The mineral dust-induced gene, *mdig*, regulates angiogenesis and lymphangiogenesis in lung adenocarcinoma by modulating the expression of VEGF-A/C/D via EGFR and HIF-1α signaling

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Abstract. Mineral dust-induced gene (*mdig*) is a novel lung cancer-related oncogene. The aim of this study was to explore the effects of mdig on angiogenesis and lymphangiogenesis by vascular endothelial growth factor (VEGF) in lung adenocarcinoma. mdig-overexpressing A549, H1299 and 293T cells, mdig-silenced A549, human umbilical vein endothelial cells (HUVECs) and human lymphatic endothelial cells (HLECs) were cultured under normoxic and hypoxic conditions. Protein expression levels of mdig, epidermal growth factor receptor (EGFR), phospho(p)-EGFR Tyr1068, hypoxia-inducible factor-1α (HIF-1α), VEGF-A/C/D and VEGF-R1/R2/R3 were assessed using western blotting. mRNA expression levels of mdig, EGFR and HIF-1α were measured using RT-qPCR. Tube formation and xenograft tumor experiments were performed to examine the mechanism of mdig in angiogenesis and lymphangiogenesis. Protein expression levels of EGFR, HIF-1α and VEGF-A/C/D were significantly upregulated in cells cultured under hypoxic conditions compared with those cultured under normoxic conditions, whereas the levels of mdig were decreased. Protein expression levels of EGFR, p-EGFR and VEGF-A/R1/R2 were significantly increased in the mdig-overexpressing cells, whereas the levels of HIF-1α and VEGF-C/D/R3 were decreased compared with those in control cells, all of which were reversed in mdig-silenced cells. Tumor volumes and density of angiogenesis in the mdig-overexpressing group were significantly increased compared with those in the control group, whereas the density of lymphangiogenesis was decreased. No tumors formed in the mdig-silenced group after 3 weeks of assessment *in vivo*. Protein expression levels of EGFR, p-EGFR, VEGF-A and angiogenesis density were significantly reduced in the mdig-overexpressing cells treated with an EGFR inhibitor, whereas the levels of HIF-1α, VEGF-C/D and the lymphangiogenesis density were significantly increased in mdig-overexpressing cells treated with a HIF-1α agonist. All changes in protein expression were reversed in EGFR agonist and HIF-1α inhibitor treated mdig-silenced cells. In conclusion, mdig is an oxygen-sensitive protein that promotes tumor growth and angiogenesis by activating the EGFR/p-EGFR/VEGF-A/VEGF-R1/R2 pathway and inhibits lymphangiogenesis by blocking the HIF-1α/VEGF-C/D/VEGF-R3 pathway.

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Introduction

Lung cancer is one of the most common malignant tumors in the world (1). Previous studies have shown that ~2.1 million individuals have been diagnosed with lung cancer, accounting for 11.6% of all new cancer cases in 2018. In addition, the number of deaths from lung cancer is ranked first among cancer-associated deaths, accounting for 18.4% of the total cancer deaths in 2018 (1,2). Due to untimely diagnoses and limited effective treatment options, particularly for later stage cancers, the 5-year survival rate of lung cancer is only 5% (3). Lung adenocarcinoma accounts for >50% of all cases of lung cancer (4). Mineral dust-induced gene (*mdig*) is a newly discovered lung cancer-related gene, which was first found in alveolar macrophages of coal miners (5,6). mdig contains a Jumonji C domain with demethylase function of histone H3K9me3, and can promote the activation of proto-oncogenes (7,8). mdig is also called myc-induced nuclear antigen with a molecular weight of 53 kDa (MINA53) or nuclear protein 52 (NO52) (6,9,10). A previous study has shown that compared with advanced lung cancer tissues, the expression levels of mdig in early lung cancer tissues are significantly higher (11). Therefore, it is speculated that overexpression of mdig may be an early event in lung cancer (6,11). It was reported that mdig possesses oncogenic properties via antagonization of tri-methyl lysine 9 on histone H3 and promoting ribosomal

RNA synthesis (7). mdig can promote lung cancer cell proliferation by accelerating cell cycle transition from the G1 phase to S phase (10,12), and it can also inhibit cell invasion and migration via regulating the glycogen synthase kinase-3β/β-catenin signaling pathway (13). However, the mechanism underlying this paradoxical phenomenon of mdig is unclear.

The formation of tumors is typically divided into two stages. First, normal cells are transformed into malignant cells by the continuous activation of proto-oncogenes. Subsequently, transformed malignant cells continue to proliferate to form solid tumors (14). Angiogenesis and lymphangiogenesis serve important roles in the second stage, during which the interaction of several complex signaling pathways is required. In addition, lack of nutrition and oxygen within the tumors creates a hypoxic microenvironment as the tumors continuously proliferate (15), further stimulating the activation of proto-oncogenes, through which they synergistically regulate angiogenesis and lymphangiogenesis (15,16). Previous studies have shown that epidermal growth factor receptor (EGFR) can induce angiogenesis by promoting the secretion of vascular endothelial growth factor (VEGF)-A via both a hypoxia-inducible factor-1α (HIF-1α)-dependent and HIF-1α-independent manner (14,17,18). In addition, studies have previously shown that VEGF-C and VEGF-D can induce both angiogenesis and lymphangiogenesis (19,20). Although it has been demonstrated that the effect of EGFR on HIF-1α can occur under hypoxic and normoxic conditions (17,18,21-23), the role and mechanism of mdig in tumor angiogenesis and lymphangiogenesis have not been previously reported.

The aim of the present study was to explore the effects of mdig on angiogenesis and lymphangiogenesis in lung adenocarcinoma under normoxic and hypoxic conditions. The results revealed that mdig is an oxygen-sensitive protein that promotes tumor growth and angiogenesis by activating an EGFR/p-EGFR/VEGF-A/VEGF-R1/R2 pathway, whilst also inhibits lymphangiogenesis by blocking a HIF-1α/VEGF-C/D/VEGF-R3 signaling pathway.

Materials and methods

Cell culture. Human lung adenocarcinoma cell lines A549 and H1299, as well as 293T cells were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. Human umbilical vein endothelial cells (HUVECs) and human lymphatic endothelial cells (HLECs) were purchased from the Cancer Institute of Peking University Cell Bank. All the cell lines were tested for mycoplasma using a MycoBlue™ Mycoplasma Detector according to the manufacturer's protocol (Vazyme Biotech Co., Ltd.), which confirmed that there was no mycoplasma contamination. All cell lines except for 293T were cultured in RPMI-1640 medium (Hyclone; Cytiva) supplemented with 10% FBS (Hyclone; Cytiva). 293T cells were cultured in DMEM (Hyclone; Cytiva) containing 10% FBS. All cell lines were maintained at 37˚C with 5% CO_2. Normoxic conditions (21% O_2) were achieved using an incubator (Thermo Fisher Scientific, Inc.), whereas hypoxic conditions (1% O_2) were induced in a hypoxic chamber (model no. C-42; Biospherix Oxycycler; BioSpherix, Ltd.). Signaling pathway agonists and inhibitors used in the present study were all obtained from Selleck Chemicals: EGFR agonist, NSC228155; EGFR inhibitor, erlotinib; HIF-1α agonist, IOX2; and HIF-1α inhibitor, BAY 87-2243.

Lentivirus transfection. The mdig (accession no. NM_032778; GenBank) overexpression lentiviral vector (LV-mdig) and its control vector (LV-con), in addition to the mdig knockdown lentiviral vectors (LV-mdig-RNAi 1, sequence, 5'-GGGTGATTTGTTGTACTTT-3'; LV-mdig-RNAi 2, sequence, 5'-AACGATTCAGTTTCACCAA-3') and their control vector (LV-mdig-RNAi-con, sequence, 5'-TTCTCCGAACGTGTCACGT-3') were purchased from Shanghai Genechem Co., Ltd. These vectors, which were mixed with HitransG P transfection enhancement solution (Shanghai Genechem Co., Ltd.), were transfected into A549, H1299 and 293T cells at multiplicities of infection of 50, 20 and 20, respectively, in T12.5 flasks (Corning, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol® reagent (Ambion; Thermo Fisher Scientific, Inc.) was used to extract total RNA from cells. The cDNA templates were then reverse transcribed using PrimeScript™ RT reagent kit with gDNA Eraser according to the manufacturer's protocol (Takara Bio, Inc.). The primers used for measuring the expression levels of mdig, EGFR, HIF-1α and the normalization control ACTB, were purchased from Takara Bio, Inc. The sequences of the primers were: mdig forward, 5'-GCAACGATTCAGTTTCACCAACC-3' and reverse, 5'-ATGTACACATTCGAGCCAACCAAG-3'; EGFR forward, 5'-TGCATACAGTGCCACCCAGAG-3' and reverse, 5'-GCACACTGGATACAGTTGTCTGGTC-3'; HIF-1α forward, 5'-CTCATCAGTTGCCACTTCCACATA-3' and reverse, 5'-AGCAATTCATCTGTGCTTTCATGTC-3'; and ACTB forward, 5'-CCTGGCACCCAGCCAAT-3' and reverse, 5'-GGGCCGGACTCGTCATAC-3'. qPCR was subsequently performed using SYBR® Premix Ex Taq™ (Takara Bio, Inc.) in a LightCycler® 480 system (Roche Diagnostics). The thermocycling conditions were as follows: Initial denaturation for 5 sec, followed by 40 cycles of 95˚C for 15 sec, 59˚C for 30 sec and 70˚C for 30 sec to detect the cycle threshold value (Cq). The $^{2-\Delta\Delta Cq}$ method was used to calculate the relative ratio of genes, and expression was presented normalized to ACTB expression (5,24-26).

Western blotting. Cells were lysed using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing 10% PMSF (Beijing Solarbio Science & Technology Co., Ltd.). Equivalent amounts (40 μg) of protein samples, which were quantified using a bicinchoninic acid protein assay, were loaded on an 8% or 12% SDS-gel, resolved using SDS-PAGE and subsequently transferred to PVDF membranes (Millipore Co., Ltd.). Membranes were blocked for 2 h at room temperature using 5% nonfat dried milk, washed with TBST, and then incubated with primary antibodies at 4˚C overnight. The primary antibodies (all at 1:1,000) used in the present study were: Anti-mdig (mouse mAb; cat. no. sc-398521; Santa Cruz Biotechnology, Inc.), anti-EGFR (rabbit mAb; cat. no. 4267; Cell Signaling Technology, Inc.), anti-phospho (p)-EGF receptor (Tyr1068; rabbit mAb; cat. no. 3777; Cell Signaling Technology, Inc.), anti-HIF-1α (rabbit mAb; cat. no. 36169; Cell Signaling Technology, Inc.), anti-VEGFA (mouse mAb; cat. no. ab1316; Abcam), anti-VEGFC (rabbit

polyclonal antibody; cat. no. ab9546; Abcam), anti-VEGFD (rabbit mAb; cat. no. ab155288; Abcam), anti-VEGFR1 (rabbit mAb; cat. no. ab32152; Abcam), anti-VEGFR2 (rabbit mAb; cat. no. 9698; Cell Signaling Technology, Inc.), anti-VEGFR3 (rabbit mAb; cat. no. 33566; Cell Signaling Technology, Inc.), anti-histone H3 (rabbit polyclonal antibody; cat. no. ab1791; Abcam) and anti-β-actin (mouse mAb; cat. no. ab8224; Abcam). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. 31460) or goat anti-mouse IgG (cat. no. 31430) secondary antibodies (1:5,000; both from Thermo Fisher Scientific, Inc.) at room temperature for 2 h. Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.) was used to detect the bands in a chemiluminescence detector (MicroChemi 4.2; DNR Bio-Imaging Systems, Ltd.). Densitometry analysis was performed using ImageJ version 1.8.0 (National Institutes of Health).

Co-immunoprecipitation. The mdig-overexpressing A549 cells cultured under either normoxic or hypoxic conditions were lysed using immunoprecipitation lysis buffer (Beyotime Institute of Biotechnology). Primary antibodies against mdig, EGFR or HIF-1α were then added into the lysate to a final concentration of 5 μg/ml. In total, ~5 μl protein A/G immunoprecipitation magnetic beads (cat. no. B23202; Bimake) were washed with immunoprecipitation lysis buffer before being mixed with the diluted antibodies. These mixtures were subsequently placed on a mixer for incubation at 4˚C overnight. The magnetic beads were collected, and the proteins were denatured by the addition of 25 μl 1X SDS loading buffer. Western blotting was then performed to assess mdig, EGFR and HIF-1α expression.

Nuclear and cytoplasmic fractionation. The mdig-overexpressing cells were lysed using cytoplasmic protein lysis buffer (Invent Biotechnologies, Inc.). The lysate was centrifuged at 10,000 x g at 4˚C for 5 min to separate the proteins of the nuclear (pellet) and cytoplasmic (supernatant) fractions. Nuclear protein lysis buffer (Invent Biotechnologies, Inc.) was then used to lyse the precipitates, before centrifuging again at 16,000 x g at 4˚C for 30 sec, and the supernatant was collected which contained the nuclear protein.

Conditioned medium. The conditioned medium was prepared as described previously (23). Transfected A549 and H1299 cells, which were cultured in either normoxic or hypoxic conditions, were first washed twice with PBS. RPMI-1640 medium without serum was used to culture the cells further for 24 h before collecting the culture supernatants. The cell-conditioned media was centrifuged at 1,000 x g at 4˚C for 10 min, following which the supernatant was subsequently used for culture of HUVECs and HLECs.

Tube formation assays of angiogenesis and lymphangiogenesis. Tube formation assays to assess angiogenesis and lymphangiogenesis were performed using HUVECs and HLECs, respectively, as described previously (17,23,27). Subsequently, 96-well plates coated with cold Matrigel (50 μl/well; cat. no. 356234; BD Biosciences) were incubated at 37˚C in the incubators for 30 min. HUVECs and HLECs ($2x10^4$ cells/well) suspended in conditioned media from the mdig-overexpressing A549 cells were seeded into 96-well plates pre-coated with Matrigel at 37˚C for 4-6 h. Images of the tube-like structures were taken at a magnification of x100 using an inverted fluorescence microscope (Carl Zeiss AG).

Xenograft tumor studies. Female athymic nu/nu mice (aged, 4-6 weeks) were purchased from Charles River Laboratories, Inc. All mice (n=4 mice/group) were bred in independently ventilated cages and were provided with sterilized food and water. mdig-overexpressing A549 cells, mdig-silenced A549 cells, mdig-overexpressing A549 cells treated with EGFR inhibitor erlotinib and mdig-overexpressing A549 cells treated with HIF-1α agonist IOX2 ($5x10^6$ cells/mouse) were suspended in PBS and then injected subcutaneously into the axilla of the mice. After 3 weeks, the mice were sacrificed and tumors were harvested for subsequent experiments. The experiments involving animals were performed in accordance with the Ethical Guidelines for Animal Care of the Institutional Animal Care and Use Committee of the China Medical University.

Immunohistochemistry. Xenograft tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned into 5-μm thick sections. The sections were then deparaffinized in xylene followed by hydration in a descending series of ethanol solutions before being subsequently incubated in sodium citrate buffer and blocked with endogenous peroxidase (cat. no. SP KIT-A1; Fuzhou Maixin Biotech Co., Ltd.) and non-specific staining blocker (cat. no. SP KIT-B1; Fuzhou Maixin Biotech Co., Ltd.) at room temperature. These sections were incubated with primary antibodies at 4˚C overnight. The primary antibodies used for this experiment included: Anti-VEGFA (rabbit polyclonal antibody; cat. no. ab39250; 1:100; Abcam), anti-VEGFC (rabbit polyclonal antibody; cat. no. ab9546; 1:100; Abcam), anti-VEGFD (rabbit mAb; cat. no. ab155288; 1:100; Abcam), anti-CD31 (rabbit polyclonal antibody; cat. no. ab28364; 1:50; Abcam), anti-LYVE1 (rabbit polyclonal antibody; cat. no. ab33682; 1:100; Abcam) and anti-mdig (rabbit polyclonal antibody; cat. no. ab126282; 1:100; Abcam). Biotinylated goat anti-mouse/rabbit IgG (cat. no. SP KIT-C1; Fuzhou Maixin Biotech Co., Ltd.) and streptavidin-peroxidase (cat. no. SP KIT-D1; Fuzhou Maixin Biotech Co., Ltd.) were then applied for 10 min, respectively, at room temperature to the sections, which were stained with DAB solution and counterstained with hematoxylin at room temperature. Images of the sections were taken in ≥3 random fields of view at the magnification of x200 and x400 using an upright light microscope (Carl Zeiss AG).

Statistical analysis. All experimental data are presented as the mean ± standard deviation of ≥3 experimental repeats. Data were compared using a Student's t-test or a one-way ANOVA followed by a Dunnett's post-hoc test in GraphPad Prism version 7.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of hypoxia on the protein expression levels of mdig, EGFR, HIF-1α and the VEGF family. A549, H1299 and 293T cells were cultured in either normoxic (21% O_2) or hypoxic (1% O_2) conditions for 24 h before the expression levels of these proteins were measured by western blotting. The protein

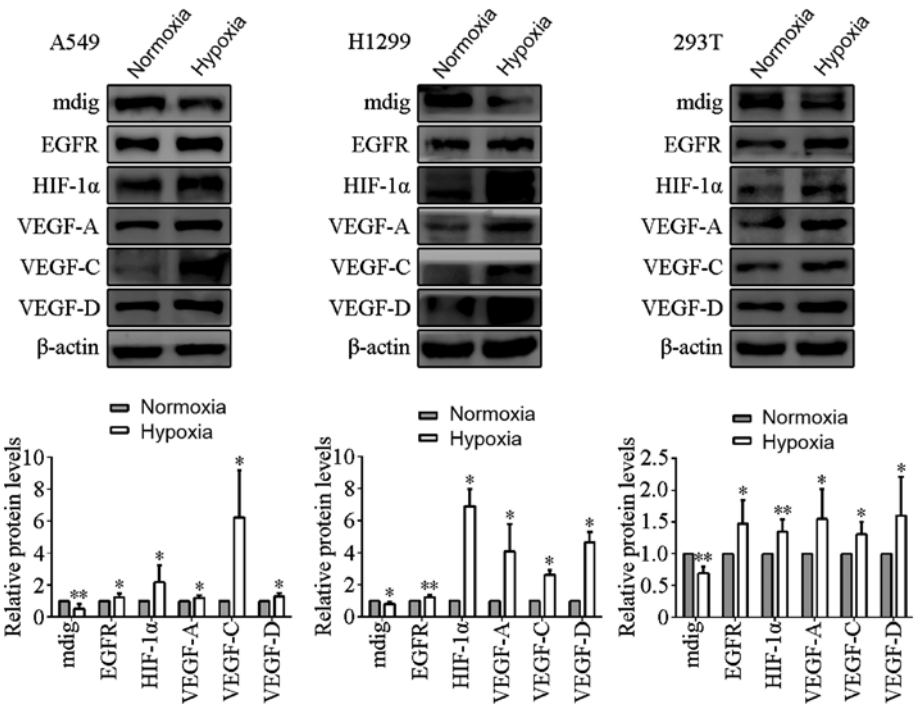

Figure 1. Effects of hypoxia on the protein expression levels of mdig, EGFR, HIF-1α and the VEGF family members. A549, H1299 and 293T cells were cultured under normoxic and hypoxic conditions. Hypoxia increased the protein expression levels of EGFR, HIF-1α and the VEGF family members, but reduced the expression of mdig. β-actin was used as the loading control. *P<0.05, **P<0.01, normoxia vs. hypoxia. EGFR, epidermal growth factor receptor; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; mdig, mineral dust-induced gene.

expression levels of EGFR, HIF-1α and VEGF-A/C/D were found to be significantly higher in the cells cultured under hypoxic conditions compared with those in cells cultured under normoxic conditions (P<0.05; Fig. 1). By contrast, mdig protein expression levels were significantly reduced by culturing under hypoxic conditions compared with those in cells cultured under normoxic conditions (P<0.05; Fig. 1).

mdig promotes the protein expression of EGFR and p-EGFR. To investigate the relationship between mdig and EGFR, A549 cells were first transfected with mdig-overexpressing LV-mdig and mdig-silencing LV-mdig-RNAi vectors, whereas H1299 and 293T cells were transfected with LV-mdig vector. The transfected cells were then cultured under either normoxic or hypoxic conditions. Western blotting results showed that the protein expression levels of EGFR, in addition to the autophosphorylation of one of its most important residues, Tyr1068 (28,29), were shown to be significantly increased in the LV-mdig group compared with those in the LV-con group (P<0.05). These two aforementioned parameters were found to be significantly reduced in the LV-mdig-RNAi group compared with those in the LV-mdig-RNAi-con group (P<0.05; Figs. 2 and 3). However, these changes were not significantly different between cells cultured under hypoxic and normoxic conditions.

Next, the mRNA expression levels of mdig and EGFR were measured in the transfected cells using RT-qPCR. There were no significant changes in EGFR mRNA expression levels in the LV-mdig and LV-mdig-RNAi transfected cells compared with those in the LV-con and LV-mdig-RNAi-con transfected cells, respectively, under both normoxic and hypoxic conditions (Fig. S1A). Subsequently, the protein expression levels of p-EGFR and EGFR were compared in the mdig-overexpressing A549 and H1299 cells. The LV-mdig/LV-con ratio of p-EGFR protein was significantly higher compared with that of the EGFR protein (Fig. S1B). Following the culturing of mdig-overexpressing A549 cells under normoxic and hypoxic conditions, their lysates were subjected to co-immunoprecipitation, and it was found that EGFR did not form complexes with mdig (Fig. S1C).

mdig inhibits the protein expression of HIF-1α and prevents its entry into the nucleus. To study the functional relationship between mdig and HIF-1α, the protein expression levels of mdig and HIF-1α in mdig-overexpressing and mdig-knockdown cells cultured under normoxic and hypoxic conditions were measured by western blotting. HIF-1α expression levels were significantly lower in the LV-mdig group compared with those in the LV-con group (P<0.05), whereas HIF-1α expression levels were significantly higher in the LV-mdig-RNAi group compared with those in the LV-mdig-RNAi-con group (P<0.05; Figs. 2 and 3). These changes were not statistically significant between the cells cultured under hypoxic and normoxic conditions. In addition, the mRNA expression levels of mdig and HIF-1α were not statistically significant when comparing the LV-mdig and LV-mdig-RNAi groups with LV-con and LV-mdig-RNAi-con groups, under normoxic and hypoxic conditions (Fig. S1A). Mdig-overexpressing A549 cells were also cultured under normoxic and hypoxic conditions prior to co-immunoprecipitation analysis, and the results showed that mdig did not interact with HIF-1α (Fig. S1C).

Since HIF-1α serves an important role in promoting the transcription of a number of genes in the nucleus (27,30), the nuclear and cytoplasmic proteins were isolated from A549 and H1299 cells overexpressing mdig, after culturing under normoxic and hypoxic conditions. Protein expression of mdig was found to

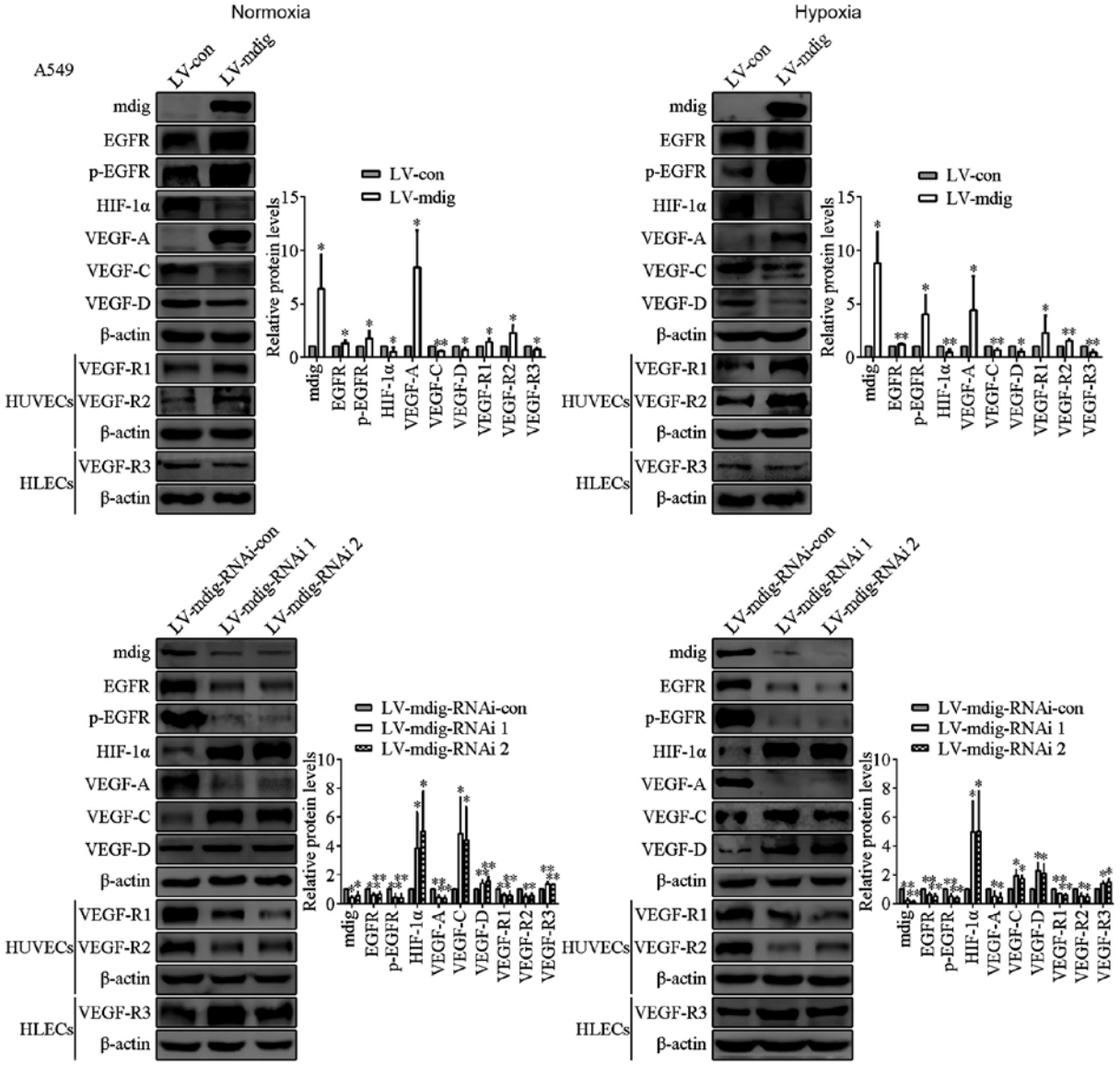

Figure 2. Regulation of EGFR, p-EGFR, HIF-1α, the VEGF family members and their receptors by mdig at the protein levels in transfected A549 cells, and HUVECs and HLECs cultured with conditioned media of LV-mdig A549 cells under normoxic and hypoxic conditions. Mdig upregulated the expression of EGFR, p-EGFR, VEGF-A and VEGF-R1/R2, and reduced the expression of HIF-1α, VEGF-C/D and VEGF-R3. β-actin was used as the protein loading control. *P<0.05, **P<0.01, LV-mdig vs. LV-con and LV-mdig-RNAi vs. LV-mdig-RNAi-con. mdig, mineral dust-induced gene; EGFR, epidermal growth factor receptor; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; p-, phospho; LV, lentivirus; RNAi, RNA interference; con, control; HUVECs, human umbilical vein endothelial cells; HLECs, human lymphatic endothelial cells.

be primarily distributed in the nucleus, with little localization observed in the cytosol. HIF-1α was also primarily localized in the nucleus, although the protein expression levels of HIF-1α in the cytosol were significantly higher in cells in the LV-mdig group compared with those in the LV-con group (P<0.05). By contrast, the protein expression levels of HIF-1α in the nucleus of cells in the LV-mdig group were significantly lower compared with those in the LV-con group (P<0.05; Fig. 4).

mdig regulates the protein expression of VEGF and VEGF receptors. To investigate the effects of mdig on tumor angiogenesis and lymphangiogenesis, the influence of mdig on the expression of VEGFs and their receptors was explored. The protein expression levels of VEGFs and their receptors in the mdig-overexpressing and mdig-silenced cells were measured by western blotting. Under normoxic and hypoxic conditions, the expression levels of VEGF-A were found to be significantly higher in the LV-mdig group, whereas those of VEGF-C and VEGF-D were significantly lower when compared with those in the LV-con group (P<0.05). Opposite results were observed in the LV-mdig-RNAi group compared with those in the LV-mdig group (Figs. 2 and 3).

Based on these observations, conditioned media were obtained from transfected A549 and H1299 cells cultured under normoxic and hypoxic conditions, which were then used to treat HUVECs and HLECs for 24 h. Western blotting was subsequently performed to measure the expression levels of VEGF-R1/R2 in HUVECs and the expression levels of VEGF-R3 in HLECs. The expression levels of VEGF-R1/R2 were shown to be significantly higher in the HUVECs cultured with conditioned media of cells from the LV-mdig group compared with those in HUVECs cultured with the conditioned media of cells from the LV-con group (P<0.05). The expression

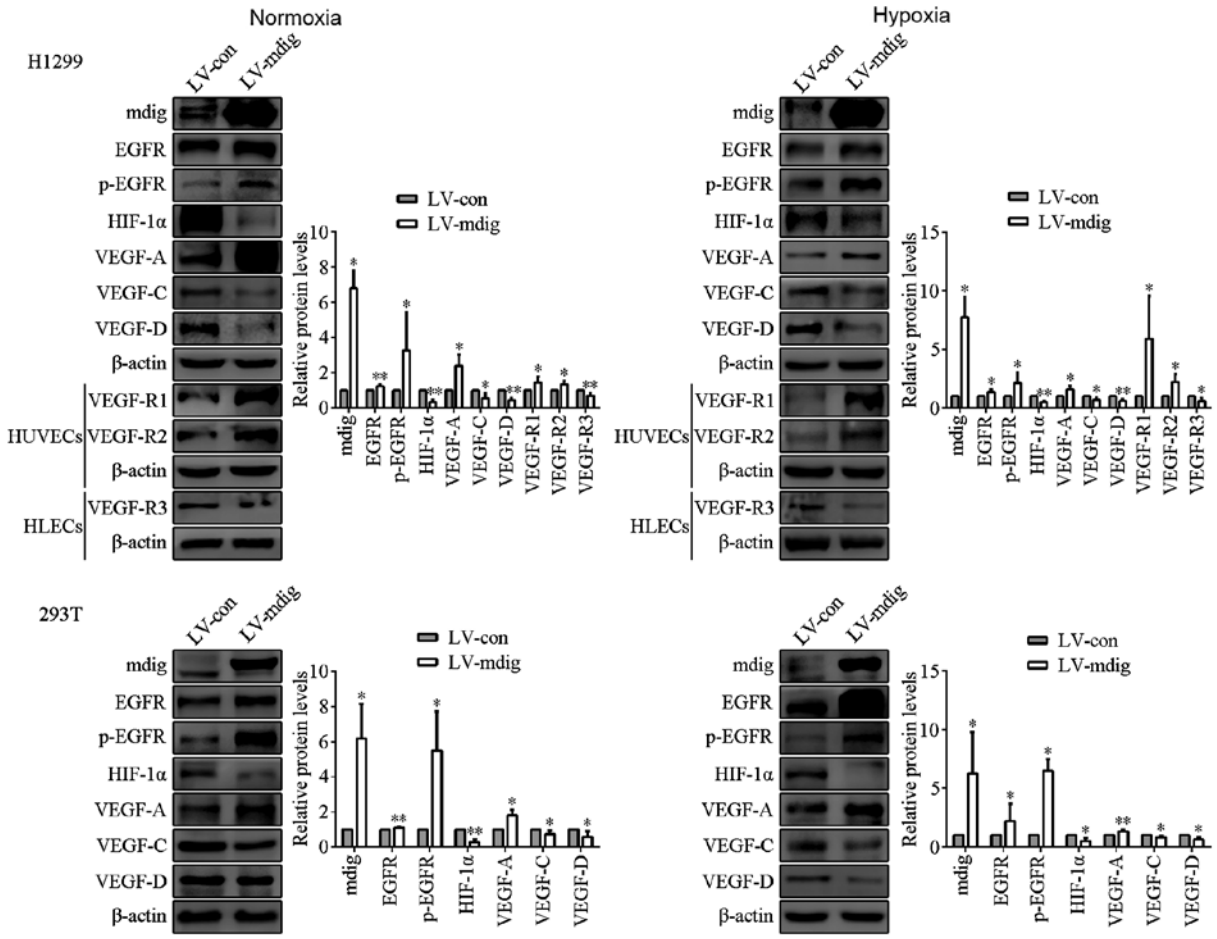

Figure 3. Regulation of EGFR, p-EGFR, HIF-1α, the VEGF family members and their receptors by mdig at the protein levels in mdig-overexpressing H1299 and 293T cells, and HUVECs and HLECs cultured with conditioned media of LV-mdig H1299 cells under normoxic and hypoxic conditions. mdig upregulated the expression of EGFR, p-EGFR, VEGF-A and VEGF-R1/R2, and reduced the expression of HIF-1α, VEGF-C/D and VEGF-R3. β-actin was used as the protein loading control. *P<0.05, **P<0.01, LV-mdig vs. LV-con. mdig, mineral dust-induced gene; EGFR, epidermal growth factor receptor; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; p-, phospho; LV, lentivirus; con, control; HUVECs, human umbilical vein endothelial cells; HLECs, human lymphatic endothelial cells.

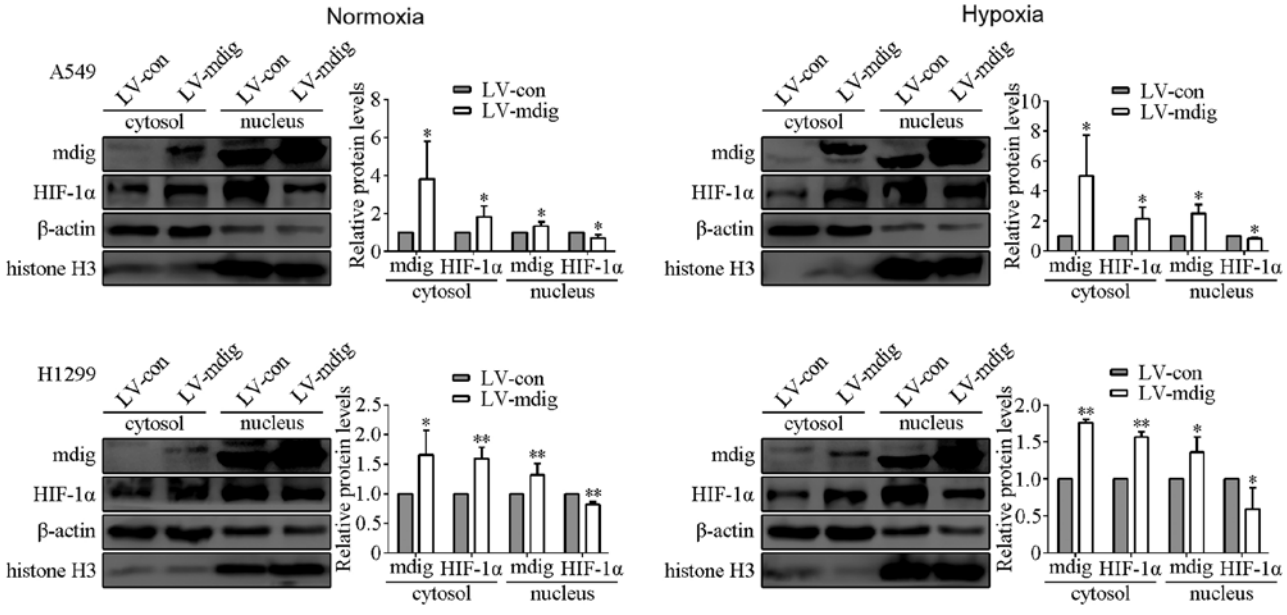

Figure 4. Regulation of intracellular HIF-1α distribution by mdig under normoxic and hypoxic conditions. Nuclear and cytoplasmic proteins were first isolated from A549 and H1299 cells transfected with LV-mdig. Western blotting was used to assess the effects of mdig on the distribution of HIF-1α. β-actin and histone H3 were used as cytoplasmic and nuclear loading control proteins, respectively. *P<0.05, **P<0.01, LV-mdig vs. LV-con. mdig, mineral dust-induced gene; HIF-1α, hypoxia-inducible factor-1α; LV, lentivirus; con, control.

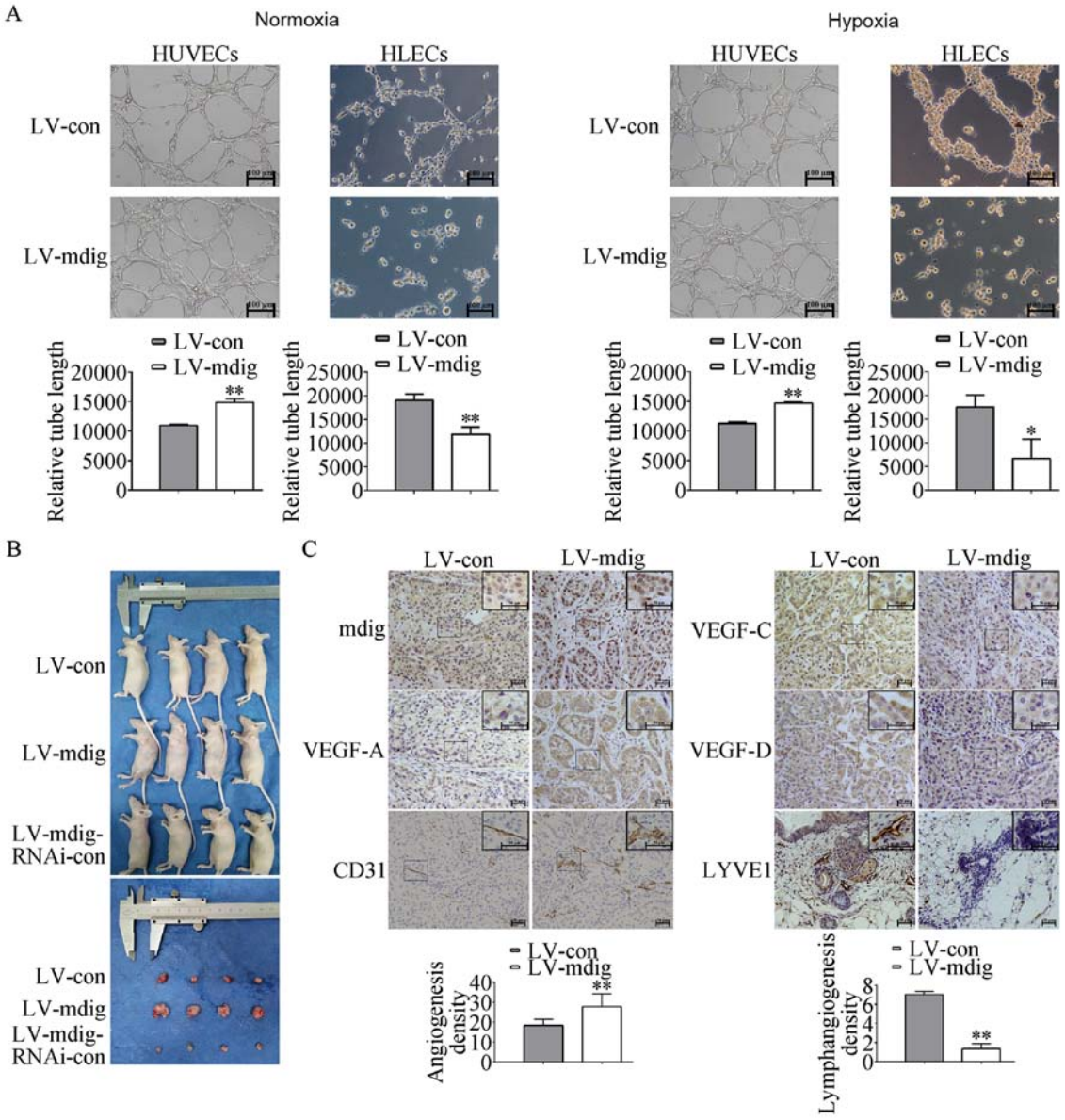

Figure 5. Regulation of tumor growth, angiogenesis and lymphangiogenesis by mdig *in vitro* and *in vivo*. (A) Density of angiogenesis and lymphangiogenesis regulated by mdig in tube formation assays. HUVECs and HLECs were suspended in conditioned media obtained from the mdig-overexpressing A549 cells cultured under normoxic and hypoxic conditions for the tube formation assays. The length of capillary-like tube structures was analyzed as the density of angiogenesis and lymphangiogenesis. Magnification, x100. *P<0.05, **P<0.01, LV-mdig vs. LV-con. (B) The volumes of the tumors formed in the LV-mdig group were significantly larger compared with those in the LV-con group 3 weeks after subcutaneous injection of transfected A549 cells into nude mice. (C) Immunohistochemistry analysis was performed using antibodies against mdig, CD31, LYVE1 and VEGF-A/C/D. The density of angiogenesis and the expression of mdig and VEGF-A in the LV-mdig group were significantly increased compared with those in the LV-con group. The density of lymphangiogenesis and the expression levels of VEGF-C/D were reduced in the LV-mdig group compared with those in the LV-con group. Magnification, x200 and x400. **P<0.01, LV-mdig vs. LV-con. mdig, mineral dust-induced gene; VEGF, vascular endothelial growth factor; LV, lentivirus; RNAi, RNA interference; con, control; HUVECs, human umbilical vein endothelial cells; HLECs, human lymphatic endothelial cells.

levels of VEGF-R3 were revealed to be significantly lower in the HLECs cultured with the conditioned media of cells from the LV-mdig group compared with those in the HLECs cultured with the conditioned media of cells from the LV-con group (P<0.05). By contrast, opposite observations were seen in HUVECs and HLECs cultured with conditioned media of cells from the LV-mdig-RNAi group compared with those in the LV-mdig group (Figs. 2 and 3).

mdig promotes tumor growth and angiogenesis, and inhibits lymphangiogenesis in vitro and in vivo. To verify the aforementioned findings, conditioned media, obtained from the mdig-overexpressing A549 cells cultured under normoxic and hypoxic conditions, were used to suspend HUVECs and HLECs for the tube formation assay of angiogenesis and lymphangiogenesis, respectively. The density of angiogenesis was significantly increased in HUVECs cultured using the conditioned media of cells from the LV-mdig group compared with that in HUVECs cultured using the conditioned media of cells from the LV-con group (P<0.05). However, the density of lymphangiogenesis was notably decreased in HLECs cultured using conditioned media of cells in the LV-mdig group compared with that in HLECs cultured using the conditioned media of cells in the LV-con group (P<0.05; Fig. 5A).

To verify further the above findings *in vitro*, mdig-overexpressing and mdig-silenced A549 cells were subcutaneously injected into nude mice. A total of 3 weeks after injection, the tumor volumes in the LV-mdig group were significantly larger compared with those in the LV-con group, and cells in the LV-mdig-RNAi group were not tumorigenic compared with those in the LV-mdig-RNAi-con group (Fig. 5B). To assess the effects of mdig on tumor angiogenesis and lymphangiogenesis, immunohistochemistry was performed on tissue sections using the antibodies of angiogenesis endothelial cell marker CD31, and lymphangiogenesis endothelial cell marker LYVE1. Compared with the LV-con group, the density of angiogenesis in tissues from the LV-mdig group was significantly increased, whereas the density of lymphangiogenesis was significantly decreased ($P<0.05$; Fig. 5C).

Subsequently, it was found that compared with cells in the LV-con group, the expression of mdig was significantly enhanced in the cells of the LV-mdig group, and expression was primarily observed in the nucleus, with limited expression in the cytosol. In addition, VEGF-A expression in the cytosol was significantly increased, but the expression levels of VEGF-C and VEGF-D in the cytosol were significantly reduced in cells in the LV-mdig group compared with those in the LV-con group (Fig. 5C).

mdig induces angiogenesis via the EGFR/p-EGFR/VEGF-A pathway. It was suggested that mdig lies upstream of EGFR, p-EGFR (Tyr1068), VEGF-A and VEGF-R1/R2, and that it promotes the expression of these proteins. Thus, the EGFR agonist NSC228155 [EGFR (+)] was therefore used in mdig-knockdown A549 cells, whereas the EGFR inhibitor erlotinib [EGFR (-)] was used to treat mdig-overexpressing A549 cells under normoxic and hypoxic conditions. The protein expression levels of EGFR, p-EGFR and VEGF-A were demonstrated to be significantly increased in the mdig-silenced A549 cells following treatment with NSC228155 under both normoxic and hypoxic conditions compared with those in the LV-mdig-RNAi group ($P<0.05$). The expression levels of these proteins were found to be significantly reduced in the mdig-overexpressing A549 cells following treatment with erlotinib compared with those in the LV-mdig group under normoxic and hypoxic conditions ($P<0.05$; Fig. 6A).

To verify these findings *in vitro*, mdig-overexpressing A549 cells treated with/without the EGFR inhibitor erlotinib were subcutaneously injected into nude mice. It was found that the tumor volumes in the LV-mdig group treated with erlotinib [EGFR (-)] were significantly smaller compared with those in the LV-mdig group 3 weeks after injection (Fig. 6B). To assess the effects of EGFR on tumor angiogenesis, immunohistochemistry was performed on tumor tissue sections using CD31 antibody. It was found that the density of angiogenesis in cancer tissues from the LV-mdig group treated with erlotinib was significantly decreased compared with that in the LV-mdig group ($P<0.05$; Fig. 6C).

mdig inhibits lymphangiogenesis by blocking the HIF-1α/VEGF-C/D pathway. The above findings suggested that under both normoxic and hypoxic conditions, mdig functions upstream of HIF-1α, VEGF-C and VEGF-D to inhibit the expression of these proteins. Previous studies have shown that HIF-1α upregulates the expression of VEGF (21,23,27), such that VEGF-C and VEGF-D serve important roles in tumor lymphangiogenesis (19,20). Therefore, it was subsequently hypothesized that mdig may inhibit the expression of HIF-1α, thereby reducing the levels of VEGF-C and VEGF-D to ultimately inhibit lymphangiogenesis in lung adenocarcinoma. To test this hypothesis, mdig-knockdown A549 cells were treated with the HIF-1α inhibitor BAY 87-2243 [HIF-1α (-)], while the mdig-overexpressing A549 cells were treated with the HIF-1α agonist IOX2 [HIF-1α (+)]. Western blotting results showed that the expression levels of HIF-1α, VEGF-C and VEGF-D were significantly reduced in cells in the LV-mdig-RNAi group following treatment with BAY 87-2243 compared with those in cells in the LV-mdig-RNAi group ($P<0.05$). Conversely, expression levels of these proteins were significantly increased in the mdig-overexpressing cells treated with IOX2 compared with those in the LV-mdig group ($P<0.05$; Fig. 7A).

To verify these findings *in vitro*, mdig-overexpressing A549 cells treated with/without the HIF-1α agonist IOX2 were subcutaneously injected into nude mice (Fig. 7B). Immunohistochemistry was performed on tissue sections using LYVE1 antibody. It was found that the density of lymphangiogenesis in tissues from the LV-mdig group treated with IOX2 [HIF-1α (+)] was significantly increased compared with that in the LV-mdig group ($P<0.05$; Fig. 7C).

Discussion

Upregulation of mineral dust-induced (mdig) mRNA and protein is a common feature of all types of lung cancer clinically, particularly in the early stages (6). Previous studies have found that mdig can promote tumor cell proliferation (6,12), but it inhibits tumor cell invasion and migration (9,13). The mechanism of this paradoxical phenomenon is unclear, which suggests that the role of mdig is different in different stages of carcinogenesis. Hence, the role of mdig in the regulation of lung adenocarcinoma requires further study.

Tumor angiogenesis is a prerequisite for tumor growth, which not only provides oxygen and nutrition for tumor cell growth in the early stages, but also activates a pathway together with lymphangiogenesis for tumor cell metastasis in the advanced stages. With the rapid proliferation of tumor cells, oxygen consumption increases, eventually leading to hypoxic conditions in the local microenvironment of the tumor, which further stimulates tumor angiogenesis (31-33). It has been confirmed that epidermal growth factor receptor (EGFR), hypoxia-inducible factor-1α (HIF-1α) and the vascular endothelial growth factor (VEGF) family serve very important roles in tumor angiogenesis and lymphangiogenesis (17,21,34). However, the role and molecular mechanism of mdig in tumor angiogenesis and lymphangiogenesis have not been reported previously.

The present study first investigated the effects of hypoxia on the protein expression levels of mdig, EGFR, HIF-1α and the VEGF family. A549, H1299 and 293T cells were cultured under normoxic (21% O_2) and hypoxic (1% O_2) conditions, and subsequently, protein expression levels were determined by western blotting. It was found that the protein expression levels of EGFR, HIF-1α and VEGF-A/C/D in each group of cells cultured under hypoxic conditions were significantly increased compared with those in cells cultured under

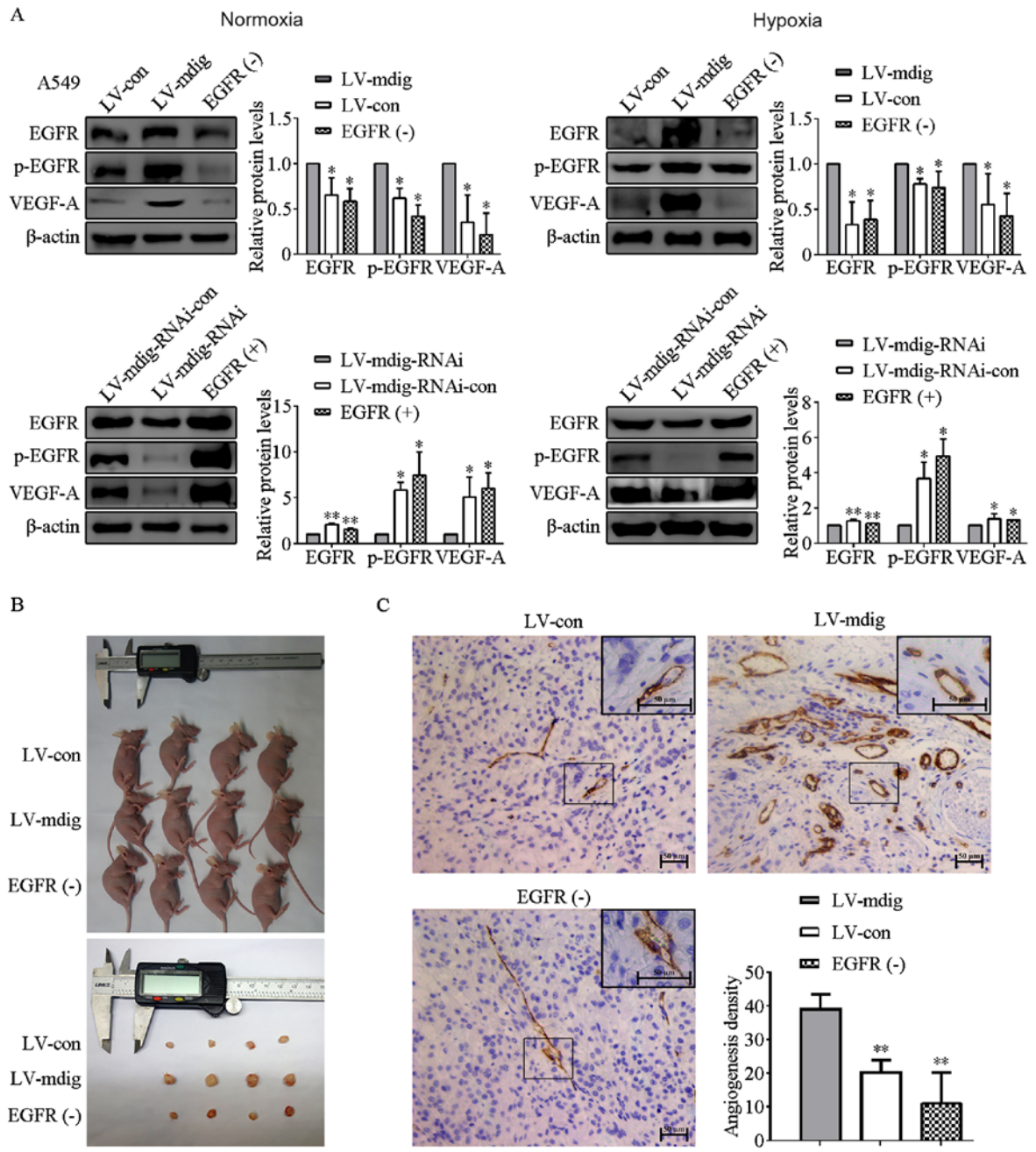

Figure 6. Regulation of VEGF-A expression and tumor angiogenesis by mdig via the EGFR pathway. (A) LV-mdig A549 cells were treated with the EGFR inhibitor [EGFR (-)] whereas LV-mdig-RNAi A549 cells were treated with the EGFR agonist [EGFR (+)]. The expression levels of EGFR, p-EGFR and VEGF-A were measured by western blotting. β-actin was used as the loading control. *P<0.05, **P<0.01, LV-mdig vs. LV-con; EGFR (-) vs. LV-mdig; LV-mdig-RNAi vs. LV-mdig-RNAi-con; and EGFR (+) vs. LV-mdig-RNAi. (B) Tumor volumes formed in the LV-mdig group treated with EGFR inhibitor erlotinib [EGFR (-)] were significantly smaller compared with those in the untreated LV-mdig group 3 weeks after injection into nude mice. (C) Immunohistochemistry was performed using antibodies against CD31. The density of angiogenesis in the LV-mdig group treated with erlotinib was significantly decreased compared with those in the untreated LV-mdig group. Magnification, x200 and x400. **P<0.01, LV-mdig vs. LV-con and EGFR (-) vs. LV-mdig. Mdig, mineral dust-induced gene; EGFR, epidermal growth factor receptor; p-, phospho; VEGF, vascular endothelial growth factor; LV, lentivirus; RNAi, RNA interference; con, control.

normoxic conditions, which were consistent with previous studies (34-36). These results further confirmed that hypoxia can upregulate the protein expression levels of EGFR, HIF-1α and VEGF-A/C/D. In addition, it was observed in the present study that the protein expression levels of mdig in cells cultured under hypoxic conditions were significantly lower compared with those in cells cultured under normoxic conditions, suggesting that mdig is an oxygen-sensitive protein, and its expression is negatively regulated by hypoxia (Fig. 8A). However, the regulatory mechanism remains unclear.

Subsequently, the relationship between mdig and EGFR in normoxia and hypoxia were explored in the present study. The results showed that under both normoxic and hypoxic conditions, mdig promoted the protein expression of EGFR and phosphorylated (p)-EGFR, indicating that mdig exerts its effects upstream of EGFR. However, mdig had no effect on mRNA levels of EGFR. Therefore, these results suggested that mdig regulates the expression of EGFR at the protein level, but not at a transcriptional level. Changes in p-EGFR and EGFR protein expression regulated by mdig were subsequently

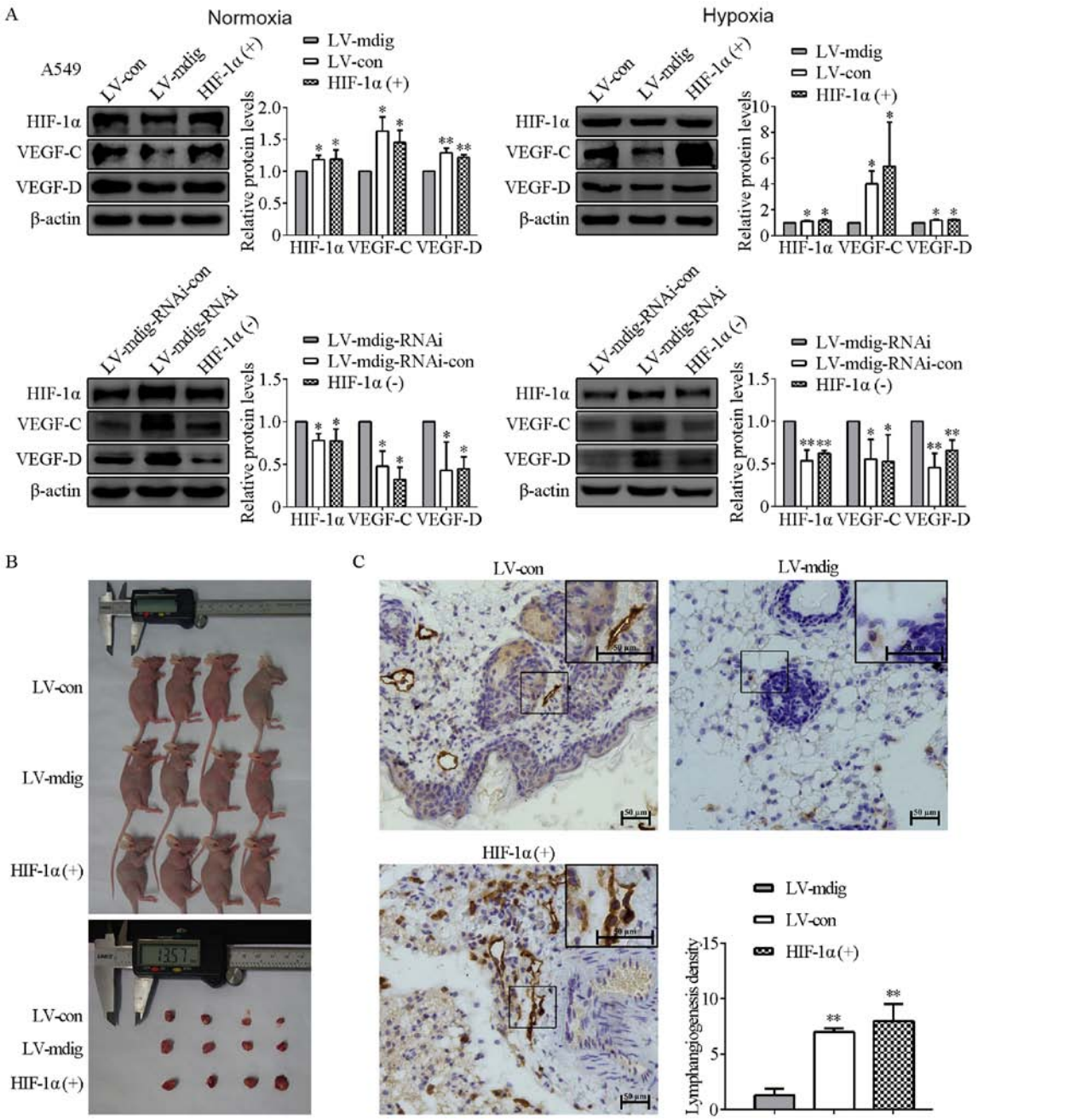

Figure 7. Regulation of VEGF-C/D expression and lymphangiogenesis by mdig via the HIF-1α pathway. (A) LV-mdig A549 cells were treated with the HIF-1α agonist [HIF-1α (+)] whereas LV-mdig-RNAi A549 cells were treated with the HIF-1α inhibitor [HIF-1α (-)]. The expression levels of HIF-1α and VEGF-C/D were measured by western blotting, with β-actin used as the loading control. *P<0.05, **P<0.01, LV-mdig vs. LV-con; HIF-1α (+) vs. LV-mdig; LV-mdig-RNAi vs. LV-mdig-RNAi-con; and HIF-1α (-) vs. LV-mdig-RNAi. (B) Tumor volumes formed in the LV-mdig group treated with HIF-1α agonist [HIF-1α (+)] 3 weeks after subcutaneous injection into nude mice. (C) Immunohistochemistry was performed using antibodies against LYVE1. The density of lymphangiogenesis in the LV-mdig group treated with HIF-1α agonist [HIF-1α (+)] was significantly increased compared with that in the untreated LV-mdig group. Magnification, x200 and x400. **P<0.01, LV-mdig vs. LV-con and HIF-1α (+) vs. LV-mdig. mdig, mineral dust-induced gene; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; LV, lentivirus; RNAi, RNA interference; con, control.

compared. It was found that the LV-mdig/LV-con ratio of p-EGFR protein was significantly higher compared with that of the EGFR protein. These observations suggested that mdig upregulated the protein expression of not only EGFR, but also the phosphorylation of its Tyr1068 residue, indicating that mdig regulates the expression of EGFR by post-translational modification at Tyr1068. To further explore the relationship between mdig and EGFR, co-immunoprecipitation analysis was performed. The results showed that no direct interactions were observed between mdig and EGFR. Combined with the results from RT-qPCR, these results indicated that mdig may increase the protein expression of EGFR through post-translational modification or signaling pathways.

HIF-1α serves a pivotal role in tumor angiogenesis and lymphangiogenesis (14,17,27,34). Studies have previously shown that HIF-1α functions downstream of EGFR. However, EGFR can promote tumor angiogenesis and lymphangiogenesis in both a HIF-1α-dependent and -independent manner (17,18,22). Therefore, the relationship between mdig and HIF-1α under normoxic and hypoxic conditions was also

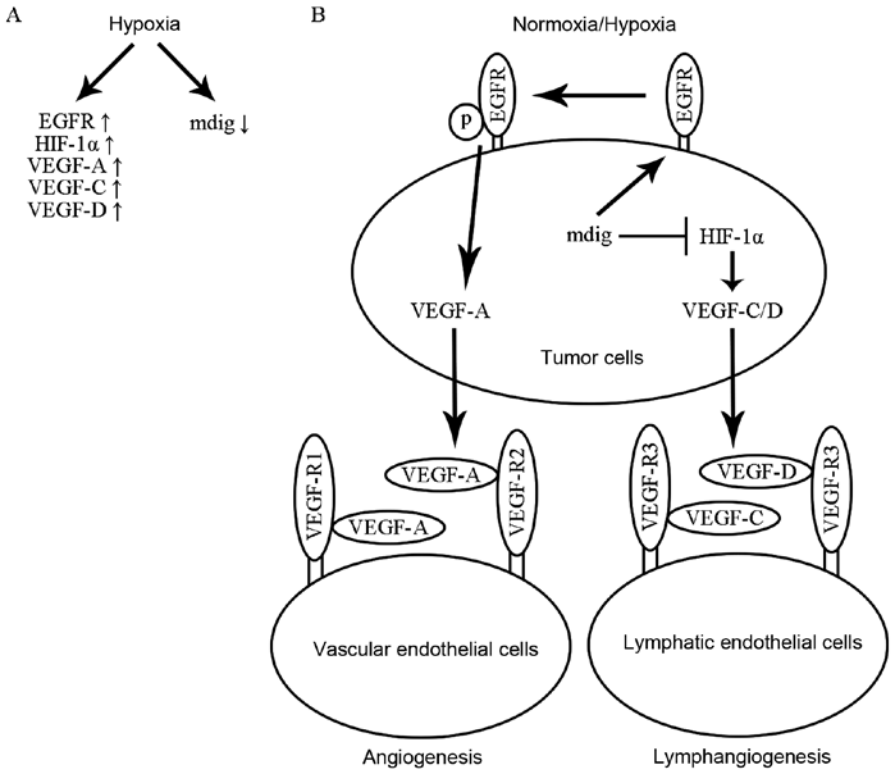

Figure 8. Regulatory mechanism of mdig on lung adenocarcinoma angiogenesis and lymphangiogenesis under normoxic and hypoxic conditions. (A) Hypoxia increases the expression levels of EGFR, HIF-1α and VEGF-A/C/D protein but reduces the expression levels of mdig protein. (B) mdig promotes tumor angiogenesis through the EGFR/p-EGFR/VEGF-A/VEGF-R1/R2 pathway but suppresses lymphangiogenesis via the HIF-1α/VEGF-C/D pathway under normoxic and hypoxic conditions. mdig, mineral dust-induced gene; EGFR, epidermal growth factor receptor; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; p-, phosphorylated.

explored in the present study. The results showed that mdig significantly inhibited HIF-1α protein expression under both normoxic and hypoxic conditions, but had no effect on mRNA levels of HIF-1α. In the subsequent co-immunoprecipitation experiment, no direct interactions were found between mdig and HIF-1α. These results suggested that mdig may inhibit HIF-1α protein expression by modulation of signaling pathways or by post-translational modification. In the nuclear and cytoplasmic fractionation experiment, the expression levels of HIF-1α protein were shown to be significantly reduced in the nucleus but were significantly increased in the cytosol following overexpression of mdig, suggesting that mdig prevents HIF-1α entering the nucleus from the cytosol, blocking HIF-1α function in the nucleus.

The VEGF family serves an important role in tumor angiogenesis and lymphangiogenesis (19,20,35). Specifically, VEGF-A has been reported to serve a major role in tumor angiogenesis (14,17,36), whereas VEGF-C/D is primarily associated with lymphangiogenesis (19,20). The relationship between mdig and VEGF family was also explored in the present study. It was found that mdig promoted the expression of VEGF-A and VEGF-R1/R2 protein but inhibited the expression of VEGF-C/D and VEGF-R3 protein both under normoxic and hypoxic conditions. These results suggested that mdig serves functionally distinct regulatory roles on tumor angiogenesis and lymphangiogenesis by regulating different members of the VEGF family and their receptors; namely, mdig promotes tumor angiogenesis by inducing protein expression of VEGF-A and VEGF-R1/R2, and suppresses lymphangiogenesis by reducing the expression of VEGF-C/D and VEGF-R3 protein. To confirm this hypothesis, tube formation assays of angiogenesis and lymphangiogenesis were also performed. The results showed that mdig significantly increased the density of angiogenesis but significantly decreased the density of lymphangiogenesis, suggesting that mdig promotes tumor angiogenesis but suppresses lymphangiogenesis.

Previous studies have shown that the EGF/RHIF-1α/VEGF(17),HIF-1α/Notch1/VEGF-A(22),WISP-1/FAK/c-Src/VEGF-A (37), EGFR/p38/MMP-1 (14) and c-myc/HIF-1α/VEGF-A (38) signaling pathways can affect tumor angiogenesis, and HIF-1α/PDGF-B/PDGFRβ (34,39), CCL21/CCR7/ERK/VEGF-D (40), EGFR/HIF-1α/VEGF (41) and CXCL12/CXCR4 (42) signaling pathways can regulate tumor lymphangiogenesis. Because the EGFR/HIF-1α/VEGF signaling pathway is one of the classical pathways associated with tumor angiogenesis and lymphangiogenesis (14,17,41), it was explored in the present study. An EGFR agonist/inhibitor and a HIF-1α agonist/inhibitor were used in the present study to assess whether mdig also regulated tumor angiogenesis and lymphangiogenesis via the EGFR/HIF-1α/VEGF signaling pathway. The results showed that the regulatory role of mdig on EGFR, p-EGFR and VEGF-A protein expression was significantly reversed by the EGFR agonist/inhibitor, suggesting that mdig increased the secretion of VEGF-A by promoting EGFR expression, indicating that mdig promotes angiogenesis in lung adenocarcinoma via the EGFR/p-EGFR/VEGF-A/VEGF-R1/R2 pathway. The present

study also showed that the regulatory role of mdig on HIF-1α and VEGF-C/D protein expression was reversed by HIF-1α agonist/inhibitor, suggesting that mdig reduces the secretion of VEGF-C/D by inhibiting HIF-1α expression, indicating that mdig can inhibit lymphangiogenesis by blocking the HIF-1α/VEGF-C/D/VEGF-R3 pathway.

Previous studies have shown that EGFR can upregulate VEGF-A expression not only in a HIF-1α-dependent manner (17,43), but also in a HIF-1α-independent manner, including via the PI3K/Akt (18,44), Akt/NF-κB (45,46) and K-Ras (47) signaling pathways. The results of the present study showed that mdig upregulated the protein expression levels of EGFR, VEGF-A and VEGF-R1/R2, but downregulated the protein expression levels of HIF-1α, indicating that mdig may promote angiogenesis through increasing the expression of VEGF-A and VEGF-R1/R2 by EGFR in a HIF-1α-independent manner in lung adenocarcinoma.

To confirm these aforementioned findings *in vitro*, mdig-transfected A549 cells were subsequently injected into nude mice. The results showed that mdig promoted tumor growth and tumor angiogenesis, but suppressed tumor lymphangiogenesis. In addition, the changes in expression of VEGF-A and VEGF-C/D *in vivo* were consistent with those observed *in vitro*, further supporting the conclusion that mdig promotes tumor angiogenesis by increasing the expression of VEGF-A, and inhibits lymphangiogenesis by suppressing the expression of VEGF-CD.

To further verify the results that mdig promotes tumor growth and angiogenesis via the EGFR signaling pathway and suppresses tumor lymphangiogenesis via the HIF-1α signaling pathway *in vitro*, an EGFR inhibitor (erlotinib) and a HIF-1α agonist (IOX2) were also used in the xenograft tumor experiments *in vivo*. The results showed that both tumor volumes and angiogenesis density were significantly decreased by the EGFR inhibitor erlotinib, and that the lymphangiogenesis density was significantly increased by the HIF-1α agonist IOX2. These results further confirmed that mdig promotes tumor growth and angiogenesis via the EGFR signaling pathway, and inhibits lymphangiogenesis by blocking the HIF-1α signaling pathway (Fig. 8B).

In conclusion, the present study confirmed that mdig is an oxygen-sensitive protein and hypoxia can inhibit the expression of mdig, that mdig induces tumor growth and angiogenesis by activating the EGFR/p-EGFR/VEGF-A/VEGF-R1/R2 pathway, and that mdig inhibits tumor lymphangiogenesis by blocking the HIF-1α/VEGF-C/D/VEGF-R3 pathway, as well as suppresses the translocation of HIF-1α from the cytosol to the nucleus. The present study lays the foundation for future research of lung adenocarcinoma angiogenesis and lymphangiogenesis, and highlights potentially novel targets for the development of novel therapies. In addition, previous studies have demonstrated that varieties of tyrosine kinase receptors can regulate tumor angiogenesis and lymphangiogenesis, such as MET, IGFR and the FGFR family (48-51). The effects of mdig on angiogenesis and lymphangiogenesis through other tyrosine kinase receptors will be explored in our subsequent studies.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HZ and HZ designed the study and wrote the manuscript. HZ, FG, YC, JD, XZ and BL performed the experiments and collected the data. HZ, HH and DS performed the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee of the China Medical University (approval no. 2019135).

Patient consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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