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Role of MEF2C in the Endothelial Cells Derived from Human Induced Pluripotent Stem Cells

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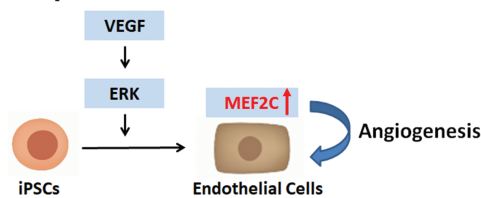
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Abstract

Human induced pluripotent stem cells (hiPSCs) not only provide an abundant source of vascular cells for potential therapeutic applications in vascular disease but also constitute an excellent model for understanding the mechanisms that regulate the differentiation and the functionality of vascular cells. Here, we reported that myocyte enhancer factor 2C (MEF2C) transcription factor, but not any other members of the MEF2 family, was robustly upregulated during the differentiation of vascular progenitors and endothelial cells (ECs) from hiPSCs. Vascular endothelial growth factors (VEGF) strongly induced *MEF2C* expression in endothelial lineage cells. The specific upregulation of *MEF2C* during the commitment of endothelial lineage was dependent on the extracellular signal regulated kinase (ERK). Moreover, knockdown of *MEF2C* with shRNA in hiPSCs did not affect the differentiation of ECs from these hiPSCs, but greatly reduced the migration and tube formation capacity of the hiPSC-derived ECs. Through a chromatin immunoprecipitation-sequencing, genome-wide RNA-sequencing, quantitative RT-PCR, and immunostaining analyses of the hiPSC-derived endothelial lineage cells with MEF2C inhibition or knockdown compared to control hiPSC-derived ECs, we identified TNF-related apoptosis inducing ligand (TRAIL) and transmembrane protein 100 (TMEM100) as novel targets of MEF2C. This study demonstrates an important role for MEF2C in regulating human EC functions and highlights MEF2C and its downstream effectors as potential targets to treat vascular malfunction-associated diseases.

Key words: induced pluripotent stem cells; endothelial cells; MEF2; angiogenesis; MAPK.

Graphical Abstract



Significance Statement

Elucidating the mechanisms that regulate vasculogenesis and angiogenesis has important ramifications for understanding vascular disease and guiding therapeutic interventions. Endothelial cells (ECs) derived from human induced pluripotent stem cells (iPSCs) provide a relevant in vitro model for probing these mechanisms. This study shows that the transcription factor MEF2C is specifically and robustly induced during the differentiation of ECs from human iPSCs and plays a critical role in the regulation of human angiogenesis.

Introduction

Vasculogenesis and angiogenesis are the fundamental processes of blood vessel formation from progenitors and from existing vessels, respectively.¹ Angiogenesis occurs after

vasculogenesis during embryogenesis and is the primary process of vascular growth and remodeling during post-natal life.^{2,3} Promoting vasculogenesis and angiogenesis has been used as a viable therapeutic strategy to treat disease where blood flow is compromised. For example, occlusive

atherosclerotic disease is the primary cause of myocardial infarction, limb ischemia, and stroke,³ the leading causes of death and disability worldwide. On the other hand, excessive vessel growth is associated with tumorigenesis, intraocular disorder, and inflammatory disease.³ Thus, understanding the mechanisms that regulate vasculogenesis and angiogenesis is critical for identification of potential targets to treat vascular malfunction-associated diseases.

The specialization of ECs during development and angiogenesis requires extrinsic signals and intrinsic regulatory events. It is well known that vascular endothelial growth factor (VEGF) and its receptor VEGF receptor 2 (VEGFR2), also named kinase insert domain receptor (KDR) or fetal liver kinase 1 (Flk-1) in mice, are required for establishment and maintenance of EC lineage.⁴⁻⁶ The coordinated action of specific transcription factors functions as intrinsic instruction to drive the elaborate multi-step process of EC differentiation as well as angiogenesis. Transcription factor forkhead box C1 (FOXC1), FOXC2, and the E-twenty six (ETS) family transcription factors, including ETS variant transcription factor 2 (ETV2) (also known as ER71), ETS-related gene (ERG), friend leukemia integration 1 (FLI1) and ETS1, have been found to be critical regulators of these processes.⁷⁻¹¹

Myocyte enhancer factor 2 (MEF2) proteins are transcription factors of the MADS box family. In vertebrates, the MEF2 family consists of 4 isoforms, MEF2A, MEF2B, MEF2C, and MEF2D. MEF2 are expressed in ECs,^{12,13} but their roles in endothelial cells are not completely understood. EC-specific knockout of *mef2c* in mice resulted in no vascular defect under physiological conditions but promoted vascular regrowth in oxygen-induced retinopathy.^{14,15} Impaired angiogenic sprouting was detected in mice with simultaneous knockout of *mef2a* and *mef2c* but not in mice with single knockout of these *mef2* genes.¹⁶ The role of the MEF2 family in human EC lineage determination and function is not fully understood.

Since MEF2C is also a key regulator for vascular smooth muscle, cardiac muscle, and neural tissue during development,^{17,18} it is critical to understand how MEF2C interacts with EC-specific growth factors and transcription factors to enhance the EC lineage or functionality. Human induced pluripotent stem cells (hiPSCs) can be differentiated into ECs.¹⁹⁻²² Here, using the in vitro EC differentiation from hiPSCs, we identified MEF2C as the only member of the MEF2C family that was robustly upregulated during the differentiation of ECs from hiPSCs. This study elucidates a novel role for MEF2C in mediating angiogenesis by human ECs.

Materials and Methods

Cell Cultures

IPS(IMR90)-4 is a female iPSC line purchased from WiCell Research Institute (Madison, WI). Dura6.9 iPSC is a male iPSC line provided by the Stem Cell Core at University of California Davis and was previously characterized.²³ Both hiPSC lines were cultured on Matrigel (Corning, #8774552)-coated tissue culture plates in StemFlex medium (Thermo Fisher, #A3349401) per manufacturer's instructions with medium refreshment every 1-2 days. When cells reached 70%-80% confluence, they were passaged by using cell dissociation solution ReLeSR (STEMCELL Technologies, #05872) per manufacturer's instructions or by manually picking with 1-mL pipet tips. Human coronary artery endothelial

cells (HCAECs), human umbilical vein endothelial cells (HUVECs), and human cardiac microvascular endothelial cells (HMVECs) were purchased from Lonza (#CC-2585) and cultured in the EC Growth Medium MV2 (ECGM-MV2) (PromoCell, #C-22111).

Cell Differentiation and Treatments

hiPSCs were seeded onto Matrigel-coated plates in StemFlex medium and cultured for 24 h. The hiPSCs were progressively differentiated into ECs as we previously described.²² In some experiments, the differentiating cells were exposed to 50 ng/mL VEGF (R&D System, #293-VE-500), 25 ng/mL BMP4 (R&D System, #314BP-050), 10 ng/mL FGF2 (Invitrogen, #PHG0023) or a combination of these growth factors from days 2 to 4. The differentiating cells were also treated with an inhibitor for a particular MAP kinase in some experiments. The ERK MAPK pathway inhibitors U0126 (EMD4Biosciences, #662005) and GDC-0994 (Selleckchem, #S7554) were used at 10 and 5 μ M, respectively. To evaluate a characteristic inflammatory response, hiPSC-derived ECs were treated with 0.6 or 10 ng/mL of TNF α (R&D System, #10291-TA) for 4 h and subsequently assessed by flow cytometry.

Knockdown of MEF2C in hiPSCs with shRNA Lentiviruses

The lentiviruses for scrambled control shRNA and MEF2C shRNA were purchased from Applied Biological Materials Inc (#LVP015-G) and Santa Cruz Biotechnology (#sc-38062-V), respectively. The hiPSCs were lifted with ReLeSR and incubated with the lentivirus in 50 μ L of StemFlex medium supplemented with 8 μ g/mL polybrene for 25 min. The cell/virus suspensions were subsequently plated into a Matrigel-coated 48-well plate with an additional 50 μ L of the same medium. Two-three days later, the cells were passaged into a new plate with StemFlex and 0.6 μ g/mL puromycin (Thermo Fisher Scientific, #A1113803) to select the transduced cells. Multiple puromycin resistant colonies were manually cloned for several rounds before use.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

To quantify the gene expression of interest, qRT-PCR was conducted as we previously described.²² Primer sequences for tested genes are listed in [Supplementary Table S1](#). For each condition, 3-5 independent samples were tested. The experiments were repeated in 2 lines of hiPSCs and data with the same trend were shown.

Chromatin Immunoprecipitation-Sequencing (ChIP-Seq)

The Dural6.9 iPSCs without (control) or with the MEF2C shRNA (shMEF2C) were differentiated into ECs. These ECs were cross-linked in 1% formaldehyde for 10 minutes at room temperature and the reaction was stopped with 0.125 M glycine. Cross-linked cells were lysed with ChIP lysis buffer (5 mM PIPES pH8, 85 mM KCl, 1% Igepal) with a protease inhibitor (PI) cocktail (Millipore Sigma, #11697498001). Nuclei were collected by centrifugation at 769 g for 5 minutes at 4 $^{\circ}$ C and lysed in nuclei lysis buffer (50 mM Tris pH8, 10 mM EDTA, 1% SDS) supplemented with the PI cocktail. Chromatin was fragmented using the Bioruptor Pico (Diagenode, Denville, NJ) and diluted with 5 volumes RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA

pH8, 1% Igepal, 0.25% deoxycholic acid). ChIP enrichment was performed by incubation with 3 µg MEF2C antibody (Abcam, #ab211493) or 2 µg normal rabbit IgG (ab46540, Abcam) for 16 h at 4 °C. Immune complexes were bound to 20 µL magnetic protein A/G beads (Bio-Rad, Hercules, CA) for 2 h at 4 °C. Beads were washed 2× with RIPA (Thermo Fisher Scientific, #J62524.AE) and 3× with ChIP wash buffer (100 mM Tris pH8, 500 mM LiCl, 1% deoxycholic acid). The final wash was performed in ChIP wash buffer with 150 mM NaCl. Cross-links were then reversed by heating beads in 100 µL ChIP elution buffer (50 mM NaHCO₃, 1% SDS) overnight at 65 °C. DNA was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). ChIP-seq library preparation was performed by Novogene using PE150 on the Novaseq 6000 sequencer (Illumina). FASTA sequencing reads were aligned to the Ensembl GRCh38_r108 reference genome with bowtie2/2.4.2. SAM files were converted to BAM files and sorted using samtools/1.15.1. Unmapped and duplicate reads were removed using sambamba/0.6.7. Peaks were called using macs3/3.0.0a6, filtered with hg38.blacklist and merged using the bedtools/2.29.2 intersect function. For data visualization, bigwig files were generated using deeptools/3.5.1 and plotted using the IGV web app. Replicate reproducibility of narrowPeak files was assessed using idr/2.0.4.2 and significant peaks were called with an IDR < 0.05. Bed files were annotated using the ChIPseeker R package.²⁴ Data were uploaded into NCBI Gene Expression Omnibus.

RNA-Sequencing

HiPSC line Dura6.9 was differentiated into vascular progenitors over 4 days. A class II histone deacetylase (HDAC) inhibitor MC-1568 (TOCRIS, #407710) at 5 µM was used to treat the differentiating cells for 24 h in EC differentiation medium. The differentiated cells not treated with MC-1568 were controls. RNA was isolated using QIAshredder (Qiagen, #79656) and RNeasy Mini Kit (Qiagen, #74106) according to the manufacturer's instructions. RNA-seq analysis was performed by Novogene (Sacramento, CA) using Illumina HiSeq 4000. Data analysis was provided by Novogene using a combination of programs including STAR, HTseq, Cufflink, and their wrapped scripts. Alignments were parsed using Tophat program and differential expression was determined through DESeq2/edgeR.

Immunofluorescence Staining

Immunostaining was performed as previously described.²⁵ Antibodies against human CD31 (Bethyl, #IHC-00055), MEF2C (Cell Signaling Technologies, #5030) and Trail (Cell Signaling Technologies, #37020) were used at a 1:200 dilution. Antibodies against TMEM100 (Invitrogen, #MA5 24949) were used at a 1:100 dilution. Alexa Fluor 594 or Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, #A21207, #A21206) targeting the primary antibodies were used at a 1:500 dilution. Images were taken with a Nikon Eclipse Ti microscope (Nikon Instruments Inc., Melville, NY).

Flow Cytometry

Cells were lifted with TrypLE (Thermo Fisher Scientific, #2167268) and stained with phycoerythrin-(PE)-conjugated mouse antihuman CD144 (BD Biosciences) or PE-conjugated mouse anti human VCAM-1 antibodies (BD Bioscience, #555647) and Alexafluor 488-conjugated mouse antihuman ICAM-1 antibodies (Biolegend, #322714) as we previously

described.²² The cells were analyzed using an Attune Flow Cytometer (Thermo Fisher Scientific).

Tube Formation and Cell Migration In Vitro

In vitro tube formation was performed as we previously described.²² To assess cell migration, hiPSC-ECs were grown to confluence. A scratch line was made with a 1-mL pipette tip. The cells were grown in ECGM-MV2 medium with 50% of all the growth factors. Phase images for the wound area were taken with a Nikon Eclipse Ti microscope (Nikon Instruments Inc.) daily for 3 days. The cells were immunostained with CD31 antibodies (Bethyl, #IHC-00055) on day 3. Distance migrated was analyzed using ImageJ (National Institutes of Health).

Statistics

Each group in an experiment had at least 3-5 samples. Data were presented as average with standard deviation. Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnett's Tukey test were performed to detect differences between 2 and more treatment groups, respectively.

Results

MEF2C Expression was Prominently Induced during EC Differentiation from hiPSCs

In this study, we first aimed to determine whether a particular member of the MEF2 family transcription factors is upregulated during EC differentiation from hiPSCs. We induced the step-wise differentiation of the hiPSC line Dura6.9 to mesoderm cells, vascular progenitors, and finally ECs, according to our previously established protocol (Fig. 1A).²² As demonstrated by RT-PCR analysis, transcript expression of the endothelial transcription factors ERG and ETS1 markedly increased approximately 40-fold and 20-fold, respectively, during the differentiation (Fig. 1B). Furthermore, expression of the EC markers platelet and endothelial cell adhesion molecule 1 (PECAM1, also called CD31 antigen) and VE-Cadherin robustly increased approximately 24 100 and 7000-fold by the end of the differentiation, respectively (Fig. 1B). Flow cytometry analysis showed that the percentage of VE-cadherin⁺ cells at days 4 and 8 was 78% and 86%, respectively (Fig. 1C), suggesting high efficiency in EC differentiation. Our data confirmed that vascular progenitors and ECs were sequentially and successfully induced from hiPSCs.

Next, we assessed the expression of MEF2 members in the differentiated cells in comparison to the same genes in hiPSCs. The expression levels of *MEF2A* and *MEF2B* did not change significantly during the differentiation and the expression level of *MEF2D* increased only approximately 3- to 4-fold during the differentiation (Fig. 1D). The *MEF2C* mRNA level, however, was robustly upregulated to more than 120-fold during the differentiation of ECs (Fig. 1D). Similar robust upregulation of *MEF2C* but not the other *MEF2s* during EC differentiation was observed in another independent hiPSC line iPSC(IMR-90)-4 (data not shown). Furthermore, immunostaining of the hiPSC-derived ECs (hiPSC-ECs) revealed that MEF2C was present in the nuclei of the CD31 positive iPSC-ECs (Fig. 1E). Therefore, our data demonstrated that *MEF2C* is the only MEF2 family member that is specifically and robustly upregulated during EC differentiation from hiPSCs.

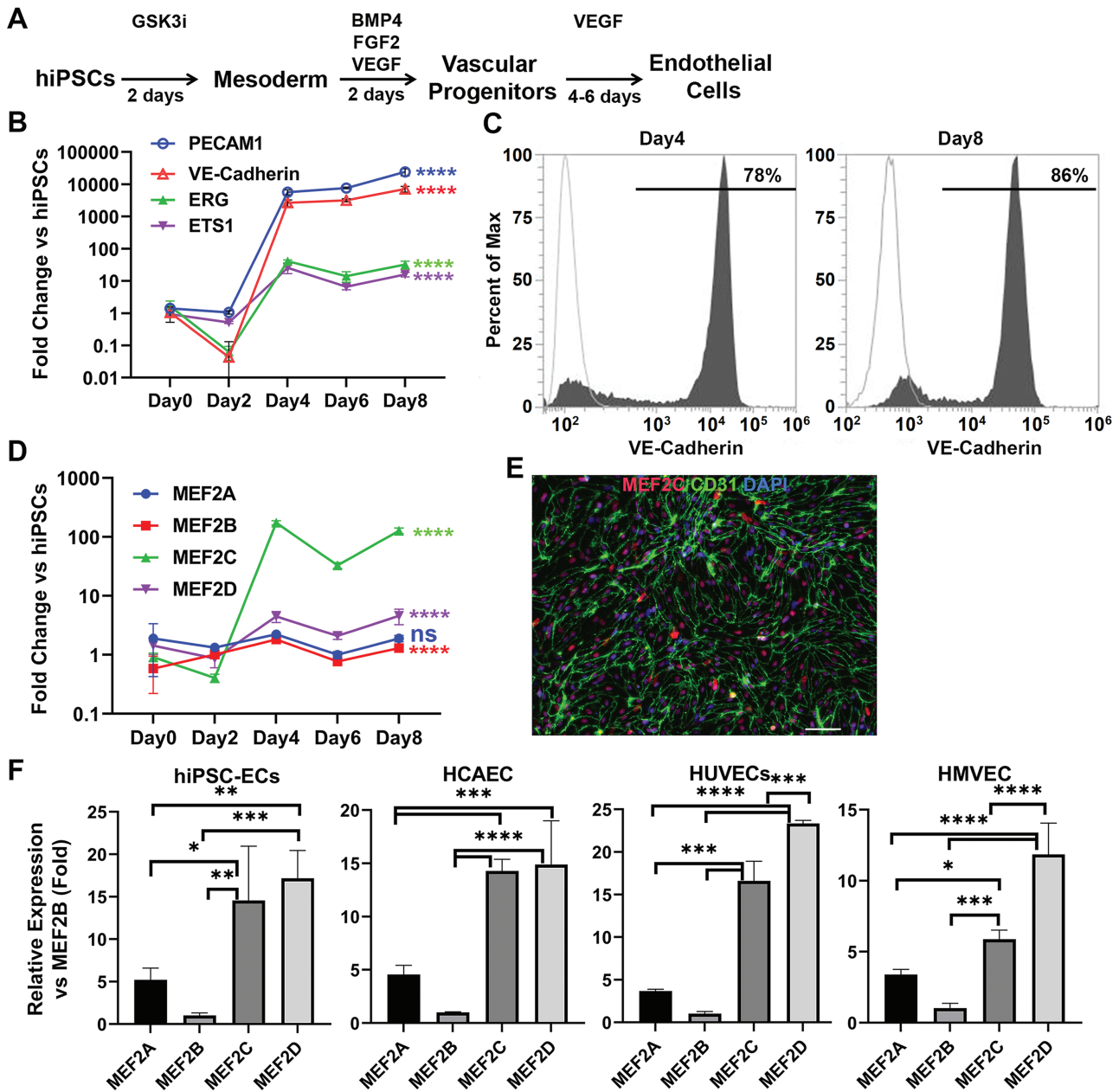


Figure 1. The expression of the endothelial specific genes and the MEF2 family genes during differentiation of hiPSCs toward endothelial cells (ECs). (A) Schematic representation of the protocol for differentiation of ECs from hiPSCs. (B) The expression of the endothelial marker PECAM1 and VE-Cadherin and transcription factors ERG and ETS1 during the differentiation was assessed by qRT-PCR ($n = 4$ or 5). (C) Flow cytometry analysis of the days 4 and 8 cells for VE-Cadherin. (D) The expression of the MEF2 family genes during the differentiation was assessed by qRT-PCR ($n = 4$ or 5). (E) Immunostaining of hiPSC-derived ECs (hiPSC-ECs) with antibodies targeting MEF2C (red) and CD31 (green). Nuclei were stained with DAPI (blue). Scale bar = 100 μm . (F) Relative expression of the MEF2 family genes in the hiPSC-derived ECs (iPSC-ECs), human coronary artery endothelial cells (HCAECs), human umbilical vein endothelial cells (HUVECs), and human cardiac microvascular endothelial cells (HMVECs) was assessed by RT-PCR. ($n = 4$) * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$.

To confirm the relevance of the expression of MEF2 family members in human ECs, the relative expression levels of the MEF2 family genes in hiPSC-ECs and several human primary ECs were compared by qRT-PCR. For comparison, the mRNA level of each MEF2 member was compared to the level of *MEF2B* in the same cell type. Both *MEF2C* and *MEF2D* were expressed at markedly higher levels than *MEF2A* and *MEF2B* in hiPSC-ECs as well as human primary ECs including coronary artery endothelial cells (HCAECs), human umbilical vein endothelial cells (HUVECs) and human cardiac microvascular endothelial cells (HMVECs) (Fig. 1F). Thus, MEF2C

and MEF2D are the predominant MEF2 proteins in both hiPSC-ECs and various human primary ECs.

MEF2C Expression was Induced by VEGF in Endothelial Lineage Cells

Since *MEF2C* expression was greatly upregulated during the induction of vascular progenitors at the differentiation stage 2 (Fig. 1C), we examined which growth factor(s) used at this differentiation stage induced the expression of *MEF2C*. The cells were differentiated from hiPSC line iPSC(IMR90)-4 over

4 days with or without exposure to FGF2, BMP4, VEGF, or any of their combinations (Fig. 2A) from days 2 to 4. qRT-PCR analysis of these cells showed that VEGF alone markedly induced *MEF2C* expression by more than 60-fold (Fig. 2B). Interestingly, although BMP4 or FGF2 by itself did not markedly upregulate *MEF2C* expression, the combination of VEGF, BMP4, and FGF2 further enhanced *MEF2C* expression by 135-fold (Fig. 2B). A similar synergistic effect of these 3 growth factors was previously shown by our group on the expression of endothelial lineage markers VEGFR2 and PECAM1.²² In contrast, the expression of *MEF2D* was not significantly induced in response to either VEGF, BMP4, or FGF2, and only marginally induced by approximately 3-fold when stimulated with both VEGF and BMP4 regardless of the presence or absence of FGF2 (Fig. 2B).

We also determined how several known endothelial-enriched transcription factors responded to VEGF, BMP4, and FGF2 in those stage 2 cells. RT-PCR analysis of these cells showed that *ETV2* was robustly induced by VEGF (Fig. 2C), but FGF2 and/or BMP4 attenuated VEGF-induced upregulation of *ETV2* (Fig. 2C). The expression of *FOXC2* was suppressed by either BMP4, FGF2, VEGF, or their combinations (Fig. 2C). *ETS1* moderate increased (approximately 3- to 6-fold) with either BMP4 or VEGF, but no synergistic effect of these growth factors on *ETS1* expression was detected (Fig. 2C). Interestingly, similar to the expression of *MEF2C*, the expression of both *FLI1* and *ERG* was upregulated by VEGF and optimally upregulated by the combination of VEGF, BMP4, and FGF2 (Fig. 2B).

To determine whether *MEF2C* was upregulated in vascular progenitors but not the other types of cells, the day 4 differentiated cells with or without exposure to VEGF or a combination of VEGF, BMP4, and FGF2 were immunostained for CD31 and *MEF2C*. VEGF increased the population of not only CD31⁺ cells but also *MEF2C*⁺ cells (Fig. 2D). Importantly, the *MEF2C* expression was detected in the CD31⁺ cells but not the CD31⁻ cells (Fig. 2C). Furthermore, the combination of VEGF, BMP4, and FGF2 generated many more cells that were both CD31⁺ and *MEF2C*⁺ (Fig. 2C). Moreover, immunostaining the day 8 ECs also showed that VEGF increased the protein level of *MEF2C*s (Fig. 2D). Thus, our data demonstrated that *MEF2C* upregulation strongly correlates with the differentiation to EC lineage, along with the endothelial transcription factors *FLI* and *ERG*.

The ERK Pathway Regulated the Expression of *MEF2C* But Not the Other *MEF2* Members

Our previous study revealed that the MAPK/ERK pathway is crucially required for the commitment of vascular lineage as well as the later stage differentiation of ECs from human pluripotent stem cells.²² Here we investigated whether the ERK pathway is also required for the expression of the *MEF2* family genes. The hiPSCs were first differentiated to vascular progenitors with or without exposure to specific ERK inhibitors from days 2 to 4 (Fig. 3A). RT-PCR analysis of these cells showed that the ERK inhibitor GDC-0994 blocked the induction of vascular progenitors from hiPSCs as demonstrated by the absence or diminished expression of *PECAM1*, *ERG*, *FLI*, and *ETS1* in the cells treated with the inhibitor (Fig. 3B). The expression of *MEF2A*, *MEF2B*, and *MEF2D* was not significantly reduced by the ERK inhibitor. In contrast to other *MEF2*s, the expression of *MEF2C* was substantially reduced by inhibition of the ERK pathway (Fig.

3A). Consistent inhibition of the expression of *MEF2C* by the MAPK inhibitor was also detected in another hiPSC line Dura6.9 (data not shown).

To assess the effect of the ERK pathway on *MEF2C* expression at the protein level, the day 4 differentiated cells that were exposed to another ERK inhibitor U0126 from days 2 to 4 were immunostained for *MEF2C*. U0126 markedly reduced *MEF2C* expression (Fig. 3C). Thus, the ERK pathway specifically promoted the expression of *MEF2C* but not the other members of the *MEF2* family at both the transcription and the protein levels. This evidence further validates the expression of *MEF2C* as a marker that correlates strongly with the differentiation of endothelial lineage in an ERK-dependent manner.

MEF2C Did Not Affect the Differentiation of ECs From hiPSCs

To test whether upregulation of *MEF2C* plays a role in determination of endothelial lineage, hiPSCs were transduced with lentiviruses encoding a scrambled shRNA or an shRNA targeting *MEF2C*. The *MEF2C* knockdown hiPSCs and the scrambled control hiPSCs were subjected to the EC differentiation. We first confirmed that *MEF2C* expression was reduced by more than 80% by the sh*MEF2C* compared to those in the control cells as demonstrated by qRT-PCR analysis (Fig. 4A). No significant change in the level of the *MEF2A* or the *MEF2B*, and only less than 40% reduction of the *MEF2D* were detected in the *MEF2C* knockdown cells compared to those in the scrambled control cells (Fig. 4A). The reduction of *MEF2C* in the sh*MEF2C* cells was further validated by immunostaining of these differentiated cells (Fig. 4B). The ECs derived from either the *MEF2C* knockdown or the scrambled control hiPSCs expressed endothelial marker CD31/PECAM-1 as shown by immunostaining (Fig. 4B). Furthermore, flow cytometry analysis showed that the cells differentiated from the *MEF2C* knockdown hiPSCs were 89.3 ± 3.9% CD144⁺/VE-cadherin⁺, comparable to those differentiated from the scrambled control hiPSCs (89.6 ± 0.7% CD144⁺/VE-cadherin⁺) (Fig. 4C). Thus, our data demonstrated that *MEF2C* knockdown does not affect the expression of typical endothelial markers in hiPSC-ECs.

To determine whether the ECs derived from either the *MEF2C* knockdown or the scrambled control hiPSCs exhibit characteristic endothelial functions, we assessed these cells for upregulation of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in response to tumor necrosis factor α (TNF α). These adhesion molecules play a central role in leukocyte transendothelial migration during inflammation.²⁶ The ECs derived from the hiPSCs with either *MEF2C* or the scrambled shRNA were exposed to TNF α at 2 doses (0.6 ng/mL and 10 ng/mL) or not exposed to TNF α (NS control). Flow cytometry analysis of these cells showed that both VCAM-1 and ICAM-1 were upregulated in a dose-dependent manner by TNF α (Fig. 4D). While no difference in the VCAM-1 levels between the *MEF2C* knockdown ECs and the scrambled ECs when compared at the same treatment of TNF α (Fig. 4D), the level of ICAM-1 was markedly lower in the *MEF2C* knockdown ECs than those in the scrambled ECs regardless of the TNF α treatment (Fig. 4D). It suggests that *MEF2C* may regulate the basal level of ICAM. Together our data demonstrate that upregulation of *MEF2C*, although a marker of mature EC, is not required for EC differentiation.

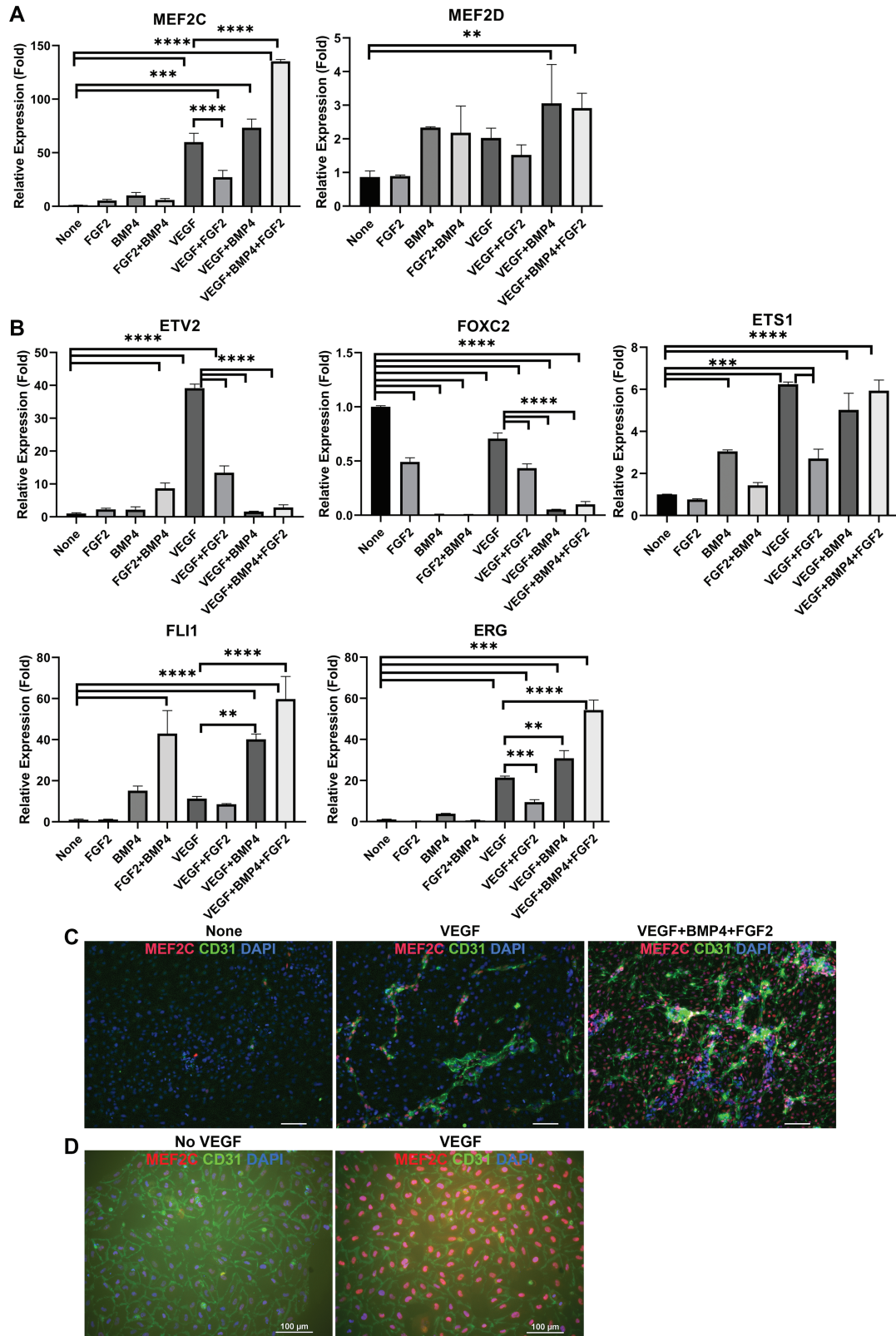


Figure 2. The effects of VEGF on the expression of the MEF2 genes and other endothelial transcription factors during EC differentiation. **(A, B, C)** hiPSCs were differentiated to vascular progenitors in 4 days with or without FGF2, BMP4, and VEGF from days 2 to 4. The cells were assessed by qRT-PCR for the expression of *MEF2C* and *MEF2D* (A) or the endothelial-enriched transcription factors (B). ($n = 4$). $**P < .01$, $***P < .001$, $****P < .0001$. **(C)** The day 4 cells were also immunostained for MEF2C (red) and CD31 (green). Nuclei were stained with DAPI (blue). Scale bar = 100 μm . **(D)** hiPSCs were differentiated into ECs in 8 days. These iPSC-ECs were serum starved (0.5% FBS) for 48 h and subsequently treated with or without 50 ng/mL VEGF for 24h. The cells were immunostained for MEF2C (red) and CD31 (green). Nuclei were stained with DAPI (blue). Scale bar = 100 μm .

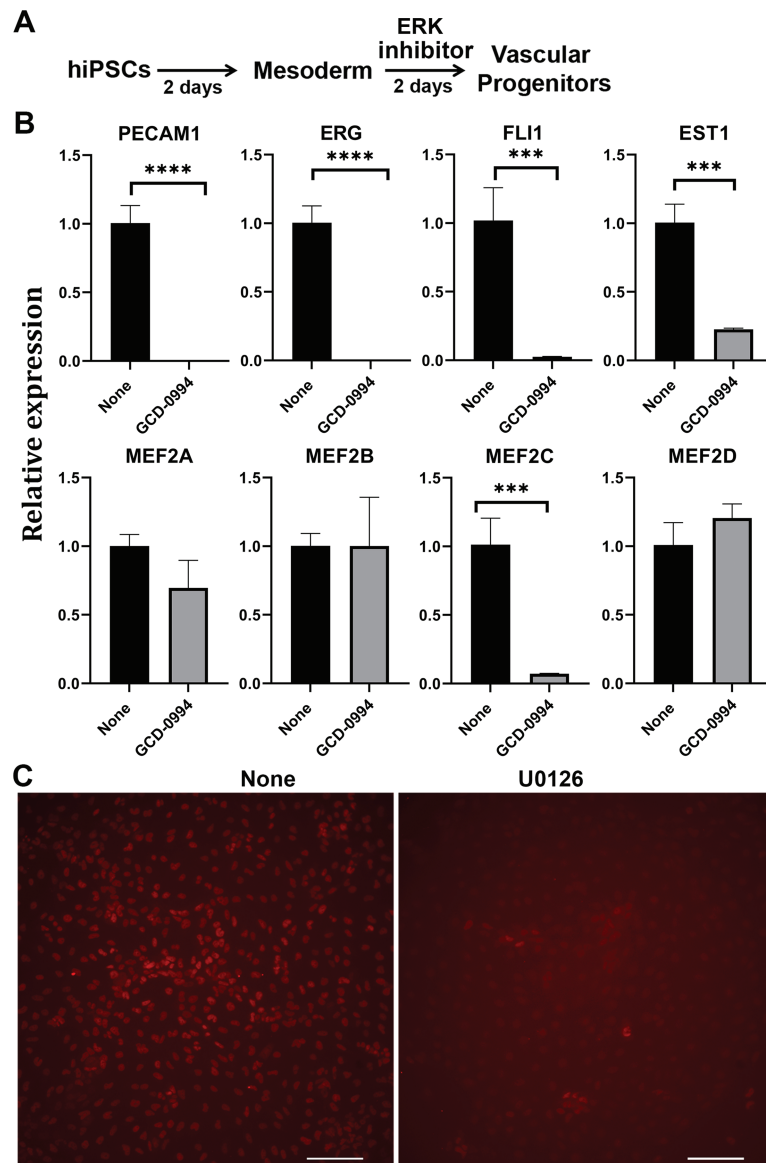


Figure 3. The effects of the ERK pathway on the expression of endothelial markers and *MEF2C*. **(A)** Schematic representation of the differentiation of vascular progenitor cells from hiPSCs with the treatment of the ERK inhibitor GCD-0994 (5 μ M) or U0126 (10 μ M). Control cells (None) were not treated with any inhibitor. **(B)** The expression of endothelial marker *PECAM1* and transcription factor *ERG*, *FLI*, *ETS1*, and the *MEF2* family genes were assessed by qRT-PCR. ($n = 4$) * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ vs. None. **(C)** The cells were immunostained with *MEF2C* (red). Scale bar=100 μ m.

MEF2C was Critical for Angiogenesis by hiPSC-ECs

Angiogenesis is the fundamental process of blood vessel growth from existing blood vessels. Angiogenesis is characterized in vitro by migration of ECs and formation of tubes composed of ECs. To test whether *MEF2C* plays a role in EC-mediated angiogenesis, we first assessed the migration of the *MEF2C* knockdown iPSC-ECs and the scrambled control hiPSC-ECs in response to a scratch-wound assay. Remarkably, the *MEF2C* knockdown hiPSC-ECs migrated much slower than the control scrambled hiPSC-ECs, leaving a larger gap between the opposite front of the ECs (Fig. 5A). Quantitation of the migrated distance of these ECs with ImageJ software further demonstrated that the *MEF2C* knockdown iPSC-ECs migrated significantly slower (<50%) than the control iPSC-ECs at both days 2 and 3 post scratch-wound (Fig. 5B). Therefore, *MEF2C* is critical for hiPSC-EC migration.

Next, we investigated the role of *MEF2C* in tube formation of hiPSC-ECs. The *MEF2C* knockdown hiPSC-ECs and the scrambled control hiPSC-ECs were grown on Matrigel. The hiPSC-ECs with *MEF2C* knockdown generated fewer tubes than the scrambled control scrambled hiPSC-ECs (Fig. 5C). *MEF2C* knockdown significantly reduced both the number of tubes and the total length of tubes per image field generated by the hiPSC-ECs (Fig 5D, 5E). Therefore, our data demonstrated that *MEF2C* plays an important role in not only hiPSC-EC migration but also tube formation, which are in vitro measures of angiogenesis.

Novel Potential Targets of *MEF2C* in ECs Are Identified

To identify potential downstream targets of *MEF2C* in hiPSC-ECs, we first analyzed the ECs derived from Dura6.9 hiPSCs

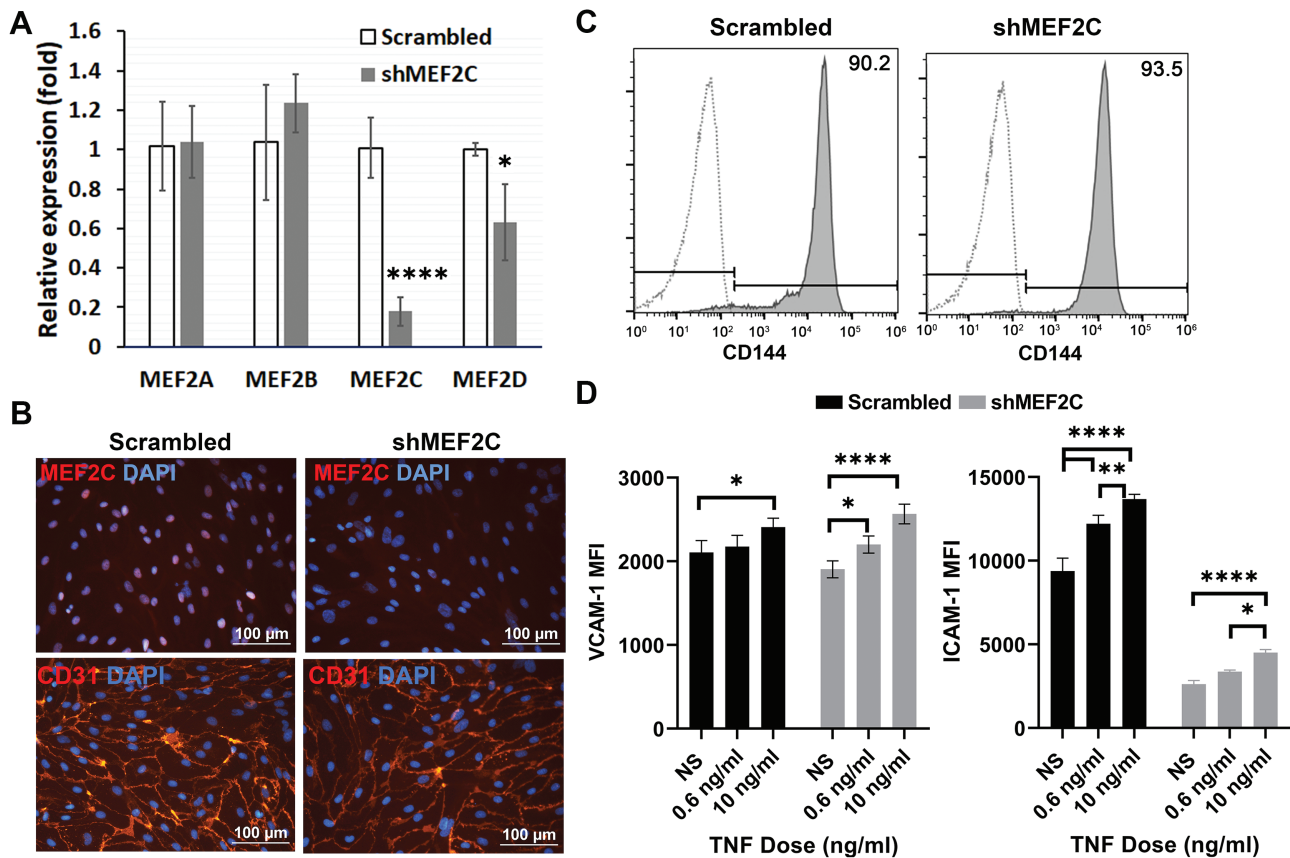


Figure 4. Effect of MEF2C knockdown on the differentiation of hiPSC-ECs. hiPSCs were transduced with lentiviruses encoding either a scrambled shRNA (Scrambled) or MEF2C shRNA (shMEF2C) and subsequently differentiated into ECs. **(A)** qRT-PCR assessment of the MEF2 family genes in the hiPSC-ECs with either MEF2C or the scrambled shRNA ($n = 4$). **(B)** Immunostaining of the hiPSC-ECs with either MEF2C or the scrambled shRNA with antibodies targeting MEF2C (red) or CD31 (red) as indicated. Nuclei were stained with DAPI (blue). Scale bar = 100 μ m. **(C)** Flow cytometry analysis of these cells for endothelial marker CD144. Dash line was the isotope control and solid line was CD144-stained cells. The number on the top right corner indicates the percentage of CD144⁺ cells ($n = 4$). **(D)** The ECs derived from the hiPSCs with either MEF2C or the scrambled shRNA were treated without (NS) or with TNF α at 0.6 ng/mL or 10 ng/mL for 4 h. These cells were analyzed by flow cytometry for their surface expression of VCAM-1 (A) and ICAM-1. MFI: Mean fluorescence intensity ($n = 4$). * $P < .05$, ** $P < .01$, **** $P < .0001$.

(control) and the MEF2C knockdown Dura6.9 hiPSCs (shMEF2C) using ChIP-seq analysis for MEF2C. Genes that had MEF2C binding peaks within the promoter region defined as -2.5 to $+0.5$ kb around transcription start sites were counted as potential MEF2C target genes. The control and shMEF2C samples showed 2459 and 3 MEF2C binding genes unique to the group, respectively, while both groups shared 314 genes (Fig. 6A). Gene ontology analysis of the MEF2C binding genes unique in the control group showed that 11 pathways were enriched in this group (Fig. 6B). Several pathways associated with neuron development and functions were targeted by MEF2C (Fig. 6B). Cell adhesion, cell-cell adhesion, small GTPase mediated signal transduction and cell differentiation were also targeted by MEF2C and may regulate EC migration (Fig. 6B).

To determine the transcriptional regulation of MEF2C on its binding targets, we performed RNA-Seq analysis of the hiPSC-derived vascular progenitors treated for 24 h without (control) or with a MEF2C inhibition molecule, MC1568 at 5 mM concentration. MC1568 was shown previously to inhibit class II HDACs and stabilize the HDAC-MEF2 complex, leading to repression of the MEF2 target genes.²⁷ By comparison of the MEF2C binding genes identified by the ChIP-seq assay and the transcriptionally regulated genes by MEF2C identified by the RNA-seq assay, we found that 79 MEF2C

binding genes were either upregulated or downregulated by at least 2-fold in the MC1568 treated cells compared to the control cells (Supplementary Table S2). Among these 79 genes, 15 genes were upregulated and 5 genes were downregulated by 3-fold or more (Fig. 6C). NFASC, SHANK2, KCNAB2, UNC13A, and KCNQ3 are associated with neural functions and were upregulated in the MEF2C-inhibited cells. The most downregulated genes in the MEF2C inhibited cells were transmembrane protein 100 (TMEM100) and TNF-related apoptosis inducing ligand (TRAIL) (Fig. 6C). TMEM100 and TRAIL have been implicated in regulating angiogenesis,²⁸⁻³¹ but have not been identified as MEF2C targets. Our ChIP-seq data showed that MEF2C binding peaks on the promoter region of *TMEM100* and *TRAIL* were present in the control hiPSC-ECs but absent in the shMEF2C hiPSC-ECs (Fig. 6D). Our data demonstrate that MEF2C directly binds to the promoter region of *TMEM100* and *TRAIL*.

To further confirm that the expression of *TMEM100* and *TRAIL* is regulated by MEF2C, we assessed the expression level of these 2 genes in the MEF2C knockdown iPSC-ECs and the scrambled control hiPSC-ECs by qRT-PCR. Knockdown of MEF2C in iPSC-ECs significantly reduced the expression of *TMEM100* and *TRAIL* to the level of 25% and 43% relative to those in the control scrambled hiPSC-ECs, respectively (Fig. 6E). In addition, immunostaining of the MEF2C

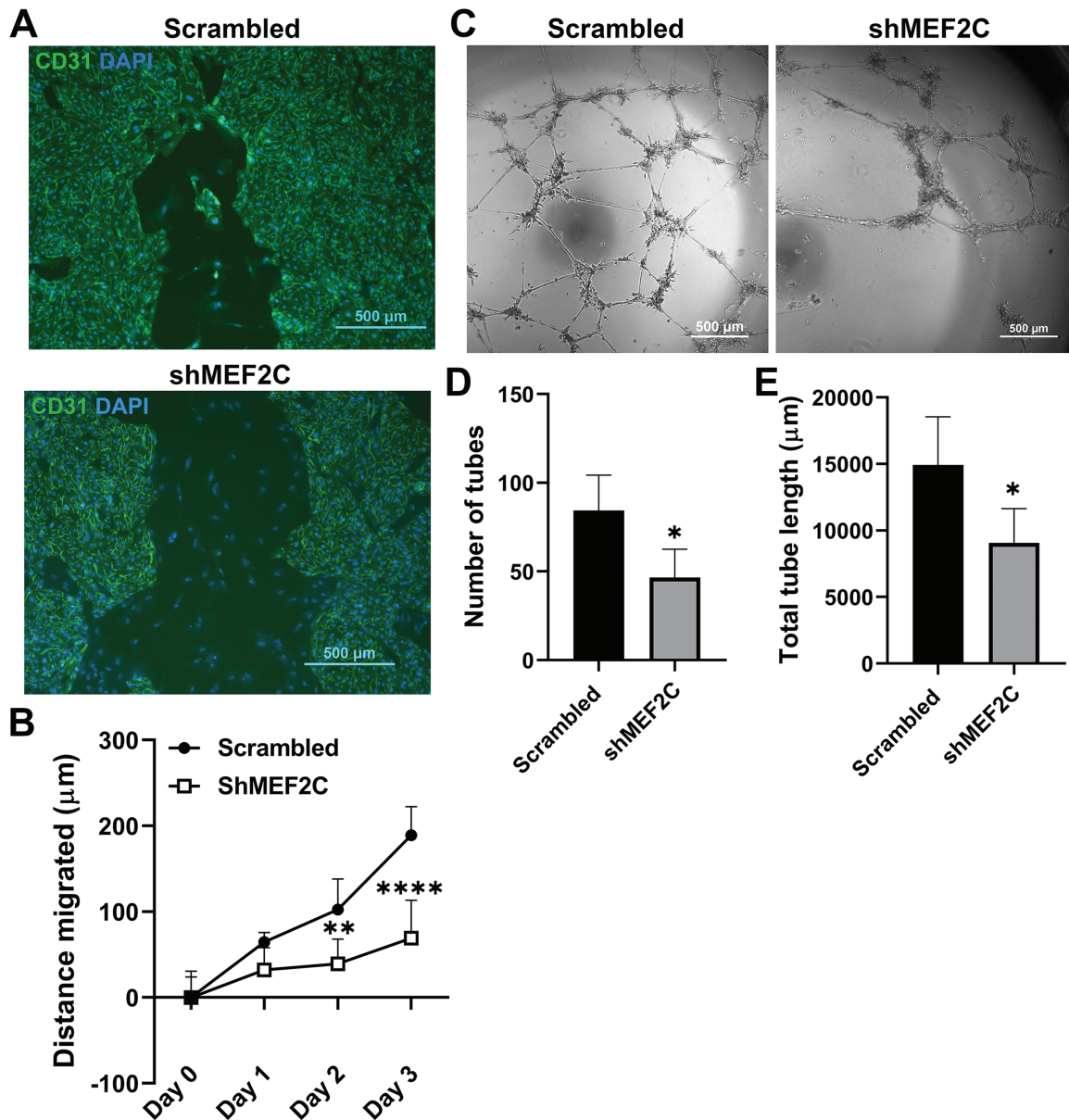


Figure 5. Effect of MEF2C knockdown on migration and tube formation of hiPSC-EC. (A, B) Confluent hiPSC-ECs with either an MEF2C or a scrambled shRNA were wounded with a scratch from a pipette tip. Phase images were taken daily. The cells were immunostained with CD31 antibodies (green) at day 3 (A). scale=500 µm. The distance migrated was measured using ImageJ (B) ($n = 8$). (C, D, E) The hiPSC-ECs with either MEF2C or the scrambled shRNA were grown on Matrigel beds. Phase images were taken (1 image/well) at 15-16 h after the cells were plated (C). Scale = 500 µm. The number of tubes (D) and total tube length (E) in a photo were measured using ImageJ ($n = 5$). * $P < .05$, ** $P < .01$, **** $P < .0001$ vs. Scrambled.

knockdown iPSC-ECs and the scrambled control hiPSC-ECs further showed that TMEM100 was substantially reduced by MEF2C knockdown (Fig. 6F). Although TRAIL was present in both the MEF2C knockdown iPSC-ECs and the scrambled control hiPSC-ECs, its location was mostly in the cytosol of the control cells but in the nuclei in the MEF2C knockdown iPSC-ECs (Fig. 6F). Thus, our studies identify TMEM100 and TRAIL as novel potential targets of MEF2C that may contribute to the MEF2C-mediated regulation of angiogenesis.

Discussion

Vascular dysfunction is a major cause of severe illness and death worldwide. Understanding the mechanisms that regulate blood vessels is important for identification of potential

targets to treat vascular malfunction-associated diseases. Loss of function mutations in *MEF2C* are associated with congenital double-outlet right ventricle as well as ventricular septal defect in patients.³² To our knowledge, we are the first to investigate the role of the MEF2 family proteins in endothelial differentiation from human iPSCs and angiogenesis by these hiPSC-derived ECs. Although MEF2A, MEF2C, and MEF2D were previously shown to be expressed in mouse endothelial cells,¹⁶ we found that MEF2C was the only MEF2 family member that was robustly induced during the differentiation of ECs from hiPSCs (Fig. 1). We further showed that MEF2C played a critical role in angiogenesis of hiPSC-ECs (Fig. 5).

Our data showed that the expression of *MEF2C* was induced by VEGF and was further increased in the presence of FGF2 and BMP4 during the differentiation of vascular

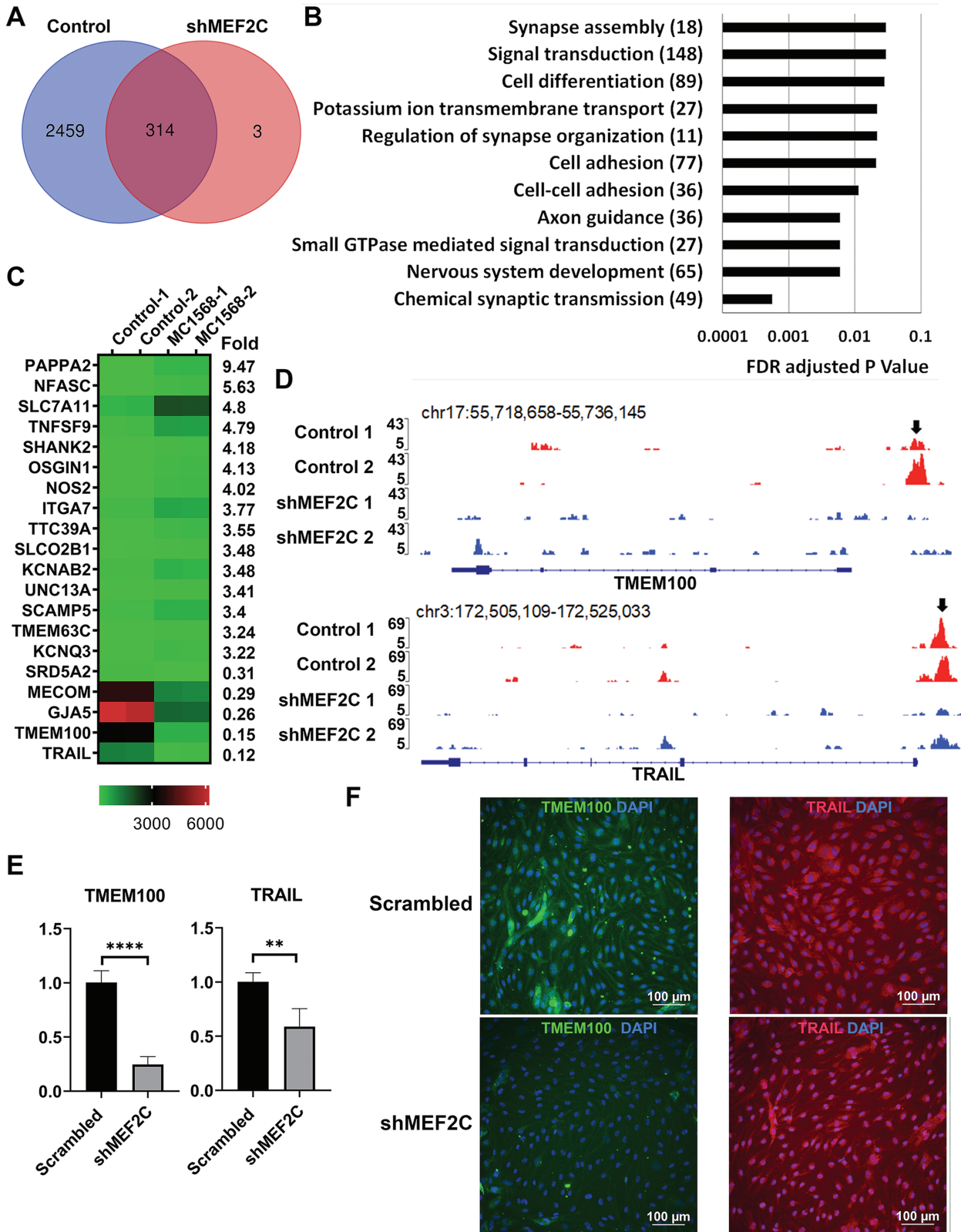


Figure 6. Analysis of the MEF2C regulated genes. (**A, B, D**) Chromatin immunoprecipitation with sequencing (ChIP-seq) analysis of the hiPSC-ECs (control) and the MEF2C knockdown hiPSC-ECs (shMEF2C) for MEF2C binding sites: A Venn diagram displaying the number of overlapping and nonoverlapping peaks between the 2 groups (A); Gene Ontology (GO) Biological Process analysis of the pathways enriched in the control samples compared to the shMEF2C samples (B); The MEF2C peaks on the *TMEM100* and the *TRAIL* gene in the control and shMEF2C ECs. Arrows indicate the peaks in their promoter regions (−2.5 to 0.5 kb around transcription start sites) (D). (**C**) RNA-Sequencing analysis of the hiPSCs-derived vascular progenitors cultured for 24 h with or without (control) 5 μ M MC1568, which functionally represses MEF2C. The differentially expressed genes with >3-fold changes and MEF2C binding sites identified by the ChIP-seq data are shown. The numbers on the right indicate the fold change in MEF2C-inhibited samples. (**E**) qRT-PCR analysis of the *TMEM100* and the *TRAIL* gene in the hiPSC-ECs with either the scrambled or MEF2C shRNA (n = 4). *P < .05, ****P < .0001. (**F**) Immunostaining of the hiPSC-ECs with either the scrambled or MEF2C shRNA for TMEM100 (green) or TRAIL (red). Nuclei were stained with DAPI (blue). Scale bar = 100 μ m.

progenitors (Fig. 2). Furthermore, VEGF upregulated MEF2C expression in hiPSC-ECs (Fig. 2). VEGF acts by binding to VEGF receptors at the plasma membrane and subsequently activating its downstream signaling pathways including the ERK pathway inside the cell.³³ We showed that the ERK pathway specifically regulated *MEF2C* expression at both the mRNA and protein levels, while no other member of MEF2s was regulated by ERK (Fig. 3). Therefore, the ERK pathway is at least partially responsible for VEGF-mediated upregulation of *MEF2C*.

It remains to be determined how VEGF/ERK pathway upregulates *MEF2C* expression. An endothelial specific enhancer of *MEF2C* with ETS binding site was previously identified by De Val et al.³⁴ The ETS family transcription factor FoxC2 and ETV2 were previously shown to bind and activate the *MEF2C* promoter.³⁵ However, we found that *ETV2* and *FOXC2* exhibited a completely different expression pattern in response to VEGF, FGF2, and BMP4 compared to *MEF2C* and thus are unlikely the primary inducers of *MEF2C* during EC differentiation from hiPSCs. Remarkably, *MEF2C* showed a similar pattern of expression to that of ETS family transcription factors ERG or FLI1 in response to VEGF, FGF2, and BMP4 (Fig. 2). Furthermore, inhibition of ERK markedly reduced the expression of not only *MEF2C* but also *ERG* and *FLI1* (Fig. 3). ERK was previously shown to directly phosphorylate ERG and thus to activate the transcriptional activity of ERG.^{36,37} It is highly possible that VEGF upregulates *MEF2C* through ERK and subsequently ERG.

The effect of MAPK pathways on the post-translational modification of *MEF2C* was previously shown. The transactivation properties of *MEF2C* were shown to be enhanced by the p38 MAP kinase^{38,39} and BMK1/ERK5 MAP kinase⁴⁰ through phosphorylation of *MEF2C* by these signaling molecules. ERK1/2 and JNK do not directly phosphorylate *MEF2C* in an in vitro kinase assay.^{38,40,41} However, ERK1/2 is required for the neuroprotective activity of *MEF2C* in cortical neurons stimulated by brain-derived neurotrophic factor (BDNF).⁴² The downstream target of ERK1/2, p90 ribosomal S6 kinase 2 (RSK2), can directly phosphorylate *MEF2C* on Ser192.⁴²

Using a *MEF2C* shRNA knockdown approach, we demonstrated that *MEF2C* was not required for the differentiation of ECs from hiPSCs and for the TNF α -induced upregulation of VCAM-1 and ICAM-1 in hiPSC-ECs (Fig. 4). However, we found that *MEF2C* was critical for the angiogenesis of the ECs derived from hiPSCs. The *MEF2C* knockdown ECs showed markedly reduced tube formation as well as migration (Fig. 5). Although *MEF2D* was at the level comparable to *MEF2C* in hiPSC-ECs and in human primary ECs (Fig. 1E), *MEF2D* failed to compensate for the loss of *MEF2C* in hiPSC-ECs in migration and tube formation assays. In mice, on the other hand, the loss of *MEF2C* in ECs could be compensated by other members of the MEF2 family.¹⁴⁻¹⁶ It remains to be determined whether the exclusive role of *MEF2C* in angiogenesis is human specific. Zhang et al reported that overexpression of *MEF2C* reduced the migration of vascular smooth muscle cells.⁴³ Apparently, the effect of *MEF2C* on cell migration can be cell-type specific.

Using ChIP-Seq and RNA-seq analysis, we identified TRAIL and TMEM100 as novel potential targets of *MEF2C*. We showed that *MEF2C* directly binds to the promoter region of TRAIL and TMEM100 (Fig. 6D). Furthermore, *MEF2C* inhibition or knockdown substantially reduced

the expression of *TMEM100* and *TRAIL* at the mRNA level (Fig. 6C, 6E). Moreover, *MEF2C* knockdown markedly reduced the *TMEM100* protein level and resulted in nuclear accumulation of TRAIL (Fig. 6E, 6F). TRAIL has been shown to both inhibit angiogenesis in tumors^{28,29} and promote angiogenesis in non-tumor cells or tissues.^{30,31} Inhibition of nuclear export of TRAIL was shown to enhance TRAIL-sensitivity in osteosarcoma.⁴⁴ *TMEM100* null mice showed embryonic lethality due to impaired differentiation of arterial endothelium and defects of vascular morphology.⁴⁵ Further studies are warranted to determine whether TRAIL and *TMEM100* mediate the effects of *MEF2C* on angiogenesis.

Conclusion

We have demonstrated that *MEF2C* is specifically upregulated during the differentiation of ECs from hiPSCs and plays a critical role in angiogenesis.

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Conflict of Interest

The authors declare no potential conflicts of interest

Author Contributions

TL: conception and design, data collection, analysis and interpretation, financial support, manuscript writing; KLC, KG, AMK, EM, JH: data collection and analysis; AGP, AW, JA: data interpretation, financial support, manuscript writing; PZ.: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript.

Data Availability

The authors will comply with the NIH and the existing policies on sharing data. The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary Material

Supplementary material is available at *Stem Cells* online.

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