Single cell sequencing reveals endothelial plasticity with transient mesenchymal activation after myocardial infarction

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Endothelial cells play a critical role in the adaptation of tissues to injury. Tissue ischemia induced by infarction leads to profound changes in endothelial cell functions and can induce transition to a mesenchymal state. Here we explore the kinetics and individual cellular responses of endothelial cells after myocardial infarction by using single cell RNA sequencing. This study demonstrates a time dependent switch in endothelial cell proliferation and inflammation associated with transient changes in metabolic gene signatures. Trajectory analysis reveals that the majority of endothelial cells 3 to 7 days after myocardial infarction acquire a transient state, characterized by mesenchymal gene expression, which returns to baseline 14 days after injury. Lineage tracing, using the Cdh5-CreERT2;mT/mG mice followed by single cell RNA sequencing, confirms the transient mesenchymal transition and reveals additional hypoxic and inflammatory signatures of endothelial cells during early and late states after injury. These data suggest that endothelial cells undergo a transient mesenchymal activation concomitant with a metabolic adaptation within the first days after myocardial infarction but do not acquire a long-term mesenchymal fate. This mesenchymal activation may facilitate endothelial cell migration and clonal expansion to regenerate the vascular network.

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the endothelium forms the inner layer of blood vessels and shows remarkable plasticity and heterogeneity in structure and function, in health and disease. Endothelial cells (ECs) form a vascular plexus during early development and differentiate into arterial, venous or lymphatic fates to establish a circulatory network. At later stages, endothelial cells acquire organ specific properties essential for maintaining the function of the individual organs. Certain ECs within the endocardium undergo a process named endothelial-mesenchymal transition (EndMT), to give rise to mesenchymal cells which are necessary for proper heart and valve development. Transitions of endothelial cells to hematopoietic cells also occurs in embryonic development.

Throughout life, endothelial cells are exposed to various mechanical, inflammatory, and metabolic environments. Phenotypic changes are facilitated by high low-density lipoprotein (LDL) cholesterol, a pro-inflammatory state and turbulent flow patterns leading to endothelial activation. This contributes to the impairment of the vasodilatory activity (so called “endothelial dysfunction”), which plays a key role in the development of atherosclerotic lesions. Switches in endothelial phenotypes additionally occur during blood vessel growth, which involves a critically fine-tuned and transient specialization of endothelial cells. Endothelial “tip” cells migrate at the forefront and are followed by “stalk” cells, which elongate the sprout. The tip-stalk cell phenotype dynamically switches under control of vascular endothelial growth factor (VEGF) and Notch and involves metabolic reprogramming.

Recent single-cell RNA sequencing studies provide further insights into the heterogenic gene expression signatures of ECs in tumors, leading to the identification of metabolic plasticity and collagen modification as potential critical angiogenic players. Under pathological pro-inflammatory conditions associated with high TGF-β2 levels, endothelial cells can undergo EndMT in adulthood and myocardial fibrosis after cardiac stress or injury. However, recent fibroblast lineage tracings analyses suggest that the overall contribution of endothelial cells to the cardiac fibroblast population is limited. A partial mesenchymal transition or mesenchymal activation may also contribute to (patho)physiological vessel growth by facilitating a pro-migratory and pro-invasive EC phenotype. In support of a role in mesenchymal activation of ECs in vessel growth, we recently demonstrated that clonally expanding ECs in ischemic tissue express mesenchymal markers. However, the molecular signatures and plasticity of endothelial cells in response to ischemia in vivo are not well established. Here we show the adaptive responses of ECs to cardiac ischemia by using single-cell technology, a powerful tool capable of deciphering individual cellular responses and transcriptional signatures within tissue.

Results
Kinetics of EC inflammation, survival, and proliferation. Single-cell RNA sequencing of the non-cardiomyocyte fraction of murine hearts was performed at day 0 (homeostasis) and at day 1, 3, 5, 7, 14, 28 post myocardial infarction (MI). This revealed 19 cell clusters including 4 clusters of endothelial cells (demarcated by Cdhd5 and Pecam1 expression), 6 fibroblast clusters, and 7 clusters of hematopoietic cells. Consistent with induction of myocardial damage, total cell numbers declined from day 1 to 7 but controls confirmed that the quality of the sequencing and cell type annotations is comparable in all samples. The relative abundance of endothelial cells and mesenchymal cells declined during the first week after MI, corresponding with an influx of immune cells at early time points. Significantly augmented GO terms in EC clusters at day 1 post MI include the cellular response to hypoxia, positive regulation of inflammatory response, programmed cell death and angiogenesis. Representative genes of these pathways include the apoptosis related genes Bax and Trp53, the hypoxia induced factors Hif1α and Ldha, as well as the inflammatory cytokines Il1b, Il6, and Tnf.

Between day 1 and 7, genes associated with epithelial mesenchymal transition, extracellular matrix organization and cellular proliferation were significantly enriched. The increase in EC proliferation was confirmed by bioinformatic analysis of cell cycle phases showing an increase in expression of cell cycle genes and number of ECs in S-phase at day 3, which is returned to homeostatic levels at day 14 after MI. Phospho histone H3 staining and published data confirm an increased proliferation of endothelial cells at day 3. Interestingly, the cellular response to nitric oxide was repressed between days 1 and 14.

ECs acquire a transient state of mesenchymal transition after MI. Next we used single-cell trajectory analysis to identify in an unbiased manner the different states which ECs acquired after MI. We could identify 5 states that are populated by Cdhd5 and Pecam1 expressing ECs. MI induced an increase in ECs associated with state 3, which is a predominantly pro-inflammatory state characterized by pathways involved in neutrophil activation, inflammatory response and cytokine signaling. Specific genes enriched in state 4 include mesenchymal genes, cell cycle and proliferative genes, whereas transcripts involved in fatty acid up-take and signaling were down-regulated. The expression of these genes resembles the signature of EndMT or ECs undergoing mesenchymal activation. Representative upregulated genes of state 4 including Col3a1, Fln1, Serpine1 and others as shown in Fig. 2c. Endothelial marker genes, such as Cdhd5 were down-regulated (Fig. 2c) in state 4. The induction of mesenchymal markers at day 3 after MI was confirmed by bulk RNA sequencing of isolated Cdhd5+ cells. Interestingly, cell numbers in state 4 were only increased between days 1 and 7 and returned to baseline levels after 14 days (Fig. 2a). A similar kinetic was observed when analyzing the total EC population (clusters 6, 7, 17, and 18) for changes in EC or mesenchymal marker expression across MI time points. This suggests that EC acquire a transient mesenchymal activation (EndMA) state in the first week after MI.

Using a different bioinformatic analysis strategy by re-clustering the EC population, cells were separated into ECs lacking mesenchymal markers, lymphatic ECs (cluster 9), and ECs expressing mesenchymal genes (cluster 4–6; Supplementary Fig. 4a, b). We observed a similar loss of endothelial gene expression and gain of mesenchymal genes when excluding lymphatic ECs and the other non-EC cluster 11 and 12 from the analysis. At early time points (d1–d7), ECs dominantly populated in clusters with mesenchymal genes and constitute the majority of EndMA cells. Moreover, this population showed more cells in state 4 of the trajectory analysis (Supplementary Fig. 4e–g), confirming the transient nature of EndMA. Consistently, mesenchymal protein expression in endothelial cells at days 3–7 after infarction was induced as assessed by immunostaining (Supplementary Fig. 5 and Supplementary Fig. 6a, b) and FACS analysis (Supplementary Fig. 6c). Furthermore, we exclude the possibility that EndMA cells may be simply removed by cell death, since we could not find Caspase3-positive endothelial cells expressing mesenchymal marker (Sm22).
at d3 and d7 after infarction (Supplementary Fig. 7a) or enrichment for apoptosis specific gene signatures in EndMA cells (Supplementary Fig. 7b).

To provide further evidence that mesenchymal cells can revert to an endothelial cell phenotype, we performed collagen-tracing studies in Col1a2-CreERT2;mT/mG mice, traced at day 3 to day 7 after infarction (Supplementary Fig. 8a). Then we performed single-cell RNA sequencing and histology at day 28. Indeed, GFP+ cells were detectable in the endothelial cluster (Supplementary Fig. 8b), demonstrating that Col1a2 traced cells can become transcriptionally similar to endothelial cells between day 7 and day 28 after infarction. These results were confirmed by histology showing GFP expressing cells, which co-express the endothelial marker Isolectin B4 (Supplementary Fig. 8c).
To test if mesenchymal activation may contribute to endothelial proliferation and expansion, we induced mesenchymal transition with the TGF-β-inhibitor Galunisertib (LY2157299) 23. We then assessed clonal expansion of ECs after MI using Confetti mice as previously described 29. TGF-β-inhibitor treatment reduced the incidence of clonal expansion in Confetti mice 7 days after MI in the border zone by 79.1% compared to infarcted controls (p = 0.03; Supplementary Fig. 9), suggesting that mesenchymal activation contributes to endothelial expansion.

Characterization of EndMA cells. To further characterize the ECs undergoing EndMA, we compared the transcriptome of endothelial cells, which express mesenchymal markers, to endothelial cells lacking the expression of these marker genes. As expected, EndMA cells showed a high expression of mesenchymal and extracellular matrix genes (Supplementary Fig. 3a), enriched expression of genes associated with epithelial mesenchymal expression (Supplementary Fig. 10a, b) and GO terms associated with extracellular matrix organization, Pdgf binding, collagen synthesis, and organization (Fig. 3b). In contrast, ECs which are not undergoing EndMA showed high expression of genes involved in fatty acid signaling, ECs enriched transcription factors and GO terms associated with VEGF and VEGF-receptor response, carbohydrate transport, Wnt signaling, vascular development, and negative regulation of EC proliferation (Fig. 3a, b). The expression of metabolism-associated genes differs between EndMA positive and negative ECs. Since EC metabolism is known to play a critical role in endothelial plasticity and EndMT 24, we performed a detailed analysis of the gene expression signatures related to glycolysis, citric acid cycle, glutamine, pentose phosphate pathway and fatty acid signaling (Fig. 3c). EndMA cells showed a high expression of glycolysis genes (Fig. 3c and Supplementary Fig. 10a), whereas the expression of fatty acid signaling, and tricarboxylic acid (TCA) cycle genes was reduced compared to ECs, which do not express mesenchymal markers (Fig. 3c), suggesting a metabolic switch from fatty acid towards a glycolytic metabolism during EndMA. Interestingly, analysis of the time course after MI demonstrates a transient switch of EC metabolism during days 1–3. Early induction of glycolysis genes at day 1 and a reduction of fatty acid signaling associated genes, such as Fabp4 at day 3 (Fig. 3d and Supplementary Fig. 11), documented an adaptation of EC metabolism during the time frame in which mesenchymal activation occurs. In vitro studies confirmed the induction of glycolysis upon TGF-β2-stimulation of endothelial cells (Supplementary Fig. 12a). Interestingly, carbon-13 glucose tracing confirmed the increasing utilization of glucose for glycolysis without effects on glucose uptake (Supplementary Fig. 12b).

EndMA may not only change intrinsic cell functions but also may affect cell-to-cell communication. To explore whether EndMA affects the interaction of ECs with other cells in the heart, we used bioinformatic assessment of ligand-receptor relations 25. Weighted analysis of such interactions revealed that EndMA cells showed higher ligand-interaction relations compared to EndMA negative cells (Supplementary Fig. 13a, b). EndMA cells showed increased outgoing signals involved in genes associated with the GO terms of extracellular matrix organization, Pdgf binding, and collagen fibril organization (Supplementary Fig. 13b–d). Analysis of the specific cell communication signatures between the cells revealed that EndMA cells overall have higher outgoing signals, particularly to ECs lacking mesenchymal markers (Supplementary Fig. 13a), suggesting that EndMA may affect the cell-to-cell communication in the heart after MI.

Cdh5-lineage tracing confirms the transient nature of EndMA after MI. Our data suggest that MI induces a transient metabolic and phenotypic switch in endothelial cells associated with EndMA. However, in the absence of lineage tracing, we may have missed ECs which fully transit to a mesenchymal phenotype and lost EC marker gene expression. To characterize the long-term fate of ECs, we used endothelial fate-mapped mT/mG reporter mice (Cdh5-CreERT2mT/mG). Tamoxifen injection led to the expected induction of GFP in endothelial cells (Fig. 4a, b and Supplementary Fig. 14a). Analysis of GO terms associated with GFP+ cells after MI showed an early increase in pathways associated with cell death, response to hypoxia, and proliferation in early stages, as well as GO terms associated with augmented extracellular matrix and epithelial mesenchymal transition at later stages (Supplementary Fig. 14b). Thus, GFP+ cells showed similar gene expression signatures as the non-traced endothelial cells (Fig. 1d), supporting the hypothesis that EndMA is a transient phenomenon.

To determine the kinetics of EndMA, we assessed the expression of mesenchymal markers in GFP+ cells at each timepoint. Interestingly, the number of GFP+ cells expressing the endothelial marker Cdh5 and mesenchymal markers, such as Col1a1, Col3a1, or Serpin1, augmented transiently with a maximum between days 3 and 7 (Fig. 4d). At later timepoints, we did not observe an increase in fully transited mesenchymal GFP+ cells lacking ECs markers (Fig. 4d, right panels).

In addition, we used an unbiased approach to determine the occurrence of GFP-traced EndMA cells after MI. Re-clustering of GFP+ cells led to the identification of a cluster of EndMA resembling cells (cluster 6), which showed a high expression of collagens but low expression of endothelial marker genes (Fig. 4e–g). Interestingly, the proportion of cells in cluster 6...
was higher at day 7 (Fig. 4g), consistent with a transient mesenchymal transition of ECs after MI.

**Mesenchymal transition is reversible in vitro.** Our data suggest that ECs transiently acquire a reversible partial mesenchymal state after MI in vivo. To gain further insights into the reversibility of EndMT in vitro, we incubated ECs with TGF-β2 for 3 days and withdrew the stimulus. After 3 days of TGF-β2 treatment, ECs express the mesenchymal marker genes calponin (CNN1) and SM22 (TAGLN) (Fig. 4h, i, and Supplementary Fig. 15a–c). The withdrawal of TGF-β2 induced the reversion of mesenchymal marker gene expression to baseline levels within 7 days, whereas continuous incubation with TGF-β2 led to a
Fig. 2 Endothelial cells gain mesenchymal markers at day 1–7 after myocardial infarction. a Pseudo-time trajectory analysis of endothelial cells (ECs), using significantly regulated genes between timepoints. Pie charts showing how many cells were assigned to each cluster relative to the total number of cells per timepoint. *(n = 2905 cells, ECs were selected from data shown in Fig. 1b).* b Dot plot showing fold-change (blue to red) of marker genes associated with fatty acid oxidation, mesenchymal identity, and cell cycle proliferation between trajectory states shown in a. Size indicates adjusted p-value for each gene in log10 scale. c Violin plot showing increased values of normalized UMI counts for mesenchymal markers (*Col3a1* and *Serpine1*) and decreased values for endothelial marker (*Cdh5*) in trajectory state 4 (*n = 694 cells*), compared to other states (*n = 2111 cells,* *p = 3.3 × 10−76* (*Col3a1*), *p = 2.6 × 10−27* (*Serpine1*), *p = 1.7 × 10−75* (*Cdh5*), bimodal likelihood-ratio test, Bonferroni adjusted p-values) d EndMT marker show significant upregulation between d1–d7 in ECs compared to baseline (homeostasis). Data is presented in average fold-change to homeostasis, mean ± standard deviation. e Bulk-RNA sequencing analysis of isolated endothelial cells comparing homeostasis (*n = 4*) and d3 after acute myocardial infarction (AMI; *n = 3*). Color indicates column-wise z-score (blue to red). f Heatmap of various endothelial marker, showing reduction of mean UMI levels at d1–d7 after infarction in ECs. Upper panel represents the mean z-score of all four endothelial markers per time point. Data is presented as mean ± SEM. g Comparison of *Pecam1* (endothelial marker) and *Fn1* (mesenchymal marker) levels. Every point indicates an individual cell. Boxes (quadrants, Q1 and Q3) show cells which are expressing the marker strongly (top 50% of all non-zero values). Percentage values are indicative of how many cells per timepoint are grouped in the respective box. Data represent *n = 1* mouse per timepoint, Homeostasis *n = 592 cells*, d1 *n = 71 cells*, d3 *n = 70 cells*, d5 *n = 181 cells*, d7 *n = 277 cells*.

Fig. 3 Activated endothelial cells show distinct transcriptional phenotypes. a Dot plot showing significant change (Bonferroni adjusted *p < 0.05*) of various mesenchymal, metabolism, and transcription factor genes in *Pecam1*/*Cdh5*/*Serpine1*/*Fn1* cells (EndMA+) compared to other endothelial cells (*Pecam1*/*Cdh5*/mesenchymal marker−; EndMA−) cells. Color indicates log-fold change (red to gray), size depicts significance level (log10 p-value). b Top 10 enriched GO-terms ranked by Enrichr’s combined score for genes, significantly upregulated in EndMA+ and EndMA−, respectively. c, d Heatmap showing gene regulation of genes associated with fatty acid (FA) signaling, pentose phosphate pathways (PPP), glutamine metabolism, tricarboxylic acid (TCA) cycle and glycolysis pathway comparing EndMA+, and EndMA− and all other cells in the dataset (c) at the indicated time points in endothelial cells (d). Data is presented in row-wise z-score.
further increase in mesenchymal marker gene expression (Fig. 4h, i). Similar findings were observed when inducing mesenchymal transition by a combination of TGF-β1, IL-1β and the nitric oxide synthase inhibitors (N-Nitroarginine methyl ester; L-NAME) (Supplementary Fig. 15d, e). Notably, mesenchymal gene expression induced by prolonged incubation with TGF-β2 for up to 10 days was reversed by replacement of the stimulus to control medium without TGF-β2 (Supplementary Fig. 15f, g). The reversible nature of EndMA was further supported by the coinciding metabolic adaptation of endothelial cells. TGF-β2 treatment induced the upregulation of glucose metabolism, which was fully reversed upon withdrawal to control medium (Supplementary Fig. 12b). To exclude that mesenchymal marker expressing endothelial cells are outcompeted by remaining endothelial cells,
we additionally treated TGF-β2 stimulated cells with a cell cycle inhibitor and assessed the effect on the reversibility. However, cell cycle inhibition did not affect reversibility of EndMT (Supplementary Fig. 16).

To assess if TGF-β2 treatment leads to a true fate change, we analyzed DNA methylation of mesenchymal genes. We did not observe changes in DNA methylation levels in gene bodies within the mesenchymal genes COL4A1, TAGLN, and CNN1 after TGF-β2 stimulation and withdrawal (Supplementary Fig. 17a–c). However, we observed a reversible demethylation of regulatory regions upon TGF-β2 stimulation (Supplementary Fig. 17d). These observations confirm that mesenchymal gene expression induced by several different protocols is reversible and is associated with changes in DNA methylation of regulatory regions.

Discussion

The presented study provides a detailed analysis of EC gene expression signatures after cardiac ischemia. We demonstrate that ECs undergo a transient mesenchymal activation that is associated with profound metabolic adaptations (Supplementary Fig. 18). The transient nature of mesenchymal gene expression in endothelial cells was supported by Cdhs5-lineage tracing studies, which demonstrated that Cdhs5-traced cells transiently express transcripts typically associated with EndMT. Mesenchymal transcript expression in Cdhs5-traced cells returns to levels detected in homeostasis condition after more than 10 days post-myocardial infarction. Interestingly, the majority of mesenchymal gene expressing GFP+ traced cells retain a low expression of endothelial genes (Fig. 4d), suggesting that they undergo a transient EndMA rather than full EndMT. In support of a partial transition, our data show that most EndMA cells express modest levels of mesenchymal marker genes in comparison to bona fide fibroblasts (Supplementary Fig. 19). Consistent with our data demonstrating that only few GFP-traced cells fully lose their endothelial signature and express high levels of mesenchymal genes, recent single-cell RNA sequencing studies also failed to detect significant numbers of cells undergoing a full mesenchymal transition after myocardial infarction26. Initial studies have imposed the concept, that the conversion of endothelial cells to mesenchymal cells was a permanent shift between two differentiated cellular states. However, in the context of cardiac ischemia our study suggests that EndMA likely represents a reversible continuum in response to a hypoxic and inflammatory injury environment, instead of a differentiation process in its classical sense. This model is consistent with our in vitro study clearly documenting a reversible nature of EndMA after removal of EndMT promoting stimuli. Interestingly, similar findings on endothelial cells were already reported in 1992, demonstrating that withdrawal of TGF-β1 after 10 days incubation resulted in the re-appearance of polygonal cells with an endothelial phenotype27. However, the mechanisms by which this switch back to an endothelial phenotype occurs and if this includes epigenetic changes is unclear. Our in vitro experiments utilizing endothelial cells did not show changes in DNA methylation patterns at promoters of mesenchymal genes after TGF-β2 stimulation, suggesting that a true transition to a mesenchymal phenotype driven by epigenetic changes at the level of DNA methylation is a rare event under these conditions. We also did not observe a hypermethylation of RASAL1, which was shown to be induced after long-term treatment of ECs with TGF-β1 for 12 days28. Instead, we found a reversible methylation pattern at several regulatory elements, but the potential functional implications of those methylation events need to be studied in more detail.

What is the physiological or pathophysiological relevance of this transient EndMA? The partial EndMA may contribute to new blood vessel growth, by promoting a pro-migratory and pro-invasive state. Indeed, EndMA cells show signatures of genes associated with extracellular matrix organization, migration and proliferation, and the inhibition of TGF-β signaling reduced expansion of endothelial cells. Reports of the literature also support such a model, as it has been demonstrated that inhibition of EndMT driving transcription factors such as Snail or Slug prevents EC migration and in vivo angiogenesis29 and clonally expanded ECs showed a high expression of EndMT marker genes20. On the other hand, even the partial induction of EndMA may have detrimental effects. Although, our studies suggest that the direct contribution of EndMA to cardiac fibrosis by transition of ECs to cardiac fibroblasts is limited, we cannot exclude that cells undergoing EndMA may secrete extracellular matrix and inflammatory proteins, which affect the wound healing response and may thereby indirectly contribute to fibrosis. A transient mesenchymal activation may also contribute to other pathological states in vascular disease, such as atherosclerosis. As mesenchymal transition is associated with cellular detachment30 one might envision a role in the acute events leading to plaque erosion. Together, our study demonstrates a transient mesenchymal activation of endothelial cells and may clarify some controversies in the literature. While our RNA single-cell sequencing study clearly confirms the existence of mesenchymal marker expressing cells, and therefore supports the many studies reporting endothelial-mesenchymal transitions31, this process appears reversible and therefore was not found to occur in long-term fate studies using lineage tracing. Future investigations may provide insights as to what extent EndMA (as opposed to EndMT) occurs in other injury or disease conditions and how risk factors affecting the stability of the endothelial epigenome may be able to interfere with the reversibility of the process.

Methods

Animals. All animal experiments were executed in agreement with the animal welfare guidelines and German national laws. All animal experiments and study protocols were authorized by the competent authority (Regierungspräsidium Darmstadt, Hessen, Germany). Mice were held at 23 °C ambient temperature and...
60% humidity in 10 h/14 h light/dark cycle. C57BL/6 J Cdh5-CreERT2 mice (obtained from The Jackson Laboratory, MGI:3849882) or C57BL/6 J Col1a2-CreERT2 mice (obtained from The Jackson Laboratory), were bred with mTmG reporter mice (obtained from The Jackson Laboratory) to generate Cdh5-CreERT2/mTmG or Col1a2-CreERT2/mTmG mice. For Cdh5-CreERT2/mTmG mice were used at an age of 10–12 weeks (n = 6, male mice). Cre expression was induced by daily intraperitoneal injections of 2 mg/kg of tamoxifen (Sigma Aldrich) for 7 days and sacrificed at high (upper 5% of the area) or low (lower 5% of the area). Three days after tamoxifen injection, left anterior descending (LAD) coronary artery ligation surgery was performed, inducing myocardial infarction (MI) as previously described. The animals were anesthetized with isofluran and analgesia was delivered inter mitral injection of buprenorphine (0.03 mg/kg BW). For GFP stainings, we used hearts isolated from Cdh5 and Pecam1 floxed (UML > 0) mice. In data from lineage tracing experiments we used a dynamic threshold to separate EGFP positive cells. Based on the density distribution of EGFP normalized UMI for each sample we calculated a cutoff that separated real GFP expressing cells (population with highest EGFP normalized UMI counts) from false positive based on a local minimum. We successfully validated this method and estimated the number of GFP positive cells for a sample which had not been injected with tamoxifen at almost 0.

Gene ontology enrichment analysis has been performed with Enrichr (R package version 1.0). We used GO_Biological_Process_2018 and GO_MolecularFunction_2018 as ontology sources and provided from the gene ontology consortium as our reference gene-set libraries. GO terms have been ranked and displayed by their “Combined Score” as implemented in Enrichr, which combines the z-score of the term’s deviation to its expected rank and the p-value from Fisher’s exact test and their specificity score as the fraction of genes added to the total number of genes in the respective GO term. Gene set enrichment analysis was performed using clusterProfiler (R package version 3.10.1) and gene sets from Molecular Signatures Database (MsigDB, gsea-msigdb.org).

For visualizing possible sub-states of endothelial cells, we applied trajectory analysis to our data, identifying pseudotemporal ordering of cells along the actual time course. We therefore used the Monocle (Version 2.6.0) toolbox and followed the standard pipeline, as described in their tutorial. In brief, Seurat objects were converted into Monocle using the “importCDS” function and size factors and dispersion were estimated. We then defined genes as expressed, when exceeding a threshold of 0.1 and where at least expressed in 10 cells. We then based our trajectory on differential genes between the individual timepoints, as calculated with “differentialGeneTest”. Cells where then accordingly filtered using “setOrderFilter”. Pseudotime reduction of dimension was applied with the “DDRTree” method, as implemented in the “reduceDimension” function. Finally, we ordered cells by using “orderCells” and plotted trajectories split by state and timepoints.

Ligand-receptor interaction analysis was performed on transcriptomic profiles. Briefly, a weighted directed graph with four layers of nodes was built linking source cell types (layer 1), defined by expression of a ligand (layer 2), to target cell types (layer 4) expressing a corresponding receptor (layer 3), after reference to a curated map of ligand-receptor pairs. Source-ligand and receptor-target edges were weighted according to the ligand-expression levels and receptors, respectively. Edges were further weighted by mouse-specific association scores from the STRING database. Permutation testing (100,000 permutations) of randomized network connections was applied to determine significant source-target network connections following Benjamini–Hochberg multiple-testing correction (adjusted q < 0.01).

Immunohistochemistry. Male C57BL/6 J mice were sacrificed by cervical dislocation on d3 (n = 6) and d14 (n = 5) post LAD surgery. Age-matched non-operated animals (homeostatic) have been sacrificed on the same day (n = 4, respectively). Hearts were flushed with HBSS (14025-050, Gibco) by injection into the Vena cava caudalis, cut in half transversally and washed in HBSS and DPBS (14190-094, Gibco). Tissue was cut in half transversally and embedded in O.C.T medium (14190-094, Gibco). Tissue was frozen at –80 °C overnight and cut in 50 μm sections. Sections were thawed at room temperature for 30 min. Antigen retrieval was performed by boiling the sections for 15 min in citrate buffer (pH 6.0, 0.1 M). Quenching of endogenous peroxidases was performed in 1% methanol with 1% H2O2 for 10 min. Sections were blocked with 3% BSA (10163.2, Roth), 5% donkey serum (7475, abcam), 20 mM MgCl2, and 0.3% Tween 20 (P1379, Sigma). Primary antibodies were incubated at 4 °C overnight followed by secondary antibody incubation for 1 h at room temperature.

For GFP stainings, we used hearts isolated from Cdh5-CreERT2/mTmG or Col1a2-CreERT2/mTmG reporter mice and embedded them in O.C.T medium
To the cultivation medium and subsequently frozen at −80 °C and stained as described above with respective antibodies.

HUVECs were cultured in the normal cell culture medium as described in the section "Cell Culture" (Control), or in a medium without epidermal growth factor and bovine brain extract, supplemented with either 10 ng/mL TGF-β2 (302-B2, R&D System), or 10 ng/mL TGF-β1 (240-B, R&D System) and 10 ng/mL IL-1β (200-01B, Peprotech)38. Nitric oxide synthase was inhibited by 1 mM N-α-Nitroarginine methyl ester (L-NAME). To reverse EndMT induction, the medium containing the named stimuli or inhibitor was replaced after 3, 7, or 10 days of culture with normal control medium and cultured for 4 days. Medium and treatment was replenished every second day. After day 1, 2, 3, 7, 10, or 14 RNA was isolated. For cell cycle inhibition assay, we treated the cells with 2.5 μM Vincristine (CENP8172, Chem Faxes) or equal amounts of DMSO after TGF-β2 treatment for 4d.

RNA analysis. RNA was isolated using the mirNeasy kit (27100; Qiagen). Additional DNAse I (79254; Qiagen) digestion according to the manufacturer’s protocol was performed. 1 μg of RNA from each sample was reverse-transcribed by using the reverse transcriptase M-MLV Reverse Transcriptase (N8080018; Life Technologies), using random hexanucleotides as primers (10 min at 25 °C, 15 min at 42 °C, 5 min at 99 °C) as previously described39. We performed qPCR analysis using Fast SYBR Green Mastermix (Q32851, Invitrogen). 200 ng of DNA was used as input for DNA methylation analysis.

Endothelial-to-mesenchymal transition assay. For the induction of endothelial-to-mesenchymal transition (EndMT), HUVECs were cultured either in the normal cell culture medium as described in the section "Cell Culture" or in a medium without epidermal growth factor and bovine brain extract, supplemented with either 10 ng/mL TGF-β2 (302-B2, R&D System), or 10 ng/mL TGF-β1 (240-B, R&D System) and 10 ng/mL IL-1β (200-01B, Peprotech)38. Nitric oxide synthase was inhibited by 1 mM N-α-Nitroarginine methyl ester (L-NAME). To reverse EndMT induction, the medium containing the named stimuli or inhibitor was replaced after 3, 7, or 10 days of culture with normal control medium and cultured for 4 days. Medium and treatment was replenished every second day. After day 1, 2, 3, 7, 10, or 14 RNA was isolated. For cell cycle inhibition assay, we treated the cells with 2.5 μM Vincristine (CENP8172, Chem Faxes) or equal amounts of DMSO after TGF-β2 treatment for 4d.

RESULTS

FACS Analysis. For FACS analysis, we used hearts from C57BL6/CrETERTm1/TmG and processed them as described above for single-cell sequencing. Cells were first fixed for 20 min in methanol on ice and blocked in 3% BSA, 5% donkey serum and 0.1% Triton-X100. We incubated cells with primary antibodies (Supplementary Table 2) for 20 min on ice and with secondary antibodies for 30 min on ice. Additionally, FACS was performed on 1 h 1 mM BrdU treated HUVECs using a BrdU kit (559691BD Pharmpingen). Here, cells were fixed and permeabilized and treated with DNAse to expose BrdU labeled DNA. Cells were stained using anti-BrdU antibody and 7-AAD dye. All reagents supplied by the kit. Analysis was performed on BD LSRefortessa X-20 using FACS Diva software.

Active glycolytic flux. HUVECs were seeded on a fibronectin coated Seahorse XF96 culture plate (Agilent Technologies) at a density of 6 × 104 cells per well in the respective endothelial growth medium. The medium was changed to Krebs Henseleit buffer (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 2 mM MgSO4, 1.2 mM Na2HPO4 containing 1 mM sodium pyruvate 1 h prior to the measurement with the XF96 Delta Flux analyzer (Agilent Technologies)). Activators and inhibitors were used at the following final concentrations: 10 mM glucose, 1.5 μM oligomycin A, 100 mM 2-deoxy-D-glucose. Each measurement was averaged from three technical replicates of three different biological replicates.

13C Glucose metabolic analysis. HUVECs were seeded in 10 cm culture dishes and treated with the respective media. In all, 48 h prior to collection, media were substituted with glucose free media, supplemented with 13 C uniformly labeled glucose (Sigma) in a final concentration of 17 mM. Samples were collected and prepared as previously described40. LC-MS/MS experiments were performed on an Agilent 1290 Infinity UPLC system (Agilent, Waldbronn, Germany) coupled to a QTrap550 mass spectrometer (Sciex, Darmstadt, Germany), equipped with an ESI TurbolonSpray source. The flow rate was 0.22 mL/min, autosampler temperature was set at 6 °C, and the mass spectrometer was operated on an Aspahpip NH2-40 (250 × 2 mm, 4 μm particle size: Shouwa Denko, Munich, Germany). The mobile phase was composed of 20 mM ammonium carbonate in H2O/35% acetonitrile (ACN), pH = 10 and ACN. After an initial 3.5 min isocratic elution of 99.9% ACN, the percentage of ACN decreased to 85% at 5 min, to 95% at 8 min, to 0% at 14 min, back to 99.9% at 34 min. The composition was maintained at 99.9% ACN until the 42 min. The QTrap5500-MS system was operated in triple quadrupole mode with positive/negative ion switching. Ion spray voltage of 5500/−4500 V were applied, respectively. Curtain gas was set to 30 psi, collision gas was set to 0.1 psi, ion source gas 1 to 35 psi, ion source gas 2 to 3 psi. Analyst 1.6.2 and MultiQuant 3.0 (both from Sciex, Darmstadt, Germany), were used for data analysis and acquisition, respectively.

DNA methylation. For assessment of DNA methylation, HUVECs were cultured as described above and DNA was isolated using the QIAamp DNA Micro Kit (56304, Qiagen), according to the manufactures protocol.

DNA concentration was quantified using the Qubit dsDNA HS Assay kit (Q32851, Invitrogen). 200–500 ng of DNA was used as input for DNA methylation analysis.

The Infinium Human-Methylation EPIC BeadChip (850k) (WG-317, Illumina) was used to determine the DNA methylation status of more than 850,000 CpG sites, respectively following the producer’s guidelines. On-chip quality metrics of all samples were carefully controlled.

Infinium human methylation EPIC array analysis was performed by using RnBeads (version 2.341 and ADMIRE42. Raw intensities were taken from IDAT files and quality control was performed on all probes and samples. During preprocessing, probes and samples have been filtered for SNP-enrichment and highest impurity using Greedycut (FDR adjusted p < 0.05). The data was normalized using dase method43. Individual site-based methylation levels were calculated and subsequently used for region-based methylation level assessment (Cpg island/promoter/ tails/gene body). Differential methylated regions were determined by comb-p method44 and the combined rank score of RnBeads. For illustration of beta values ADMIRe visualization functions were used.

For regulatory element analysis, 848,254 Cpg probes that were retained after EPIC array quality filtering were overlapped with 2,4 million annotated regulatory elements (REMs) of the EpiRegion database45 and analyzed using RnBeads software. Cpg methylation was averaged over all Cpg probes overlapping a REM. Differentially methylated regulatory elements were defined as CpG sites with a methylation difference ≥0.1 between Control and +TGF-β2 (d3) or +TGF-β2 (d3) -TGF-β2 (d7) respectively, and FDR corrected p-values ≤ 0.05 were selected. These significantly differentially methylated REMs were clustered by their DNA methylation pattern using hierarchical clustering.

Histone and gene expression analysis. For single-cell RNA Sequencing statistical analysis of differential gene expression was performed with Seurat’s FindMarkers or FindAllMarkers functions by using a bimodal likelihood estimator test suitable for zero-inflated scRNA-seq data. Data is always shown in p-values corrected for multiple testing (Bonferroni).

For GO-Term enrichment of significantly regulated genes we used enrichR (version 2.246. Data is always shown in p-values corrected for multiple testing (Bonferroni).

For qPCR data, ANOVA non-parametric Kruskall Wallis multiple comparison test with posthoc Dunn’s test was used for comparison of multiple groups or a two-sided Student’s t test with Welch’s correction. A value of p < 0.05 was considered statistically significant.

We used a χ2-test of independence to indicate significant changes in ratios of cell cycle assignment and considered p < 0.01 as significant.

Representative images shown have been chosen from n = 3 independent experiments.

For ConfettiC576-CrETERT2 we prepared an endo-regional enrichment of significantly regulated genes we used enrichR Software suite48. We used the combined score to report on enrichment, which combines the term’s deviation to its expected rank and the p-value of Fisher’s exact test.

For qPCR data, ANOVA non-parametric Kruskall–Wallis multiple comparison test with posthoc Dunn’s test was used for comparison of multiple groups or a two-sided Student’s t test with Welch’s correction. A value of p < 0.05 was considered statistically significant.

Data availability

The single-cell RNA-seq datasets generated in this study are available at ArrayExpress (E-MTAB98916 and E-MTAB98917). Bulk RNA-seq has been deposited at Sequencing Read Archive (PRJNA697225). RnBeads output files are available (10.6084/m9.8paper.1342710.v1). All other data are included within the article, source data, supplementary data or can be made available upon request. Source data are provided with this paper.

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