Clear cell renal cell carcinoma (ccRCC) patients are highly angiogenic and treated by targeted therapies against VEGFA/VEGFR signaling pathway. However, tumors with such targeted therapies remain a significant clinic challenge. Understanding the underlying mechanism against angiogenesis is highly desired. Here, we demonstrated that the lncRNA DMDRMR serves as a sponge of miR-378a-5p to increase EZH2 and SMURF1 expression, thus promoting EZH2-mediated transcriptional repression of DAB2IP and SMURF1-mediated degradation of DAB2IP. Consequently, this axis activates VEGFA/VEGFR2 signaling pathway, resulting in angiogenesis and resistance of tumor cells to sunitinib in ccRCC. Moreover, the competing endogenous RNA regulatory axis of DMDRMR is clinically relevant to ccRCC pathogenesis and prognosis of patients with ccRCC. Our results support that the DMDRMR/miR-378a-5p/DAB2IP axis may serve as a novel target for combination diagnosis or therapy of ccRCC patients. Our findings may have highly clinical relevance for future translation to develop the targeted therapies for patients with ccRCC.

**INTRODUCTION**

Clear cell renal cell carcinoma (ccRCC) is a major type of RCCs and characterized by high angiogenesis and dense vascularization [1, 2]. A common genetic mutation in ccRCC is loss of the von Hippel-Lindau gene, which results in stabilization of hypoxia-inducible factors (HIFs), and contributes to the activation of HIF target genes, including vascular endothelial growth factor (VEGF) [3]. The deregulated VEGFA/VEGF receptor (VEGFR) signaling pathway represents an ideal therapeutic target for advanced ccRCC treatment. However, most patients acquire drug resistance with the targeted angiogenesis therapy [3]. Therefore, further understanding of tumor angiogenesis is highly desired.

Long non-coding RNAs (lncRNAs) with a minimum 200 bases in length have been shown to regulate gene expression in multiple layers, including transcription, translation, and post-transcriptional/translational modification through multiple ways, such as binding regulatory proteins and acting as microRNA (miRNA) sponges [4, 5]. Emerging evidence indicates that lncRNAs may act as new modulators in angiogenesis [6], which need to be further dissected.

In this study, we expanded the function of our previously reported DMDRMR [7] and revealed that DMDRMR activates the VEGFA/VEGFR2 signaling pathway by sponging miR-378a-5p to promote EZH2 and SMURF1-mediated repression of DAB2IP expression, resulting in enhanced angiogenesis and sunitinib resistance. These findings further highlight the key roles of DMDRMR in ccRCC.

**MATERIALS AND METHODS**

**Cell culture**

The human umbilical vein endothelial cells (HUVECs) were obtained from Prof. YF Zhou (Soochow University) and cultured in HUVECs specialized medium (Procell, CM-0122). The human embryonic kidney HEK293T (293T) cell line was cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS). 786-O and 769-P cell lines were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS. 293T, 786-O, and 769-P cell lines were purchased from the Shanghai Cell Bank Type Culture Collection Committee (Shanghai, China). All these cells were maintained in a 37°C incubator in a humidified atmosphere containing 5% CO2 and were previously examined negative for mycoplasma contamination.

**Enzyme-linked immuno sorbent assay**

The concentration of VEGFA in the supernatants of cell cultures were measured using Human VEGFA ELISA Kit (ABclonal Technology, RK00023) according to the guidelines of the manufacturer. Briefly, 100 μl/well of standard and test samples were loaded into 96-well plates. After incubating with biotin-conjugate antibody, streptavidin-conjugated horse-radish peroxidase (HRP) was added to each well and reacted with the HRP substrate solution. Detect the optical density within 5 minutes (min) under 450 nm and correct the wavelength set at 570 nm.
Matrigel tube formation assay
In all, 10 µl of matrigel (Corning Inc., NY, USA) was thawed on ice at 4 °C overnight, added into each well of a precooled μ-Slide Angiogenesis plate (Ibidi), and incubated at 37 °C for 30 min for hardening. In total, 1 × 10⁴ HUVECs in 100 µl conditioned cell culture medium were plated onto the precoated matrigel for 24 hours (h). The resulting capillary-like structures in each well were then photographed with a microscope and counted with ImageJ software.

In vivo matrigel plug angiogenesis assay
The conditioned cell medium from DMDRMR KD cells was collected and concentrated, 300 µl of concentrated medium mixed with 400 µl of BD Matrigel®Basement Membrane Matrix and 1 × 10⁵ HUVECs were injected subcutaneously into the back of the 5-week old nude male mice. After 10 days, the skin was pulled back with scissors to expose intact Matrigel plugs, and plug images were taken. The hemoglobin content of the matrigel plugs were determined using Hemoglobin Assay kit (Abcam, ab234046). Histological section on slides were stained with hematoxylin and eosin (H&E). All protocols involving animals were previously approved by the Ethics Committee for the Use of Experimental Animals of the Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences (Suzhou, Jiangsu, China).

Statistical analyses
All data were presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). All experiments with statistical analysis have been repeated at least three times. Two-tailed Student’s t test was performed to analyze the difference between two groups. A two-sided χ² test was used to assess the statistical significance of the association between the expression of RNA or protein levels and the clinico-pathological parameters of patients. For the kaplan-meier survival analysis, a log-rank test was performed. For the correlation analysis, spearman’s correlation was performed. "PROC" package in R software was used to construct receiver operating characteristic (ROC) curves and then to calculate area under curve (AUC). All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad software, Inc.) or R software (version 3.5.2). A value of p < 0.05 was considered statistically significant difference. Additional methods can be found in the Supplementary Material and Methods.

RESULTS
DMDRMR Induces VEGFA production
In our previous study, the RNA-sequence and quantitative real-time polymerase chain reaction (qRT-PCR) data from DMDRMR knockdown (KD) and control 786-O cells showed that KD of DMDRMR decreases the transcriptional level of VEGFA [7]. Here, we further confirmed the effects of DMDRMR on VEGFA expression. qPCR analyses showed that KD of DMDRMR decreased and overexpression (OE) of DMDRMR increased the transcriptional level of VEGFA (Figs. 1A and S1A, B). Immunoblot and enzyme-linked immuno sorbent assay (ELISA) assays confirmed that both KD and knockout (KO) of DMDRMR reduced, whereas OE of DMDRMR upregulated protein and secreted levels of VEGFA (Fig. 1B, C). In our previous mouse xenograft models [7], DMDRMR KD reduced VEGFA expression levels (Fig. 1D, E). Consistently, DMDRMR expression level was positively correlated with VEGFA expression in The Cancer Genome Atlas (TCGA) ccRCC cohort (Fig. 1F). These results suggest that DMDRMR increases VEGFA expression and induces VEGFA production.

DMDRMR drives angiogenesis
Given that VEGFA is an inducer of angiogenesis and endothelial cell tube formation [8], we wondered whether DMDRMR regulates angiogenesis in ccRCC. Firstly, we performed gene ontology (GO) pathway analysis and found that 1039 differentially expressed genes (DEGs) from DMDRMR KD and control 786-O cells [7] were enriched for angiogenesis-related pathways, such as angiogenesis, sprouting angiogenesis, and regulation of VEGF production (FDR < 0.05) (Fig. S1C, D). Consistently, based on the median value of DMDRMR expression in ccRCC patients from TCGA, Gen Set

Enrichment Analysis (GSEA) revealed that DMDRMR expression is associated with VEGF-related pathways including positive regulation of VEGF production and VEGF pathway (FDR < 0.25) (Fig. S1E, F), suggesting that DMDRMR might be an important modulator of angiogenesis in ccRCC. To functionally validate these findings, we evaluated the effect of DMDRMR expression on in vitro angiogenesis activities of vascular endothelial cells. HUVECs were incubated with conditional medium collected from ccRCC cells expressing variable amounts of DMDRMR, and the capillary tube formation of HUVECs were measured. Both DMDRMR KD and KO reduced, whereas DMDRMR OE increased the amounts of capillary tube formation (Fig. 2A–C). In vivo matrigel plug angiogenesis assay showed that DMDRMR KD reduces blood vessel formation as evidenced by decreased redness and less numbers of capillaries (Fig. 2D). Similarly, the amounts of hemoglobin were markedly reduced in DMDRMR KD (Fig. 2E). In our previous mouse xenograft models [7], the vascular endothelial cell marker CD31 staining of tumors indicated that DMDRMR KD inhibited the generation of microvessels (Fig. 2F, G). Taken together, these results indicate that DMDRMR promotes angiogenesis in ccRCC.

VEGFA is an indirect-regulated gene of DMDRMR
To define the molecular mechanisms of DMDRMR in driving angiogenesis, we firstly investigated whether DMDRMR directly regulates VEGFA expression. Given DMDRMR transcriptionally regulates VEGFA expression, we examined whether DMDRMR affects the transcription of VEGFA or the mRNA stability of VEGFA transcript. The nuclear run-on assay showed that the transcription efficiency of VEGFA was not significantly altered after DMDRMR KD and KO (Fig. S2A, B), which excludes the regulation of DMDRMR to VEGFA at transcriptional level. We further treated 786-O cells with the transcriptional inhibitor actinomycin D and assessed the mRNA stability of VEGFA. The half-lives of VEGFA mRNA remained similar in DMDRMR KD and KO cells (Fig. S2C, D). Moreover, MS2-based GFP RNA immunoprecipitation (RIP) revealed that VEGFA was not enriched for DMDRMR (Fig. S2E). Furthermore, it is known that DMDRMR regulates VEGFA expression without utilizing its binding protein IGF2BP3 [7]. These data suggest that VEGFA serves as an indirect downstream effector of DMDRMR to regulate angiogenesis.

DMDRMR functions as a ceRNA for miR-378a-5p
We showed that DMDRMR partially localizes to the cytoplasm [7], suggesting a possible function as a competing endogenous RNA (ceRNA) to sequester miRNAs and regulate angiogenesis in ccRCC [9, 10]. The miRNA sponge is expected to form a complex with Argonaute2 (Ago2) [11], thus we first assessed whether DMDRMR forms a complex with Ago2. RIP qRT-PCR assays showed that DMDRMR was indeed enriched in the Ago2 immunoprecipitates, which was associated with IncRNA H19 as a positive control [12] and EMS as a negative control [13] (Fig. 3A), suggesting that DMDRMR acts as a miRNA sponge. To identify potential target miRNAs of DMDRMR, we performed bioinformatic analysis by TargetScan, miRanda and PicTar databases [14–16] and found 137 miRNAs that have at least a site on DMDRMR (Fig. 3B), among which 3 miRNAs including miR-378a-5p, miR-532-5p and miR-199a-5p were significantly downregulated in TCGA ccRCC tissues versus adjacent tissues and further validated (Fig. S3A–C). Luciferase reporter assays (LRAs) showed miR-378a-5p mimic repressed and miR-378a-5p inhibitor enhanced the luciferase activity of full-length DMDRMR (DMDRMR-WT), which was not changed by miR-532-5p and miR-199a-5p (Fig. S3D, E), suggesting that DMDRMR might selectively sponge miR-378a-5p.

To further examine whether DMDRMR binds to miR-378a-5p, introduction of miR-378a-5p mimic or inhibitor selectively reduced or increased reporter activities of DMDRMR-WT, respectively, but not a construct with the two mutated miR-378a-5p binding sites (DMDRMR-MUT) (Fig. 3C–E). Further seeking evidence for this
interaction, we performed MS2-based GFP RIP (Fig. S3F) and demonstrated that DMDRMR-WT was enriched for miR-378a-5p, but not DMDRMR-MUT (Fig. 3F). The specific association was further validated by affinity pull-down of endogenous DMDRMR using in vitro-synthesized biotin-labeled miR-378a-5p (Fig. 3G), collectively supporting the specific interaction between DMDRMR and miR-378a-5p. Furthermore, qRT-PCR assays showed that DMDRMR and miR-378a-5p inhibited each other’s expression (Fig. S3G, H), miRNAs are known to bind their targets and cause translational repression and/or RNA degradation in an Ago2-dependent manner [17]. To further determine whether DMDRMR was regulated by miR-378a-5p in such a manner, we performed RIP qRT-PCR and found that endogenous DMDRMR pull-down by Ago2 was largely enriched in miR-378a-5p-transfected cells (Fig. 3H). Also, endogenous miR-378a-5p pull-down by Ago2 was specifically enriched in DMDRMR-WT OE cells, but not in DMDRMR-MUT OE cells (Fig. 3I), indicating that miR-378a-5p bound to DMDRMR and then induced the degradation of DMDRMR in an Ago2-dependent manner. These results indicate that DMDRMR may function as a ceRNA for miR-378a-5p.

To elucidate whether miR-378a-5p mediates the promotional effect of DMDRMR on angiogenesis, we firstly performed GSEA analysis in TCGA ccRCC dataset and found that the gene signatures of angiogenesis and VEGF signaling pathways were enriched in patients with low miR-378a-5p expression (Figs. 4A, B and S4A–C), indicating that miR-378a-5p might inhibit angiogenesis and VEGF signaling pathways. Furthermore, rescue experiments showed that miR-378a-5p inhibitor reversed the inhibition of DMDRMR KD on the capillary tube formation of HUVECs, as well as protein and secreted levels of VEGFA (Fig. 4C, E). Conversely, miR-378a-5p mimic prevented the increased capillary tube formation of HUVECs and upregulated protein and secreted levels of VEGFA induced by DMDRMR OE (Fig. 4D, F). All these data suggest that an interaction with miR-378a-5p is necessary for DMDRMR to induce angiogenesis.

DMDRMR increases EZH2 and SMURF1 expression through competitively binding miR-378a-5p

To understand how DMDRMR promotes angiogenesis via miR-378a-5p, we used TargetScan [14] and miRwalk [18] to identify
downstream targets of the miR-378a-5p, resulting in a set of 1298 target genes. 190 candidate target genes were selected according to the following criteria: upregulated in ccRCC tissues and positively correlated with both DMDRMR and VEGFA expression (Fig. 5A). By further considering well-studied oncogenes, we focused on SMAD specific E3 ubiquitin protein ligase 1 (SMURF1) and Enhancer of zeste homolog 2 (EZH2) that extensively regulate gene expression and thereby promote tumorigenesis in various carcinomas, including renal cancer [19, 20]. To explore whether miR-378a-5p could target EZH2 and SMURF1, we performed LRAs and revealed that miR-378a-5p mimic repressed and miR-378a-5p inhibitor enhanced the luciferase activities of the 3′ UTRs of both EZH2 and SMURF1, but not 3′ UTRs with mutations in miR-378a-5p targeting sites (Figs. S5B-E and S5A, B). Moreover, in vitro-synthesized biotin-labeled miR-378a-5p pulldown assays showed the direct binding of miR-378a-5p to the EZH2 and SMURF1 transcripts (Fig. 5F), suggesting that miR-378a-5p specifically targets the 3′ UTR regions of EZH2 and SMURF1 transcripts. Furthermore, miR-378a-5p mimic decreased and miR-378a-5p inhibitor increased the expression levels of EZH2 and SMURF1 (Figs. 5G and S5C), confirming that miR-378a-5p inhibits expressions of EZH2 and SMURF1 through directly targeting the two transcripts.

We further assessed whether DMDRMR is involved in the regulation of miR-378a-5p to EZH2 and SMURF1. Strikingly, DMDRMR KD downregulated, whereas its OE upregulated, EZH2 and SMURF1 expression (Fig. 5H). Furthermore, we performed RIP qRT-PCR assays and found that DMDRMR-WT OE, but not DMDRMR-MUT OE led to the reduced enrichment of Ago2 on EZH2 and SMURF1 transcripts (Fig. 5I). Conversely, DMDRMR KD elicited a significant increase in the recruitment of Ago2 to EZH2 and SMURF1 transcripts (Fig. 5J). The EZH2 small-molecule inhibitor is being evaluated in clinical trials for the treatment of cancers, including tazemetostat [21], thus we further evaluated the relevance of DMDRMR/miR-378a-5p axis in the efficacy of tazemetostat. Cell proliferation assay showed that miR-378a-5p mimic partially weakened the resistance of DMDRMR OE to tazemetostat (Fig. 5D), suggesting that miR-378a-5p could potentiate the efficacy of tazemetostat in ccRCC. These results demonstrate that DMDRMR increases EZH2 and SMURF1 expression by competitively binding miR-378a-5p.

**DMDRMR represses DAB2IP expression through its ceRNA**

DAB2IP, also known as a tumor suppressor in ccRCC, has been reported to be epigenetically repressed by EZH2-mediated methylation of lysine 27 in histone H3 (H3K27me3), and also be degraded by SMURF1-mediated ubiquitin-proteasome regulation [22–24]. Moreover, DAB2IP functions as an endogenous inhibitor in VEGFR2-mediated angiogenesis [25]. Thus, we hypothesized that DMDRMR antagonizes DAB2IP via its ceRNA. The qRT-PCR and immunoblot assays showed that both DMDRMR KD and KO increased, whereas DMDRMR-WT OE inhibited the transcriptional and protein levels of DAB2IP, which was not affected by DMDRMR-MUT OE (Figs. 6A and S6A, B). To further evaluate whether DMDRMR inhibits DAB2IP expression via EZH2 and SMURF1, we performed rescue experiments and showed that DMDRMR KD reversed the transcriptional level of DAB2IP depressed by EZH2 OE (Fig. S6C). Moreover, EZH2 OE or SMURF1 OE reduced DAB2IP protein level and abrogated the effects of DMDRMR silencing on DAB2IP expression (Fig. 5D, E). Similar effects were also observed in DMDRMR KD cells transfected with miR-378a-5p inhibitor (Fig. S6F). Conversely, EZH2 KD or SMURF1 KD promoted DAB2IP protein level and reversed the effects of DMDRMR OE on DAB2IP expression (Fig. S7A, B), which were also observed in DMDRMR OE cells transfected with miR-378a-5p mimic (Fig. S7C). Meanwhile, the increased and decreased expression levels of H3K27me3 were consistent with EZH2 (Figs. S6D–F and S7A–C). These results indicate that DMDRMR inhibits DAB2IP expression through EZH2 and SMURF1.

To further dissect underlying mechanisms of the decreased DAB2IP upon the increased DMDRMR, ChiP qRT-PCR and LRAs were first performed. Two putative sites of DAB2IP with potential promoter activities had been identified [26], thus we detected the effects of DMDRMR on the two sites of DAB2IP promoter. ChiP qRT-PCR assay revealed that DMDRMR KD reduced EZH2 and H3K27me3 occupancies on the two sites of DAB2IP promoter (Fig. 6B). Moreover, DMDRMR-WT OE but not DMDRMR-MUT OE inhibited, DMDRMR KD increased the luciferase activities of the
two sites (Fig. S7D, E), suggesting that DMDRMR repressed the gene transcription of DAB2IP through facilitating the recruitment of EZH2 to DAB2IP promoter region accompanied with H3K27me3. Next, we examined whether DMDRMR mediates SMURF1-dependent DAB2IP degradation. We treated ccRCC cells with the protein synthesis inhibitor cycloheximide and assessed the protein levels of DAB2IP over time. Strikingly, DMDRMR OE decreased and DMDRMR KD increased the half-lives of DAB2IP protein (Fig. 6C, D). Following treatment with a proteasome inhibitor MG132, the inhibitory effects of DMDRM-WT OE on DAB2IP protein level could be reversed (Fig. 6E). Conversely, the accumulation of endogenous DAB2IP in DMDRMR KD cells was increased (Fig. 6F), indicating that DMDRMR might accelerate the proteasome-dependent degradation of DAB2IP. Furthermore, DMDRMR OE enhanced the interaction between SMURF1 and DAB2IP (Fig. 6G), resulting in the increased ubiquitination level of DAB2IP (Fig. 6H, I), suggesting that DMDRMR represses the protein stability of DAB2IP through increasing the interaction of SMURF1 with DAB2IP and thereby promoting ubiquitination-dependent DAB2IP degradation. Collectively, these results indicate that DMDRMR represses DAB2IP expression through its ceRNA.

**DAB2IP is involved in DMDRMR-mediated angiogenesis**

We next determined whether DAB2IP was functionally involved in DMDRMR-mediated angiogenesis. DAB2IP KD remarkably reversed the capillary tube formation of HUVECs and the protein and secreted levels of VEGFA which had been reduced by DMDRMR KD (Fig. 7A, C). Conversely, restoration of DAB2IP could impair the capillary tube formation of HUVECs and the protein and secreted levels of VEGFA induced by DMDRMR OE (Fig. 7B, D), supporting that DAB2IP is involved in DMDRMR-mediated angiogenesis.

DAB2IP is recruited to the VEGFR2-PLC-γ complex to inhibit VEGFR2-dependent angiogenic signaling [25], thus we explored whether DMDRMR/miR378a-5p/DAB2IP regulatory axis promotes angiogenesis through activating VEGFR2 signaling. We incubated HUVECs with conditional medium collected from ccRCC cells expressing variable amounts of DMDRMR, miR378a-5p or DAB2IP and performed immunoblot assays, the results showed that the inactive effects of DMDRMR KD on the phosphorylation of VEGFR2 and PLC-γ could be partially reversed by DAB2IP KD and miR-378a-5p.

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**Fig. 3  DMDRMR binds to miR-378a-5p.** A Ago2 RIP qRT-PCR was performed to analyze the interaction of DMDRMR with Ago2 in 786-O cells. H19 as a positive control and EMS as a negative control. B Flowchart showing stepwise approach to identify the potential DMDRMR bound to miRNAs. C Schematic representation shows two predicted binding sites for miR-378a-5p in DMDRMR. DMDRMR wild type (WT) sequence (upper) and mutant (MUT) sequence (lower). D, E Relative luciferase activities of DMDRMR-WT and MUT luciferase reporters in miR-378a-5p mimic (D) and inhibitor (E)-transfected 293T cells. F GFP RIP qRT-PCR assay showing the interaction of miR-378a-5p with DMDRMR in 293T cells. NC, negative control. G In vitro-synthesized biotin-labeled miR-378a-5p pull down assay showing the interaction of DMDRMR with miR-378a-5p in 786-O cells. H, I Ago2 RIP qRT-PCR assay showing the interaction of DMDRMR with Ago2 in miR-378a-5p mimic-transfected 786-O cells (H) and DMDRMR-WT and MUT OE 769-P cells (I). *p < 0.05, **p < 0.01, ***p < 0.001. ns, no significant.
5p inhibitor (Figs. 7E and S8A). Conversely, DAB2IP OE and miR-378a-5p mimic partially repressed the effect of DMDRMR OE on the phosphorylation of VEGFR2 and PLC-γ (Figs. 7F and S8B). These results demonstrated that DMDRMR activates VEGFA/VEGFR2 signaling through inhibiting DAB2IP expression, resulting in angiogenesis. Consequently, we further examined whether DMDRMR and DAB2IP regulate resistance of ccRCC cells to sunitinib, which targets angiogenic pathways. Cell proliferation assay showed that both DMDRMR KD and DAB2IP OE cells reduced sunitinib resistance (Fig. S8C, D). In contrast, DMDRMR OE and DAB2IP KD cells increased the resistance to sunitinib (Fig. S8C, D). Moreover, DAB2IP KD reversed the reduction of DMDRMR KD on the resistance to sunitinib (Fig. 7G). Similarly, DMDRMR OE also increased the other VEGFR inhibitor pazopanib resistance (Fig. S8E). Furthermore, in vivo xenograft experiments showed that DMDRMR KD increased ccRCC sensitivity to sunitinib as evidenced by the decreased volume and weight of tumors from mice treatment with sunitinib (Figs. 7H, I and S8F). These results suggest that DMDRMR enhances the resistance of ccRCC cells to sunitinib through DAB2IP.

The ceRNA regulatory axis of DMDRMR is critical to ccRCC pathogenesis

To investigate the clinical relevance of the above findings, the same tissue microarray with our previous study [7] was assessed to evaluate the relationships among DMDRMR, miR-378a-5p, and DAB2IP. miRNA in situ hybridization (ISH) result revealed that, miR-378a-5p expression in the cytoplasm reduced in the ccRCC tissues versus the adjacent tissues (Fig. 8A, B). Similar result was observed in DAB2IP immunohistochemistry (IHC) staining (Fig. 8C, D). DMDRMR level was inversely correlated with miR-378a-5p expression in the cytoplasm reduced in the ccRCC tissues versus the adjacent tissues (Fig. 8A, B). DMDRMR OE and DAB2IP expression (Fig. 8E, F). Meanwhile, miR-378a-5p expression was positively correlated with DAB2IP expression (Fig. 8G).

Furthermore, receiver operating characteristic (ROC) analysis showed that combined use of DMDRMR, miR-378a-5p and DAB2IP expression could improve the area under curve (AUC) value for the

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Fig. 4  DMDRMR induces angiogenesis through sequestering miR-378a-5p. A, B GSEA showing the enrichment of “angiogenesis” (Hallmark database, A) and “vegf signaling pathway” (KEGG database, B) in high (red) and low (blue) miR-378a-5p expression. C, D Representative bright-field images (left) and quantifications (right) of matrigel tube formation of HUVECs incubated with conditioned medium from DMDRMR KD 786-O cells transfected with miR-378a-5p inhibitor (C) and DMDRMR OE 769-P cells transfected with miR-378a-5p mimic (D) (scale bars, 200 µm). E, F ELISA assay detecting the secreted VEGFA levels in DMDRMR KD 786-O cells transfected with miR-378a-5p inhibitor (E) and in DMDRMR OE 769-P cells transfected with miR-378a-5p mimic (F). The results are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. ns, no significant.
diagnosis of ccRCC (Fig. 8H), suggesting that the combination of DMDRMR and VEGFA expression can serve as a diagnostic biomarker of ccRCC.

Next, qRT-PCR assay was used to determine the association between DMDRMR and its ceRNA in 48 pairs of ccRCC tissues and adjacent tissues used in our previous study [7]. Downregulated miR-378a-5p levels and upregulated EZH2, SMURF1, and VEGFA transcriptional levels were observed in ccRCC tissues versus adjacent tissues (Fig. 8I–L). Moreover, spearman correlation analysis showed that DMDRMR was positively correlated with EZH2, SMURF1, and VEGFA, whereas miR-378a-5p was negatively correlated with DMDRMR, EZH2, SMURF1, and VEGFA (Fig. 8M).

However, DAB2IP transcriptional level was increased in ccRCC tissues (Fig. S9A). Furthermore, we also analyzed the TCGA ccRCC dataset, high expression levels of EZH2 and SMURF1 and low expression of DAB2IP predicted poor overall survival of ccRCC patients (Fig. S9B–D). High EZH2 and low DAB2IP expression was associated with poor outcomes, including pathologic stage, tumor size, metastatic status and Fuhrman grade (Fig. S9B, D). These results supported the oncogenic role of EZH2, SMURF1, and...
Fig. 6  DMDRM represses DAB2IP expression through its ceRNA. A The qRT-PCR (top) and immunoblot (bottom) analysis of DAB2IP in DMDRM KD 786-O cells (left) and in DMDRM-WT and MUT OE 769-P cells (right). B ChIP qRT-PCR analysis showing the binding efficiencies of EZH2 and H3K27me3 to the two regions of DAB2IP promoter in DMDRM KD 786-O cells. P1, the first region of DAB2IP promoter; P2, the second region of DAB2IP promoter. C, D Immunoblot of DAB2IP in the DMDRM OE 769-P cells (C) and KD 786-O (D) cells treated with cycloheximide (CHX) for indicated times. The densitometry analysis showing the relative DAB2IP expression levels that normalized to the reference protein αTubulin (Red numbers). E, F Immunoblot of DAB2IP in the DMDRM-WT and MUT OE 769-P cells (E) and DMDRM KD 786-O cells (F) treated with MG132 for 10 h. G, J Immunoprecipitation showing the association between DAB2IP and SMURF1 in DMDRM OE 769-P cells (G) and KD 786-O cells (J). H, K Immunoprecipitation showing the associations between DAB2IP and HA-tagged ubiquitin (HA-UB) in SMURF1 KD with DMDRM OE 293T cells (H) and in DMDRM KD with SMURF1 OE 293T cells (K). I, L Immunoprecipitation showing the associations between DAB2IP and HA-UB in DMDRM OE transfected with miR-378a-5p mimic 293 T cells (I) and in DMDRM KD transfected with miR-378a-5p inhibitor 293T cells (L). The results are presented as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.
VEGFA and the tumor suppressive role of miR-378a-5p and DAB2IP in ccRCC. Consistent with our findings, DAB2IP was negatively correlated with DMDRMR and EZH2 (Fig. S9E, F). Interestingly, the combination of high DMDRMR and low DAB2IP predicted the poorest overall survival of patients (Fig. 8N). These results demonstrated that the ceRNA regulatory axis of DMDRMR is clinically relevant to ccRCC pathogenesis and prognosis of patients with ccRCC.

**DISCUSSION**

Angiogenesis is a hallmark of cancer, which supplies enough oxygen and nutrients to promote the progression of ccRCC [27]. Consistent with our study, several other studies had reported that IncRNAs promote tumor angiogenesis or sunitinib resistance in RCC, including HOTAIR and IncARSR [28, 29], supporting that IncRNAs may provide new targets for therapy and predictive biomarkers for anti-angiogenesis therapy response in RCC. It is established that IncRNAs function as ceRNA to increase VEGFA expression by competing with miRNAs, resulting in tumor angiogenesis [30–32]. However, our results revealed that VEGFA is an indirect-regulated gene of DMDRMR/miR-378a-5p axis and contribute to the effect of this axis on angiogenesis in ccRCC. Similar findings have been reported that miR-378a-5p can suppress angiogenesis of oral squamous cell carcinoma by targeting KLK4 and indirectly reducing VEGFA expression [33] or promotes tumor angiogenesis by directly and indirectly upregulating VEGFA, including metastatic melanoma [34, 35], which may be explained by the cancer-type specificity, cellular-context dependence, or selection of signaling pathway. Recent study demonstrated that miR-378a-5p can attenuate cell proliferation, migration, invasion and promote cell apoptosis in RCC, and is associated with good prognosis of patients with RCC [36], which further verify our finding that miR-378a-5p acts as a tumor suppressor in ccRCC. It has been reported that both EZH2 and SMURF1 exert oncogenic functions in ccRCC [19, 20, 37]. Loss of DAB2IP could enhance tumor growth and resistance to mTOR-targeted therapies and ionizing radiation in RCC [22]. However, the dysregulated mechanism of EZH2, SMURF1 and DAB2IP expression remains enigmatic. Herein, we discovered that DMDRMR/miR-378a-5p axis decreases DAB2IP expression through directly upregulating EZH2 and SMURF1 in ccRCC. Moreover, we extend the role of miR-378a-5p, EZH2, SMURF1, and DAB2IP in ccRCC angiogenesis.
DAB2IP functions as an endogenous inhibitor of adaptive angiogenesis in part by binding directly to VEGFR2 and limiting PI3K activation. Although we did not define how the DMDRMR/miR-378a-5p/DAB2IP axis regulates VEGFA expression, we revealed that the DMDRMR/miR-378a-5p axis inhibits the inactivation of DAB2IP-regulated VEGFA/VEGFR2 signaling pathway, which can explain our notion that the DMDRMR/miR-378a-5p axis could serve as an angiogenic activator in ccRCC. Given the known function of DMDRMR and miR-378a-5p in ccRCC, the proposed model for the DMDRMR/miR-378a-5p/DAB2IP axis promoting the angiogenesis of ccRCC is shown in Figure 8.
378a-5p in ccRCC [37, 36], and angiogenesis has been shown to be responsible for tumor growth and metastasis [38], we also explored whether DMDRMR/miR-378a-5p/DAB2IP axis regulates the cell proliferation, migration, invasion of ccRCC and found that DMDRMR KD partially abrogated the stimulative effect of miR-378a-5p inhibitor and DAB2IP KD on these phenotypes (Fig. S10A–F), suggesting that DMDRMR promotes the cell proliferation, migration, invasion of ccRCC through selectively repressing miR-378a-5p and DAB2IP. Therefore, this study further reinforces our previous finding that DMDRMR promotes tumor growth and metastasis [7]. Clearly, this study will improve our understanding of the mechanistic, functional, and pathological roles of DMDRMR in ccRCC. But whether the function and molecular mechanism of DMDRMR is involved in other cancers need to be further investigated.

Exosomal IncRNAs were identified as promising biomarkers for cancers [39]. ExoRBase, a repository of extracellular vehicles (EVs) IncRNAs derived from blood of healthy human and cancer cohorts [40], showed that DMDRMR was expressed in EVs and higher expression levels in the esophageal squamous cell carcinoma, gastric cancer and melanoma relative to healthy cohorts (Fig. S11). However, EVs DMDRMR was not statistically significant in ccRCC cohort, which may be explained by small sample size. Targeted nucleic acid-based therapeutics are emerging as a promising approach [41, 42], therefore, we need to further explore the clinical value of oncogenic DMDRMR in exosomal biomarkers and nucleic acid therapeutic targets for cancers in future.

Overall, our study reveals that DMDRMR is a pro-angiogenic IncRNA that promotes angiogenesis and sunitinib resistance, by competitively binding miR-378a-5p to promote EZH2 and SMURF1-mediated repression of DAB2IP expression and thereby activating VEGFA/VEGFR2 signaling pathway (Fig. 8O). This pathway might provide novel clinical markers and therapeutical targets for ccRCC patients.

**Reporting Summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**DATA AVAILABILITY**

The data and materials during this study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS
S.G. and Y.G. designed research, wrote the paper; Y.Z., X.L., Y.G., Y.W., Y.P., X.H., B.P., and H.W. performed research; X.Z. and S.N. provided samples; Y.G., Y.Z., and X.L. analyzed data; Q.Y. review the paper.

COMPETING INTERESTS
The authors declare no competing interests.

ETHICAL APPROVAL
All animal experiments were conducted under an appropriate animal project license approved by Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences (Suzhou, Jiangsu, China). The Department of Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences has a well-equipped animal room of SPF level, equipped with ultra-clean bench, anesthesia machine, etc. The purchase and feeding of experimental animals are in accordance with national standards, ensuring the smooth conduct of animal experiments.

ADDITIONAL INFORMATION
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