Identification of DEAD-box RNA Helicase 6 (DDX6) as a Cellular Modulator of Vascular Endothelial Growth Factor Expression under Hypoxia*\(5\)

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Background: We explore post-transcriptional control of VEGF expression.

Results: DDX6 specifically binds to the VEGF mRNA 5′-UTR and regulates VEGF mRNA translation in vitro and in vivo. Decline of DDX6 under hypoxia leads to enhanced VEGF secretion, which increases angiogenesis in vitro.

Conclusion: In MCF-7 breast cancer cells DDX6 modulates VEGF translation in response to oxygen supply.

Significance: Hypoxia-induced DDX6 reduction positively affects proangiogenic VEGF expression.

Vascular endothelial growth factor A (VEGF) is a crucial proangiogenic factor, which regulates blood vessel supply under physiologic and pathologic conditions. The VEGF mRNA 5′-untranslated region (5′-UTR) bears internal ribosome entry sites (IRES), which confer sustained VEGF mRNA translation under hypoxia when 5′-cap-dependent mRNA translation is inhibited. VEGF IRES-mediated initiation of translation requires the modulated interaction of trans-acting factors. To identify trans-acting factors that control VEGF mRNA translation under hypoxic conditions we established an in vitro translation system based on human adenocarcinoma cells (MCF-7). Cytoplasmic extracts of MCF-7 cells grown under hypoxia (1% oxygen) recapitulate VEGF IRES-mediated reporter mRNA translation. Employing the VEGF mRNA 5′-UTR and 3′-UTR in an RNA affinity approach we isolated interacting proteins from translatable active MCF-7 extract prepared from cells grown under normoxia or hypoxia. Interestingly, mass spectrometry analysis identified the DEAD-box RNA helicase 6 (DDX6) that interacts with the VEGF mRNA 5′-UTR. Recombinant DDX6 inhibits VEGF IRES-mediated translation in normoxic MCF-7 extract. Under hypoxia the level of DDX6 declines, and its interaction with VEGF mRNA is diminished in vivo. Depletion of DDX6 by RNAi further promotes VEGF expression in MCF-7 cells. Increased secretion of VEGF from DDX6 knockdown cells positively affects vascular tube formation of human umbilical vein endothelial cells (HUVEC) in vitro. Our results indicate that the decrease of DDX6 under hypoxia contributes to the activation of VEGF expression and promotes its proangiogenic function.

VEGF is a potent inducer of blood vessel formation. It is involved not only in the control of embryonic development and vascular differentiation, in wound healing, and reproduction, but also in tumor growth, arthritis, and psoriasis (1–4).

Under hypoxic conditions VEGF expression is induced by increased mRNA synthesis, stability, and translation. Enhanced VEGF mRNA synthesis under hypoxia is mediated by transcription factor HIF-1α that is composed of an O2-labile α-subunit (HIF-1α) and a constitutive β-subunit (5). Under normoxia proline hydroxylation of HIF-1α leads to pVHL-E3 ligase binding and proteosomal degradation of HIF-1α (6–8).

In MCF-7 cells VEGF\(_{121}\), VEGF\(_{165}\), and VEGF\(_{189}\) are generated as preferentially expressed isoforms (9). VEGF mRNA stabilization under hypoxia is controlled by proteins that interact with its 3′-untranslated region (3′-UTR) (10–15). HuR, a member of the ELAV family (16), and heterogeneous nuclear ribonucleoprotein (hnRNP L) (17) were identified as VEGF mRNA-stabilizing factors, whereas TIS11b (18) and tristetraprolin (19) function in destabilization.

Whereas under hypoxic conditions global protein synthesis is attenuated, VEGF mRNA is preferentially translated. It has been demonstrated that IRES elements in the VEGF mRNA 5′-UTR promote efficient 5′-cap-independent translation under hypoxia (20–22). Pyrimidine tract binding protein has been identified as a 5′-UTR trans-acting factor, but no function in translation regulation could be assigned (21).

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4 The abbreviations used are: HIF-1α, hypoxia-inducible factor 1α; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CAT, chloramphenicol acetyltransferase; DDX6, DEAD-box RNA helicase 6; hnrNP, heterogeneous nuclear ribonucleoprotein; HUVEC, human umbilical vein endothelial cell; IRES, internal ribosome entry sites; mRNP, messenger ribonucleoprotein particle; qRT-PCR, quantitative RT-PCR.
Because the regulation of VEGF mRNA translation by 5’- and/or 3’-UTR-interacting proteins has not been investigated in detail so far, our studies focused on the identification and characterization of cellular factors that modulate VEGF mRNA translation under hypoxia. For this purpose, we have established an in vitro translation system based on cytoplasmic MCF-7 cell extract that recapitulates VEGF IRES-mediated translation under hypoxic conditions. Through the use of tobramycin RNA aptamer affinity chromatography we identified AUFl-1 (hnRNP D), hnRNP K, and DDX6 as VEGF mRNA trans-acting factors. Employing RNA interaction assays we identified the VEGF mRNA cis-element that is required for DDX6 binding. The DDX6-dependent modulation of VEGF mRNA translation and its functional consequences under hypoxia were addressed in vitro and in MCF-7 cells by siRNA-mediated DDX6 knockdown.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction*—Primers used for cloning are summarized in supplemental Table 1. Cloning procedures are described in supplemental Materials and Methods.

*Cell Culture and Hypoxia Treatment*—MCF-7 cells (DSMZ, ACC 115) were grown in DMEM supplemented with heat-inactivated FBS (10%), nonessential amino acids, penicillin, and streptomycin. For hypoxia treatment, cells were incubated at 1% O2, 5% CO2 for 24 h. For tube formation assays, human umbilical vein epithelial cells (HUVECs) were grown in supplemented endothelial cell growth medium (Promocell).

*Cell Lysate Preparation*—Nuclear/cytoplasmic fractionation was performed according to Ref. 23 and total cell lysate preparation as in Ref. 24.

*Cytoplasmic Extract Preparation*—MCF-7 extract was prepared as in Ref. 25. Subconfluent cells were harvested with trypsin-EDTA, washed with ice-cold isotonic buffer (35 mM Hepes/ KOH, pH 7.6, 146 mM NaCl, 11 mM glucose), and collected by centrifugation (300 × g, 2 min, 4 °C). The pellet was resuspended in an equal volume of hypotonic buffer (10 mM Hepes/ KOH, pH 7.6, 10 mM KCl, 0.5 mM Mg(CH3CO2)2, 5 mM DTT) with protease inhibitors (Roche Applied Science), and incubated for 10 min on ice. Cells were broken by expelling through a 26-gauge needle, and nuclei were removed by centrifugation (20,000 × g, 10 min, 4 °C).

*RNA Affinity Purification*—Tobramycin aptamer RNA affinity purification according to (26) was modified: 4 pmol Tobramycin-VEGF mRNA were incubated with 600 μg cytoplasmic extract under translation initiation conditions (1.5 mM cycloheximide, 5 min, 37 °C), cooled on ice, incubated with tobramycin matrix in 200 μl of binding buffer (20 mM Tris-HCl, pH 8.0, 1 mM CaCl2, 60 mM KCl, 3.5 mM MgCl2, 0.05% Nonidet P-40, 0.2 mM DTT, 1 h, 4 °C) and washed three times with binding buffer. Complexes were eluted with SDS sample buffer and subjected to mass spectrometry and Western blotting. For the interaction studies shown in Fig. 4A, recombinant DDX6 (20 pmol) or hnRNP K (40 pmol) were incubated with 2 pmol of the indicated RNAs in binding buffer supplemented with 0.5 mg/ml BSA for 10 min at room temperature prior to immobilization at the tobramycin matrix. Washing and elution were performed as above.

*Mass Spectrometry*—Proteins were separated on a NuPAGE 4–12% Bis-Tris gel (Invitrogen). Entire lanes were cut in 23 slices, proteins were in gel-digested with trypsin and extracted according to (27). The extracted peptides were analyzed under standard conditions in a nano-HPLC-coupled electrospray ionization quadrupole time of flight (ESI-Q-TOF; Ultima, Waters) mass spectrometer. Proteins were identified by database search against human NCBI database (nr_08092010, 518493 human proteins) using MASCOT (Version 2.2) as search engine. Data were further evaluated and annotated with Scaffold software version 3.4.5 (Proteome Software).

*Expression of Recombinant Proteins*—His-hnRNP K and His-DDX6 were expressed and purified as described (28, 29) with the exception that His-hnRNP K was dialyzed against 20 mM Tris, pH 8.0, 300 mM potassium chloride, 10 mM sodium citrate, 10% sucrose and His-DDX6 against 20 mM Hepes, pH 7.4, 150 mM potassium chloride, 5% glycerol, 1 mM DTT.

*In Vitro Translation*—MRNAs were transcribed with T3 MEGAscript® kit (Applied Biosystems) in the presence of 6 mM γGppG-cap or ApppG-cap (KEDAR) for in vitro translation and affinity purification.

*In Vitro Translation and Micrococcus Nuclease Treatment*—Prior to in vitro translation cytoplasmic extracts were treated with Micrococcus nuclease (0.4 unit/60 μg of extract, 0.2 mM Ca(OAc)2, 8 min at 25 °C, stopped with 0.4 mM EGTA on ice). Translation reactions contained 60 μg of cytoplasmic extract, 100 μm amino acids, 16 mM Hepes, pH 7.6, 2–2.5 mM Mg(CH3CO2)2, 60–90 mM KCl, 200 μg/ml tRNA, 0.8 mM ATP, 0.1 mM GTP, 40 μg/ml creatine kinase, 20 mM creatine phosphate, and 100 fmol of bicistronic, 200 fmol of monocistronic, or 50 fmol of 5’- cap-Luc mRNA. Reactions were incubated for 30 min at 37 °C. Luciferase activity was measured with the DualGlo luciferase system or the luciferase assay system (Promega). For the experiments shown in Fig. 4C, 0.9 pmol of His-DDX6, His-hnRNP K, or BSA was preincubated with MCF-7 cell extract for 10 min.

*Polysome Analysis*—Cells were incubated with 100 μg/ml cycloheximide for 10 min, washed twice, and harvested in ice-cold PBS supplemented with 100 μg/ml cycloheximide and centrifuged at 300 × g, 2 min, 4 °C. Lysis was performed with a Dounce homogenizer in gradient buffer (150 mM KCl, 20 mM Hepes, pH 7.6, 6.5 mM MgCl2) supplemented with 1 mM DTT, 100 μg/ml cycloheximide, 0.5% Nonidet P-40, protease, and RNase inhibitors. Nuclei and cell debris were removed by centrifugation (20,000 × g, 10 min, 4 °C). 600 μg of total protein was loaded on a linear 15–45% sucrose gradient and centrifuged 35,000 rpm, 4 °C, SW41 Ti. 20 fractions were collected from the bottom to the top of the gradient.

*RNA Preparation and Quantitative RT-PCR*—Prior to RNA extraction 5 ng of CAT mRNA was added per reaction. Total RNA was prepared using TRIzol. For reverse transcription random primers and M-MLV RT (Promega) were used (30). Quantitative RT-PCR was performed with SYBR Green (Sigma) (Taq-Master mix; Promega) on a 7300 Real Time PCR System (Applied Biosystems) (Figs. 2C and 5B) or with Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus (Applied Biosystems) (Fig. 5E). Primers are summarized in supplemental Table 1. For mRNA analysis in sucrose gradients
(Figs. 1C and 5E) RNA was prepared from 300 μl of individual fractions, and equal volumes were used in RT-PCR (Fig. 1C) or qRT-PCR (Fig. 5E). For reporter mRNA stability determination in in vitro translation experiments (Fig. 2C) or analysis of endogenous mRNA (Fig. 5B) equal amounts of RNA. For the analysis of VEGF mRNA stability, cells were treated with actinomycin D (5 μg/ml) for 0, 1, and 2 h. Equal amounts of RNA were analyzed by qRT-PCR. mRNA amounts were determined by the ΔΔCt method (31), normalized for CAT mRNA (Figs. 2C and 5E) or endogenous rpL0p mRNA (Fig. 5B). mRNAs in Fig. 1C were detected by RT-PCR performed with GoTaq Flexi DNA Polymerase (Promega) according to the manufacturer’s protocols, and products were analyzed on GelRed-stained (Biotium) 1% agarose gels.

Antibodies—Antibodies were purchased from Abcam (GAPDH, Histone H3), Millipore (AUF-1), Santa Cruz Biotechnology (hnRNK, hnRNPL, HuR, G3BP1, KDEL-ER, rpL19), Abnova (Dcp1A), Sigma-Aldrich (vinculin), Novus Biologicals (DDX6), R&D Systems (VEGF), BD Transductions (HIF-1α), and GE Healthcare (HRP-conjugated antibodies).

Immunochemistry—Immunofluorescence staining was essentially performed as described in Ref. 30. FISH (probe sequences in supplemental Table 1) combined with immunofluorescence staining was performed as described (32), except that FISH was carried out before immunofluorescence staining. Microscopy was performed with an Apotome 2 (Zeiss), and images were acquired with AxioVision (Zeiss) and intensity profiles with ImageJ.

Immunoblot Analysis and ELISA—Western blot assays were performed as described previously and analyzed on a LAS-4000 system (FujiFilm) (32). Detection of VEGF in MCF-7 culture supernatants was performed with human VEGF DuoSet ELISA reagents (R&D Systems).

RNAi—For RNAi, MCF-7 cells (1 × 10⁶ cells in DMEM without FBS, nonessential amino acids, and antibiotics) were transfected by electroporation at 0.36 kV, 500 microfarads (GenePulser II, Bio-Rad) with 500 pmol of siRNAs (MWG) against DDX6 (32) or a nonspecific control siRNA (30). Cells were harvested 72 h after transfection.

Tube Formation Assay—Tube formation assays (33) were performed in 96-well plates. The wells were coated with 40 μl of Matrigel (BD Bioscience) and polymerized for 30 min, 37 °C. 3 × 10⁴ HUVECs/well were seeded in 100 μl of medium, consisting of 25% HUVEC medium and 75% conditioned MCF-7 cell medium and incubated for 16 h, 37 °C. For VEGF neutralization, MCF-7 medium was preincubated with 500 ng/ml antivegf antibody for 20 min at room temperature. Tube formation was analyzed by light microscopy.

RESULTS

MCF-7 Cells Recapitulate Increased VEGF mRNA Synthesis and Translation under Hypoxia—MCF-7 cells were cultured for 24 h under normoxic (21% O₂) or hypoxic (1% O₂) conditions. HIF-1α is undetectable under normoxia due to rapid degradation by the ubiquitin/proteasome system (6, 34), whereas under hypoxia, this HIF-1 subunit is stabilized. Specifically, under hypoxia nuclear HIF-1α could be detected by immunofluorescence microscopy (Fig. 1A). When nuclear and cytoplasmic extracts were analyzed, HIF-1α could specifically be detected in nuclear extracts of MCF-7 cells grown under hypoxic (H) but not normoxic (N) conditions. Histone H3 served as control for the nuclear compartment, vinculin and GAPDH as cytoplasmic controls (Fig. 1B).

To investigate whether endogenous VEGF mRNA translation is enhanced under hypoxia, we characterized its co-localization with polysomes by fractionation of cytoplasmic extracts on 15–45% sucrose gradients (Fig. 1C). mRNA translation was stalled by cycloheximide treatment before MCF-7 cells were harvested for extract preparation. 18 S and 28 S rRNA indicated the distribution of ribosomal complexes and mRNPs to the gradient fractions (Fig. 1C). The A₂₆₀ nm profiles revealed an rRNA increase in the 80 S peak and reduction in polysomal fractions when hypoxic extracts (Fig. 1C, right) were characterized. To analyze endogenous mRNAs, total RNA was extracted from individual fractions, to which CAT mRNA was added as extraction control. The comparison of VEGF mRNA and β-actin mRNA distribution, which was analyzed by RT-PCR, showed a different pattern under normoxia and hypoxia. VEGF mRNA localization to monosome- and polysome-containing fractions was augmented under hypoxia, indicating increased VEGF mRNA translation (Fig. 1C, compare fractions 4–15, left and right), whereas polysomal association of β-actin mRNA, which is translated in a 5’-cap-dependent manner, was reduced (Fig. 1C, compare fractions 3–14, left and right). Consistently, immunofluorescence microscopy revealed an increased colocalization of VEGF mRNA with the 60 S ribosomal subunit protein rpL19 under hypoxia and diminished β-actin mRNA containing with rpL19 compared with normoxia, as indicated by the corresponding overlapping signal amplitudes of the intensity profiles (Fig. 1D and supplemental Fig. S1A). RpL19 was chosen as localization marker, because in contrast to the ribosomal 40 S subunit, the 60 S subunit is not present either in translational silent stress granules (35) or in P-body-like mRNPs (32). These data demonstrate that under hypoxic conditions VEGF mRNA translation is induced in MCF-7 cells, which can be employed as an in vivo system to identify and characterize cellular factors required for VEGF expression.

Cytoplasmic Extract from MCF-7 Cells Grown under Hypoxia Recapitulates VEGF IRES Activity—To analyze VEGF IRES activity in vitro, we generated translation-competent cytoplasmic extracts from MCF-7 cells cultured under normoxic or hypoxic conditions. The IRES containing VEGF mRNA 5’-UTR was inserted in a polyadenylated (A₉₈) bicistronic Renilla and firefly luciferase encoding reporter mRNA (RVF) or was replaced by a nonrelated sequence in a control mRNA (RF) (Fig. 2A). To ensure that firefly luciferase translation initiated at the VEGF 5’-UTR did not result from reinitiating ribosomes that failed to dissociate from the upstream Renilla luciferase open reading frame, a stable stem-loop was inserted in both mRNAs (Fig. 2A). Insertion of the stem-loop strongly inhibited 5’-cap-mediated Renilla translation in normoxic and hypoxic extracts (Fig. 2B, left), whereas VEGF 5’-UTR-dependent translation of firefly luciferase is increased 2-fold in normoxic and nearly 4-fold in hypoxic extract com-

DDX6 Regulates VEGF Expression under Hypoxia
DDX6 Regulates VEGF Expression under Hypoxia
pared with the control RNA (Fig. 2B, right). The comparable stability of all four mRNAs isolated from the translation reactions indicates that the newly established MCF-7 in vitro translation system recapitulates enhanced translation initiation at the VEGF 5′-UTR under hypoxia (Fig. 2C). From these results, we conclude that MCF-7 cell extract represents a potent in vitro system to study hypoxia-mediated regulation of VEGF mRNA translation.

RNA Affinity Purification and IF-FISH Analysis Revealed DDX6 as a New VEGF mRNA-interacting Protein—The hypoxia-mediated increased association of VEGF mRNA with monosomes and polysomes in vivo and enhanced VEGF IRES-dependent translation in vitro (Figs. 1 and 2) motivated us to identify cellular factors that modulate VEGF synthesis under hypoxic conditions systematically. For this purpose, we made use of the tobramycin aptamer RNA affinity chromatography, which is based on high affinity binding of the RNA aptamer J6f1 to tobramycin (KD 5n M) (36) and proved to be highly efficient and specific in the analysis of RNA-protein complexes (26, 37–39). To purify proteins under translation initiation conditions we incubated the reporter mRNA comprising the VEGF 5′-m7GpppG-cap, 3′-(A)98 tail and open reading frames encoding Renilla reniformis and firefly luciferase, spaced by an unrelated sequence (RF) or the VEGF 5′-UTR (RFV). Where indicated, RF and RVF contain a stable 5′-stem-loop.

FIGURE 1. Characterization of the hypoxic response in MCF-7 cells. A, detection of HIF-1α with a specific antibody, actin staining with phalloidin-TRITC, and detection of nuclei with DAPI in MCF-7 cells grown under normoxic or hypoxic conditions. B, Analysis of proteins in nuclear and cytoplasmic fractions from cells grown under normoxia (N) or hypoxia (H) with the indicated antibodies by Western blotting. C, A260 nm profiles of normoxic or hypoxic MCF-7 cytoplasmic extracts fractionated on 15–45% sucrose density gradients. Polysomes, 80 S ribosomes, 60 S and 40 S ribosomal subunits and mRNPs are indicated. Total RNA extracted from 20 gradient fractions was analyzed on a 1% agarose gel; 28 S and 18 S rRNAs are indicated. Endogenous VEGF and β-actin mRNA distribution on sucrose gradients was analyzed by RT-PCR. CAT mRNA served as extraction control. D, IF-FISH analysis of MCF-7 cells cultured under normoxic and hypoxic conditions. Cells were hybridized with FISH probes against VEGF or β-actin mRNA (FITC, green), as indicated. Costaining of endogenous rpl19 (Cy3, red) was detected with a specific antibody. Dotted squares indicate the magnified area represented in the enlargement. Cy3 and FITC fluorescence monitored along the dashed lines is shown as relative signal intensity in the bottom panel.

FIGURE 2. VEGF IRES-dependent translation in the MCF-7 cell-derived in vitro translation system. A, schematic representation of bicistronic mRNAs that bear a 5′-m7GpppG-cap, 3′-(A)98 tail and open reading frames encoding Renilla reniformis and firefly luciferase, spaced by an unrelated sequence (RF) or the VEGF 5′-UTR (RFV). Where indicated, RF and RVF contain a stable 5′-stem-loop. B, in vitro translation reactions with RF or RVF mRNA in the absence or presence of the stem-loop in normoxic or hypoxic cytoplasmic MCF-7 cell extracts. Renilla and firefly luciferase activity (in percentage) is depicted. Error bars represent three independent experiments. Data were analyzed with Student’s t test (two-tailed); ***, p < 0.005. C, total RNA was extracted from three independent in vitro translation reactions, and RF and RVF mRNA stability was determined by qRT-PCR with primers detecting the firefly open reading frame.
DDX6 Regulates VEGF Expression under Hypoxia

Tobramycin beads alone with translation competent cytoplasmic extracts of MCF-7 cells cultured under normoxic or hypoxic conditions in the presence of cycloheximide. Eluted proteins were separated on a 4–12% NuPAGE gel and subjected to LC/tandem MS (32). Mascot-analysis identified proteins specifically enriched on the VEGF matrix are summarized in Table 1. Five of them were analyzed further. In addition to hnRNP L and HuR, which served as positive controls (16, 17), we focused on AUF-1, hnRNP K, and DDX6. Eluates from the tobramycin purification were subjected to Western blot analysis to verify their interaction with the VEGF matrix (Fig. 3B, lanes 1–6). Under hypoxia the hnRNP L level increased, and its interaction with the VEGF matrix was enhanced. HuR was not detectable in the input, but accumulated strongly on the VEGF matrix both under normoxia and hypoxia (Fig. 3B, lanes 1, 2, 5, and 6). G3BP1, GAPDH and vinculin, not identified by mass spectrometry, showed no binding (Fig. 3B, lanes 5 and 6). AUF-1 represents a family of four polypeptides (p37, p40, p42, and p45), which originate from alternative splicing (40). p45 more prominent in the input and is found more efficiently to the VEGF matrix when hypoxic extract was used. HnRNP K levels were equal under both conditions, and the interaction with VEGF was comparable in both extracts. Furthermore, we identified the DEAD-box helicase DDX6. Interestingly, the DDX6 signal was slightly reduced in cells grown under hypoxic conditions, but equal amounts bound to the VEGF matrix under normoxia and hypoxia (Fig. 3B, lanes 1–6). The reduced level of DDX6 under hypoxia prompted us to analyze its impact under normoxia and hypoxia (Fig. 3C, left). Noticeable, the level of DDX6, which was localized to granular structures, decreased under hypoxia (Fig. 3C, left). A quantification of DDX6 in the cytoplasmic fraction from cells grown under hypoxia revealed a decrease to 72% (Fig. 3C, right). Consistently, cytoplasmic colocalization of DDX6 and VEGF mRNA under normoxia was reduced under hypoxia, indicated by a reduced overlap of signal amplitudes (Fig. 3C, left, and supplemental Fig. S1B). In contrast, β-actin mRNA appeared to be mainly excluded from DDX6 containing granular structures (Fig. 3C, left). G3BP1 colocalized with VEGF mRNA only to a minor extent (Fig. 3D and supplemental Fig. 1B), consistent with the results of the RNA binding studies (Fig. 3B). Because DDX6 or its orthologs Rck/p54/CGH-1 localize to stress granules (41), P-bodies (42), and P-body like RNP granules (32), we characterized the nature of DDX6 containing granular structures by co-staining of respective marker proteins (Fig. 3E). Colocalization of P-body marker Dcp1A (43) with DDX6, preferentially in large granular structures under normoxia, disappeared with the decrease of DDX6 under hypoxia. The stress granule marker G3BP1 (44) was not localized to DDX6-positive granules (Fig. 3E and supplemental Fig. S1B). Together, this indicates that under normoxia translational silenced VEGF mRNA, but not actively translated β-actin mRNA, colocalizes with DDX6 in granular structures.

Recombinant DDX6 Interacts with the VEGF mRNA 5'-UTR and Inhibits VEGF-IREs-mediated Translation in Vitro—To characterize the function of DDX6 in VEGF mRNA translation, we first investigated whether DDX6 directly interacts with the VEGF mRNA 5' and/or 3'-UTR. For this purpose, tobramycin RNA affinity purification was performed with respective sequence elements and recombinant DDX6. The β-globin mRNA 5'-leader sequence and recombinant hnRNP K served as controls (Fig. 4A). DDX6 showed increased binding to the VEGF mRNA 5'-UTR (Fig. 4A, lane 4), whereas hnRNP K

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**TABLE 1**

Proteins specifically enriched on the VEGF matrix

| Protein                                             | Gene name              | Protein ID | Synonym | Molecular mass (kDa) | Peptides matched to VEGF matrix (N, H) | Sequence coverage (N, H, PIP) |
|-----------------------------------------------------|------------------------|------------|---------|----------------------|----------------------------------------|-----------------------------|
| DEAD (Asp-Glu-Ala-Asp) box helicase 6               | DDX6                   | NP_001244120.1 | RCK       | 54.4                 | 21 18                                 | 52 41 100                   |
| Heterogeneous nuclear ribonucleoprotein A3          | HNRNP A3               | NP_0019223.1 |         | 39.6                 | 10 11                                 | 27 32 100                   |
| Heterogeneous nuclear ribonucleoprotein K           | HNRNP K                | NP_002131.2 | TUNP     | 51.0                 | 16 11                                 | 43 30 100                   |
| Autoantigen La                                      | LA                     | NP_001333.1 |          | 36.8                 | 7 11                                  | 19 35 100                   |
| Eukaryotic translation elongation factor 1 epsilon-1 | EEF1E1                 | NP_004271.1 | API3M    | 19.8                 | 4 5                                   | 24 38 100                   |
| Heterogeneous nuclear ribonucleoprotein D           | HNRNP D                | NP_011278.1 | AU-1F     | 38.4                 | 6 3                                   | 23 14 100                   |
| Heterogeneous nuclear ribonucleoprotein L           | HNRNP L                | NP_001524.2 |          | 61.9                 | 4 4                                   | 10 11 100                   |
| Heterogeneous nuclear ribonucleoprotein A0          | HNRNP A0               | NP_006796.1 |          | 30.8                 | 4 4                                   | 17 21 100                   |
| Cellular nucleic acid-binding protein, isoform 3    | CNBP                   | NP_003409.1 | ZNF9      | 18.4                 | 2 4                                   | 15 32 100                   |
| Embryonic lethal, abnormal vision)-like 1           | ELAVL1                 | NP_001410.2 | HUR       | 36.1                 | 5 4                                   | 20 14 100                   |
| Eukaryotic translation initiation factor 2 subunit 2 | EIFIS2                 | NP_003899.2 | EIF2B     | 38.4                 | 3 3                                   | 13 13 100                   |
| Human pumilio homolog 2                             | PIHMH2                 | NP_056132.1 | PUM1L2    | 114.2                | 3 3                                   | 4.3 3.7 100                  |

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**FIGURE 3**

Tobramycin aptamer RNA affinity purification and IF-FISH analysis reveal that DDX6 interacts with VEGF mRNA. A, schematic representation of the transcript applied in the tobramycin RNA affinity purification employing normoxic (N) or hypoxic (H) cytoplasmic extracts of MCF-7 cells. B, Western blot analysis of proteins isolated by RNA affinity purification. The AUF-1 antibody detected four bands representing isoforms p45, p40, p40, and p37. C, left, and D, IF-FISH analysis of MCF-7 cells cultured under normoxic or hypoxic conditions. Cells were hybridized with VEGF mRNA and β-actin mRNA probes (FITC, green), as indicated. Co-staining of endogenous proteins (Cy3, red) was detected with antibodies against DDX6 (C) or G3BP1 (D). C, right, Western blot analysis of different amounts of recombinant His-DDX6 and cytoplasmic extract of MCF-7 cells cultured under normoxic or hypoxic conditions with antibodies against DDX6, GAPDH, and vinculin. The relative amount of DDX6 is indicated at the bottom. E, containing of DDX6 (Cy3, red) and Dcp1A or G3BP1 (FITC, green) with specific antibodies. Dotted squares indicate the magnified area represented in the enlargement. Cy3 and FITC fluorescence monitored along the dashed lines is shown as relative signal intensity in the bottom panel of C–E.
DDX6 Regulates VEGF Expression under Hypoxia

**FIGURE 4.** Recombinant DDX6 interacts with the VEGF mRNA 5′-UTR and inhibits VEGF IRES-mediated translation in vitro. A, tobramycin RNA affinity purification was performed employing the VEGF mRNA 5′-UTR or 3′-UTR, respectively, or the β-globin mRNA 5′-leader as control sequence (ctrl.) and recombinant DDX6 or hnRNP K. Proteins eluted from the RNA matrix were subjected to Western blot analysis with antibodies as indicated. B, schematic representation of the reporter mRNAs employed in vitro translation experiments: 5′-cap-LUC (F), VEGF-IRES-LUC (VF), inverted VEGF-IRES-LUC (ViF). C, in vitro translation reactions with the indicated mRNAs in normoxic MCF-7 cell extracts in the presence of 0.9 pmol of recombinant DDX6, recombinant hnRNP K, or BSA as control protein. Firefly luciferase activity was expressed as fold repression normalized to luciferase activity in the presence of BSA. Error bars represent three independent experiments. Data were analyzed with Student’s t test (two-tailed); ***, p < 0.001.

Bound exclusively to the 3′-UTR (Fig. 4A, lane 5). To investigate whether 5′-UTR binding of DDX6 contributes to translation inhibition under normoxia, we generated monocistronic reporter mRNAs bearing a 5′-AappG-cap and the VEGF mRNA 5′-UTR (VF) or the inverted 5′-UTR (ViF). Reporter mRNAs bearing a 5′-m7GpppG-cap (F) served as control (Fig. 4B). All mRNAs were translated in normoxic extracts in the absence or presence of recombinant DDX6 or hnRNP K (Fig. 4C). BSA was used as control to express the impact of DDX6 or hnRNP K on reporter mRNA translation. DDX6 repressed VF mRNA translation 3.9-fold compared with 5′-cap-dependent (F) or inverted VEGF 5′-UTR (ViR)-mediated translation, which were repressed 1.6- or 1.5-fold, respectively (Fig. 4C). The reduced translation was not due to declined mRNA stability (data not shown). Increasing amounts of DDX6 led to a reduction of control (F and ViF) mRNA translation and diminished specific VF mRNA translation inhibition (data not shown). Our results indicate that under normoxia the interaction of DDX6 with the VEGF mRNA 5′-UTR inhibits translation.

**DDX6 Depletion Results in Enhanced VEGF Expression under Hypoxia**—The observed decline of DDX6 under hypoxia (Fig. 3, B and C), led us to investigate the influences of DDX6 reduction on endogenous VEGF expression. DDX6 could efficiently be depleted by two independent siRNAs in MCF-7 cells cultured under normoxic and hypoxic conditions (Fig. 5A). To assess VEGF mRNA stability under DDX6 depletion, transcription was blocked by actinomycin D treatment. VEGF mRNA stability that is enhanced by hypoxia (13) remained unchanged after 2 h when DDX6(2) siRNA was applied compared with control siRNA under normoxia and hypoxia (Fig. 5B). Whether DDX6 depletion modulates endogenous VEGF mRNA translation was addressed by colocalization analysis of VEGF mRNA with rPL19 of the ribosomal 60 S subunit employing FISH and immunostaining (Fig. 5C and supplemental Fig. S1C). siRNA-mediated DDX6 depletion under normoxia resulted in an increased colocalization of VEGF mRNA and rPL19 compared with control siRNA (Fig. 5C, columns 1 and 2). The enhanced overlap of the intensity profiles in the presence of DDX6(2) siRNA under normoxia was comparable with control siRNA treatment under hypoxia (Fig. 5C, columns 2 and 3). Under hypoxia, DDX6 declined, and VEGF mRNA colocalization with rPL9 was further enhanced by DDX6 depletion (Fig. 5C, columns 1, 3, and 4). Colocalization of β-actin mRNA with rPL9 was reduced under hypoxia, but not affected by DDX6 siRNA (Fig. 5C, columns 5–8).

A potential impact of DDX6 depletion on VEGF protein expression was analyzed by immunofluorescence microscopy (Fig. 5D). Knockdown of DDX6 resulted in a slight increase of the VEGF protein signal under normoxia (Fig. 5D, left) that was strongly enhanced under hypoxia (Fig. 5D, right). Predominant localization of VEGF protein to the endoplasmic reticulum, detected by a KDEL antibody, could be demonstrated in the merge (Fig. 5D). To prove that endogenous VEGF mRNA translation is enhanced under hypoxia in cells transfected with the DDX6 siRNA 2, we characterized VEGF mRNA cosedimentation with polysomes by fractionation of cytoplasmic extracts on 15–45% sucrose gradients (Fig. 5E, bottom). mRNA translation was stalled by cycloheximide treatment before MCF-7 cells were harvested for extract preparation. The efficiency of the DDX6 knockdown in MCF-7 cells grown under hypoxia was analyzed by Western blotting (Fig. 5E, top). The A260 nm profile indicates the position of ribosomal complexes and polysomes (Fig. 5E, middle). The analysis of endogenous VEGF mRNA distribution by qRT-PCR illustrates that DDX6 knockdown resulted in a strong accumulation of VEGF mRNA in polysomal fractions (Fig. 5E, bottom), indicating an enhanced translation of VEGF mRNA in vivo. The results of our studies suggest that DDX6 down-regulation enhances endogenous VEGF mRNA
DDX6 Regulates VEGF Expression under Hypoxia

A) Western

B) 

C) 

D) 

E)
DDX6 Regulates VEGF Expression under Hypoxia

Vascular Tube Formation Is Activated by Increased VEGF Secretion under DDX6 Depletion—To ascertain whether the detected increase in intracellular VEGF in MCF-7 cells adds to secretion of the protein, its accumulation in the culture supernatant was determined by ELISA (Fig. 6A). Under hypoxic growth conditions, the amount of secreted VEGF increased 2.5-fold and was even enhanced to 4.5-fold when DDX6 was depleted by siRNA transfection compared with control siRNA (Fig. 6A). The angiogenic function of elevated VEGF expression following DDX6 depletion was directly addressed in vascular tube formation assays employing HUVECs. HUVEC culture medium was supplemented with supernatants from DDX6 or control siRNA-treated MCF-7 cells grown under normoxia or hypoxia (Fig. 6A). Enhanced vascular tube formation could specifically be detected when supernatants from hypoxic DDX6 siRNA knockdown cells were applied (Fig. 6B). Importantly, increased vascular tube formation could be reversed in the presence of a neutralizing VEGF antibody (Fig. 6B). Enhanced vascular tube formation mirrors the increased VEGF levels in the supernatant of DDX6 siRNA-transfected MCF-7 cells cultured under hypoxic conditions, which were detected by ELISA (Fig. 6A). These assays show that elevated VEGF mRNA translation under DDX6 depletion results in the synthesis and secretion of VEGF protein that exhibits prototypical proangiogenic function.

DISCUSSION

We focused on the identification of cellular factors that regulate VEGF mRNA translation under hypoxic conditions, when global 5’-cap-dependent mRNA translation is suppressed (45). For this purpose we employed VEGF-expressing MCF-7 cells that show hypoxia-dependent enhanced polysomal association of VEGF mRNA (Fig. 1C) and increased colocalization with rpL19 (Fig. 1D). Based on cytoplasmic MCF-7 cell extracts we generated an in vitro translation system, which shows increased VEGF IRES mediated translation of a bicistronic reporter mRNA when cells were grown under hypoxic conditions (Fig. 2, A–C). Similar results were obtained with transiently transfected bicistronic constructs in NIH3T3 cells subjected to hypoxia (46).

Through the use of tobramycin aptamer RNA chromatography we isolated trans-acting factors that associated with the VEGF mRNA 5’-UTR and 3’-UTR under normoxia and hypoxia (Fig. 3). On a VEGF 5’-UTR- and 3’-UTR-bearing mRNA matrix hnrPN L and HuR were specifically purified. In addition we identified AUF-1, hnrPN K, and DDX6.

A specific increase of AUF-1 isoform p45, which is enriched on the VEGF matrix, could be detected under hypoxia (Fig. 3B).

FIGURE 5. Depletion of DDX6 enhances VEGF expression under hypoxia, but does not influence VEGF mRNA stability. A, Western blot analysis of MCF-7 cells, which were transfected with a control siRNA (ctrl) (lanes 1 and 4) or two siRNAs directed against DDX6 (lanes 2, 3, 5, and 6) and cultured under normoxic (lanes 1–3) or hypoxic conditions (lanes 4–6). Antibodies detecting DDX6 and vinculin were applied. B, analysis of VEGF mRNA stability by qRT-PCR. Cells were transfected with control siRNA (si-ctrl, circles, dashed line) or DDX6 siRNA (si-DDX6(#2), triangles, solid line), cultured under normoxic (light gray) or hypoxic (dark gray) conditions and treated with actinomycin D for 0, 1, and 2 h. VEGF mRNA was normalized to rpL10 mRNA (0 h time point 100%); means from two independent experiments are shown. C, IF-FISH analysis of MCF-7 cells, which were transfected with control siRNA (ctrl) or DDX6 (2) siRNA and cultured under normoxic or hypoxic conditions. DDX6 was visualized with a specific antibody (Cy5, cyan). Cells were hybridized with VEGF mRNA (left) or β-actin mRNA (right) probes (FITC, green). Costaining of endogenous rpL19 (Cy3, red) was detected with a specific antibody. Dotted squares indicate the magnified area represented in the enlargement. Cy3 and FITC fluorescence monitored along the dashed lines is shown as relative signal intensity in the bottom panel. D, immunofluorescence microscopy of MCF-7 cells, which were untreated (−) or transfected with control siRNA (ctrl) or siRNAs directed against DDX6 cultured under normoxic or hypoxic conditions. Endogenous VEGF was stained with a specific antibody (FITC, the endoplasmic reticulum with an antibody directed against the KDEL endoplasmic reticulum retention signal (Cy3) and nuclei with DAPI. The merge represents an overlay of VEGF and KDEL endoplasmic reticulum staining. E, top, Western blot analysis of cytoplasmic extracts generated from MCF-7 cells, which were transfected with a control siRNA (ctrl) or DDX6 (2) siRNA and cultured under hypoxic conditions with antibodies specific for DDX6 and vinculin. E, middle, representative A_{260} profile of cytoplasmic extracts characterized in the top panel fractionated on 15–45% sucrose density gradients is shown. Polysomes, 80 S ribosomes, 60 S and 40 S ribosomal subunits, and mRNPs are indicated. E, bottom, endogenous VEGF mRNA distribution under hypoxic conditions in cytoplasmic extracts generated from cells transfected with control (ctrl) or DDX6 (2) siRNA was determined by qRT-PCR using the ΔΔCt method and normalized to CAT mRNA extraction control and endogenous β-actin mRNA. The percentage of VEGF mRNA in each fraction is shown.

FIGURE 6. Supernatants of DDX6-depleted MCF-7 cells enhance vascular tube formation by HUVECs. A, VEGF secretion into the culture supernatant was measured by ELISA in three independent transfection experiments designed as in Fig. 5D. Changes of VEGF levels were expressed as -fold change compared with untreated cells (−) under normoxia, which were set to 1. Data were analyzed with Student’s t test (two-tailed): *, p < 0.05; **, p < 0.01. B, analysis of vascular tubes formed by HUVECs in the presence of 75% culture supernatant from MCF-7 cells transfected with control siRNA (ctrl) or DDX6 (2) siRNA under hypoxia in absence and presence of the neutralizing VEGF antibody. The number of vascular tubes counted was set to 100% when supernatants from control siRNA transfections of cells grown under normoxia or hypoxia were applied. Data were analyzed with Student’s t test (two-tailed): **, p < 0.01.
Reduced pVHL-mediated ubiquitination of peptide p45 contributes to its stabilization under hypoxic conditions. AUF-1 p45 was suggested to modulate VEGF mRNA stability in human carcinoma cells and acts in concert with HuR via 3'-UTR interaction (47). Consistent with that, siRNA-mediated AUF-1 knockdown does not affect VEGF expression (data not shown), probably due to the stabilizing function of HuR.

HnRNP K that controls translation of specific mRNAs (28, 30, 48) interacts directly with the VEGF mRNA 3'-UTR (Fig. 4A). HnRNP K knockdown results only in a slight increase in VEGF expression and secretion, without affecting VEGF mRNA synthesis (data not shown).

The DEAD-box RNA helicase DDX6, which was also identified in the present study, has a strong binding preference for the VEGF mRNA 5'-UTR. DDX6 has been reported to be involved in the regulation of mRNA storage and translation in different systems (32, 49, 50). Under hypoxic conditions the cellular level of DDX6 decreases (Fig. 3, B and C). Reduction of DDX6 and subcellular rearrangement under hypoxia result in diminished interaction of DDX6 with VEGF mRNA (Fig. 3C). Colocalization of DDX6 with Dcp1A (Fig. 3E) suggested that DDX6-containing granular structures represent P-bodies, distinct cytoplasmic granules, in which translation of colocalized mRNAs is repressed or mRNA decay takes place (51–54). In contrast to hnRNP K, DDX6 binds preferentially to the 5'-UTR of VEGF mRNA (Fig. 4A). Addition of recombinant DDX6 to cytoplasmic extracts prepared from cells cultivated under normoxic conditions significantly inhibits the translation of a reporter mRNA bearing the VEGF 5'-UTR (Fig. 4C). Interestingly, recently an interaction of DDX6 with HIF-1α mRNA has been reported in murine hippocampus cells, and in HEK293 cells transfected with a bicistronic reporter translation initiated at the IRES of HIF-1α mRNA was shown to be activated when DDX6 depleting siRNAs were cotransfected (55).

Furthermore, biochemical and structural analysis indicated that DDX6 (Rck/p54) unwinds the IRES of c-myc mRNA, which might reduce c-myc IRES activity (56). Overexpression of DDX6 in HeLa cells led to down-regulation of endogenous c-myc mRNA expression (56). The elucidation of the mechanism by which DDX6 interferes with the translation of specific IRES-containing mRNAs will be an interesting task for further analyses in reconstituted in vitro translation systems.

Studies on translation initiation mediated by the c-myc and VEGF IRES provide evidence for a selective functional requirement of domains of the eukaryotic initiation factor (eIF) 4G (57). A direct contribution of the C-terminal fragment of eIF4G that lacks the N-terminal PABP and eIF4E interaction domain but retains RNA binding capacity was shown employing inducible expression systems (57). The direct interaction of the eIF4G C-terminal fragment with the VEGF IRES potentially promotes 43S complex formation under conditions like hypoxia (58) when phosphorylation of eIF2α compromises the formation of active ternary complexes (59) and mTOR pathway inhibition leads to withdrawal of 5'-cap-binding eIF4E due to accumulation of nonphosphorylated eIF4E binding protein 1 (4E-BP1) (60). Translational active cytoplasmic extracts of MCF-7 cells grown under normoxia or hypoxia will provide helpful tools to study the impact of eIF4G fragments and DDX6 on VEGF IRES-mediated translation in vitro.

Efficient siRNA-mediated reduction of DDX6 expression did not affect VEGF mRNA stability (Fig. 5B), but resulted in increased VEGF mRNA colocalization with rpL19. That colocalization could already be detected under normoxia, when the level of DDX6 is reduced by RNAi (Fig. 5C), indicating that VEGF mRNA could be translated under normoxia in the absence of DDX6. Furthermore, synthesis and secretion of VEGF protein were enhanced under hypoxia when DDX6 was depleted as detected by immunofluorescence microscopy and ELISA, respectively (Figs. 5D and 6A). This is consistent with the finding that endogenous VEGF mRNA accumulated in polysomal fractions when DDX6 was depleted by siRNA treatment from MCF-7 cells grown under hypoxia (Fig. 5E). Importantly, enhanced VEGF secretion from MCF-7 DDX6 knockdown cells increased the formation of vascular tubes by HUVEC cells when they were cultured in the presence of DDX6 knockdown supernatants (Fig. 6, A and B). This suggests that reduction of DDX6 under prolonged hypoxia activates expression of proangiogenic VEGF that is critical in fast growing tissues during normal development and in pathologic processes.

In future studies we will investigate how the level of DDX6 in MCF-7 cells is decreased under hypoxia. An increase of miR-130 under hypoxic conditions was shown to be involved in DDX6 down-regulation in murine hippocampus cells (55), but in MCF-7 cells miR-130 is hypoxia-insensitive (61).

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