Vascular endothelial growth factor A (VEGFA) is a critical proangiogenic factor that is activated by hypoxia at both the transcriptional and post-transcriptional levels. In hypoxia conditions, stabilized hypoxia-inducible factor 1α (HIF1A) is the key regulator for transcriptional activation of VEGFA. However, the post-transcriptional control of VEGFA expression remains poorly understood. Here, we report that the eukaryotic translation initiation factor 3i (eIF3i) is required for VEGFA protein expression in both normal embryonic and tumorigenic angiogenesis. eIF3i is dynamically expressed in the early stages of zebrafish embryogenesis and in human hepatocellular carcinoma tissues. eIF3i homozygous mutant zebrafish embryos show severe angiogenesis defects and human hepatocellular cancer cells with depletion of eIF3i to induce less angiogenesis in tumor models. Under hypoxia, the HIF1A protein can interact with its binding sequence in the eIF3i promoter and activate eIF3i transcription. The expression of VEGFA, which should rise in hypoxia, is significantly inhibited by eIF3i siRNA treatment. Moreover, eIF3i knockdown did not cause a general translation repression but specifically reduced the translation efficiency of the VEGFA mRNAs. Taken together, our results suggest that eIF3i is induced by HIF1A under hypoxia and controls normal and tumorigenic angiogenesis through regulating VEGFA protein translation.

Protein synthesis plays an important role in the control of cell proliferation, and its deregulation is associated with cancer susceptibility (1). The translation initiation of specific mRNAs is one of the key steps for the regulation of protein synthesis (2). eIF3 is the largest and most complicated translation initiation factor with a molecular mass of about 550–700 kDa, including 13 nonidentical protein subunits in mammalian cells known as eIF3a-eIF3m (3–5). eIF3 binds to the 40 S ribosome and interacts with other eIFs and mRNA, stimulating the generation of the 43 S pre-initiation complex. The eIF3 complex is critical for both 5’ cap-dependent and internal ribosome entry site (IRES)3-dependent translation initiation. Accumulating evidence suggests that altered expression of eIFs is associated with a wide range of human tumors and cancer cell lines (4–9). eIF3i was initially identified as a WD40 repeat-containing protein that is bound and phosphorylated by the TGF-β type receptor II and modulates the TGF-β signaling pathway, so it was first named TGF-β receptor II interacting protein 1 (Trip1) (10–12). Later, this gene was also named eIF3i, eIF3S2, or eIF3b, because it is a part of the eIF3 complex. Elevated expression of eIF3i was found in various human cancers, including breast tumor, head and neck squamous cell carcinomas, and HCC tissues (13–15). Overexpression of eIF3i in NIH3T3 cells induced malignant transformation, and the knockdown of eIF3i reversed cadmium-induced carcinogenesis (13, 16). In addition to being a translation initiation factor, eIF3i can also interact with Akt1 and prevents PP2A-mediated Akt1 dephosphorylation, resulting in constitutively active Akt1 oncogenic signaling (17). Thus, these observations suggested that eIF3i is a proto-oncogene. However, how the expression of eIF3i is regulated during carcinogenesis remains unknown.
VEGFA is the major pro-angiogenesis factor in tumor angiogenesis and is enhanced in tumor cells by hypoxia (18). Rapid tumor growth leads to localized hypoxia, which induces VEGFA expression at both transcriptional and post-transcriptional levels (19). The accumulation of HIF1A is one of the major factors that trigger VEGFA transcription through binding to the hypoxia-responsive element (HRE) in the 5’-upstream sequence of VEGFA gene (20). At the post-transcriptional level, hypoxia increases VEGFA mRNA stability (21) and increases the IRES-directed translation of VEGFA but impairs the 5’ cap-dependent translation (22). Recently, the DEAD-box RNA helicase 6 (DDX6) has been identified as an IRES trans-acting factor that can particularly interact with the 5’-untranslated region (5’UTR) of VEGFA mRNA and inhibit VEGFA IRES-mediated translation under normoxic conditions. While under hypoxia, the level of DDX6 declines, and its interaction with VEGFA mRNA is diminished, which enhances VEGFA translation and promotes angiogenesis (23).

Here, we report that elf3i is required for VEGFA protein expression in both normal embryonic and tumorigenic angiogenesis. In zebrafish, elf3i is dynamically expressed at the early stage of embryonic development. elf3i-/- mutant zebrafish embryos show severe angiogenesis defects, and human hepatocellular cancer cells with depletion of elf3i induce less angiogenesis in tumor models. Besides, overexpression of elf3i promotes colony formation, and knockdown of elf3i inhibits cancer cell proliferation. In hepatocellular cells, hypoxic conditions can enhance elf3i expression, and HIF1A directly regulates elf3i transcription by binding to the HRE in the elf3i promoter. Taken together, these findings demonstrate that elf3i is critical for VEGFA protein expression in embryonic and tumorigenic development, offering a regulatory mechanism for elf3i expression in cancer cells.

**EXPERIMENTAL PROCEDURES**

**Identification of elf3i Mutant Zebrafish—elf3i mutation was identified in a large scale retrovirus-mediated insertion as described before (24–26). Genotype of elf3i was determined by the presence of retrovirus insertion with follow- ing primers: elf3i WT F, 5’-ATGACTTACTGGTCTGCTTG-3’; elf3i WT R, 5’-GACAGTGTAGTGGGAGCTTCTT-3’; Virus R, 5’-GACCTTGTGTGCTCCTGTTCTTG-3’.**

**Cell Culture—Human embryonic kidney cell line 293T, human liver cell line Lo2, and human hepatocellular carcinoma cell line HepG2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. All of the cells were maintained in a 37 °C incubator with a humidified 5% CO2 atmosphere, and the medium was changed every other day. CoCl2 (Sigma) was added at a final concentration of 200 μM for 5 h to mimic hypoxia. In parallel, 5% CO2 and 95% N2 in the cell culture incubator were also designed to mimic hypoxia. In parallel, 5% CO2 and 95% N2 in the cell culture incubator were also designed to mimic hypoxia.**

**siRNA Interference—elf3i siRNA (5’-GATGCTTACAGCCA- GATTA-3’ and 5’-GACAGAACGTCCTGCAAC-3’) and c-myc siRNA (5’-CATCATCATCCAGACTGTAT-3’ and 5’-CGAGCTAAAACGGAGCTT-3’) were purchased from Ribobio, and the corresponding control siRNAs with scrambled sequences were also prepared. The siRNA transfection was performed into the HepG2 cell line using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.**

**Establishment of Stable Cell Line—The elf3i coding region was amplified by PCR with primers 5’-AAGAATTCGCCACCATGAAAGCAGATC-3’ and 5’-GCTCTAGATCAAGCCT-3’ and was then cloned into pLVX-IRES-ZsGreen1 to get recombinant plasmid, which was transformed into Escherichia coli TOP10. After positive screening, the recombinant plasmids were transfected into 293T cells with the packaging and envelop plasmids to make lentivirus. To obtain the elf3i overexpression cells, the lentivirus infection was conducted into Lo2 cell line, and the fluorescent cells were sorted by flow cytometry. The elf3i knockdown cell was constructed by the infection of lentivirus-mediated elf3i RNAi. Cells were selected by puromycin (Sigma) at 2 μg/ml for 2 weeks and maintained in growth medium supplemented with puromycin (1 μg/ml).**

**Cell Viability Assay—After transfection of elf3i siRNAs for different time intervals (24, 48, 72, and 96 h), the cell viability of HepG2 was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) colorimetric assay. The working concentration of MTT was 1 mg/ml, and the spectrometric absorbance at 570 nm was measured on Multiscan MK3 ELISA reader (Thermo Scientific, Waltham, MA). The cell survival rate was assessed as percent cell viability in terms of non- treated control cells.**

**Colony Formation Assay—elf3i overexpression cells were digested with trypsin to obtain a single cell suspension, plated onto a 10-cm dish, and incubated for 2–3 weeks. Then the colonies were fixed with 4% paraformaldehyde for 15 min and stained using 1% methylene blue. The residual methylene blue was slowly removed with flowing water. The colony formation ability was measured into control Lo2 cells.**

**Real Time PCR—For real time PCR, the total RNA of HepG2 cells, which were treated with CoCl2, or HepG2 cells (control or elf3i shRNA), was extracted by using TRIzol reagent (Invitrogen) and quantified by spectrophotometry (Nanodrop 2000; Thermo Scientific). The cDNA was synthesized using RevertAid first strand cDNA synthesis kit (Thermo Scientific). The SsoAdvanced SYBR Green Supermix (Bio-Rad) was used for real time PCR on a CFX96 PCR System (Bio-Rad). Relative change in expression level was determined using the 2-ΔΔCt method (27). The primers were designed as follows: elf3i RT F, 5’-CTGTGGCAAACGGACCTAC-3’ and elf3i RT R, 5’-CCAGTGAGACCACCTGGTT-3’; VEGFA RT F, CTACCTCCACCATGCACCAGT, and VEGFA RT-R, GCAG- TAGCTGCCGCTGATAGA. Human GAPDH was used as internal control (F, 5’-AGAAGGCTGGGGCTCATT-3’ and R AGGGGCACTCCACACGTTC).**

**Western Blot—Protein extraction and Western blot analysis were performed as described previously (28). Briefly, cells were
elf3i Controls VEGFA Signaling during Angiogenesis

lysed by RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China), and the total proteins resolved by SDS-PAGE were electroblotted onto polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Billerica, MA). After blocking with 5% nonfat milk and 0.1% Tween 20, the PVDF membrane was incubated with primary antibodies overnight at 4 °C. Then, the membrane was washed with TBST and incubated for 1 h at 37 °C with secondary horseradish peroxidase (HRP)-conjugated antibody. Signals were visualized by exposure to x-ray films after treatment with ECL detection kit (Invitrogen). The following antibodies were used for Western blot: elf3i (Abcam, Cambridge, MA), HIF1A (Abcam), VEGF (Abcam), c-Myc (Epitomics, Burlingame, CA), α-tubulin (Tianjin Sunge Biotech Co., Tianjin, China), and β-actin (Tianjin Sunge Biotech Co.).

Histological Analysis—The human hepatocellular carcinoma tissue microarrays (TMA) were stained with immunohistochemistry for testing elf3i expression. The TMA slides were dewaxed and rehydrated. Then high pressure cooking was conducted for antigen retrieval. After blocking with 3% hydrogen peroxide and 1% goat serum, the slides were incubated with rabbit polyclonal antibodies against elf3i at 4 °C overnight, and immunoreactivity was visualized using peroxidase/3,3′-diaminobenzidine. The hematoxylin was used as a counterstain. For Ki67 immunofluorescence assay, the HepG2 cells were plated in 24 wells at a density of 20,000 cells/well in a total volume of 0.5 ml of complete medium. After interfering with elf3i siRNAs for 48 h, cell medium was removed, and the cells were fixed, blocked, incubated with Ki67 (Abcam) at 4 °C overnight, and then stained with goat anti-rabbit IgG/FITC. DAPI was used to stain cell nuclei (blue). The TMAs and HepG2 cells were viewed under a fluorescence microscope (Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany).

Cell Counting—For cell counting, the embryos of wild type or elf3i−/− zebrafish were anesthetized with Tricaine and washed using sterilized fish water. Then the embryos were exposed to proteinase K and incubated at 37 °C until complete embryonic digestion. After dilution with L-15 medium, the embryo cells were counted using a hemocytometer.

Body Size Calculation—The heterozygous elf3i zebrafish were in-crossed, and the embryos were collected. Then the chorion envelopes of embryos were removed manually at 2 dpf, and the sizes of embryos were measured with Leica fluorescence microscopy.

Acridine Orange Staining—Acridine orange staining was performed on zebrafish embryos at 3 dpf to quantify apoptosis in zebrafish embryos as described previously (29). Briefly, living embryos were incubated in a solution of 5 μg/ml acridine orange (Sigma) in fish water for 30 min and washed twice with fish water. For imaging, fish were embedded in 2% methylcellulose supplemented with Tricaine (Sigma). The green nuclei were counted in the whole spinal cord of the embryos under a Leica fluorescence microscopy.

In Situ Hybridization—Antisense probes for elf3i, cp, foxa3, and fli were synthesized with a digoxigenin labeling kit in the presence of digoxigenin (Roche Applied Science). Whole mount in situ hybridizations were performed as described previously (30). Hybridized transcripts were recognized in situ by antidigoxigenin antibodies linked to alkaline phosphatase and visualized with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solutions. Then the embryos were photographed using a microscope (Leica Mikrosysteme Vertrieb GmbH) with an attached camera.

Luciferase Reporter Assay—Dual-luciferase reporter assays were performed as described previously (31). Briefly, a proximal human elf3i promoter, referred to as wild type elf3i promoter that flanks the −593 to −24 of elf3i gene, was cloned into pGL3 luciferase vector for reporter assays. The HIF1A-binding site in this promoter was mutated by overlap extension PCR using the primers described below. The reporter constructs were co-transfected with the pRL-CMV plasmid that contains the CMV-driven Renilla luciferase gene as internal control. 24 h after transfection, 200 μM CoCl2 was added to mimic hypoxia for 5 h. Then cells were harvested for dual-luciferase assay. Primers used to construct the elf3i promoter were as follows: pelf3i F-593, 5′-GGTACCCTTTTTCACAGAGACGCACCT-3′; pelf3i R1–24-bam, 5′-GGATCCGCAACGTGAGTAAAGACCGGA-3′; pelf3i-MT F, 5′-AGGCGGTGATCGGCACCCCAAGAGCGG-3′; and pelf3i-MT R, TGGGGTGTGGCA TCAGCCGCTGCTAAGCGG.

Chromatin Immunoprecipitation (CHIP)—CHIP assays were performed as described previously with modifications (32). Briefly, the treated hepatocytes were cross-linked with 1% formaldehyde for 10 min and were stopped by the addition of glycine to a final concentration of 0.125 M. The cross-linked hepatocytes were rinsed in cold PBS and scraped. Then cells were homogenized, and nuclei were pelleted. The extract was sonicated to generate 300–500-bp fragments. Fragmented chromatin was immunoprecipitated overnight at 4 °C with 3 μg of HIF1A antibody or the control isotype IgG2b (Lab Vision Corp., Fremont, CA) and then pulled down by Mabselect beads (GE Healthcare). Precipitates were washed and extracted with 1% SDS and 0.1 M NaHCO3. Eluates were pooled and heated. DNA fragments were purified and used as template for PCR. The primers used for the amplification of elf3i promoter containing HRE were 5′-CTTTTCACAGAGAGCCACTT-3′ and 5′-TCAGGGTGACTCACTCTTCT-3′; the primers flanking the junction of the 2nd intron and 3rd exon were 5′-GGCTAGAAGCGATACCTCC-3′ and 5′-CCAGTGAGGACAGTGCTTGGT-3′.

Cell Microinjection—The wild or elf3i−/− green fluorescence-labeled HepG2 cells with stable expression of either control shRNA or elf3i shRNA were harvested at a concentration of 107 cells/ml. The cell mixture was then loaded into a boro-silicate glass needle pulled by a Flaming/Brown micropipette puller (Nari-shige, Japan, PN-30). About 50–100 cells were implanted into the perivitelline space of Flk:mcherry transgenic zebrafish embryos by using an electronically regulated micro-injector (Harvard Apparatus, NY, PL1-90). After injection, the zebrafish embryos were maintained under normal fish husbandry conditions, examined for the presence of fluorescent cells, and subsequently documented photographically.

Polysome Analysis—Polysome analysis was performed as described previously (33). Briefly, control and elf3i shRNA HepG2 cells were treated with cycloheximide at a final concen-
tration of 100 μg/ml in growth media for 15 min before being harvested. After that, the cells were washed twice with cold PBS, scraped, and collected in a 50-ml tube. Then the cells were lysed with lysis buffer containing 10 mM HEPES (pH 7.8), 150 mM NaCl, 10 mM KCl, 1 mM EDTA (pH 8.0), 2 mM MgCl2, 10% glycerol, 0.5% Triton X-100, 1 mM DTT, 1 IU/µl RNase inhibitor. The lysates were spun, and the supernatants were transferred to premade 10–50% sucrose gradients and centrifuged in a Beckman SW40Ti rotor at 35,000 rpm for 4 h at 4 °C with an Optima L80XP ultracentrifuge (Beckman Coulter). The gradients were then manually fractioned into 80 aliquots at a volume of 150 µL. The absorbance at 260 nm of each aliquot was measured by a spectrophotometer to obtain the ribosome profile. Each six aliquots were combined into one fraction to extract RNAs for electrophoresis and reverse transcription-PCR (RT-PCR) analysis. Primers used for RT-PCR analysis were as follows: VEGFA RT-F, 5′-CTACCTCCACCACATGCAAAGT-3′, and VEGFA RT-R, 5′-GCA-GTAGCTGCGCTATAGA-3′; GAPDH-F, 5′-AGAAGGCTGGGCTCATTTG-3′, and GAPDH-R, AGGGGCACTCCACAGTCTTC; β-actin-F, 5′-AGCGAGACATCCCCCAAAGTT-3′, and β-actin-R, 5′-GGGCACGAAGGCTCATCATT-3′. The PCR products were analyzed by DNA electrophoresis and then quantified with ImageJ software.

Statistical Analysis—Data were assayed by analysis of variance and unpaired Student’s t test using SPSS statistics 18.0 analysis software. Differences between means or ranks as appropriate were considered significant when p value was < 0.05.

RESULTS
eIF3i Is Dynamically Expressed in Early Embryonic Development—Molecular mechanisms of embryonic development often overlap with those in cancer, and various signaling pathways are shared between the two processes (34, 35). Although overexpression of eIF3i has been reported in several cancer types (15, 17), no data are available so far on its expression in embryonic development of any model animals. We used zebrafish to analyze the expression pattern of eIF3i mRNA was maternally expressed as it can be detected at the one-cell stage (Fig. 1A). The zygotic transcripts were expressed dynamically (Fig. 1, B–D). After 24 h post-fertilization, eIF3i mRNAs were detected in the anterior neural tissue and the somites at high levels. G, expression of eIF3i in the somites was dramatically reduced and anterior neural tissue expression domain was limited to the retina and ependymal layer. H–K, eIF3i began to be expressed in endodermal organs such as liver, pancreas, and intestine at 3 dpf, and these expression domains were maintained at 4 dpf. L and M, endodermal expression of eIF3i withered at 5 dpf. Arrows point to the liver region.
eIF3i Controls VEGFA Signaling during Angiogenesis

A

B

C

D

E

F

G

WT eIF3i-/-

β-actin

Cell number per embryo (10^4)

Probable insertion

PCR1: 344bp

PCR2: 379bp

PCR3: over 2000bp

Wildtype

Mutant

Phenotype

Genotype

Number of apoptotic cells

eIF3i+/± eIF3i-/-

cp

foxo3
Elf3i Controls VEGFA Signaling during Angiogenesis

Elf3i Mutations Affect Zebrafish Embryonic Development—

To assess elf3i function in vivo, we used an elf3i mutant line identified in a large scale zebrafish retrovirus-mediated insertional mutagenesis. This line harbors a retroviral insertion in the 2nd exon of the elf3i gene causing a premature stop codon in the elf3i open reading frame (Fig. 2A). We crossed heterozygous elf3i fishes and observed no obvious morphogenetic defects in the embryos before 24 hpf. At 2 dpf, 25% embryos (n > 500) showed reduced body size and less pigment as compared with the wild type embryos (p < 0.01) (Fig. 2B). Genotyping of the incrossed embryos revealed that all the embryos with morphological defects were homozygous for elf3i insertion (Fig. 2E). In parallel, we determined elf3i protein levels in homozygous elf3i zebrafish at 2 dpf and did not observe any detectable elf3i proteins, suggesting that this line was likely an elf3i null mutant (Fig. 2C). Because elf3i mutation caused a small body size in zebrafish, we next sought to examine whether cell proliferation and apoptosis are abnormal in these mutant embryos. We found that elf3i mutant embryos had decreased cell numbers as compared with wild type siblings (Fig. 2D). We also conducted the acridine orange staining and found that a higher level of apoptosis was induced in elf3i−−/− mutant embryos (p < 0.01) (Fig. 2F). The expression pattern of elf3i during embryonic development also suggested a role of elf3i in liver organogenesis. To address this issue, we examined the expression of two liver-specific marker cp and hepatocyte nuclear factor 3 (foxa3) by WISH. We found that the mRNA expression of both genes in the liver was present but greatly reduced, indicating that elf3i mutation mainly affected the proliferation of hepatocytes and the outgrowth of the liver bud, but it did not affect the initial hepatocyte specification (Fig. 2G).

Elf3i Mutations Affect Zebrafish Angiogenesis—In comparison with normal zebrafish embryos (supplemental movie 1), elf3i−−/− mutant embryos nearly lacked blood circulation in their intersegmental regions at 2 dpf (supplemental movie 2). To assess whether mutation of elf3i affects angiogenesis, expression of fl1, a vascular endothelial marker gene, was analyzed by WISH at 28 hpf. At this stage, no general morphological defects were observed in embryos derived from elf3i heterozygous parents. Fli expression in the dorsal aorta and pectoral vein was not changed in all embryos examined, but its expression in the inter-segmental vessels showed defects in about 25% embryos (n = 126), consistent with the Mendelian ratio (Fig. 3A). These defects ranged from morphological defects of the tip cells to lacking tip cells or even lacking both stalk and tip cells, suggesting that specification and function of tip cells were disrupted. After WISH and photography, individual embryos (n = 32) were genotyped by PCR. The results showed that all the embryos with defects in fl1 expression were elf3i homozygous mutants, and the rest were either elf3i heterozygous or wild type, confirming that the phenotype was specific to elf3i homozygous mutants. To further confirm the data, we crossed the elf3i+/− zebrafish with the Flk:GFP transgenic line expressing GFP in the vascular endothelial cells to obtain elf3i+/−:flk:GFP+/− double transgenic fishes. Then we inter-crossed the double transgenic fishes and collected the embryos for time-lapse observation using a fluorescence microscopy. At 24 hpf, we found that 25% GFP-positive embryos exhibited similar angiogenesis defects (n > 100, Fig. 3B). Again, the embryos with angiogenesis phenotypes were genotyped by PCR, and the results showed that embryos with angiogenesis defects were all elf3i homozygous mutant. We separated live 24 hpf mutant embryos from their siblings for cell number and apoptosis analysis. However, we did not find obvious differences in cell number and apoptosis levels between homozygous mutant embryos and their wild type or heterozygous siblings (data not shown). Together, these data suggested that elf3i has specific roles in the sprouting and specification of endothelial tip cells during zebrafish embryonic angiogenesis.

Because VEGFA is the major factor that induces angiogenic sprouting and tip cell specification (36), we next analyzed whether VEGFA expression was affected in elf3i mutant embryos. As shown in Fig. 3C, compared with wild type, elf3i−−/− mutant embryos expressed much less VEGFA proteins. We then tested whether ectopic forced expression of VEGFA could rescue angiogenesis caused by elf3i mutation. We injected 5 pg of human VEGFA plasmids or empty pcDNA3.1 plasmids into one-cell stage embryos that were produced by in-crossing elf3i−−/−:flk:GFP+/− double transgenic fishes. The presence of proper sprouting tip cells was scored to evaluate rescue. In the pcDNA3.1-injected control group at 24 hpf, angiogenesis defects were observed in 25% embryos (n = 64), whereas only 6.4% embryos (n = 78) showed obvious angiogenesis defects in the VEGFA-injected group (p < 0.01) (Fig. 3D), suggesting a partial rescue by VEGFA ectopic overexpression. These data demonstrated that elf3i controls embryonic angiogenesis by regulating VEGFA expression.

Elf3i is Highly Expressed in Human Hepatocellular Carcinoma—According to the expression pattern of elf3i and the phenotype caused by elf3i mutation, we reasoned that elf3i might be associated with liver carcinogenesis. Consistent with this hypothesis, elf3i overexpression has been observed in several cancer types, including HCC (15, 17). To confirm the data, we performed immunohistochemistry analysis of elf3i expression.

FIGURE 2. elf3i mutation affects zebrafish embryo development. A, position of retroviral insertion in the elf3i gene was revealed by sequencing the ensemble database. The dark dashed ellipse indicates the insertion. B, elf3i−−/− homozygous embryos showed reduced body size (**, p < 0.01) and less pigment as compared with their siblings and wild type embryos at 2 dpf. Scale bar, 500 μm. C, no obvious elf3i protein was detected in elf3i−−/− embryos. Western blot analyses of elf3i−−/− homozygous zebrafish at 2 dpf were done with equivalent amounts of protein. D, elf3i−−/− zebrafish embryos showed significantly lower cell proliferation than wild type ones (**, p < 0.01). The wild type or elf3i−−/− zebrafish embryos were anesthetized and digested, and embryonic cells were counted using a hemocytometer. E, embryos with mutant or wild type phenotypes were subjected to genotyping. All the embryos with mutant phenotypes were elf3i homozygous mutant, and embryos with normal phenotypes were either elf3i heterozygous or wild type. F, elf3i−−/− mutation caused increased apoptosis in zebrafish embryos. Acridine orange staining was performed on zebrafish embryos at 3 dpf, and the green nuclei were counted in the whole spinal cord of the embryos. Obvious higher levels of apoptosis were observed in elf3i−−/− mutant embryos (**, p < 0.01). G, expression of liver-specific marker cp and hepatocyte nuclear factor 3 (foxa3) were examined by WISH. Both of cp and foxa3 expression domains in the liver were greatly reduced (arrows).
eIF3i Controls VEGFA Signaling during Angiogenesis

We found that eIF3i proteins were very low in noncancerous tissues but increased in HCC tissues (Fig. 4, A and B). Of 100 TMA, 66% were eIF3i protein-positive (17% weakly positive, 25% moderately positive, and 24% strongly positive, Fig. 4C). These data suggested that aberrant expression of eIF3i is associated with human hepatocellular carcinoma.

**eIF3i Promotes Cell Proliferation and Colony Formation**—To investigate the function of eIF3i in tumorigenesis, we analyzed loss-of-function of eIF3i by transfection of two different eIF3i-specific siRNAs into HepG2 cells. Both siRNAs displayed com-
Majority of HCC samples. The HCC samples were subclassified according to strength of eIF3i expression (negative, and strong positive, intervals (24, 48, 72, and 96 h). Transfection of either eIF3i expression by Western blot in cells with either over- or under-expression of these factors. As expected, hypoxia conditions (95% N2 + 5% CO2) or 200 μM CoCl2 treatment significantly induced eIF3i expression (Fig. 6B), and knockdown of c-Myc with two published siRNAs mildly reduced eIF3i expression (Fig. 6C). However, adjustment of NF-κB signaling, either by LPS and/or BAY 11-7082, had no effect on eIF3i expression (Fig. 6D). Because eIF3i expression is more sensitive to hypoxia conditions and the eIF3i promoter region that contains HRE is very conserved during evolution, we focused our study on elucidating whether eIF3i is a direct target of HIF1A. We observed that eIF3i mRNA was up-regulated upon CoCl2 treatment (Fig. 6F), suggesting that eIF3i transcription was regulated by hypoxia. To test whether HIF1A can bind to the eIF3i promoter through these conserved sequences, we performed ChIP analysis of the eIF3i promoter in HepG2 exposed to CoCl2. Using the chromatin pulled down by anti-HIF1A or normal IgG antibody as templates, a DNA fragment that flanks −593 to −395 of the eIF3i promoter containing HRE was analyzed. A region flanking +1773 to +1993 in the eIF3i gene locus that contains no HRE was used as a negative control. HIF1A antibody efficiently immunoprecipitated the HRE-containing region of the eIF3i proximal promoter when compared with control IgG antibody, although it did not show any specific affinity for the region that does not contain HRE (Fig. 6G). To further confirm the direct interaction of HIF1A with HRE in the proximal eIF3i promoter, we constructed luciferase reporter vectors that either contain the wild type (WT) eIF3i promoter or the mutant promoter with a 3-bp substitution in the HRE core sequence (named pGL3-pE1F3i-WT and pGL3-pE1F3i-MT, respectively, Fig. 6H). The reporter constructs were co-transfected with an internal control vector, pRL-CMV, into HepG2 cells, which were then subjected to normoxia or hypoxia for 5 h. The lucif-
erase activity of the constructs containing wild type HRE was significantly higher than that of HRE mutant promoter in the HepG2 cell under normoxia. Under hypoxia, the wild type eIF3i promoter showed increased activity, although the mutant promoter did not (Fig. 6I). The ChIP and luciferase reporter assays demonstrated that HIF1A directly interacts with the HIF1A-binding sequence in eIF3i promoter and activates eIF3i transcription during hypoxia.

eIF3i Controls VEGFA Signaling during Angiogenesis—To determine whether eIF3i also helps to induce tumorigenic angiogenesis, we constructed stable HepG2 cell lines that expressed either negative control shRNA or eIF3i shRNA. The stable cell lines were selected by puromycin and FACS taking advantage of co-expression of GFP and the puromycin-resistant gene with shRNA. To evaluate the effects of eIF3i knockdown on the tumorigenic angiogenesis, the control or eIF3i shRNA green fluorescence-labeled HepG2 cells were implanted into the perivitelline space of 48-hpf flk:mcherry transgenic zebrafish embryos. The transplanted embryos bearing hepatocellular carcinoma xenografts were allowed to grow at 33 °C for 1 week. As shown in Fig. 7A, the embryos bearing HepG2 tumors expressing negative control shRNA had obvious tumor angiogenesis. However, the embryos bearing HepG2 tumors expressing eIF3i shRNA had much less or

FIGURE 5. eIF3i promotes cell proliferation and colony formation. A, eIF3i siRNAs transfection significantly suppressed eIF3i expression as determined by Western blot. B, both eIF3i siRNAs inhibited cell proliferation compared with the negative control. After transfection of siRNAs, the cell viability of HepG2 was evaluated by MTT assay. C, cells transfected with either eIF3i siRNA displayed reduced Ki-67 expression (**, p < 0.01). D, stable Lo2 cell lines expressing eIF3i-IRES-zsgreen or IRES-ZsGreen were constructed by lentivirus infection and sorted by FACS. The expression level of eIF3i protein in stable transfected cells was determined by Western blot. E, Lo2 cells overexpressing eIF3i formed more colonies than control cells (**, p < 0.01).
even no obvious angiogenesis induction. These results suggested that eIF3i also control tumorigenic angiogenesis.

As eIF3i controls embryonic angiogenesis by regulating Vegfa expression in zebrafish, we next sought to determine whether this mechanism also functions in human cells. We first checked the expression levels of VEGFA protein in eIF3i shRNA and control HepG2 cells. As expected, VEGFA protein expression was dramatically reduced in the eIF3i shRNA cell (Fig. 7B). In addition, because VEGFA is also a direct target of HIF1A (37), we also determined whether eIF3i depletion also reduced the VEGFA expression in the hypoxia environment. We first used eIF3i siRNAs to interfere with the eIF3i expres-
expression in HegG2 cells for 48 h, and these cells were then exposed to hypoxia. After 5 h, these cells were collected to analyze the expression of HIF1A, eIF3i, and VEGFA through Western blot. As shown in Fig. 7D, hypoxia induced the expression of HIF1A, eIF3i, and VEGFA. The treatment of eIF3i siRNAs effectively interferes with the expression of eIF3i even in hypoxia. Also, the expression of VEGFA, which should rise in hypoxia, is significantly inhibited by eIF3i siRNAs. According to these results, we revealed a new mechanism for VEGFA expression regulation involving eIF3i, which itself is also a target of hypoxia.
eIF3i Silencing Reduces Translation of VEGFA mRNA—To study the detailed mechanism that eIF3i controls VEGFA expression, we examined whether eIF3i silencing decreased the transcription or the translation of VEGFA. Total RNAs of HepG2 cells expressing control or eIF3i shRNA were extracted and analyzed with quantitative PCR. As shown in Fig. 7C, the eIF3i mRNA was markedly down-regulated by eIF3i shRNA treatment (p < 0.01), although no obvious change in the VEGFA mRNA level was detected, suggesting that knockdown of eIF3i did not alter VEGFA transcription. Then we examined whether eIF3i silencing reduced VEGFA translation by using polysome analysis (Fig. 7E). Cell extracts from control and eIF3i shRNA HepG2 cells were fractionated using 10–50% sucrose gradient to isolate polysome-bound mRNAs from ribosome-free mRNAs. Then the levels of VEGFA, β-actin, and GAPDH mRNAs were analyzed by RT-PCR and quantified with ImageJ software. As shown in Fig. 7, F and G, in eIF3i shRNA, treatment led to a significant shift of VEGFA mRNAs from the heavy polysome fractions toward light polysome and monosome fractions. However, no significant redistribution of β-actin or GAPDH mRNAs was detected in eIF3i shRNA cells. Taken together, these analyses suggested that eIF3i knockdown did not cause a general translation repression but specifically reduced the translation efficiency of the VEGFA mRNAs.

**DISCUSSION**

Molecular mechanisms of embryonic development are demonstrated to correlate with those in cancer, and many embryonic genes that are activated during embryonic organogenesis and silenced in adult tissues are re-expressed in malignant tumors. In this work, we studied the expression and function of eIF3i in embryonic development. We found that the expression of eIF3i is dynamic during zebrafish embryogenesis and is accumulated in endodermal organs during organogenesis. We isolated a mutant line with retrovirus inserting to the second exon of eIF3i and found that deletion of eIF3i induces angiogenesis defects characterized by the lack of or disrupted tip cells, which we demonstrated is the result of insufficient Vegfa production. WISH experiments revealed that depletion of eIF3i also results in defected liver organogenesis. Thus, our results indicated that eIF3i expression is critical for embryogenesis especially for angiogenesis and liver organogenesis.

For a long time, eIFs have been thought to be constitutively expressed housekeeping genes, and some of them, such as eIF1a, eIF2a, and eIF4a, are even considered as internal controls in RT-PCR experiments (38–40). However, the dysregulation of eIFs expression in cancer tissues suggests that at least a part of eIFs are not constitutively expressed. Actually, the aberrant expression of eIF3i has been identified in several diseases, including a broad spectrum of tumors, such as breast, esophagus, cervix, lung, stomach, and liver cancers, suggesting expression of eIF3i is under specific control (3, 17, 41–45). We reported that 66% (66/100) of human hepatocellular carcinoma tissues is eIF3i-positive staining. Then, we analyzed the proximal promoter of eIF3i and found several cis-elements recognized by oncogenic transcriptional factors, including c-MYC, NF-κB, and HIF1A. We also showed that HIF1A and c-MYC, but not NF-κB, activate eIF3i expression, which provides solid evidence demonstrating expression of eIF3i requires specific signals in cancer cells. In line with this notion, eIF3i is also expressed in a tissue-specific manner during embryogenesis.

Angiogenesis is essential for proper organ growth and repair. The imbalance in the regulation of this process correlates with inflammatory, ischemic, and malignant disorders (46). In our study, we noticed that the blood circulation is quite abnormal in eIF3i−/− mutant zebrafish embryos as some somites of these embryos have no blood flow. We found the expression of endothelial marker fl is defective in the homozygous mutant, and the specification and function of tip cells are disrupted in these embryos. Consistent with these observations, the mutant offspring of the double transgenic line (eIF3i+/−; Flk:GFP+/−) showed similar angiogenesis defects. These angiogenesis defects are quite similar to that caused by insufficient Vegf signaling. As expected, we detected that eIF3i−/− mutant embryos express much less Vegfa proteins than wild type embryos. Besides, the injection of hVEGFA plasmids effectively rescued angiogenesis defects in mutant embryos, suggesting Vegfa is a downstream target of eIF3i in angiogenesis control. In addition, tumor angiogenesis primarily relies on VEGFA-driven responses, which result in a complex tumor vasculature to coordinate tumor progression and metastasis (47). Then we tested whether eIF3i also promotes tumorigenic angiogenesis in human cancer and found that the stable knockdown deletion of eIF3i greatly reduced the expression of VEGFA in HepG2 cells. The zebrafish embryos bearing eIF3i shRNA-HepG2 tumors have much less angiogenesis induction in comparison with embryos bearing control HepG2 tumors. Together, these data demonstrated that eIF3i controls embryonic and tumorigenic angiogenesis by regulating VEGFA expression.

The expression of VEGFA is modulated by a variety of signaling, including a number of cytokines, growth factors, and steroid hormones (22). However, in solid tumors, the up-regulation of VEGFA by hypoxia is very significant (48). Hypoxia
regulates VEGFA expression at both the transcriptional and post-transcriptional levels (19). The transcriptional activation of VEGFA in hypoxia is mainly mediated by HIF1A, which accumulates during hypoxia and activates VEGFA transcription through binding to HRE within the VEGFA promoter (37, 49). Hypoxia also increases VEGFA mRNA stability and promotes VEGFA IRES-mediated translation (50, 51). In this study, we revealed a new mechanism for VEGFA expression regulation involving eIF3i, which itself is also a target of hypoxia. Because eIF3i belongs to the eIF3 complex that is critical for IRES-mediated translation initiation, it is reasonable to hypothesize that elevated expression of eIF3i in hypoxia helps to promote the IRES-mediated translation initiation of VEGFA. Very recently, a study reported that eIF3i can bind to and activate Akt1 signaling. The binding of eIF3i to Akt1 prevents PP2A-mediated Akt1 dephosphorylation and results in constitutively active Akt1 oncogenic signaling (17). Previous studies have revealed important roles of Akt1 in the regulation of VEGFA expression, and therefore it is also reasonable that eIF3i promotes VEGFA production through activating Akt1 signaling (52, 53). In addition, it has been shown that hypoxia induces Akt1 phosphorylation, and activated Akt1 can also induce HIF1A expression (54). Consistent with this, we also observed knockdown of eIF3i reduces HIF1A expression (data not shown). Thus, this evidence places eIF3i in a positive feedback loop that involves multiple oncogenic molecules and suggests eIF3i can be an oncotarget in anticancer therapies.

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