Meox2/Tcf15 Heterodimers Program the Heart Capillary Endothelium for Cardiac Fatty Acid Uptake

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Background—Microvascular endothelium in different organs is specialized to fulfill the particular needs of parenchymal cells. However, specific information about heart capillary endothelial cells (ECs) is lacking.

Methods and Results—Using microarray profiling on freshly isolated ECs from heart, brain, and liver, we revealed a generic signature for microvascular heart ECs and identified Meox2/Tcf15 heterodimers as novel transcriptional determinants. This signature was largely shared with skeletal muscle and adipose tissue endothelium and was enriched in genes encoding fatty acid (FA) transport–related proteins. Using gain- and loss-of-function approaches, we showed that Meox2/Tcf15 mediate FA uptake in heart ECs, in part, by driving endothelial CD36 and lipoprotein lipase expression and facilitate FA transport across heart ECs. Combined Meox2 and Tcf15 haplodeficiency impaired FA uptake in heart ECs and reduced FA transfer to cardiomyocytes. In the long term, this combined haplodeficiency resulted in impaired cardiac contractility.

Conclusions—Our findings highlight a regulatory role for ECs in FA transfer to the heart parenchyma and unveil 2 of its intrinsic regulators. Our insights could be used to develop new strategies based on endothelial Meox2/Tcf15 targeting to modulate FA transfer to the heart and remedy cardiac dysfunction resulting from altered energy substrate usage.

Key Words: endothelium ■ fatty acids ■ heart ■ metabolism ■ transcription factors

Endothelial cells (ECs) lining capillaries of different organs have specialized gene expression patterns, morphologies, and functions related to the specific needs of tissue parenchymal cells with which they communicate. This heterogeneity is determined by environmental cues and intrinsic regulators (eg, transcription factors or TFs), which remain largely unknown. Although specific characteristics of capillary ECs have been described for liver and brain, such information for heart ECs is lacking, with the exception of their documented modulating role in myocardial contractile performance. As a continuously contracting organ, the heart daily produces ≈20 times its own weight in adenosine triphosphate (ATP). Mainly glucose and fatty acids (FAs) serve as substrates for ATP production. Under physiological conditions, FAs account for 60% to 90% of ATP generation, whereas glucose contributes for 10% to 40%.

A broad range of cardiomyopathies leading to cardiac hypertrophy and failure are associated with chronic alterations in cardiac energy metabolism, in general, and FA use, in particular. Besides the demonstrated role of molecules involved in FA metabolism in cardiomyocytes, recent findings suggest that proteins involved in FA uptake and transport in ECs...
and cardiomyocytes may also play a role in these pathologies. Nevertheless, it has not been established whether cardiac ECs are specialized to fulfill the high FA delivery demanded by the heart.

Recent studies identified vascular endothelial growth factor (VEGF)-B as a paracrine mediator of FA uptake through ECs in the heart, brown adipose tissue, and skeletal muscle and showed that VEGF-B inhibition protected against type 2 diabetes mellitus by decreasing FA uptake and reverting muscle metabolism toward glucose consumption. However, EC-intrinsic (transcriptional) regulators determining FA uptake and transfer are unknown, with the exception of peroxisome proliferator–activated receptor (PPAR) γ, whose activities are not restricted to the heart. We identified 2 novel transcriptional regulators of the gene signature and metabolic substrate transport in heart ECs, that is, Meox2 and Tcf15. They both have been described to play a role in the specification of paraxial mesoderm to somitic dermomyotome, and a defect in heart development has not been reported in Meox2- or Tcf15-deficient mice. Meox2 participates in EC homeostasis, whereas Tcf15 has never been studied in ECs, except that it was cloned from an endothelial library. Here, we demonstrate that, within the adult heart, Meox2 and Tcf15 are exclusively expressed in ECs, and that they are critical regulators of FA transport across cardiac ECs.

Methods
An extended Methods text is available online, which includes Table I in the online-only Data Supplement (antibodies) and Table II in the online-only Data Supplement (quantitative real-time polymerase chain reaction primers).

Animal/Human Biopsies
ECs for expression profiling were sorted from Tie2-GFP mice. Meox2+/− mice (referred to as Meox2−/−; C57Bl/6) were obtained from the Jackson Laboratories and Tcf15−/− mice (129S7/SvEvBrd/C57Bl/6) were provided by E. N. Olson/A. Rawls (Dallas, TX/Tempe, AZ). Human biopsies obtained under informed consent were plated on laminin-coated dishes in protein-supplemented M199 EGM2-MV bullet-kit on gelatin-coated plates, and cardiomyocytes were cultured by using a Langendorff perfusion setup. For in vitro studies, human heart ECs and human umbilical vein ECs were grown in EB2 medium supplemented with EGM2-MV bullet-kit on gelatin-coated plates, and cardiomyocytes were plated on laminin-coated dishes in protein-supplemented M199 medium.

Microarray
Mouse genomewide microarrays on ECs were performed by the VIB Nucleomics Core, as described in the online-only Data Supplement. Functional annotation analysis was done on the validated heart EC gene list with the use of DAVID software (http://david.abcc.ncifcrf.gov/).

RNA/Protein/cDNA Preparation, Quantitative Real-Time Polymerase Chain Reaction, Western Blot, Enzyme-Linked Immunosorbent Assay, and Enzymatic Activity Assays
Total RNA was extracted with RLT or TRIzol, retrotranscribed using SuperScriptIII Reverse Transcriptase, and quantitative real-time polymerase chain reaction was performed with SYBR-Green master mix. For Western blot, total proteins were extracted with radioimmuno-precipitation assay buffer containing protease inhibitors. Human apoapolipoprotein B and mouse lipoprotein lipase (Lpl) protein were measured by enzyme-linked immunosorbent assay, and Lpl activity was measured with a colorimetric kit.

Lentiviral Overexpression/siRNA Knockdown
The construct for overexpressing human MEOX2 was purchased (Genecopoeia). Open reading frames for human WT1, EBF3, and murine Tcf15 were cloned from cDNA-containing plasmids (Thermo Scientific), and PPARG1 (further referred to as PPARG) was cloned from human heart EC cDNA. Lentiviruses were produced in HEK293 cells. Lentiviral transduction, lysate harvesting, and siRNA knockdown of CD36 or LPL were performed as described in the online-only Data Supplement.

Proximity Ligation Assay/GST Pull-Down Assays
Proximity ligation assay was performed as previously described in human umbilical vein ECs overexpressing MEOX2 and FLAG-tagged Tcf15 or PROX1. Glutathione S-transferase (GST) pull-down assays were performed in HEK293 cells transfected with plasmids encoding GST-tagged Tcf15, FLAG-tagged MEOX2, or both.

In Vitro/Ex Vivo FA Uptake/Transport
FA uptake was analyzed as described in the online-only Data Supplement in cultured human heart ECs 72 hours posttransduction or after siRNA treatment, in freshly isolated magnetically selected murine heart ECs or in adult murine cardiomyocytes, 4 hours after plating. FA transport in EC mono- or coculture with cardiomyocytes was analyzed as described in the online-only Data Supplement.

Histology
Immunofluorescence and Sirius red staining were performed on 3- to 6-μm paraffin sections. Oil Red O staining was performed on 10-μm cryosections as described in the online-only Data Supplement.

Echocardiography
Mice (n=8–9, analyzed at 3–4 and 11 months of age) were sedated with 1.5% isoflurane and standard views were obtained in 2 dimensions by transthoracic echocardiography by using an MS400 transducer on a Vevo2100 scanner. Image analysis was performed by a blinded investigator using VisualSonics software.

FA/Glucose Uptake
For FA uptake assessment in vivo, mice were injected intravenously with 14C-oleic acid (OA) or [3H]OA, and, after 30 minutes, radioactivity in different organs/plasma was measured on an LKB-Wallac Rackbeta-1214 Counter or hearts were harvested and processed for semithin sectioning and autoradiography. Glucose uptake was determined by positron emission tomography imaging during 1 hour after intravenous injection of 2-deoxy-2-[18F]fluoro-D-glucose.

Statistics
Data are expressed as mean±standard error of the mean from pooled data of 3 to 12 independent experiments. Data normality was supported by the Shapiro-Wilk test (larger samples) or evidence from literature.
Figure 1. The heart EC fingerprint is enriched in lipid transport–related genes. A, Principal component diagram and heat map from a microarray on freshly isolated ECs from murine (m) heart, brain, and liver (n=5). B, Log2 probe set intensity of validated genes from the heart EC fingerprint in murine heart, liver, and brain ECs (n=5; *P<0.05 vs heart ECs). C, Functional annotation of significantly enriched gene categories from the heart EC fingerprint. D, mRNA expression (qRT-PCR) of heart EC fingerprint genes in SkM, BAT, or WAT relative to heart ECs (n=3–4; *P<0.05 vs heart ECs and a minimum 4-fold difference). E, Western blots of Meox2, Tcf15, housekeeping protein expression (α-Tubulin/Gapdh), or loading control (Ponceau) in sorted ECs and non-ECs from the heart or freshly isolated cardiomyocytes. F, Immunofluorescence stainings for Meox2 or Tcf15 (red; white arrowheads) in murine heart, liver, and brain tissue (ECs stained by lectin in green; arrows indicate EC nuclei). Scale bars, 20 μm. Quantitative data represent mean±standard error of the mean. BAT indicates brown adipose tissue; EC, endothelial cell; qRT-PCR, quantitative real-time polymerase chain reaction; SkM, skeletal muscle; and WAT, white adipose tissue.
reporting on analogous data sets (smaller samples). For single comparisons, the homogeneity of group variances was determined with the Levene test and \(P\) values were calculated with the Welch \(t\) test or the 2-tailed (un)paired Student’s \(t\) test where appropriate. For time courses of a single variable comparison, repeated-measures analysis of variance was applied, followed by Student’s \(t\) test for each time point. \(P<0.05\) was considered significant. For multiple comparisons, \(P\) values were calculated with a priori nonorthogonal contrast analysis (with the type of comparisons predetermined based on biological relevance and indicated in the figure legends), after 1-way analysis of variance and the Levene test. The significance level \(\alpha\) (0.05) was corrected for multiple comparisons with the Keppel-modified Bonferroni test and the resulting \(\alpha'\) is indicated in the figure legends where appropriate.

**Results**

**Gene Signature of Heart Capillary ECs**

To obtain a heart microvascular EC signature, we performed a microarray comparison of pure populations of GFP+ ECs freshly isolated from the hearts, livers, and brains of adult Tie2-GFP mice (Figure IA through IC and Note I in the online-only Data Supplement) revealing a high degree of heterogeneity between vascular beds (Figure 1A). After filtration (by defining as a threshold a minimum \(\text{Log}_2\) probe set intensity of 6 and a minimum 4-fold difference in comparison with brain and liver ECs \([P<0.05]\)) and quantitative real-time polymerase chain reaction validation of the gene list (only genes significantly enriched in the EC fraction in comparison with non-ECs and/or cardiomyocytes were retained \([P<0.05]\), data not shown), we obtained a set of 31 genes enriched in heart capillary ECs (Figure 1B). Functional annotation revealed that the main functions represented were lipid binding and lipid/FA transport (Figure 1C). To consolidate this signature, we measured its expression in ECs freshly isolated from additional tissues, ie, pancreas, lung, and kidney. We found that most genes (81% in comparison with at least 2 tissues, 48% in comparison with all 3 tissues) were at least 4-fold enriched in heart ECs (Figure ID in the online-only Data Supplement).

On the other hand, ECs isolated from tissues with continuous endothelium that, like the heart, are highly active in FA uptake for energy production or storage (ie, skeletal muscle or SkM, brown and white adipose tissue or BAT and WAT, respectively) showed for most genes an expression level similar to that in heart ECs (Figure 1D). Cross-species validation in human ECs confirmed a partially retained enrichment of the signature in human heart in comparison with brain and liver ECs (Figure IIA in the online-only Data Supplement). Finally, as for other EC types,2,31,32 human heart ECs lost their specific gene expression profile upon culturing (Figure IIB in the online-only Data Supplement).

**Transcriptional Regulation of Heart EC Identity In Vitro**

In addition to FA transport-related genes, the signature contained 5 TF-encoding genes, ie, Mox2, Tcf15, early B-cell factor 3 (Ebf3), Pparg and Wilms tumor 1 (Wt1; Figure 1B).
We confirmed by tissue immunostaining and Western blot on sorted cells that Meox2 and Tcf15 were exclusively expressed in the EC compartment of the murine heart and undetectable in liver or brain ECs (Figure 1E and 1F and Figure III in the online-only Data Supplement). We next tested whether these TFs would intrinsically regulate the heart EC signature by assessing whether their overexpression would restore the fingerprint in human heart ECs in which it was erased by culture. Overexpressing MEOX2 or Tcf15 alone did not affect signature gene expression; however, when overexpressed in combination, 31% of the genes were significantly upregulated (Figure 2A). Proximity ligation assay and GST pull-down revealed that this cooperative effect involved heterodimeric complex formation (Figure 2B and 2C). MEOX2 or Tcf15 did neither affect each other’s expression nor that of WT1, but had a small yet significant inductive effect on PPARG, EBF3 was strongly induced by MEOX2+Tcf15. Yet, its overexpression induced a limited number of signature genes and thus did not recapitulate the effect of MEOX2+Tcf15. Furthermore, combining EBF3 overexpression with MEOX2+Tcf15 did not have an additive effect (Figure IVA in the online-only Data Supplement). We also studied the inductive effect of PPARG and the most abundant WT1 isoform (Note II in the online-only Data Supplement). PPARG and WT1 overexpression partially induced the heart EC fingerprint (Figure IVB and IVC in the online-only Data Supplement). Moreover, PPARG exerted a synergistic effect with MEOX2+Tcf15, whereas WT1 did not have an additive effect on MEOX2+Tcf15+PPARG overexpression. Upregulation for a random gene subset was confirmed at protein level (Figure IVD in the online-only Data Supplement). MEOX2+Tcf15 overexpression induced the heart EC signature in human umbilical vein ECs to a similar extent as in cultured heart ECs (Figure IVE in the online-only Data Supplement).

**Figure 3.** Meox2/Tcf15 regulate heart EC identity in vivo. A, mRNA expression (qRT-PCR) of heart EC fingerprint genes in heart ECs from Meox2−/−, Tcf15−/−, or Meox2−/−:Tcf15−/− mice relative to wild-type littersmates (n=3–8; *P<0.05, **P<0.1 vs wild-type). B and C, mRNA expression (qRT-PCR) in heart (B and C) or liver ECs (B) from Meox2−/−:Tcf15−/− mice relative to wild-type littersmates (n=5–8 for B; n=3 for C; *P<0.05, **P<0.1 vs wild-type). D, Histogram overlay representative for n=3 showing reduced CD36 protein in heart ECs from Meox2−/−:Tcf15−/− mice vs wild-type littermates. Quantitative data represent mean±standard error of the mean. APC indicates allophycocyanin; EC, endothelial cell; and qRT-PCR, quantitative real-time polymerase chain reaction.

**Meox2/Tcf15 Determine Heart EC Identity In Vivo**

To address whether Meox2 and Tcf15 cooperated to affect the heart EC expression profile in vivo, mice lacking 1 allele of each factor were generated. Mice homozygously deficient for Tcf15−/− or Meox2 (online-only Data Supplement Note III) die perinatally, while combined heterozygously deficient mice are viable. ECs were isolated from hearts and, as a control, from livers of adult Meox2−/−;Tcf15−/− mice and their single-heterozygous or wild-type littersmates and their gene expression profile were analyzed by quantitative real-time polymerase chain reaction. Although haploinsufficiency for Meox2 or Tcf15 alone only slightly affected the heart EC signature, we observed a significant downregulation of 45% of the signature genes in Meox2−/−;Tcf15−/− hearts, supporting the cooperative interaction of both TFs also in vivo (Figure 3A). Complementary to our in vitro findings, Pparg and Ebf3 were significantly downregulated, whereas Wt1 was not affected, confirming the TF hierarchy we unraveled in vitro (Figure 2A). General EC markers were not affected in heart ECs, and liver sinusoidal EC-specific genes were not altered in liver ECs (Figure VA and VB in the online-only Data Supplement), suggesting that Meox2/Tcf15 specialize ECs in the heart, while neither affecting EC identity per se, nor EC specialization in unrelated tissues.

**Meox2+Tcf15 Haplodeficiency Alters Energy Substrate Transporter Gene Expression in Heart ECs In Vivo**

Because FA transport emerged as a principal function associated with the heart EC signature, we further investigated whether Meox2+Tcf15 haploinsufficiency altered endothelial expression of additional genes encoding FA or glucose transport–related.
molecules, previously described in heart ECs but not included in our signature, either because of not being enriched in heart ECs in comparison with both liver and brain ECs, or because of being <4-fold enriched (Figure VC in the online-only Data Supplement). FA translocase CD36,15,18,19 cytoplasmic FA binding protein Fabp433 and Gpihbp1, encoding the transporter and anchoring protein for Lpl on the luminal EC surface,34 were significantly downregulated in Meox2+/–:Tcf15+/– heart, but not in liver ECs, whereas Fatp3, a FA transporter regulated by VEGF-B interaction with heart ECs,21 and the glucose transporter Glut1 were upregulated in Meox2+/–:Tcf15+/– heart ECs (Figure 3B and 3C). Expression of Fabp4, another FA transporter gene induced by VEGF-B,21 was not affected by Meox2+/–Tcf15 haplodeficiency (Figure 3D). Together, these observations further suggest a functional role for Meox2+Tcf15 in FA (and glucose) transfer in and through heart ECs.

**Meox2/Tcf15 Drive Free FA Uptake in and Transport Across ECs**

To demonstrate that Meox2/Tcf15 are functionally important for FA trafficking, we next determined whether free FA (FFA) uptake in cultured human heart ECs could be enhanced by their overexpression. As measured by fluorescence-activated cell sorting, uptake of bovine serum albumin-bound fluorescently labeled dodecanoic acid was increased by 150% upon MEOX2+Tcf15 overexpression (Figure 4A). Additionally, freshly isolated heart ECs from Meox2+/–:Tcf15+/– mice showed a 19% decrease in FFA uptake in comparison with those of wild-type littermates (Figure 4B). Because CD36 expression in the heart ECs of Meox2+/–:Tcf15+/– mice was significantly lowered (Figure 3B and 3D), whereas it was upregulated upon combined MEOX2+Tcf15 overexpression in cultured heart ECs and human umbilical vein ECs (unlike other transporters; Figure V1A and V1B in the online-only Data Supplement and data not shown), and because CD36 is the FA transporter expressed at the highest level in heart ECs (>200-fold higher than any other FA transporter; Figure VIC in the online-only Data Supplement), we investigated whether CD36 was involved in Meox2/Tcf15-driven FFA uptake. CD36 knockdown reduced FFA uptake by 12% in heart ECs overexpressing MEOX2+Tcf15, meaning that CD36 accounted for ≈20% of the uptake caused by combined MEOX2+Tcf15 overexpression (Figure 4C). Using an in vitro transwell assay, we deliver proof that Meox2/Tcf15 also affected FFA transport.

![Figure 4. Meox2/Tcf15 regulate free FA uptake and transport in ECs. A, Representative histogram overlay showing BODIPY-dodecanoic acid (DA) uptake by cultured human heart ECs transduced with empty or MEOX2+Tcf15 lentiviral vectors and corresponding quantification of mean fluorescence intensity (MFI; n=3; P=0.053 vs empty vector). B, Representative histogram overlay showing BODIPY-DA-uptake by freshly isolated heart ECs from wild-type and Meox2+/–:Tcf15+/– mice and corresponding MFI quantification (n=4; *P<0.05 vs wild-type). C, Quantification of BODIPY-DA-uptake in human heart ECs overexpressing MEOX2+Tcf15 transfected with a nonsilencing (NS) or a siRNA-CD36 construct (n=4; *P<0.05 vs NS). D, Time course of Texas-Red Dextran and 14C-palmitic acid accumulation in the lower well of a transwell chamber, transported through a monolayer of HUVECs transduced with Cherry or MEOX2+Tcf15 lentiviruses. Data are expressed relative to the signal at time zero. (n=3; *P<0.05 vs Cherry). E, Relative radioactivity in cardiomyocytes (CM) cocultured with HUVECs (ECs) transduced with Cherry or MEOX2+Tcf15 lentiviruses and preloaded with 14C-OA. Data are expressed as relative signal vs CM cocultured with ECs+Cherry. (n=4; *P<0.05 vs CM+.ECs+Cherry]). Quantitative data represent mean±standard error of the mean. EC indicates endothelial cell; FA, fatty acid; HUVEC, human umbilical vein endothelial cell; OA, oleic acid; and PE, phycoerythrin.
across an endothelial monolayer (Figure 4D). Finally, with a coculture system we showed that Meox2/Tcf15 regulate the capacity of ECs to take up and transfer FFAs to cardiomyocytes (Figure 4E).

**Meox2/Tcf15 Regulate VLDL-Derived FA Uptake in Heart ECs**

FFAs reach the heart EC barrier not only bound to albumin (FFAs) but also esterified in the triacylglycerol core of circulating lipoproteins (e.g., chylomicrons or very-low-density lipoproteins [VLDL]). We determined whether Meox2/Tcf15 could regulate VLDL-derived FA uptake by measuring the uptake of 1,1’-Dioctadecyl-3,3,3’,3’-Tetramethylindocarbocyanine Perchlorate (DiI) signal incorporated in the lipidic parts of DiI-labeled VLDL. **MEOX2+Tcf15** overexpression in cultured heart ECs induced an increase of DiI uptake by 53% (Figure 5A), and, accordingly, in heart ECs freshly isolated from **Meox2+/–**:Tcf15+/– mice, DiI uptake was decreased by 22% in comparison with wild-type mice (Figure 5B). It is known that in vivo, triglyceride-derived FFAs from VLDL can be taken up by tissues either as a component of nascent or remnant lipoprotein particles or as FFAs following Lpl-mediated triglyceride lipolysis at the

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**Figure 5.** Meox2/Tcf15 regulate VLDL-derived FA uptake in heart ECs. **A** and **B**, Representative histogram overlays showing DiI-VLDL uptake in cultured human heart ECs transduced with empty or MEOX2+Tcf15 lentiviral vectors and corresponding MFI quantification (n=5; *P<0.05 versus empty vector; **A**), and in freshly isolated heart ECs from wild-type and Meox2+/–:Tcf15+/– mice and corresponding MFI quantification (n=6; *P<0.05 versus wild-type; **B**). **C**, Diagram representing qRT-PCR on freshly isolated cardiomyocytes and heart ECs (n=3; *P<0.05 versus cardiomyocytes). **D**, Quantification of LPL protein content in wild-type and Meox2+/–:Tcf15+/– hearts. (n=3–4; *P<0.05 versus wild-type). **E**, Relative LPL activity in wild-type and Meox2+/–:Tcf15+/– hearts (n=3–4; *P<0.1 versus wild-type). **F**, Bar graphs representing relative LPL activity in cultured heart ECs transduced with empty vector or MEOX2+Tcf15 (Left) or in MEOX2+Tcf15 overexpressing heart ECs transfected with a nonsilencing (NS) or a siRNA-LPL construct (Right; n=3–5; *P<0.05 versus empty vector or NS). **G**, Quantification of DiI-VLDL uptake in human heart ECs overexpressing MEOX2+Tcf15 transfected with either a nonsilencing (NS) or an siRNA-CD36 construct (n=5; *P<0.05 versus NS). Quantitative data represent mean±standard error of the mean. EC indicates endothelial cell; FA, fatty acid; LPL, lipoprotein lipase; MFI, mean fluorescence intensity; PE, phycoerythrin; qRT-PCR, quantitative real-time polymerase chain reaction; and VLDL, very-low-density lipoproteins.
luminal EC surface. In our in vitro system, VLDL particle uptake occurred and MEOX2+Tcf15 overexpression tended to increase this, as shown by the uptake of Apolipoprotein B protein, but this increase was not significant (Figure VIIA in the online-only Data Supplement). On the other hand, we found Lpl mRNA expression in pure heart ECs (Figure 1B), the levels of which were 25% of those in pure cardiomyocytes (Figure 5C). Because, in comparison with wild-type, MEOX2−/−:Tcf15−/− hearts show a downregulation of Lpl mRNA by 90% in their EC but not cardiomyocyte fraction (Figure 3A and Figure VIII A in the online-only Data Supplement) and had an ≈18% decrease in Lpl protein and activity (Figure 5D and 5E), we estimated that EC-derived Lpl activity is ≈20% of that in the total heart. Furthermore, MEOX2+Tcf15 overexpression in cultured heart ECs increased lipoprotein lipase (LPL) activity by 48%, and this increase was completely neutralized upon LPL knockdown (Figure 5F). Together, these results support the production of active Lpl by heart ECs and its regulation by Meox2/Tcf15. To demonstrate that this EC-produced LPL-hydrolyzing activity was involved in MEOX2+Tcf15-driven VLDL-derived FFA uptake, we knocked down CD36, an important downstream mediator of LPL-generated FFAs that is not involved in VLDL particle uptake (Figure VIIB in the online-only Data Supplement). CD36 knockdown in MEOX2−/−:Tcf15−/− overexpressing heart ECs decreased the Dil signal by 13% (Figure 5G). Together, these data demonstrate an EC-autonomous role for MEOX2/Tcf15-regulated Lpl in VLDL-derived FFA uptake.

**Meox2/Tcf15 Regulates Cardiac Energy Substrate Uptake In Vivo**

To evaluate whether Meox2+Tcf15 haplodeficiency altered FA uptake in the heart in vivo, we administered 14C-OA via tail vein injection to 12- to 16-week-old Meox2−/−:Tcf15−/− and wild-type males, and measured the radioactive signal in different organs after 30 minutes. FA uptake was significantly decreased in the heart (by 35%), and was also decreased (by 50%) in oxidative SkM (soleus) in Meox2−/−:Tcf15−/− mice – as expected by the fact that Meox2 and Tcf15 are also expressed in the SkM endothelium, which also expresses the other genes of the heart EC signature at high levels (Figure 6A). Unexpectedly, however, FA uptake was significantly increased (by ≈100%) in BAT of Meox2−/−:Tcf15−/− mice, although BAT ECs had similar levels of expression of the genes of the heart EC fingerprint and FA transporters than heart ECs, and showed downregulation of, eg, CD36 and Lpl in Meox2−/−:Tcf15−/− mice (Figure 1D and Figure IXA and IXB in the online-only Data Supplement). Other organs had a similar uptake in the 2 genotypes. Although we detected a 22% increase in radioactive signal in plasma of Meox2−/−:Tcf15−/− mice, which

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**Figure 6.** Meox2/Tcf15 regulate cardiac energy substrate uptake in vivo. **A**, Diagram showing the amount of radioactivity in hearts from Meox2−/−:Tcf15−/− or wild-type littermates, expressed as percentage of injected 14C-oleic acid (OA) dose (ID) per gram (n=5–14; *P<0.05, *P<0.1 vs wild-type). **B**, Representative autoradiography pictures on semithin sections (with corresponding insets on the right) of wild-type and Meox2−/−:Tcf15−/− hearts 30 minutes after [3H]OA injection. Scale bars, 10 μm. **C** and **D**, Time-lapse quantification (C) of [18F]FDG uptake into Meox2−/−:Tcf15−/− or wild-type hearts (n=11–12; *P<0.05 vs wild-type) and representative small-animal PET images (D) of wild-type and Meox2−/−:Tcf15−/− hearts (arrows) 60 minutes after injection. Quantitative data represent mean±standard error of the mean. [18F]FDG indicates 2-deoxy-2-[18F]fluoro-d-glucose; and PET, positron emission tomography.
could suggest a slower clearance of plasma FAs by peripheral tissues, this increase was not statistically significant ($P=0.24$). Autoradiography on semithin sections of $Meox2^{+/−}$:$Tcf15^{+/−}$ and wild-type littermate hearts confirmed that there was much less FA signal in $Meox2^{+/−}$:$Tcf15^{+/−}$ hearts 30 minutes after $[^{1}H]$OA injection and furthermore shows that the signal almost exclusively originated from cardiomyocytes, with no accumulation of FAs in the extracellular space, suggesting that FA uptake by cardiomyocytes was not compromised (Figure 6B). Accordingly, expression of none of the lipid or glucose transport–related genes tested was affected in $Meox2^{+/−}$:$Tcf15^{+/−}$ cardiomyocytes and they did not have an impaired FA uptake in vitro (Figure VIII A and VIII B in the online-only Data Supplement). Together, these data suggest that, in $Meox2^{+/−}$:$Tcf15^{+/−}$ mice, FA delivery from the plasma to cardiomyocytes is impaired because of a reduced passage through the endothelium. To investigate whether reduced FA delivery was compensated by increased glucose uptake, we measured 2-deoxy-2-$[^{18}F]$fluoro-d-glucose uptake in the heart, and detected a 38% increase in $Meox2^{+/−}$:$Tcf15^{+/−}$ in comparison with wild-type mice at $\approx$60 minutes postinjection (Figure 6C and 6D).

Aged $Meox2^{+/−}$:$Tcf15^{+/−}$ Mice Develop Cardiac Dysfunction

Because an altered energy substrate use could be associated with cardiac dysfunction in animal models upon aging,17,36 we performed histological and functional analysis on young and aged $Meox2^{+/−}$:$Tcf15^{+/−}$ and wild-type males. In accordance with the in vivo $^{14}$C-OA uptake assay, Oil Red O staining revealed a decrease in lipid accumulation in heart muscle of $Meox2^{+/−}$:$Tcf15^{+/−}$ in comparison with wild-type hearts both in young (Figure XA in the online-only Data Supplement) and aged mice (Figure 7A). However, although young mice displayed normal cardiac function and histology (Figure XB and XC and Table III in the online-only Data Supplement), aged (44 weeks old) $Meox2^{+/−}$:$Tcf15^{+/−}$ mice displayed heart systolic dysfunction, as evidenced by a reduced ejection fraction and fractional shortening, sporadically accompanied by fibrosis and associated with a slight decrease in capillary density, but no significant differences in cardiomyocyte size (Figure 7 and Tables III and IV in the online-only Data Supplement).

**Discussion**

To improve the understanding of EC heterogeneity between capillaries of different organs, we here report a fingerprint of genes enriched in heart microvascular ECs, which is largely shared with ECs of SkM, BAT, and WAT, all tissues highly active in FA uptake for energy production or storage. We show that $Meox2$ and $Tcf15$, by interacting as a heterodimeric complex, EC-autonomously determine the expression of these fingerprint genes both in vitro and in vivo, while individually they had only a minor effect.

Our in vitro and ex vivo mechanistic studies showed that $Meox2$/$Tcf15$ intrinsically regulate uptake by cardiac ECs of FFAs originating from both bovine serum albumin conjugates and EC-derived Lpl hydrolysis of triglycerides contained in VLDL particles (Figure 8). Although the finding of EC-derived Lpl challenges the currently prevailing view that cardiomyocytes are the sole cardiac source of Lpl,34 we estimated that EC-derived Lpl protein and activity is $\approx 20\%$ of that in the whole heart, which is well in line with the observation by Bharadwaj et al35 that there is $\approx 14\%$ residual Lpl activity in hearts of cardiomyocyte-specific $Lpl$-deficient mice. $Meox2$/$Tcf15$ also potentially regulate the expression of $Gpihbp1$,34 responsible for Lpl transfer from cardiomyocytes and for its positioning at the luminal surface of heart capillary ECs (Figure 8).
Using a combination of gain- and loss-of-function experiments, we showed that CD36 is downstream of Meox2/Tcf15-mediated uptake of FFAs originating from both bovine serum albumin conjugates and VLDL particles in heart ECs. The high CD36 expression levels in comparison with other transporter genes in heart ECs further support its important role in cardiac FA uptake. Nevertheless, although, in our DiI-VLDL assay, CD36’s contribution to Meox2/Tcf15-driven FA uptake could have been underestimated by simultaneous detection of particle uptake, the fact that CD36 knockdown only partially impaired Meox2/Tcf15-driven uptake of BODIPY-labeled FFAs suggests the existence of other Meox2/Tcf15 downstream mediators or the occurrence of compensatory mechanisms upon CD36 knockdown.

In Meox2+/–:Tcf15+/– heart ECs, Fatp3, a FA transporter regulated by the paracrine VEGF-B,21 was increased, but this was not sufficient to fully compensate for the partial loss of CD36 induced by Meox2+/–:Tcf15+/– haplodeficiency, because cardiac FA uptake was significantly compromised in the Meox2+/–:Tcf15+/– heart. On the other hand, Fatp4, another FA transporter regulated by VEGF-B,21 was not affected by the haploinsufficiency of Meox2+Tcf15 in heart ECs. Finally, MEOX2+Tcf15 overexpression did not regulate FATP3 or FATP4 in vitro (data not shown), whereas VEGF-B is not able to regulate CD36.21 Therefore, we hypothesize that these extrinsic and intrinsic pathways do not overlap in regulating FA uptake in heart ECs. On the other hand, we found that Pparg, known to be an intrinsic regulator of EC FA uptake,23 seemed to be downstream of and synergize with MEOX2+Tcf15 to determine the heart EC signature, suggesting a new TF hierarchy in charge of endothelial FA uptake.

MEOX2+Tcf15 did not only support FA uptake in, but also transport across ECs, as shown with a transwell assay. In particular, they could regulate the capacity of ECs to take up and transfer FFAs to cardiomyocytes, as shown in our in vitro coculture experiments. MEOX2+Tcf15-mediated transendothelial transport was likely dependent on CD36-mediated FA uptake and may involve certain fingerprint genes and cytoplasmic FA transporters in charge of FA shuttling from the apical to the basal side of ECs, such as Fabp4 and Fabp5, the loss of which is known to decrease FA uptake in the heart13 and which were downregulated in heart ECs of MEOX2+Tcf15+/– mice (Figure 8).

In vivo, Meox2+Tcf15 haplodeﬁciency caused decreased FA uptake in the heart. Surprisingly, despite a similar expression of signature genes and FA transporters in heart and BAT ECs and a common downregulation of CD36 in ECs of both tissues in Meox2+/–:Tcf15+/– mice, FFA uptake was increased in Meox2+/–:Tcf15+/– BAT, suggesting that compensatory uptake mechanisms were at play, eg, involving BAT EC-specific genes or environmental factors secreted by brown adipocytes.

Together, we observed that FA transport to cardiomyocytes accounts for the lower cardiac FA uptake measured in adult Meox2+/–:Tcf15+/– hearts in vivo. Additional support for such a role is given by the fact that Meox2+Tcf15 expression was undetectable in other cardiac cells and, additionally, expression of genes related to FA transport in freshly isolated Meox2+/–:Tcf15+/– cardiomyocytes was unaffected nor was their uptake of FFAs – as evidenced in vitro in isolated cardiomyocytes and in vivo by autoradiography. Nevertheless, we found in vivo FA uptake studies were performed in ubiquituous and constitutive Meox2+/–:Tcf15+/– mice. Hence, we cannot entirely exclude that Meox2+Tcf15 haplodeﬁciency during development or in other organs or even in other cell types in the heart could have also contributed to the lower cardiac FA uptake in adult Meox2+/–:Tcf15+/– mice. Definitive proof for an isolated cardiac EC-exclusive cause of the diminished cardiac FA uptake would require the generation of cardiac EC-specific, inducible knockout mice.

Interestingly, the reduced FA uptake was compensated by a higher glucose uptake in the heart of Meox2+/–:Tcf15+/– mice – possibly due to higher Glut1 (but not Glut4; data not shown) expression in cardiac ECs, suggesting a switch in energy substrate usage in Meox2+/–:Tcf15+/– hearts. Even though higher glucose consumption has been proven beneficial for the heart upon cardiac stress,36,37 chronic adaptation to impaired FA uptake toward higher glucose consumption (like in Lpl–/–,36 H-Fabp–/–,17 or CD36–/–,15,18 mice) limits the heart’s metabolic flexibility, rendering it more susceptible to events that lead to cardiac metabolic changes, like an insult or aging. Accordingly, Meox2+Tcf15 haplodeﬁciency in aged mice resulted in impaired cardiac contractility, sporadically accompanied by ﬁbrosis – likely a late manifestation of cardiac dysfunction. Our current data, however, do not provide
conclusive evidence that the heart phenotype of aged Meox2+/– Tcf15+/– mice is related to a defect in adult cardiac EC FA handling, because this would require a conditional genetic approach. Furthermore, other genes regulated by Meox2/ Tcf15-encoding proteins whose function is not directly linked to energy substrate delivery could contribute to heart dysfunction/fibrosis, eg, tissue inhibitor of metalloproteinase 4 and metalloproteinase Adamts9, which play a role in matrix remodeling in the heart.38,39

In summary, here we provide for the first time a fingerprint of genes enriched in ECs lining capillaries of tissues with high metabolic capacity for FAs, suggesting a common specialization of these vascular beds. We identified Meox2/Tcf15 heterodimers as transcriptional determinants of the heart EC fingerprint and demonstrated that they are critical regulators of FA transport across heart ECs, in part, through the regulation of CD36 and Lpl expression. Combined Meox2+/– Tcf15–/– haplodeficiency resulted in downregulation of these and other FA transport-related genes and an increase of glucose transporter GLUT1 in heart ECs, and a switch of the cardiac energy substrate balance (Figure 8). These findings have broadened our understanding of the genetic identity and physiological role of heart microvascular endothelium and provide a platform for further mechanistic studies for therapeutic exploitation of specific EC targeting aimed at modulating the energy substrate uptake in the heart or promoting heart-specific revascularization.

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Disclosures

None.

References

1. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res. 2007;100:158–173. doi: 10.1161/01. RES.0000255691.76142.4a.
2. Géraud C, Schledzewski K, Demory A, Klein D, Kaus M, Peyre F, Stiecht C, Evdokimov K, Lu S, Schneider A, Goerdet S. Liver sinusoidal endothelium: a microenvironment-dependent differentiation program in rat including the novel functional protein liver endothelial differentiation-associated protein-1. Hepatology. 2010;52:313–326. doi: 10.1002/ hep.23618.
3. Abiet NJ, Patahendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurosci Bull. 2010;37:13–25. doi: 10.1016/j.nbb.2009.07.030.
4. Brutsaert DL, Fransen P, Andries L, De Keulenaer GW, Sys SU. Cardiac endothelium and myocardial function. Cardiovasc Res. 1998;38:281–290.
5. Kuruviella L, Kartha CC. Molecular mechanisms in endothelial regulation of cardiac function. Mol Cell Biochem. 2003;253:113–123.
6. Neubauer S. The failing heart—an engine out of fuel. N Engl J Med. 2007;356:1140–1151. doi: 10.1056/NEJMra063022.
7. Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. Physiol Rev. 2005;85:1093–1129. doi: 10.1152/physrev.00006.2004.
8. Krishnan J, Suter M, Windak R, Krebs T, Felley A, Montessuit C, Tokarska-Schlatter M, Assem A, Bogdanova A, Perriard E, Perriard J, Larsen T, Pedrazzini T, Keck W. Activation of a HIF1alpha-PARalpha axis underlies the integration of glycolytic and lipid anabolic pathways in pathologic cardiac hypertrophy. Cell Metab. 2009;9:512–524. doi: 10.1016/j.cmet.2009.05.005.
9. Battiprolu PK, Hojayev B, Jiang N, Wang ZV, Luo X, Igleswki M, Shetion JM, Gerard RD, Rothermel BA, Gillette TG, Lavender S, Hill JA. Metabolic stress-induced activation of FoxO1 triggers diabetic cardiomyopathy in mice. J Clin Invest. 2012;122:1109–1118. doi: 10.1172/JCI60329.
10. Altin SE, Schulze PC. Metabolism of the right ventricle and the response to hypertension and failure. Prog Cardiovasc Dis. 2012;55:229–233. doi: 10.1016/j.pcad.2012.07.010.
11. Abel ED, O’Shea KM, Ramasamy R. Insulin resistance: metabolic mechanisms and consequences in the heart. Arterioscler Thromb Vasc Biol. 2012;32:2068–2076. doi: 10.1161/ATVBAHA.111.241984.
12. Rider OJ, Cox P, Tyler D, Clarke K, Neubauer S. Myocardial substrate metabolism in obesity. Int J Obes (Lond). 2013;37:972–979. doi: 10.1038/ijo.2012.170.
13. Glatz JF, Luiken JJ, Bonen A. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiol Rev. 2010;90:367–417. doi: 10.1152/physrev.00003.2009.
14. Hagberg C, Mehlum A, Falkevall A, Muhl L, Eriksson U. Endothelial fatty acid transport: role of vascular endothelial growth factor B. Physiology (Bethesda). 2013;28:125–134. doi: 10.1095/physiol.2012.044212.
15. Irie H, Krukenkamp IB, Brinkmann JF, Gaudette GR, Saltman AE, Jou W, Glatt JF, Abumrad NA, Ibrahimii A. Myocardial recovery from ischemia is impaired in CD36-null mice and restored by myocyte CD36 expression. Proc Natl Acad Sci U S A. 2003;100:6819–6824. doi: 10.1073/pnas.1132094100.
16. van der Vusse GJ, van Bilsen M, Glatt JF. Cardiac fatty acid uptake and transport in health and disease. Cardiovasc Res. 2000;45:279–293.
17. Binas B, Danneberg H, McWhir J, Mullins L, Clark AJ. Requirement for the heart-type fatty acid binding protein in cardiac fatty acid utilization. FASEB J. 1999;13:805–812.
18. Steinbusch LK, Luiken JJ, Vlaubom R, Chabowski A, Hoebers NT, Coumans WA, Vroegrijk IO, Voshol PJ, Ouwens DM, Glatt JF, Diamant M. Absence of fatty acid transporter CD36 protects against Western-type diet-related cardiac dysfunction following pressure overload in mice. Ann J Physiol Endocrinol Metab. 2011;301:E618–E627. doi: 10.1152/ajpendo.00106.2011.
19. Watanabe K, Ohta Y, Toba K, Ogawa Y, Hanawa H, Hirokawa Y, Kodama M, Tanabe N, Hiroko S, Okhura Y, Nakamura Y, Kato K, Aizawa Y, Fuse I, Miyajima S, Kusano Y, Nagamoto T, Hasegawa G, Naito M. Myocardial CD36 expression and fatty acid accumulation in patients with type I and II CD36 deficiency. Am J Physiol Heart Circ Physiol. 1999;286:H261–H266.
20. Heathler LC, Cole MA, Lygate CA, Evans RD, Stuckey DJ, Murray AJ, Neubauer S, Clarke K. Fatty acid transporter levels and palmitate oxidation rate correlate with ejection fraction in the infarcted rat heart. Cardiovasc Res. 2006;72:430–437. doi: 10.1016/j.cardiores.2006.08.020.
21. Hagberg CE, Falkevall A, Wang X, Larsson E, Huusko J, Nilsson I, van Meeteren LA, Samuel E, Lu L, Vanveldenmierck M, Klar J, Genove G, Pietrus K, Stone-Elander S, Claesson-Welsh L, Yls-Hertmuta S, Noth P, Eriksson U. Vascular endothelial growth factor B controls endothelial fatty acid uptake. Nature. 2010;464:917–921. doi: 10.1038/nature08945.
22. Hagberg CE, Mehlum A, Falkevall A, Muhl L, Fam BC, Otsrätter S, Scoteeny P, Nyqvist D, Samén E, Lu L, Stone-Elander S, Proietto J, Andrikopoulos S, Sjöholm A, Nash A, Eriksson U. Targeting VEGF-B as a novel treatment for insulin resistance and type 2 diabetes. Nature. 2012;490:426–430. doi: 10.1038/nature11464.
CLINICAL PERSPECTIVE

Endothelial cells lining capillaries of different organs have specialized gene expression patterns, morphologies, and functions in relation to the specific needs of the tissue parenchymal cells with which they communicate. This microvascular endothelial heterogeneity is determined by environmental cues and intrinsic regulators (eg, transcription factors), many of which remain largely unknown. Furthermore, unlike for liver or brain, information on the specific characteristics of endothelial cells in the heart is currently lacking. Understanding these characteristics and the mechanisms that determine them may form the basis of new therapeutic strategies to tackle heart disease. Here, we identified Meox2 and Tcf15 as transcriptional co-determinants of the heart endothelial gene signature that program the heart endothelium for fatty acid uptake and transport to the heart parenchyma. Combined heterozygous deficiency for these transcription factors resulted in reduced fatty acid uptake and a compensatory increase in glucose uptake in the heart. Because there are many forms of cardiac dysfunction related to changes in energy substrate usage, our insights could be used to develop new strategies based on endothelial Meox2/Tcf15 targeting to treat such dysfunction.