A MST1–FOXO1 cascade establishes endothelial tip cell polarity and facilitates sprouting angiogenesis

Hypothesis: Hypoxia is a main driver of sprouting angiogenesis, but how tip endothelial cells are directed to hypoxic regions remains poorly understood. Here, we show that an endothelial MST1–FOXO1 cascade is essential for directional migration of tip cells towards hypoxic regions. In mice, endothelial-specific deletion of either MST1 or FOXO1 leads to the loss of tip cell polarity and subsequent impairment of sprouting angiogenesis. Mechanistically, MST1 is activated by reactive oxygen species (ROS) produced in mitochondria in response to hypoxia, and activated MST1 promotes the nuclear import of FOXO1, thus augmenting its transcriptional regulation of polarity and migration-associated genes. Furthermore, endothelial MST1–FOXO1 cascade is required for revascularization and neovascularization in the oxygen-induced retinopathy model. Together, the results of our study delineate a crucial coupling between extracellular hypoxia and an intracellular ROS-MST1-FOXO1 cascade in establishing endothelial tip cell polarity during sprouting angiogenesis.
The vascular system expands its network from pre-existing vessels by sprouting angiogenesis for supplying oxygen and nutrients to avascular and hypoxic tissues. In response to numerous angiogenic cues from oxygen- and nutrient-deprived tissues, endothelial cells (ECs), the main components of the vascular lumen, adopt a series of morphogenic behaviors, such as tip ECs and stalk ECs, for coordinating sprouting angiogenesis. Tip ECs are championed cells and highly migratory, leading the sprouts in the direction of a guidance cue, while stalk ECs are proliferative, supplying building blocks for sprout elongation. Haemodynamic forces drive lumen expansion into newly formed sprouts to deliver oxygen- and nutrient-rich blood flow. These overall processes are finely regulated by various extrinsic cues and corresponding intrinsic signaling in the ECs. Lately, significant advances have been made in the understanding of intrinsic transcriptional and metabolic changes in tip ECs; however, how they are directed—the EC polarization at the vascular front—into the avascular, hypoxic area is poorly understood.

Mammalian sterile 20-like kinases 1 and 2 (MST1/2) have been characterized as mediators of oxidative stress and lately characterized as the major component of the Hippo pathway. The mammalian core Hippo pathway components encompass MST1/2, large tumor suppressor homolog 1 and 2 (LATS1/2), and Yes-associated protein (YAP) or its paralog transcriptional coactivator with PDZ-binding motif (TAZ). YAP/TAZ are transcription coactivators that mainly interact with the TEAD/TEF family of transcription factors and play crucial roles in regulating cellular proliferation, differentiation and migration, tissue growth, and organ morphogenesis. We and others lately have found that YAP/TAZ play critical roles in the morphogenesis of tip ECs and proliferation of stalk ECs by regulating cytoskeletal rearrangement and metabolic activity during sprouting angiogenesis. LATS1/2 are direct upstream regulators of YAP/TAZ, limiting their activities through phosphorylation-dependent cytoplasmic retention and destabilization. Indeed, endothelial deletion of LATS1/2 enhances activities of YAP/TAZ, leading to a dense and hyperplastic network, uncoordinated outgrowth, numerous filopodia bursts in tip ECs, and increased proliferating ECs in growing retinal vessels. Overall, this LATS1/2-YAP/TAZ cascade responds to vascular endothelial growth factor-A (i.e., VEGF) and regulates angiogenesis. MST1/2 are serine/threonine kinases that are expressed ubiquitously in most tissues and cell types. MST1/2 phosphate and activate LATS1/2, and thereby inactivate YAP/TAZ in the canonical Hippo pathway. However, these kinase–substrate relationships are highly cell type- and context-dependent. Specifically, MST1 is activated by cellular stress such as ultraviolet radiation, serum starvation, hydrogen peroxide, and reactive oxygen species (ROS), followed by phosphorylation of its cellular substrates including Forkhead box (FOXO) proteins. In fact, MST1 mediates oxidative stress-induced neuronal cell death through phosphorylation of FOXO1 at serine 212, which leads to disruption of the association between FOXO1 and 14-3-3 proteins, subsequently enhancing nuclear import of FOXO1. Of importance in ECs, FOXO1 is a crucial gatekeeper for EC quiescence mediated through reducing glycolysis, mitochondrial respiration, and proliferation by suppressing MYC during sprouting angiogenesis.

Here, we unveil that MST1 acts as an upstream regulator of FOXO1 rather than of LATS1/2 and plays key roles in sprouting angiogenesis by establishing endothelial polarity at tip ECs. Moreover, hypoxia rather than VEGF monopolizes the MST1–FOXO1 cascade in this context. Our results demonstrate a crucial coupling between extracellular hypoxia and an intracellular MST1–FOXO1 cascade, which facilitates sprouting angiogenesis.

Results

MST1 is involved in establishing endothelial polarization. Considering that MST1/2 are upstream regulators of LATS1/2 in the Hippo pathway, we hypothesized that the roles of MST1/2 could be similar to those of LATS1/2 but opposite to those of YAP/TAZ in sprouting angiogenesis, based on our previous report. To investigate the role of MST1 in sprouting angiogenesis, we generated Mst1ΔEC mice by crossing Mst1flox/flox mice with VE-cadherin Cre-ERT2 mice (Fig. 1a), confirmed EC-specific deletion of MST1 in these animals (Supplementary Fig. 1a–d), and examined retinal angiogenesis during the postnatal period. Cre-ER12-positive but flox/negative mice among the littermates for each experiment were defined as wild-type (WT) mice. EC-specific deletion of MST1 in Mst1ΔEC mice from P1 led to impaired retinal angiogenesis at P6. Compared with WT mice, Mst1ΔEC mice exhibited respectively 29 and 60% reduced vascular branching and density, and 72% reduced 5-ethyl-2’-deoxyuridine (EdU) incorporation (proliferation), but no difference in cleaved caspase3 (apoptosis) in the ECs at the vascular front (Fig. 1b, c and Supplementary Fig. 2a–c). Moreover, tip ECs at the vascular front of Mst1ΔEC mice exhibited reduced both sprout number and length, but no difference in filopodia formation per sprout compared with WT mice (Fig. 1d, f). Of special interest, Mst1ΔEC mice showed abnormally aligned VE-cadherin (marking endothelial junctions) and ETS-related gene (ERG, marking endothelial nuclei) in the vascular front of the growing retinal vessels (Fig. 1e).

These findings led us to ask whether MST1 regulates endothelial polarization during sprout elongation. To address this question, we further analyzed shapes and locations of EC nuclei in the vascular front by visualizing ERG; these nuclei are elliptical in actively migrating cells and spherical in static cells. Consistent with previous reports, the nuclei of tip ECs were largely elliptical in WT mice. In contrast, they were spherical and not directed into the avascular area although filopodia properly extended in Mst1ΔEC mice (Fig. 1d, g). Moreover, ERG+ nuclei of tip ECs overlapped each other in Mst1ΔEC mice but positioned in a planar fashion in WT mice (Fig. 1g).

We additionally assessed the orientation of Golgi apparatus in tip ECs to analyze EC polarity according to previous reports. Note, the Golgi apparatus were polarized toward the anterior or posterior of the nuclei in tip ECs of WT mice, whereas such polarization was lost in tip ECs of Mst1ΔEC mice (Fig. 1b and Supplementary Fig. 2d). These data indicate that MST1 plays a critical role in endothelial polarization during sprouting angiogenesis.

MST1 is crucial for perpendicular vascular branching. Because retinal angiogenesis analyzed at P6 represents sprouting angiogenesis in two-dimensional space, we determined the role of MST1 in angiogenesis in three-dimensional space by analyzing retinal deep vascular plexus formation and brain angiogenesis at P12. Of importance, perpendicular vascular sprouting was not extended into the retinal deep layer in Mst1ΔEC mice; instead, sprouts passed each other, displaying aberrant vessel crossings. As a result, vascular network formation in the retinal deep layer was severely impaired in Mst1ΔEC mice at P12. Nevertheless, no apparent impairments in lumen formation (outlined by ICAM2 distribution), collagen type IV deposition (basement membrane), or barrier integrity [shown by TER119+ red blood cells (RBCs) inside vascular lumen] were shown in Mst1ΔEC mice (Fig. 2f–h). Similarly, brain angiogenesis in cerebellum but not in cerebral was also impaired with accompanying reduced vascular density without affecting barrier integrity (no change in GLUT1 intensity) compared with WT.
MST1 is critical for perpendicular vascular branching. MST1 does not rely on the Hippo pathway in angiogenesis. Because MST2 is closest to MST1 among the five members of the MST kinase family, we determined whether Mst2 null mice exhibited similar phenotypes in retinal angiogenesis during the postnatal period (Supplementary Fig. 3a). Although no apparent differences were detected in retinal angiogenesis between WT and Mst2 null mice, the defective phenotypes of double Mst1ΔEC/Mst2 ΔEC null mice were similar to those of Mst1ΔEC mice (Supplementary Fig. 3b, c), indicating that endothelial MST1 but not MST2 critical in sprouting angiogenesis.

To see whether LATS1/2 are downstream regulators of MST1 in sprouting angiogenesis, we characterized the vascular phenotypes in growing retinal vessels of Lats1/2ΔEC mice and compared them with those of Mst1ΔEC mice (Supplementary Fig. 3a).
Fig. 1 MST1 plays a critical role in establishing endothelial polarization. **a** Diagram depicting the experimental schedule for endothelial cell (EC)-specific deletion of Mst1 in retinal vessels from P1 and their analyses at P6 using Mst1CreiΔEC mice. **b–g** Images of CD31+ retinal vessels and comparisons of indicated parameters in WT (n = 6) and Mst1CreiΔEC (n = 6) mice. Scale bars, 200 μm. **d** Magnified images of CD31+ vessels and ERG+ nuclei of ECs in WT and Mst1CreiΔEC mice. The insets (white dashed-line boxes) are 3D reconstructed and magnified in **g**. Scale bars, 50 μm. **e** Images showing abnormally aligned VE-cadherin (VEcad) and ERG+ nuclei of ECs in the vascular front of Mst1CreiΔEC mice compared with those of WT mice. Middle and bottom panels show VEcad and ERG signals of insets (white dashed-line boxes) in top panels. Scale bars, 100 μm. **f** Comparisons of indicated parameters in WT (n = 5) and Mst1CreiΔEC (n = 5) mice. **g** 3D reconstructed images of ERG+ nuclei of ECs from the front and a 45° angle showing that the ERG+ nuclei of ECs overlap each other in Mst1CreiΔEC mice. **h** Images of CD31+ vessels, ERG+ nuclei of ECs, and GM130+ Golgi apparatus at tip ECs of WT and Mst1CreiΔEC mice. The yellow dashed line outlines CD31+ vessels. Note that GM130+ Golgi apparatus are polarized towards the anterior or posterior of the nuclei in tip ECs of WT mice (yellow arrowheads), while such polarization is lost in tip ECs of Mst1CreiΔEC mice (yellow arrows). Scale bars, 20 μm. Data represent mean (bar) ± s.d. (error bars). P values, versus WT by two-tailed unpaired t-test. NS not significant. Source data are provided as a Source Data file.

Fig. 4a). However, in contrast to those of Mst1fl/+EC mice, Lats1/2ΔEC mice exhibited 49 and 40% increased vascular density and branching, respectively. ERG+ nuclei of tip ECs in Lats1/2ΔEC mice were elliptical and properly aligned in the direction of filopodia extension, which was identical to that of WT mice (Supplementary Fig. 4b, d). Furthermore, TAZ was 12-fold enriched in the retinal vessels of Lats1/2ΔEC mice but unchanged in those of Mst1fl/+EC mice (Supplementary Fig. 4c, d). Thus, vascular phenotypes of Mst1fl/+EC mice were completely different from those of Lats1/2ΔEC mice, meaning that MST1 does not act as an upstream regulator of LATS1/2 by taking a canonical Hippo pathway for directional sprouting angiogenesis. These findings led us to seek an alternative downstream pathway of MST1 for sprouting angiogenesis.

**MST1 regulates FOXO1 localization at tip ECs.** In seeking the alternative downstream effector of MST1 beyond the canonical Hippo pathway, we predicted that FOXO1, a critical transcriptional and metabolic regulator for ECs during sprouting angiogenesis14, can be a downstream cellular substrate of MST1, based on the findings at other cell types in previous reports19,21,22. Of note, FOXO1 was highly and specifically expressed in most ECs but showed differential subcellular localizations in different regions of growing retinal blood vessels (Fig. 3a, b). Consistent with a previous report11, a diffuse nucleoeytoplasmic localization of FOXO1 at the vascular front but relatively intense nuclear localization at the vascular plexus was shown. Beyond those findings, we identified relatively strong nuclear localization of FOXO1 at tip ECs of WT mice (Fig. 3b). However, of special note, Mst1fl/+EC mice exhibited a nucleoeytoplasmic localization of FOXO1 at tip ECs. Moreover, angiopoietin-2 (Angpt2), which is expressed at tip ECs under control of FOXO1, was markedly reduced in Mst1fl/+EC mice (Fig. 3c–e). These data imply that MST1 may promote nuclear import of FOXO1 in tip ECs during sprouting angiogenesis.

**FOXO1 is required for establishing endothelial polarization.** To address the role of nuclear FOXO1 in tip ECs during sprouting elongation, we generated Foxo1fl/+EC mice by crossing Foxo1fl/fllox/lox mice38 with VE-cadherin Cre-ERt2 mice (Fig. 4a and Supplementary Fig. 5a, b). When Foxo1 was specifically deleted in ECs of Foxo1fl/+EC mice from P1, the mice at P6 showed retarded radial vessel growth (Fig. 4b, c). Foxo1fl/+EC mice exhibited three major defective subtypes of vascular sprouts depending on the regionally variant subcellular localization of FOXO1 in growing retinal blood vessels: flat type, hill type at the peri-arterial ECs, and glomeruloid type at the peri-venous ECs (Supplementary Fig. 5c). However, regardless of these subtypes, abnormally aligned VE-cadherin and ERG+ nuclei were detected in all tip ECs of Foxo1fl/+EC mice (Fig. 4d). In fact, tip ECs were stacked with vascular front ECs and the ERG+ nuclei of tip ECs were spherical and randomly positioned, but the filopodia extension of tip ECs was directed to the avascular region in Foxo1fl/+EC mice (Fig. 4e, h). Of interest, the ERG+ nuclei of tip ECs strikingly overlapped each other in Foxo1fl/+EC mice, but arranged in a planar distribution in WT mice (Fig. 4f). Moreover, the polarization of Golgi apparatus was lost in tip ECs of Foxo1fl/+EC mice (Fig. 4g and Supplementary Fig. 5d). Thus, the vascular phenotypes of Foxo1fl/+EC mice are similar to those of Mst1fl/+EC mice, implying that FOXO1 could be a downstream substrate of MST1 for endothelial polarization during sprouting angiogenesis.

**Hypoxia activates MST1–FOXO1 cascade in primary cultured ECs.** To reveal the factors that activate MST1 during sprouting angiogenesis, we analyzed the publicly available microarray data (GSE19284) that identified enriched genes in tip ECs39. Of note, the genes related to ROS, including ROS biosynthesis and response to ROS, and those related to hypoxia were enriched in tip ECs compared to non-ECs (Fig. 5a). These findings led us to hypothesize that a hypoxic microenvironment in the vascular front constantly induces intracellular ROS synthesis, which activates the MST1–FOXO1 cascade at tip ECs. To address this hypothesis, primary cultured human umbilical vascular endothelial cells (HUVECs) were exposed to hypoxia (1% O2). After hypoxia exposure, increased MST1 phosphorylation at Thr183 (pMST1) was detected already at 30 min, gradually increased without alteration of the activity of canonical Hippo pathway, peaked at 6 h, decreased thereafter, and returned to basal level at 36 h. Under the same condition, increased changes in HIF1α protein level and FOXO1 phosphorylation at Ser212 (pFOXO1) were positively correlated with increases in pMST1 (Fig. 5b, c; Supplementary Figs. 6 and 7a–e). These data imply that hypoxia activates MST1 and FOXO1 in ECs, leading us to ask whether ROS directly contributes to activate the MST1–FOXO1 cascade and where ROS is produced in ECs under hypoxia.

The process of ROS biosynthesis by NADPH oxidase, xanthine oxidase and mitochondrial electron transport chain requires oxygen as a substrate. However, even though the exact mechanism remains elusive, ROS can be produced in hypoxia, which is mainly dependent on mitochondrial electron transport chain40,41. To delineate the relationship among hypoxia-intracellular ROS–MST1–FOXO1 in ECs, we treated Trolox (a ROS scavenger) and rotenone (a mitochondrial electron transport chain 1 inhibitor) to the HUVECs. Both Trolox and rotenone suppressed the hypoxia-induced stabilization of HIF1α protein level by 31 and 48% and pMST1 by 35 and 41%, respectively (Fig. 5d, e), indicating that hypoxia-induced MST1 activation is primarily mediated through intracellular ROS biosynthesis at the mitochondria in ECs. We then asked whether pFOXO1 depends on pMST1, according to previous report19,22. Of note,
**Fig. 2** MST1 is crucial for perpendicular vascular branching. 

**a** Diagram depicting the experimental schedule for EC-specific deletion of MST1 in retinal vessels from P1 and their analyses at P12 in Mst1ΔEC mice. 

**b, c** Images of CD31⁺ vessels in superficial and deep layers of retina and comparisons of indicated parameters in WT (n = 5) and Mst1ΔEC (n = 5) mice. Scale bars, 500 μm. 

**d** 3D reconstructed images of CD31⁺ vascular plexus and comparisons of the length of perpendicular growth in WT (n = 5) and Mst1ΔEC (n = 5) mice. Note that aberrant vessel crossings (yellow arrowheads) are shown. Scale bars, 500 μm. 

**e** Images of IB4⁺ vessels, ICAM2⁺ lumen formation and COL4⁺ collagen deposition in WT and Mst1ΔEC mice at P12. Scale bars, 500 μm. 

**f** Images of TER119⁺ RBC and CD31⁺ retinal vessels, and comparison of RBC leakage in WT (n = 5) and Mst1ΔEC (n = 5) mice. Scale bars, 100 μm. 

**g, h** Images of CD31⁺ vessels and comparison of vascular density at cerebral cortex and striatum in WT (n = 5) and Mst1ΔEC (n = 5) mice. Scale bars, 200 μm. 

**i, j** Images of CD31⁺ vessels and comparisons of indicated parameters at cerebellum in WT (n = 5) and Mst1ΔEC (n = 5) mice. Scale bars, 200 μm. Data represent mean (bar) ± s.d. (error bars). P values, versus WT by two-tailed unpaired t-test. NS not significant. Source data are provided as a Source Data file.
immunoprecipitation analysis using the HUVECs revealed that endogenous MST1 physically interacts with endogenous FOXO1 (Fig. 5f). Moreover, depletion of MST1 using its corresponding small interfering RNA (siRNA) abrogated the hypoxia-induced pFOXO1 in parallel (Fig. 5g). These results imply that MST1 directly mediates pFOXO1 through hypoxia-induced intracellular ROS biosynthesis (Fig. 5h), which is consistent with previous findings in other cell types19.

MST1 governs the nuclear import of FOXO1 under hypoxia.

Considering activation of phospatidylinositol-3-OH kinase (PI3K)/AKT signaling by VEGF promotes nuclear export of FOXO1 under hypoxia, we postulated that activated MST1 may predominantly promote nuclear import of FOXO1 through hypoxia-induced intracellular ROS biosynthesis (Fig. 5f), which is consistent with previous findings in other cell types19.

MST1 regulates nuclear localization of FOXO1 at tip ECs. a Images of CD31+ retinal vessels and distribution of FOXO1 of whole retina in WT mouse at P6. The red dashed lines separate into tip ECs, vascular front and vascular plexus from top to bottom. Scale bars, 200 μm. b Magnified images of CD31+ vessels and subcellular localization of FOXO1 at indicated portions. Scale bars, 50 μm. c Magnified images of the nuclear localization of FOXO1 (yellow arrowheads) at tip ECs in WT and Mst1ΔEC mice. Scale bars, 50 μm. d Images of angiopoietin-2 (Angpt2) expression and CD31+ vessels at vascular front in WT and Mst1ΔEC mice. Scale bars, 100 μm. e Comparisons of indicated parameters in WT (n = 5) and Mst1ΔEC (n = 5) mice. Data represent mean (bar) ± s.d. (error bars). P values, versus WT by two-tailed unpaired t-test. NS not significant. Source data are provided as a Source Data file.

Fig. 3 MST1 regulates nuclear localization of FOXO1 at tip ECs. Considering activation of phospatidylinositol-3-OH kinase (PI3K)/AKT signaling by VEGF promotes nuclear export of FOXO1 through phosphorylation at Ser256, we postulated that activated MST1 may predominantly promote nuclear import of FOXO1 through phosphorylation at Ser212 against the export stimulated by VEGF-induced PI3K/AKT activation. To decipher this postulation, we confirmed that hypoxia–MST1–FOXO1 cascade operates independently of VEGF/VEGFR2–PI3K–AKT–FOXO1 cascade (Supplementary Fig. 7 f, g). Then, the HUVECs transfected with the siRNA for control (siCont-ECs) or the siRNA for MST1 gene (siMST1-ECs) were stimulated with VEGF (200 ng/ml) for 30 min under normoxia or hypoxia (1% O2). Under normoxia, FOXO1 was mainly localized in nuclei of siCont-ECs but mostly was (96%) exported to cytoplasm upon VEGF stimulation. This VEGF-stimulated dramatic FOXO1 shuttling was largely (58%) abrogated under hypoxia. In contrast, this shuttling was consistently muted in siMST1-ECs under both normoxia and hypoxia (Fig. 6a–c). Immunoblot analysis following nuclear–cytoplasmic fractionation of the HUVECs confirmed that MST1 was stably settled in the cytoplasm regardless of hypoxia and VEGF stimulation, whereas FOXO1 was dynamically shuttled between cytoplasm and nucleus in response to VEGF under normoxia but not under hypoxia (Fig. 6d). To confirm the effect of MST1 and phosphorylation of FOXO1 at Ser212 in determining the subcellular localization of FOXO1, we transfected HEK293T cells with gene constructs encoding either GFP-tagged FOXO1 (GFP-FOXO1-WT) or non-phosphorylatable FOXO1 (GFP-FOXO1-S212A) together with either control vector (CTL) or gene construct encoding MST1 (FLAG-MST1). MST1 induced the nuclear import of FOXO1 in GFP-FOXO1-WT-transfected cells (8.8% in CTL transfected cells compared with 38.9% in FLAG-MST1 transfected cells of all GFP-expressing cells), while it was markedly reduced in GFP-FOXO1-S212A-transfected cells (3.7% in CTL transfected cells compared with 4.7% in FLAG-MST1 transfected cells of all GFP-expressing cells) (Fig. 6e). These results indicate that the phosphorylation of FOXO1 at Ser212 by MST1 mediates hypoxia-induced nuclear import of FOXO1.

Consistent with these in vitro findings, compared to those of WT mice at P6, distinctly nuclear localized FOXO1 largely
disappeared in tip ECs of growing retinal vessels of $\text{Mst}^{\text{ΔEC}}$ mice. In contrast, no apparent differences were detected in the localizations of FOXO1 in the ECs of the vascular front and plexus. These results imply that MST1 activation plays a dominant role in nuclear–cytoplasmic shuttling of FOXO1 in tip ECs that are exposed to extreme hypoxia (Fig. 6f).

MST1–FOXO1 cascade establishes cell polarity in EC migration. Given that $\text{Mst}^{\text{ΔEC}}$ and $\text{Foxo}^{\text{ΔEC}}$ mice failed to induce tip EC polarization, we postulated that the MST1–FOXO1 cascade is critical for inducing tip EC polarization into the avascular area. To evaluate the role of the MST1–FOXO1 cascade in establishing cell polarity during directional EC migration into the avascular...
area, we employed a wound scratch assay in confluent siCont-ECs, siMST1-ECs, and siFOXO1-ECs (HUVECs transfected with siRNA targeting the FOXO1 gene) and examined the EC behaviors at the wound margin (Supplementary Fig. 8a, b). While the gap was 95% closed in siCont-ECs at 15 h after scratching, it was only 35% and 43% closed in siMST1-ECs and siFOXO1-ECs, respectively. Of importance, siMST1-ECs and siFOXO1-ECs lost polarization toward the gap area in the leading edge, while siCont-ECs were polarized toward the gap (Fig. 7a–c and Supplementary Movies 1–3).

We further tracked each EC using a CellTracker and analyzed speed and directionality of the ECs. siCont-ECs showed a linear cell morphology and migrated toward the gap, while siMST1-ECs showed a mixed cell morphology of linear and cobblestone-like appearance and migrated randomly regardless of the direction of the gap, and siFOXO1-ECs exhibited a cobblestone-like appearance with small displacement between imaging intervals (Supplementary Fig. 8c and Movies 4–6). Although the average and instantaneous speeds of each EC among siCont-, siMST1-, and siFOXO1-ECs were similar for 15 h, ∼90% of siCont-ECs were displaced into the gap, while 51 and 61% of siMST1- and siFOXO1-ECs, respectively, were randomly displaced (Fig. 7d, e). Moreover, while siCont-ECs exhibited persistent and directional migration, siMST1- and siFOXO1-ECs randomly migrated without directional persistence (defined by the net displacement divided by the total migrating path of each cell) (Fig. 7f). Furthermore, at 9 h after scratching, in the leading edge of siMST1- and siFOXO1-ECs compared with those of siCont-ECs, there were randomly positioned Golgi apparatus, microtubule organizing centers including microtubules (shown by tubulin) and centrosome (shown by pericentrin), and caveolin; fewer incorporated vinculin into the focal adhesion site; and smaller lamellipodia (Fig. 7g, h and Supplementary Fig. 8d–g). These results indicate that MST1 and FOXO1 are crucial for establishing endothelial polarity in the direction of cell migration.

### MST1–FOXO1 cascade commonly regulates genes related to EC migration.

To elucidate how MST1 and FOXO1 regulate directional EC migration at the transcriptional level, we performed RNA sequencing analysis in siCont-, siMST1-, and siFOXO1-ECs. Ingenuity Pathway Analysis was used to identify Gene Ontology (GO) analysis in comparisons of siCont-ECs versus siMST1-ECs and siFOXO1-ECs versus siFOXO1-ECs. The GO term Cellular Movements was ranked first and second in the comparison of siMST1-ECs and siFOXO1-ECs to siCont-ECs, respectively (Supplementary Fig. 9a), implying that MST1 and FOXO1 commonly regulate genes related to Cellular Movements. To decipher how much MST1 and FOXO1 cooperate to regulate genes related to cellular movements, we employed the EdgeR method in R and found 659 and 3,680 differential expressed genes (DEGs) in the comparisons of siCont-ECs versus siMST1-ECs and siCont-ECs versus siFOXO1-ECs, respectively. Only 369 genes among DEGs were commonly regulated by MST1 and FOXO1 (Supplementary Fig. 9b). Furthermore, we clustered the commonly regulated 369 genes and found that 286 genes were commonly up- or down-regulated by MST1 and FOXO1 (Supplementary Fig. 9c), which were assumed to be the genes regulated by the MST1–FOXO1 cascade. Of interest, GO biological process analysis with the genes regulated by MST1–FOXO1 cascade revealed that GO terms including EC migration, sprouting angiogenesis, and cell-substrate junction assembly were ranked in the top 10 enrichment candidates, and GO terms related to angiogenesis showed significantly high Q scores (q-values) (Supplementary Fig. 9d, e). Collectively, these results delineate that MST1–FOXO1 cascade establishes endothelial cell polarity through transcriptional control of cell migration and adhesion which affects cell polarity and vice versa (Fig. 7i).

Furthermore, to identify the target genes of MST1–FOXO1 cascade, we transfected each gene constructs; GFP-FOXO1-WT, GFP-FOXO1-S212A, or Ctrl into the HUVECs. Intriguingly, CCBE1, CXCL8, RSP03, and FGF2 were upregulated in GFP-FOXO1-WT compared with Ctrl. Conversely, CCBE1 and CXCL8 were downregulated in GFP-FOXO1-S212A compared with GFP-FOXO1-WT. In addition, when MST1 was depleted in the HUVECs, CCBE1 and CXCL8 were downregulated; however, this was reversed when the HUVECs were also transfected with GFP-FOXO1-WT. Taken together, MST1-FOXO1 cascade regulates the expressions of CCBE1 and CXCL8 in a pFOXO1-dependent manner in the HUVECs (Supplementary Fig. 10a–d).

### MST1–FOXO1 cascade is required for pathological angiogenesis.

To examine whether endothelial MST1 is required to maintain retinal vessel integrity, we deleted MST1 in ECs of MST1ΔEC mice from age 8 weeks and analyzed them 4 weeks later (Supplementary Fig. 11a). No definite differences were found between WT and MST1ΔEC mice in radial length, vascular density, RBC leakage, and distributions of VE-cadherin and ERG+ nuclei of the retinal vessels (Supplementary Fig. 11b–e). These results indicate that endothelial MST1 is dispensable for maintaining integrity of retinal vessels. To see whether endothelial MST1 and FOXO1 has a significant role in pathologic angiogenesis, we employed an oxygen-induced retinopathy (OIR) model (Supplementary Fig. 11f). MST1ΔEC-OIR and Foxo1ΔEC-OIR mice exhibited impaired revascularization (196 and 167% increased avascular area, respectively) and neovascularization [60 and 85% reduced neovascular tuft (NVT) area, respectively] compared with WT-OIR mice (Fig. 8a, c). FOXO1 was enriched in the nuclei of ECs and Angpt2 was highly expressed in tip ECs and NVT ECs, where aberrant sprouting occurs, compared with adjacent ECs in WT-OIR mice. However, MST1ΔEC-OIR mice showed 70 and 71% reduced FOXO1 intensity and 64 and 71% reduced Angpt2 expression in tip ECs and NVT ECs, respectively, compared with those of WT-OIR mice. Furthermore, Angpt2 expression was nearly absent in tip ECs and NVT ECs of Foxo1ΔEC-OIR mice compared with those of WT-OIR mice.
Fig. 5 Hypoxia activates the MST1–FOXO1 cascade in primary cultured ECs. a GSEA analyses of the microarray data (GSE19284) obtained from isolated tip ECs and non-ECs by laser capture microdissection. b, c Immunoblot analyses and temporal changes of indicated proteins in HUVECs exposed to hypoxia (1% O2) for indicated times (n = 3, each group). Center line, median; Box limits, upper and lower quartiles; Whiskers, s.d. p values versus 0 h by one-way ANOVA with Tukey’s post hoc test. NS not significant. d Immunoblot analyses of indicated proteins in HUVECs under normoxia (−) and hypoxia (+) in absence (−) or presence (+) of Trolox treatment. e Immunoblot analyses of indicated proteins in HUVECs under normoxia (−) and hypoxia (+) in absence (−) or presence (+) of Rotenone treatment. f Immunoprecipitation analysis in HUVECs with control anti-IgG and anti-FOXO1 antibody followed by immunoblotting with anti-MST1 antibody. g Immunoblot analyses of indicated proteins in siCont-ECs and siMST1-ECs under hypoxia. h Schematic picture depicting a hypoxia-intracellular ROS–MST1–FOXO1 cascade and the conserved phosphorylation site of FOXO1 by MST1. Source data are provided as a Source Data file.
In addition, the Golgi apparatus were polarized towards the anterior or posterior of the nuclei in tip ECs of WT-OIR mice. On the contrary, such polarizations were lost in tip ECs of Mst1ΔEC-OIR and Foxo1ΔEC-OIR mice (Fig. 8d, e). Thus, these results imply that MST1 facilitates the nuclear import of FOXO1 in tip ECs as well as in NVT ECs during OIR progression. Moreover, endothelial MST1-FOXO1 cascade is required for revascularization and neovascularization and contributes to establishing endothelial polarity in revascularization.

**Discussion**

Rapidly growing tissues during development and solid tumors swiftly expanding into surrounding parenchymal tissues constantly face hypoxia due to inadequate angiogenesis and blood...
supply\(^{44,45}\). In turn, hypoxia is a leading driver for sprouting and intussusceptive angiogenesis, but how it guides tip ECs toward the hypoxic area has been an enigma. An ample number of previous studies\(^{14,46–49}\) have suggested that the VEGF gradient could be a major determinant for tip EC polarization during sprouting angiogenesis. However, there is devoid of sprouting angiogenesis with accompanying tip EC polarization in VEGF-rich adult organs such as endocrine glands, liver, kidney, and muscles\(^{50}\), raising the possibility that hypoxia could activate an alternative pathway for guiding tip ECs in sprouting angiogenesis into the avascular region. In this study, we uncovered a coupling between extracellular hypoxia and intracellular ROS–MST1–FOXO1 pathway, which establishes the polarity of tip EC during sprouting angiogenesis.

Among diverse roles of MST1, we have defined the roles in the behavior of tip ECs of growing blood vessels. Of note, our findings indicate that hypoxia-induced ROS biosynthesis from mitochondria could be a major upstream regulator of MST1 activation in ECs. Activated MST1 promotes planar and perpendicular vascular branching for regulating tip EC polarity and sprouting angiogenesis. In general, MST1 can be activated in two ways: cleavage and phosphorylation\(^{56}\). Cleaved MST1 under apoptotic stimuli translocates from cytosol into the nucleus, promoting chromatin condensation during cellular apoptosis\(^{56}\). On the other hand, a full-length MST1 can be autophosphorylated at Thr183 under cellular stresses by disrupting the interaction between thioredoxin-1 and SARA (Salvador/RASSFL/Hippo) domain\(^{51}\), and activated MST1 phosphorylates cytosolic substrates such as SAV, LATS1/2, and FOXOs\(^{56}\). Not surprisingly, the physiologic activity of MST1 and its cellular substrates are cell type- and context-dependent\(^{20,52,53}\).

Our analyses using the genetically modified mice revealed that endothelial MST1 does not undertake a canonical Hippo pathway. Instead, endothelial MST1 phosphorylates FOXO1 under hypoxia in HUVECs, and this kinase-substrate interaction drives nuclear import of FOXO1 in tip ECs. Of importance, this hypoxia–MST1–FOXO1 cascade operates independently of VEGF/VEGFR2 signaling in regulating sprouting angiogenesis. Thus, endothelial MST1 is involved in the regulatory cascade for nuclear transport of FOXO1 rather than its cleavage and in regulation of apoptosis and chromatin condensation or its phosphorylation to LATS1/2 for the canonical Hippo pathway for establishing tip EC polarity during sprouting angiogenesis.

Then, how does nuclear FOXO1 regulate polarity of tip ECs? Beyond the traditional roles of FOXOs as transcriptional factors for maintaining cellular homeostasis\(^{34}\), the newly identified roles of FOXOs in neuronal polarization and positioning have been highlighted\(^{55,56}\). They control axon–dendrite polarity and morphogenesis in developing granular neurons through regulating polarity-associated gene expression\(^{56}\). Thus, these novel roles of FOXOs in neurons could be shared with the roles described here for FOXO1 in tip EC polarization in terms of cell behaviors and regulating polarity-associated gene expression. Nevertheless, this involvement is apart from the deciphered role of FOXO1 in metabolic gatekeeping to maintain EC homeostasis\(^{11}\) during establishment of the vascular plexus of growing vessels. In this regard, determining specific FOXO1 transcriptional output will be of interest.

Several models have been proposed to explain the specific transcriptional regulation of FOXO1\(^{54}\). FOXO1 can bind numerous sites with members of transcriptional machinery because the length of its DNA binding motif is short\(^{57}\), on the other hand, FOXO1 interacts with other transcriptional factors or cofactors and can initiate transcriptional activity with or even without direct binding to DNA\(^{58}\). Of particular note, FOXO1 that is phosphorylated by MST1 could not bind to DNA, even if it translocates to the nucleus\(^{59}\). Together, our finding suggest that MST1–FOXO1 cascade may regulate polarity/migration-associated genes by interacting with other transcriptional machineries rather than direct binding to DNA\(^{58}\). In addition, although we identified the hypoxia–intracellular ROS-MST1 as the upstream signal of FOXO1 at tip ECs, a limitation of this study is the inability to analyze the regulatory roles of FOXO1 in gene expression and PTMs of FOXO1 in ECs in different regions of growing retinal vessels. Defined analysis at the single cell level at the different regions of ECs is warranted for further study to understand a context-dependent role of FOXO1 during angiogenesis.

In conclusion, this study demarcates a crucial coupling between extracellular hypoxia and intracellular ROS–MST1–FOXO1 pathway, which establishes the polarity of tip ECs during developmental and pathologic angiogenesis.

**Methods**

**Mice and treatment.** Specific-pathogen-free (SPF) C57BL/6J mice and R26-flTomato were purchased from the Jackson Laboratory. VE-cadherin-Cre-ER\(^{72,28}\), Mst1\(^{fl/fl}\)/Foxo1\(^{−/−}\), Mst2 null\(^{54}\), and Foxo1\(^{fl/fl}\)/Foxo3\(^{−/−}\) mice were transferred, bred and bred in SPF animal facilities at KAIST and fed with free access to a standard diet (PML Lab diet) and water. In order to induce Cre activity in the Cre-Er\(^{72}\) mice, tamoxifen (Sigma-Aldrich, C8267) was injected into the stomach daily from P1 to P3; for OIR mice, 200 μg of tamoxifen was injected intraperitoneally (i.p.) daily from P12 to P14; for adult mice aged over 8 weeks, 2 mg of tamoxifen was injected i.p. for 5 consecutive days from the indicated time point. For anesthesia, mice were injected i.p. with the anesthetic solution (ketamine 40 mg/kg and xylazine 5 mg/kg). We complied with all ethical regulations for animal testing and research and performed all animal experiments under the approval from the Institute Animal Care and Use Committee (No. KA2017-31) of KAIST.

**Histological analyses.** Briefly, eyeballs were enucleated and fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). After preparing the retinas from eyeballs, they were additionally fixed in 1% PFA for 1 h at RT. Samples were blocked with 5% donkey (or goat) serum in PBST (0.3% Triton X-100 in PBS) for 30 min, incubated in primary antibodies (diluted at a ratio of 1:200 in blocking solution) at 4 °C overnight, washed in PBS, and incubated in secondary antibodies in blocking solution at RT for 2 h. The retinas were washed in PBS and mounted on microscope glass slides with Vectashield (Vector Laboratories, H-1200). IF
staining of brain were performed as follows. Mice were anesthetized and perfusion-fixed with 4% PFA. After dissecting the brain from skull, the brain was additionally fixed in 4% PFA at 4 °C for 6 h. The samples were cut into 150 μm sections by a vibratome (Leica, VT1200 S), immunostained, and mounted. Primary cultured HUVECs were fixed in 2% PFA for 10 min at RT. After blocking, the cells were stained with primary and secondary antibodies using the same procedure as tissue staining, and mounted with DAKO mounting medium. Primary antibodies and reagent used for IF were as follows: hamster anti-CD31 monoclonal (Millipore, MAB1398Z); isolectin B4 (IB4), Alexa Fluor 594-conjugated (Thermo Fisher Scientific, I21413); rat anti-VE-cadherin monoclonal (BD Pharmingen, 555289); mouse anti-VE-cadherin monoclonal (BD Biosciences, #555661); rabbit anti-ERG monoclonal (Abcam, ab92513); rabbit anti-cleaved-caspase-3 (Cl-CASP3) polyclonal (CST, #9664); rabbit anti-pHH3 polyclonal (Millipore, 05-806); rabbit anti-collagen IV polyclonal (Bio-Rad, 2150-1470); rat anti-ICAM2 monoclonal (BD...
Fig. 7 MST1-FOXO1 cascade establishes cell polarity in EC migration. a Images of phalloidin+ actin cytoskeleton and caveolin in indicated ECs subjected to the wound scratch. The dashed lines indicate the initial margin of wound scratch. Scale bars, 200 μm. b Schematic pictures depicting cell morphology at the leading edge in indicated ECs. c Comparisons of indicated parameters in indicated ECs. n = 15, each group in the left panel. n = 10–15, each group at each time in the right panel. d Comparisons of indicated parameters in indicated ECs at 9 h after initiating cell migration. Scale bars, 50 μm. e Polar plots showing net displacement. n = 1941 (siCont), 2190 (siMST1), 2217 (siFOXO1) in the left panel. n = 24–25, each group in the right panel. e Polar plots showing net displacement. n = 94 (siCont), 98 (siMST1), 88 (siFOXO1). f Schematic picture depicting net displacement and total migrating path and comparison of single cell directional persistence defined by net displacement divided by total migrating path length in indicated ECs. n = 47 (siCont), 61 (siMST1), 52 (siFOXO1). g Images of phalloidin+ actin cytoskeleton, α-tubulin+ microtubule, GM130+ Golgi apparatus and DAPI in the leading edge of indicated ECs at 9 h after initiating cell migration. Note that Golgi apparatus and microtubule (red and yellow arrows) in siCont-ECs are localized in the direction of cell migration, while those (red and yellow arrowheads) in siMST1-ECs and siFOXO1-ECs are localized randomly. Moreover, siMST1-ECs and siFOXO1-ECs rarely show lamellipodia (white arrowheads) compared to siCont-ECs (white arrows). Scale bars, 50 μm. h Polar plots showing Golgi apparatus [n = 36 (siCont), 41 (siMST1), 38 (siFOXO1)] and microtubule organizing centre (MTOC) polarization [n = 15, each group in the left panel]. i Schematic picture summarizing predominant role of MST1 in the nuclear import of FOXO1 against VEGF/VEGFR2-PI3K/AKT pathway at tip ECs. c, d, f Box plots represent Center line, median; Box limits, upper and lower quartiles; whiskers, s.d. Right panel in c represents means (points) ± s.d. (error bars). Right panel in d represents Bar, s.d.; Points, median. P values, versus siCont by one-way ANOVA with Tukey’s post hoc test. NS not significant. e, h the bold lines indicate 120° region centered on the vector which is vertical to the wound scratch direction. The numbers indicate the frequency of dots within the 120° region of the bold line. Source data are provided as a Source Data file.
Analyses for cell migration characteristics. The cell migration characteristics were analyzed using CellTracker® implemented in MATLAB (MATLAB R2017a, MathWorks). Three images or videos were taken for each sample and three replicates were analyzed for siCont-, siMST1- and siFOXO1-ECs. In each image or video, 18–20 cells were tracked. Raw data from CellTracker were extracted in pixel unit which were converted into μm (1784.22 μm/1920 pixel = 0.93 μm/pixel). Average speed and net displacement were compared among each groups from the extracted data. Distance from origin and instantaneous speed were calculated and compared from the data measured every 30 min. Directional persistence was calculated as the net displacement divided by the total migrating path of each cell and compared among each groups.

Generation of the anti-phospho-FOXO1 (Ser212) polyclonal antibody. The rabbit antibody against phospho-FOXO1 (Ser212) was generated as follows13,19. Briefly, the phosphopeptide antigen C-SAGWKNpSIRHNLS was synthesized, HPLC purified, conjugated to keyhole limpet hemocyanine, and injected into two rabbits over an 8-week period (one primary injection and three boosting injections) (Abclon). The antiserum was obtained for affinity purification to remove non-specific antibodies, increase sensitivity and reduce background.

Immunoblotting and immunoprecipitation assays. The cells were lysed on ice in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (CST,
Fig. 8 MST1–FOXO1 cascade is required for pathologic angiogenesis. a Images of CD31+ vessels in the superficial layer of retinas and avascular area (red) in WT-OIR, Mst1ΔEC-OIR, and Foxo3ΔEC-OIR mice. Scale bars, 500 μm. b Images of subcellular localization of FOXO1 in CD31+ vessels at vascular front (revascularization) and vascular plexus (neovascularization) in WT-OIR, Mst1ΔEC-OIR, and Foxo3ΔEC-OIR mice. Scale bars, 100 μm. Note that WT-OIR mice exhibited a nuclear localization of FOXO1 (yellow arrowheads), while Mst1ΔEC-OIR mice showed a diffuse nucleocytoplasmic localization of FOXO1 (yellow arrows) in tip ECs and NVT ECs. c Comparisons of indicated parameters in WT-OIR (n = 5), Mst1ΔEC-OIR (n = 5) and Foxo3ΔEC-OIR (n = 5) mice. Data represent mean (bar) ± s.d. (error bars). Student’s t-test between 2 groups or the one-way ANOVA followed by Tukey’s test for multiple-group comparison. Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software). Statistical significance was set to P-value < 0.05.

RNA sequencing analysis. HVEGs were cultured in 60 mm dish and transfected with GL2 (siCont-ECS), Mst1ΔEC-ECS and FOXO1 (siFOXO1-ECS) siRNA. At 48 h after siRNA transfection, each siCont-, siMST1-, and siFOXO1-ECS were exposed to 10% fetal bovine serum (FBS) for 1 h. After exposing total RNA, RNA was extracted from nonvascular-ECS, hypoxia-ECS, siCont-ECS, siMST1-ECS, and siFOXO1-ECS using Trizol reagent (Invitrogen). 1 μg of total RNA was used to construct cDNA libraries with the TruSeq RNA library kit. The protocol consisted of poly A-selected RNA extraction, RNA fragmentation, random hexamer primed reverse transcription, and 100 nt paired-end sequencing by Illumina HiSeq 2500. The libraries were quantified using qPCR according to the qPCR Quantification Protocol Guide and qualified using an Agilent Technologies 2100 Bioanalyzer. The sequencing adapters were removed by Trimmomatic v0.26.1. Then, the trimmed reads were mapped to the human Ensembl reference genome (GRCh38.91) using HISAT2 aligner as so as to acquire the BAM file. Raw read counts were achieved from the following using the HT-Seq, then used to analyze the differentially expressed genes (DEG) between samples by applying EdgeR method. Hierarchically clustered heatmaps of DEG among siCont-ECS, siMST1-ECS, and siFOXO1-ECS were generated in R using the heatmap function. To analyze Gene Ontology biological process and get the Fold enrichment score and Q-score, the ingenuity pathway analysis (QIAGEN) was used and all DEGs were mapped to Gene Ontology database Released 2018-04-04 (http://www.geneontology.org)/6.84.

RNA extraction and quantitative RT-PCR. Total RNA was extracted from samples using RNeasy mini kit (Qiagen) according the manufacturer’s protocols. A total of 1 μg of extracted RNA was transcribed into cDNA using GoScriptTMD Reverse Transcription System (Promega). cDNA was mixed with primers and FastStart RT-PCR Green Master (Roche), and mRNA expression levels were measured by real-time PCR QuantStudio3 (Thermo Fisher Scientific). The primers were designed using Primer-BLAST. The list of qRT–PCR primers used in this study is described in Supplementary Table 1.

Transcriptional profile analysis of microarray data. Gene set enrichment analysis (GSEA) was performed with v6.1 of the Molecular Signature Database (http://www.broadinstitute.org/gsea/msigdb), and the gene sets which were <0.05 nominal P-value were stated.

OIR mouse model. Briefly, P7 mice were exposed to 75% oxygen in a hyperoxic chamber (CO2 laboratories, O2 Control InVovo Cabinet, Grass Lake, Michigan) for 5 days with their nursing mother and then returned to room air for 5 days.

Code availability. R scripts used in the present work are available from the authors upon request.

Statistics and reproducibility. No methods were not used to predetermine sample size. Reproducibility was ensured by performing more than five and three independent in vivo and in vitro experiments, respectively. Animals or samples were not randomized and the investigator was not blinded during experiments. Both male and female neonatal mice were analyzed at P6 and P12 and only male mice were used at 12 weeks of age. No animals were excluded from analysis. All values are presented as mean ± standard deviation (s.d.). Statistical significance was determined by the two-tailed unpaired t-test between 2 groups or the one-way ANOVA followed by Tukey’s honest significant difference (HSD) test with ranks for multiple-group comparison. Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software). Statistical significance was set to P-value < 0.05.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.
Data availability
The RNA-sequencing data generated with this study have been deposited in Gene Expression Omnibus under the accession number GSE16033. The source data underlying all Figs and Supplementary Figs are provided as a Source Data file. A Reporting Summary for this article is available as a Supplementary Information file. All other data that support the findings of this study are available from the corresponding author upon reasonable request. Expression data from the published studies were obtained from the accession number GSE19284 in the Gene Expression Omnibus.

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Author contributions
Y.H.K. and G.Y.K. designed the experiments. Y.H.K. performed most of experiments and analyzed the data. J.C. and S.P.H. contributed to immunofluorescence and FACS analysis. M.J.Y. analyzed the RNA sequencing data and provided the related methods; J.C. and C.-k.L. provided important ideas and technical support. Y.K. and D.-S.L. provided the mice. Y.H.K. and G.Y.K. generated the figures, and wrote as well as edited the manuscript. G.Y.K. directed and supervised the project.

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