTGTGGAAGGTGGACACAA
Snai1	CTTGTGTCTGCACGACCTGT	AGGAGAATGGCTTCTCACCA
Snai2	CATTGCCTTGTGTCTGCAAG	CAGTGAGGGCAAGAGAAAGG
Srf	CCACCACAGACCAGAGAATGAG	TCTTGAGCACAGTCCCGTTG
Tagln	GACTGCACTTCTCGGCTCAT	CCGAAGCTACTCTCCTTCCA
Tek	ACCTCCAGTGGATCTTGGTG	TGGAGTCAGCTTGCTCCTTT
Tcf21	CATTCACCCAGTCAACCTGA	CCACTTCCTTCAGGTCATTCTC
Tmsb4x	ATGTCTGACAAACCCGATATGGC	CCAGCTTGCTTCTCTTGTTCA
Tpm1	GAAGCCTCATGAGAACAGAACCA	CTTCCTGCTGATCCCACCAT
Twist1	CGGACAAGCTGAGCAAGAT	GGACCTGGTACAGGAAGTCG
Vcl	AACCAGCCAATGATGATGGC	TTGGCTGCTGCAATGATGTC
Vim	GACATTGAGATCGCCACCTA	GGCAGAGAAATCCTGCTCTC
Wt1	ATCCGCAACCAAGGATACAG	GGTCCTCGTGTTTGAAGGAA

qPCR parameters: denature 95°C, 10 sec; annealing 60°C, 30 sec; 50x cycles.
†RT-PCR parameters: 95°C,3 min; 95ºC,30 sec; 52ºC, 30 sec; 72ºC, 30 sec; 72ºC, 5 min 35x cycles.