TIAR and TIA-1 mRNA binding proteins co-aggregate under conditions of rapid oxygen decline and extreme hypoxia, suppress HIF-1α pathway and inhibit proliferation and angiogenesis

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III. Abbreviations

AMPK	adenosine monophosphate kinase
ARNT	aryl hydrocarbon nuclear translocator
ATCC	American Type Culture Collection
ATF6	activating transcription factor 6
ATP	adenosine triphosphate
AUR	AU-rich
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
CBP	cAMP response element binding protein
CMV	cytomegalovirus
DAPI	4', 6'- diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
FIH-1	factor inhibiting HIF-1
GCN2	serine/threonine-protein kinase GCN2
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HBSS	Hank's buffered salt solution

HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
HRE	hypoxia-responsive element
HRI	heme regulated inhibitor
HRP	Horseradish peroxidase
HUSAR	Heidelberg Unix Sequence Analysis resources
HUVEC	human umbilical vein endothelial cells
IRE1	endoplasmic reticulum-to-nucleus signaling 1
MAPK	Mitogen-activated protein kinase
MCS	multiple cloning site
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NTPs	nucleoside triphosphate
P300	E1A binding protein p300
P53	protein 53
PCR	polymerase chain reaction
PERK	pancreatic endoplasmic reticulum eIF2alpha *kinase*
PHD2	prolyl hydroxylase domain protein 2
PKR	protein kinase R
POP6	performance-optimized polymer 6
Pro	proline
RACK1	receptor for activated C-kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature

SDS	sodium dodecyl sulfate
siRNA	small interfering ribonucleic acid
TIA-1	T-cell intracellular antigen 1
TIAR	TIA-1 related protein
TK	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
TSR	template suppression reagent
UV	ultraviolet
VEGF	vascular endothelial growth factor
VHL	von Hippel–Lindau tumor suppressor protein
BCA	bicinchoninic acid
PVDF	polyvinylidene fluoride
ECL	enhanced chemi-luminescence
BrdU	5-bromo-2’-deoxyuridine
HBSS	Hank's Buffered Salt Solution

1 Introduction

Cellular hypoxia occurs under physiologic conditions during development (1, 2), physical activity (3) and exposure to hypoxic environment (4, 5). Hypoxia is also caused by various pathophysiological conditions. Among them are pulmonary (6), cardiac and vascular diseases (7), inflammation (8, 9), transplantation (10) and cancer (11, 12).

Hypoxia induces cellular adaptation processes at the level of transcription and translation. These adaptive responses often depend on the degree of hypoxia (13, 14).

1.1 Hypoxia and gene regulation by HIF-1

Hypoxia stabilizes and transactivates the transcription factor HIF-1, that initiates a wide gene expression program to handle the hypoxic situation (for review see (15)). In particular, HIF-1 induces target genes that attenuate mitochondrial respiration and induces glycolytic ATP generation.

Furthermore, HIF-1 increases oxygen transfer and induces angiogenesis a multilayer process that requires proliferation and migration of endothelial cells. HIF-1 is involved in this process since it induces several genes encoding pro-angiogenic factors and components (16). The most prominent factor represents the vascular endothelial growth factor (VEGF) and its proangiogenic receptor VEGF-R2 (17-19).

Also, HIF-1 causes a cell cycle arrest by targeting cyclin-dependent kinase inhibitors thus inhibiting proliferation (20). These effects favor cell survival and increase the resistant of cells towards hypoxia. On the other hand, HIF-1 induces apoptosis by induction of various pro-apoptotic proteins (21-23).

HIF-1 consists of an O_2-regulated HIF-1α and a constitutively expressed HIF-1β subunit (24). In well-oxygenated cells, HIF-1α is hydroxylated at proline residue 402 (Pro-402) and/or Pro-564 by prolyl hydroxylase domain protein 2 (PHD2), which uses O_2 and α-ketoglutarate as substrates in a reaction that generates CO_2 and succinate as byproducts (15). Prolylhydroxylated HIF-1α is bound by the von Hippel–Lindau tumor suppressor protein (VHL), which recruits an E3-ubiquitin ligase that targets HIF-1α for proteasomal degradation (Figure 1). Asparagine 803 in the transactivation domain is hydroxylated in well-oxygenated cells by factor inhibiting HIF-1 (FIH-1), which blocks the binding of the coactivators p300 and CBP (25, 26). Under hypoxic conditions, the prolyl and asparaginyl hydroxylation reactions are inhibited by substrate (O_2) deprivation and/or the mitochondrial generation of reactive oxygen species (ROS), which may oxidize Fe(II) present in the catalytic center of the hydroxylases (27). Stabilized HIF-1α interacts with the constitutively expressed HIF-1β (aryl hydrocarbon nuclear translocator; ARNT). The HIF-1 complex then translocates to the nucleus and activates genes with hypoxia-responsive elements in their promoters (28).

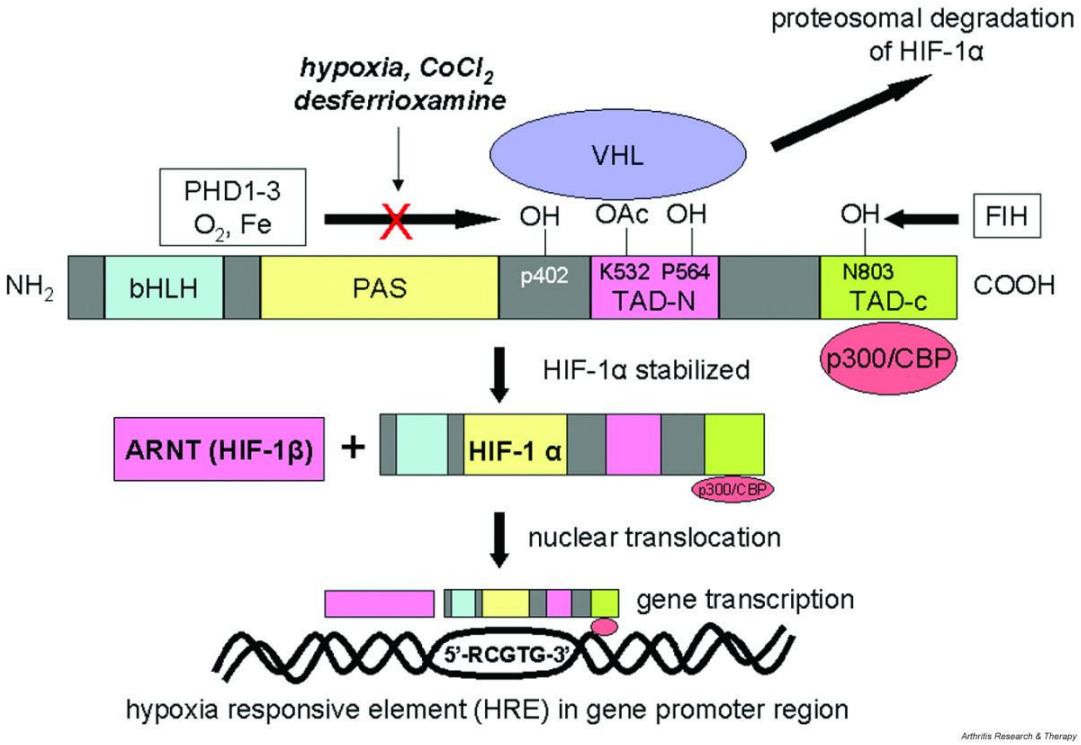


Figure 1. Hypoxic regulation of the hypoxia-inducible factor-1α (HIF-1α)
Regulation of the hypoxia-inducible factor-1α (HIF-1α) occurs primarily through inhibition of its degradation in hypoxia. Under normoxic conditions, HIF-1α undergoes rapid proteosomal degradation once it forms a complex with von Hippel–Landau tumor suppressor factor (VHL) and E3 ligase complex. This requires the hydroxylation of critical proline residues by a family of HIF-1α-specific prolyl hydroxylases (PHD-1,2,3), which requires O_2 and several cofactors, including iron. Under hypoxic conditions, or when iron is chelated or competitively inhibited, proline hydroxylation does not occur, thus stabilizing HIF-1α and allowing it to interact with the constitutively expressed HIF-1β (aryl hydrocarbon nuclear translocator; ARNT). The HIF-1 complex then translocates to the nucleus and activates genes with hypoxia-responsive elements in their promoters. bHLH, basic helix-loop-helix; CBP, cAMP response element binding protein; FIH, factor inhibiting HIF-1α; PAS, PER-ARNT-SIM; TAD, transactivation domain.(28)

1.2 Hypoxia and regulation of translation

Regulation of translation is an important aspect of controlling gene expression at a superior level, allowing cells to rapidly respond to varying intracellular and extracellular stress, such as hypoxia (29-32) thus interfering with HIF-dependent gene regulation. This is affected by mechanisms targeting the general translation efficiency. Also, the fate of specific mRNAs

is targeted by various mRNA binding proteins affecting mRNA decay (33, 34), mRNA stability (35, 36), mRNA splicing (37) and mRNA translation (38).

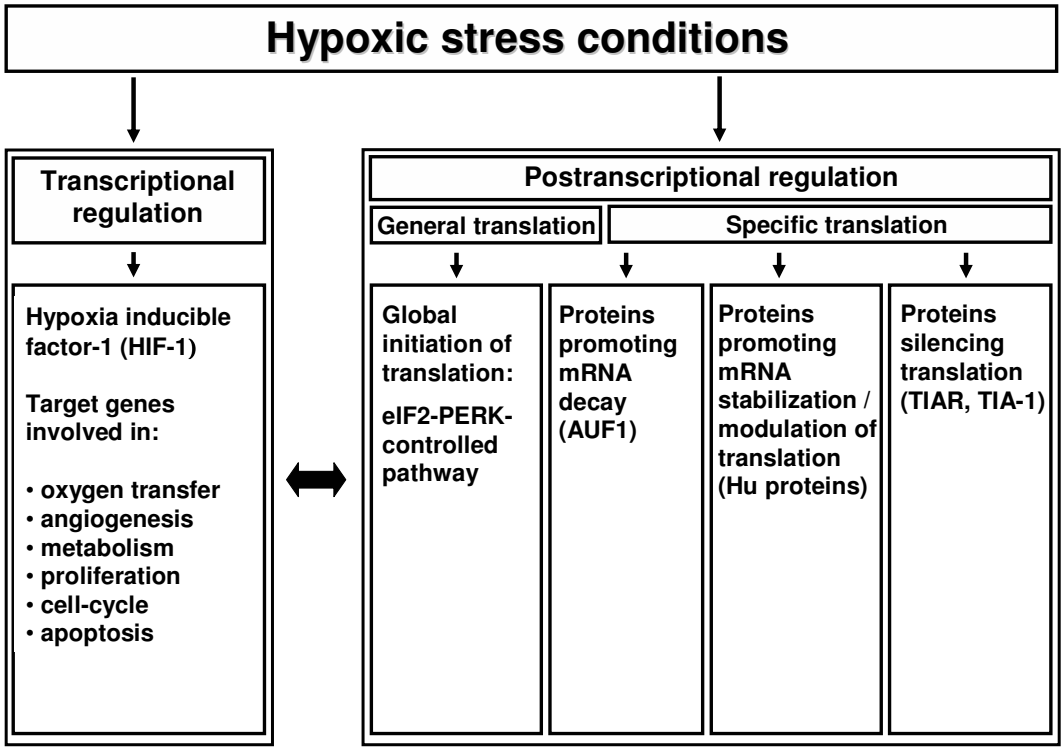


Figure 2. Transcriptional and posttranscriptional regulation under hypoxic stress conditions.
Hypoxic stress conditions affect regulation at the transcriptional by induction of HIF-1a that initiates a wide gene expression program to handle the hypoxic situation. Its target genes are involved in various processes among them are oxygen transfer, angiogenesis, metabolism, proliferation, cell-cycle and apoptosis. Induction of genes is also controlled at the posttranscriptional level. These mechanisms include modulation of general translation by the eIF2-PERK controlled pathway and also translation of specific mRNAs. This is mediated by proteins promoting mRNA decay like AUF1 (33, 34), by proteins promoting mRNA stabilization like Hu proteins (35, 36) and by proteins that silence the translation (general translation or translation of specific targets) like TIAR and TIA-1 (39, 40).

1.2.1 Regulation of general translation

Global translation is regulated by the initiation of translation, which depends on the action of the eukaryotic initiation factor (eIF) family members (41, 42). A key regulatory process that targets translation is the activity of the

endoplasmic reticulum (ER) kinase (PERK) which can be phosphorylated in response to activation of the ER stress pathway under hypoxic conditions. In turn, PERK phosphorylates eIF2α and blocks global initiation of translation (43). Other pathways can also be initiated in response to hypoxia, for example by activation of adenosine monophosphate kinase (AMPK), phosphoinositide 3-kinase, or by the ER transmembrane proteins IRE1 and ATF6, which interfere with translational control (30, 44-46).

1.2.2 TIAR and TIA-1 mRNA binding proteins

The mRNA binding proteins T-cell intracellular antigen 1 (TIA-1) and TIA-1 related protein (TIAR) affect mainly the stabilization, splicing and access for translation of mRNAs. TIAR/TIA-1 proteins can aggregate to visible stress granules under stress conditions in a reversible manner. Stress granules represent a kind of activated stage of these proteins assembled with additional components in distinct aggregates both stabilizing and silencing certain mRNAs. TIA proteins are important for determining the fate of mRNAs, including the regulation of splicing, stability, storage, and translation efficiency (39, 47-51).

The TIAR/TIA-1 proteins can selectively target specific mRNAs by binding to specific adenosine-uridine rich (AUR) elements located at the 3′ end of the target mRNAs (39, 40, 52-54).

The TIA-proteins are characterized by three RNA-binding domains and a glutamine-rich carboxyl-terminal domain that enable aggregation to insoluble aggregates.

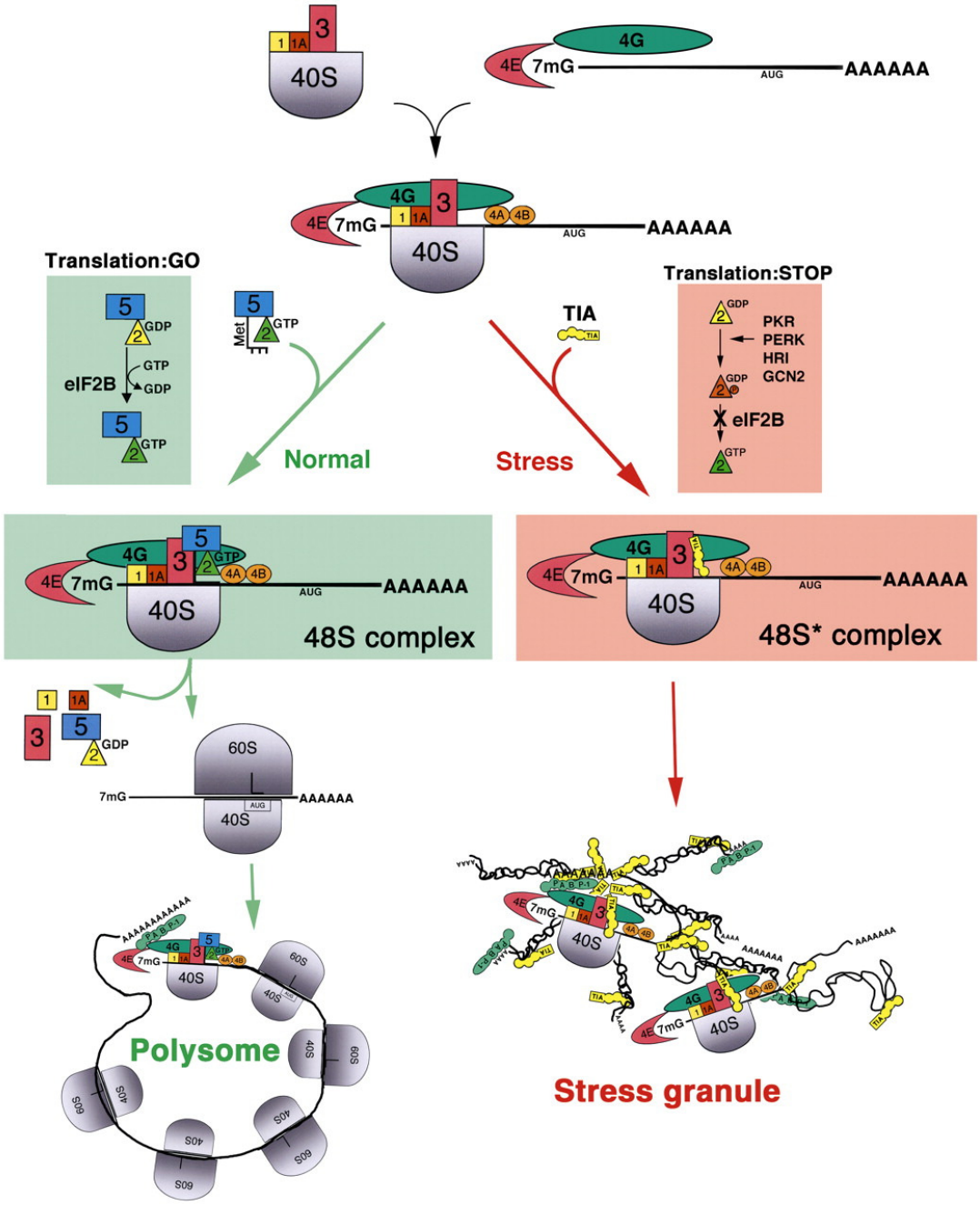


Figure 3. Translational initiation in the absence or presence of stress.

(Green panels) In the absence of stress, eIF2B promotes the charging of the eIF2-GTP-tRNAMet ternary complex by exchanging GDP for GTP. When the eIF2-GTP-tRNAMet ternary complex is available, a canonical 48S preinitiation complex is assembled at the 5' end of capped transcripts (green arrow: Normal) and scanning begins. Upon recognition of the initiation codon by the anticodon of tRNAMet, eIF5 promotes GTP hydrolysis, and early initiation factors are displaced by the 60S ribosomal subunit. As additional ribosomes are added to the transcript, the mRNA is converted into a polysome. (Red panels) In stressed cells (red arrow: Stress), phosphorylation of eIF2α by PKR, PERK, HRI or GCN2 converts eIF2 into a competitive antagonist of eIF2B, depleting the stores of eIF2/GTP/tRNAMet. Under

these conditions, TIA-1 is included in a non-canonical, eIF2/eIF5-deficient 48S* preinitiation complex (composed of all components of the 48S pre-initiation complex except eIF2 and eIF5) that is translationally silent. TIA-1 self-aggregation then promotes the accumulation of these complexes at discrete cytoplasmic foci known as stress granules. Blue square, eIF5; green triangle, eIF2 bound to GTP; yellow triangle, eIF2 bound to GDP; red triangle, phospho-eIF2 bound to GDP.(55)

Formation of stress granules has been demonstrated to display anti-apoptotic effects favoring cell survival under certain conditions. In this regard, the scaffold protein RACK1 was revealed as a crucial mediator. However, the anti-apoptotic effects related to stress granule formation can differ between certain conditions and may depend on the severity of the stress stimuli (56).

In this context it is of note, that TIAR/TIA-1 proteins were demonstrated to exhibit a pro-apoptotic role in thymocytes. This was suggested to be mediated by its effects on translation and differential splicing of certain target-mRNAs. However, in this study, the formation of stress granules by certain stress stimuli was not induced (57).

Targeted disruption of TIAR or TIA-1 result in embryonic lethality. Interestingly, the penetrance of lethality depends on the mice strains and is higher for TIAR than for TIA-1. Embryonic lethality is suggested to be related to the requirement of TIAR/TIA-1 for cell viability (58, 59).

Hypoxia has been suggested as a trigger for TIAR/TIA-1-dependent stress granule aggregation in different studies and organisms (31, 60, 61). Also, stress granules have been suggested to contribute to the development of therapy-resistant cancer cells (62, 63), in particular in hypoxic tumor areas (64, 65).

2 Aims of the Study

Hypoxia represents a condition of cellular stress that causes adaptation processes at different cellular levels such as the regulation of transcription and translation.

Hypoxia-dependent control of transcription is mainly dependent on the transcription factor HIF-1 that is specifically induced in hypoxia.

Hypoxia-dependent control of translation is mediated by different mechanisms involving regulatory components of the general translational machinery. Also, mRNA binding proteins such as TIAR/TIA-1 contribute by affecting the availability of specific mRNAs for translation. TIAR/TIA-1 proteins can aggregate to visible stress granules under stress conditions in a reversible manner representing an activated form of these proteins.

Hypoxia is a dynamic stress condition that strongly can vary in its severity with regard to the cellular responses. These responses can cause changes in cellular function with impact on proliferation and angiogenesis.

In this context, this work addressed the following questions:

1) Does hypoxia affect the formation of TIAR/TIA-1 containing stress granules in A549 cells and is the severity of hypoxia (absolute oxygen concentration and the kinetic of oxygen partial pressure decline) of importance in this regard?
2) Do TIAR/TIA-1 proteins have any effects on the abundance and activity of HIF1α and are these effects dependent on stress granules formation?
3) Do TIAR/TIA-1 proteins have any effect on proliferation and angiogenesis?

3 Materials and Methods

3.1 Cell Culture

3.1.1 Culture of the human lung adenocarcinoma A549 cell line

Human lung adenocarcinoma A549 cell line, which was used in part of the experiments, was purchased from American Type Culture Collection (ATCC). The culture of the the human lung adenocarcinoma A549 cell line was performed according to the protocol given by the American Type Culture Collection (ATCC). The cells frozen in 10 % dimethylsulfoxid (DMSO) in liquid nitrogen (approx. 5 x 10^6 cells/ml) were thawed rapidly at 37 °C and then added drop wise to 100 mm dish containing 10 ml of pre-warmed DMEM/F12 (DMEM : F-12 (1:1) 500 ml, -L-Glutamin, Gibco, Karlsruhe) (1:1) culture medium (supplemented with 10 % FCS (v/v) (fetal calf serum, heat inactivated for 30 min at 56 °C, sterile filtered, (PAA Laboratories GmbH, Pasching, Österreich), 1 % (v/v) Gentamycin/Penicillin G (lyophilized, sterile, Gibco, Karlsruhe), 1 % vitamins (MEM Vitamin solution, Gibco, Karlsruhe), 1 % L-glutamine (Gibco, Karlsruhe), and 1 % non essential amino acids (MEM Non-Essential Amino Acids, Gibco, Karlsruhe). When the cells became confluent, they were detached with 5 ml 1x trypsin (Gibco, Karlsruhe) per 100 mm plate (Greiner Bio-One, Germany) for approximate 2 min at 37 °C. The reaction was stopped by adding 1 ml of FCS which contains trypsin inhibitors. For continuous culture, about 1/4 of the medium containing A549 cells were transferred to a fresh plate and cultured at 37°C in a gas controlled incubator (IR 1500 Automatic CO_2 Incubator, Flow Laboratories GmbH, Meckenheim) with water-saturated atmosphere containing 5% CO_2 .

A549 Medium (500ml)

DME-M/F12	430 ml	86 %
FCS	50 ml	10 %
Penicillin/Streptomycin	5 ml	1 %
Vitamins	5 ml	1 %
Non-essentials Aminoacids	5 ml	1 %
Glutamine	5 ml	1 %

1 x Trypsin Volume (100ml)

10 x Trypsin	10 ml	10 %
HEPES (200 mM)	10 ml	10 %
Isotonic NaCl (0.9 %)	80 ml	80 %

3.1.2 Culture of human umbilical vein endothelial cells (HUVEC)

The endothelial cells, kindly provided by Dr. K. Mayer (University of Giessen Lung Center (UGLC), Justus-Liebig-University of Giessen, Giessen, Germany), were isolated from human umbilical veins (Human Umbilical Veins Endothelial Cells). Briefly, cells obtained from collagenase digestion were washed, pooled, centrifuged for 10 min at 210g, and resuspended in fresh medium. Before splitting, cells were grown for 2 to 3 days on T 75 culture flasks (Greiner Bio-One, Germany) coated with gelatine in an atmosphere of 95% O_2 and 5% CO_2. For the experiments HUVECs from passage 2 and 3 were used. Cells were cultured in Endothelial Cell Growth Medium (containing 10ml FCS, 2ml ECGS/H, 0.1ng EGF/ml, 0.1ng bFGF, 0.1µg Hydrocortison/ml in 500ml of medium) supplemented with Supplement Mix C-39215 (all from PromoCell GmbH, Heidelberg, Germany).

3.2 Hypoxia experiments

3.2.1 Hypoxia inducing methods

A hypoxic environment was prepared in a chamber equilibrated with a water-saturated gas mixture and set to 1% O_2, 5% CO_2 or to 0.1% O_2, 5% CO_2 (Innova CO-48, New Brunswick Scientific, Edison, NJ, USA). For generating a rapid oxygen decline, EC-Oxyrase® (Oxyrase Inc.**,** Mansfield, Ohio, USA) an enzyme that consumes O_2 was added to the cell culture medium according to the company recommendations at a dilution of 1:100 together with sodium lactate (Sigma-Aldrich, St. Louis, MO, USA) at concentration of 20 mM. For measuring of the O_2 partial pressure in the cell culture medium, the Licox Oxygen Monitoring System (Integra, New Jersey, USA) was employed.

3.2.2 Validating hypoxia within the cells using Hypoxiprobe™-1

For validating the hypoxia within the A549 cells, the Hypoxiprobe™-1 kit (HPI, Inc., Burlington, MA, USA) was employed. This kit consists of two principal components: pimonidazole hydrochloride, a small molecule hypoxia marker that selectively binds to oxygen starved cells and Hypoxyprobe™-1-Mab1, a monoclonal antibody that was used to detect pimonidazole adducts using immunocytoflorescence. During the incubation of the A549 cells in hypoxic conditions, pimonidazole hydrochloride was added in a concentration of 200 µM in the cell culture medium of the cells. After the incubation time (3 h for Oxyrase and 4 h for hypoxic gas incubator), the cells were fixed and immunostained according to the protocol described in the next section.

3.3 Immunocyto- and immunohisto-fluorescent analyses

3.3.1 Antibodies

Primary antibodies

Antibody	Company	Dilution
c-Myc, raised in rabbit	AbCam, Cambridge USA	1:500
HIF-1α raised in rabbit	LifeSpan Biosciences, Seattle, WA, USA	1:200
lamin B, raised in goat	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:250
TIA-1 raised in goat	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:200
TIAR, raised in mouse	BD Biosciences, San Jose, California USA	1:200

Fluorescencent labelled secondary antibodies

Antibody	Company	Dilution
donkey anti-goat-cy3	Chemicon, Temecula, CA, USA	1:400
donkey anti-mouse-Alexa 488	Invitrogen, Carlsbad, CA	1:1000
donkey anti-rabbit-Alexa 488	Invitrogen, Carlsbad, CA	1:1000
goat anti-mouse-Alexa 555	Invitrogen, Carlsbad, CA	1:1000

3.3.2 Immunostaining protocol

Immunocytofluorescence was performed with A549 or HUVEC. For this purpose 30000 cells were cultured per each chamber slide well (Lab-Tek Thermofisher Scientific, Denmark) and then treated as indicated.

Immunohistofluorescence was performed from cryosections (5 µm) of subcutaneous A549 xenograft tumors frozen in liquid nitrogen. The cryosection were obtained using a Cryostat set for -20°C (Leica CM1850 UV-Kryostat, Leica Microsysteme Vertrieb GmbH, Bensheim) equipped with a microtome blade (Mikrotom Messer, S-35, Feather, PFM Produkte für die Medizin AG, Köln). The cryosection were fixed on glass slides (Super Frost® Plus Objektträger, 25 x 75 x 1 mm, R. Langenbrinck, Labor- und Medizintechnik, Teningen) and stored in -20 °C.

The cells or tissues were fixed in 4 % paraformaldehyde for 20 min and then washed 3-times with phosphate buffered saline containing 0.1 % BSA and 0.2 % Triton X-100. The non-specific binding sites were blocked with the same buffer enriched with 10 % horse serum for 30 min. The primary antibody was added and incubated for 2 h. After washing, the secondary antibody was added and incubated for 1 h at room temperature. Counterstaining of the nuclei was performed by addition of 1 µM DAPI (Sigma- Aldrich Chemie GmbH, Munich, Germany) for 5 min. Images were recorded using a Leica DMLA Q550/W microscope (Leica Microsysteme Vertrieb GmbH, Bensheim, Germany) with Leica Q-Win standard software.

3.4 Western-blot analysis

3.4.1 Samples preparation

The samples for Western-blot were prepared by plating 150000 cells on 6 well-plates (Greiner Bio-One, Germany) and using 2 wells for each group. The cells were transfected with si-RNA and/or cultured under hypoxic conditions,

depending on the experiment design. Cells were lysed in Laemmli buffer and placed on ice. The samples were heated at 90 °C for 5 min and the protein concentration was measured according to the Pierce BCA protein assay. Afterwards the samples were mixed with β-mercaptoethanol and bromophenol blue, and then loaded in equal protein amounts onto a sodium dodecyl sulfate polyacrylamide gel.

Laemmli 4X (100 ml)

Tris-HCl pH 6.8 (250 mM)	3 g
40 % (v/v) Glycerol	40 ml
8 % (p/v) SDS	5 g
H_20	to 100 ml

*Add 10 % ß-mercaptoethanol and 0.005% (p/v) Bromophenol Blue only after measuring the protein concentration and before use.

3.4.2 BCA protein concentration assay

The *BCA protein assay kit* (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) was used for the measurement of the protein concentration. This method is based on peptide bonds mediated reduction of Cu^{2+} to Cu^{1+} that interacts with bicinchoninic acid (BCA) generating a purple-colored product and it is useful for the measurement of protein concentrations in buffers containing various chemical agents including SDS. Thus, samples dissolved in 1x Laemmli buffer were directly applicable for the protein concentration measurement. Preparation of standards (differently diluted bovine serum albumin), incubation conditions, spectrophotometric

measurement and calculation of protein concentration were performed according to the company's protocol. The absorbance of the purple color product was measured on a 96 well plate (Nunc, Roskilde, Denmark) at 492nm with a spectrofluorometer (FL-600, BioTek Instruments GmbH, Bad Friedrichshall, Germany). As background, values of absorbance of relevant buffer(s) were used.

3.4.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The gel electrophoresis, i.e. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was performed with self-made 10% polyacrylamid gel. Equal amount of protein (between 5 µg and 50 µg, depending on the analyzed protein) was loaded from each sample on the gel In SDS-PAGE the denatured polypeptides bind SDS and become negatively charged. The amount of bound SDS is always proportional to the molecular weight of the polypeptide and is independent of its size and charge, therefore the SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the molecular weight of the polypeptides. By using prestained SDS-PAGE protein marker, PageRuler (Fermentas Life Sciences, Burlington, Ontario, Canada), it is therefore possible to estimate the molecular weight of the polypeptide chains. The electrophoresis was performed with 100 V constant in electrophoresis chambers (Bio-Rad, Hercules, CA, USA) and the gel was run till the bromophenol blue reaches the bottom of the separating gel (for about 2 h). Then, the gel can be used for western-blot analysis.

Separating Buffer Final concentration

Tris	1.125 M
Saccharose	30 %
pH	8.8

Collecting Buffer Final concentration

Tris	0.625 M
pH	6.8

1 x Running Buffer Final concentration

Tris	25 mM
Glycine	192 mM
SDS	0.1 %

3.4.4 Electro blotting of immobilized proteins

The separated proteins on the SDS-polyacrylamide gel were electrically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA) by semi dry electro blotting. The PVDF membrane was activated by methanol before use. The transfer equipment was prepared in the following way: two layers of blotting paper (Bio-Rad, Hercules, CA, USA) soaked with transfer buffer followed by activated PVDF membrane washed with transfer buffer were placed onto the electro-blotting chamber (Bio-Rad, Hercules, CA, USA). On the PVDF membrane, the gel and the other two layers of blotting paper soaked with transfer buffer were placed. The cathode and anode from the power supply were connected with

the electro-blotting chamber. Electro blotting was performed at constant current (2 mA/cm^2) for 90 min.

Transfer Buffer Final concentration

Tris	25 mM
Glycine	192 mM
Methanol	20 %
SDS	0.01 %

3.4.5 Immunological detection of immobilized proteins

Antibodies used in the experiments are all commercially available:

Primary antibodies

Antibody	**Company**	**Dilution**
HIF-1α, raised in mouse	BD Biosciences / California, USA	1:3000
TIA-1 raised in goat	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:500
TIAR, raised in mouse	BD Biosciences, San Jose, California USA	1:1000
β-actin, raised in mouse	Abcam, Cambridge, UK	1:10000

HRP-conjugated secondary antibodies

Antibody	Company	Dilution
Donkey Anti-goat IgG	Thermo scientific, Pierce, Rockford, IL, USA	1:2000
Goat anti-mouse IgG	Thermo scientific, Pierce, Rockford, IL, USA	1:2000
Goat anti-rabbit IgG	Thermo scientific, Pierce, Rockford, IL, USA	1:2000

The membrane was blocked with 1 x NET buffer containing 0.25% skin porcine gelatin (Sigma-Aldrich, St. Louis, MO, USA) at room temperature (RT) for 2 h followed by incubation with primary antibody overnight at 4 °C. Then, the membrane was washed and incubated with horse radish peroxidase-conjugated secondary antibodies for 2 h. Immunoreactive signals were detected by ECL Plus Western Blot Detection System (GE Healthcare, Amersham™, Buckinghamshire, UK) employing an imager system (Fluorchem™ IS-8900, Alpha Innotech, San Leandro, CA, USA).

Blocking Buffer Final concentration

NaCl	150 mM
EDTA (pH = 8.0)	5 mM
Tris	50 mM
Triton X-100	0.05 %
Gelatine	0.25 %

10 x NET buffer Final concentration

NaCl	1.5 M
EDTA (pH = 8.0)	50 mM
Tris	500 mM
Triton X-100	0.5 %

3.5 Small interfering RNA

Selective targeting of TIAR and TIA-1 was performed using specific small interfering RNAs (siRNAs). As a control, a siRNA sequence (si-con) that does not target any gene in the human genome and has been tested by micro-array analysis (Dharmacon Inc., Chicago, IL, USA) was employed. The siRNAs were synthesized commercially (Biomers.net GmbH, Ulm, Germany).

The sequences were as follows:

si-con

Forward 5′-UAGCGACUAAACACAUCAA dTdT-3′

Reverse 5′- UUGAUGUGUUUAGUCGCUA dTdT -3′

si-TIAR

Forward 5′- GGGCUAUUCAUUUGUCAGA dTdT -3′

Reverse 5′- UCUGACAAAUGAAUAGCCC dTdT -3′

si-TIA-1

Forward 5′- CGAUUUGGGAGGUAGUGAA dTdT -3′

Reverse 5′- UUCACUACCUCCCAAAUCG dTdT -3′

3.6 Transfection of A549 and HUVEC cells

The liposome mediated transfection method was employed for transfection of A549 cells. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent was used for transfection of plasmid or si-RNA or both in A549 and HUVEC cells. One day before transfection, appropriate numbers of A549 or HUVEC cells was plated on respective culture dishes (all from Greiner Bio-One, Germany) with culture medium so that they will become 90-95 % confluent for plasmid and 60 % confluent for si-RNA transfection at the time of transfection. DNA and/or si-RNA was diluted in Opti-MEM® reduced serum medium and mixed gently. Lipofectamine 2000 was mixed gently before use and then diluted in the appropriate amount of Opti-MEM® medium. Mixtures were mixed gently and incubated for 5 min at room temperature. After 5 min incubation, the diluted DNA and/or si-RNA and the diluted Lipofectamine 2000 were mixed and incubated for 20 min at room temperature to allow the DNA-Lipofectamine 2000 complexes to form. DNA-lipofectamine complexes were added to each well containing cells and medium and mixed gently by rocking the plate back and forth. Cells were incubated in CO_2 incubator at 37 ℃ for 5 h and the medium was replaced. Cells were further incubated either in normoxic or hypoxic incubator according to the experiment need.

Protocol for transfection of A549 or HUVEC cells

Method	Culture vessel	Seeding cell number	Total volume per well (µl)	Plasmid DNA (µg)	Si-RNA (µl from 40nmol stock)	DNA/ RNA dilution volume (µl)	Lipid dilution volume (µl)
Western-Blot	6-wells plate	150000	2500	-	2.5	250	250
Cell-count assay	24-wells plate	5000	500	-	0.5	50	50
Reporter gene assay or immunocyto-fluorescence	48-wells plate or chamber -slides	30000	250	0.3	0.25	25	25
BrdU incorporation assay	96-wells plates	1000	150	-	0.15	25	25

3.7 Reporter gene assay

3.7.1 Recombinant plasmids

The pGL3-TK plasmid (Promega) with the thymidine kinase minimal promoter was used to construct the HRE-plasmid employing the NheI and XhoI restriction sites. For cloning, forward and reverse oligonucleotides corresponding to the HRE from the phosphoglycerate kinase (PGK) gene (66) was used after annealing and restriction digest with NheI and XhoI: HRE-PGK: CTA GCG CGT CGT GCA GGA CGT GAC AAA TAG CGC GTC GTG CAG GAC GTG ACA AAT AGC GCG TCG TGC AGG ACG TGA CA AAT. Finally, a construct with five repeats of HRE-PGK ligated to the 5′ end of the TK-mp promoter were isolated and verified by sequencing. The pGL4.13 plasmid (Promega) was used for construction of the AUR plasmid. A synthetic sequence of the HIF-1α 3′ end with the putative AUR elements (CTA GGT ATT TAA ACC ATT GCA TTG CAG TAG CAT CAT TTT AAA AAA TGC ACC TTT TTA TTT ATT TAT TTT TGG CT) was used to ligate this sequence into the XbaI site located at the 3′ end of the firefly luciferase gene.

Transfection of A549 cells by plasmids was performed employing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

3.7.2 Experiment design and measurement of the Luciferase activity

For the reporter gene assay 30000 A549 cells were plated on 48-wells plates (Greiner Bio-One, Germany) and transfected with plasmid (HRE or AUR-luciferase reporter plasmid) and si-RNA. After 24h, the experiment was stopped (AUR experiments) or the cells were cultured for 4 h under hypoxic condition and then stopped (HRE experiments).

The detection of luciferase activity in the cells transfected with reporter vectors containing the firefly luciferase gene was performed with the luciferase reporter assay kit (Promega, Mannheim, Germany). The luciferase assay is based on the enzyme-catalyzed chemiluminescence. Luciferin present in the luciferase assay reagent is oxidized by luciferase in the presence of ATP, air oxygen and magnesium ions. This reaction produces light with a wavelength of 562 nm that can be measured by a luminometer. After washing once with 1 x PBS, the transfected cells were shaken for 15 min in 100 µl of 1 x lysis buffer, then frozen in -80 °C overnight and thawed at room temperature next day for measurement. For measurement of firefly luciferase activity, 20 µl of the lysate were mixed in white and flat bottom 96 well plates with 100 µl luciferase assay reagent, which was freshly prepared by mixing substrate and the luciferase assay buffer.

The activity of luciferase in cells transfected with AUR- and HRE-reporter plasmids was measured as relative light units (RLU) with the Luciferase Assay System (Promega Corporation, Madison, WI, USA) employing a Spectrofluorometer (FL-600 BioTek Instruments GmbH).

5 x lysis buffer (pH 7.8)	Volume	Final concentration
1 M Tris	25 ml	125 mM
200 mM EDTA	10 ml	10 mM
500 mM DTT	4 ml	10 mM
85 % Glycerol	115 ml	50 %
Triton X-100	10 ml	5 %
H_2O	to 200 ml	

3.8 Cell count assay

Cell proliferation was measured by cell count assay. This method consists in counting the cells from distinct groups treated differently. On the first day 5000 A549 cells were plated on 24 wells plates (Greiner Bio-One, Nürtingen), each group consisting on 3 wells. On the second day the cells were transfected with si-RNA according to the si-RNA transfection protocol. For each time-point was prepared a 24 well plate consisting of all the analyzed groups. The considered time-points were 1 day, 2 days, 4 days and 6 days. At the specified time-point the cells from each well from the respective plate were detached using trypsine and counted.

3.9 BrdU incorporation assay

Cell proliferation was also measured by BrdU incorporation assay (Roche, Mannheim, Germany). This method is based on the incorporation of the pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. After it's incorporation into DNA, BrdU is detected by colorimetric immunoassay. One day before experiment, A549 cells were plated in 96 well plates (1000 cells/well). On the second day, transfection of si-RNA was performed as described above and medium was

replaced by DMEM low-serum medium containing antibiotics (0.1 % (v/v) FCS, 1 % (v/v) penicillin and streptomycin) and incubated overnight for cellular synchronization. On the third day, the cells were cultured with normal culture medium. On the forth day was performed the BrdU incorporation (final con. 10 µM) for 4 h. After labeling with BrdU, the cells were fixed with FixDenat (200 µl/well) for 30 min at RT, and then the FixDenat solution was removed thoroughly by tapping and washed once with 1 x PBS before 100 µl of anti-BrdU-POD working solution was added to each well for 1 h. Afterwards, the anti-BrdU-POD solution was removed and each well rinsed three times with 300 µl of washing solution, 100 µl of peroxidase substrate (tetramethyl benzidine, TMB) was added for 5 min at RT, then 25 µl of 1M H_2SO_4 was added to each well and incubated for approximately 1 min on the shaker to stop the substrate reaction. The absorbance of the samples was measured at 450 nm excitation and 690 nm emission by spectrophotometer (FL-600, BIO-TEK Instruments, Inc., USA). The values given in the figures represent the raw data subtract blank control obtained by photometric measurement at 450 nm excitation and 690 nm emission.

3.10 Tube formation assay

Tube formation assay was employed for assessing the angiogenesis in vivo. For this purpose there were employed HUVEC, which were transfected with si-RNA, according to the protocol, 24 h before the experiment. On the day of the experiment, 250 µl liquid BD Matrigel™ (BD Biosciences, Canada) was pipetted on each well from a 24 well-plate (Greiner Bio-One, Nürtingen) and let for 30 min in a 37 °C incubator to solidify. Afterwards 60000 HUVEC were plated on each BD Matrigel™ containg well in 350 µl HUVEC medium. The cells were incubated at 37 °C for 16 h. Afterwards the wells were carefully washed with Hank's buffered salt solution (HBSS) and then 300 µl Calcein AM (Fluka, BioChemika) in a dilution of 8 µg/ml was added on each well and

incubated for 30 min at 37 °C. The wells were again washed with HBSS and then pictures could be taken using the fluorescent filter and the 4x magnification of the microscope (Olympus Mikroskop, Gerätetyp JMT-2, Inverses Forschungsmikroskop, Olympus Deutschland GmbH, Hamburg).

Hank's Stock Solutions

Stock 1

Dissolve the following in 90ml of distilled H_2O

8.0 g NaCl

0.4 g KCl

qs to 100 ml with distilled H_2O

Stock 2

Dissolve the following in 90ml of distilled H_2O

0.358 g Na_2HPO_4 (anhydrous)

0.60 g KH_2PO_4

qs to 100 ml with distilled H_2O

Stock 3

Add 0.72 g $CaCl_2$ to 50ml of distilled H_2O

Stock 4

Add 1.23 g $MgSO_4x7H_2O$ to 50ml of distilled H_2O

Stock 5

Add 0.35 g $NaHCO_3$ to 10ml of distilled H_2O

Hank's Premix

Combine the solutions in following in order:

10.0 ml Solution 1

1.0 ml Solution 2

1.0 ml Solution 3

86.0 ml distilled H_2O

1.0 ml Solution 4

Hank's full strength (mix prior to use)

9.9 ml Hank's Premix

0.1 ml Stock 5

Hank's (Full Strength with carbonate) composition

0.137 M NaCl

5.4 mM KCl

0.25 mM Na_2HPO_4

0.44 mM KH_2PO_4

1.3 mM $CaCl_2$

1.0 mM $MgSO_4$

4.2 mM $NaHCO_3$

3.3 Statistical analysis

All the data in the figures and text are expressed as means ± SEM of n independent observations unless indicated otherwise. Statistical evaluation was performed by unpaired t-test.

4 Results

4.1 Formation of TIAR/TIA-1 containing stress granules under different stress conditions in A549 cells

The formation of stress granules has been described in various mammalian cells. In this study, we aimed to analyze the hypoxic conditions regarding stress granule formation in A549 cells.

As comparison, we employed stress conditions that were previously shown to induce the formation of stress granules such as heat-shock (67, 68) or H_2O_2 (69). For this purpose, A549 cells were treated with 200 µM H_2O_2 for 3 h or cultured under elevated temperature (41 °C for 30 min) and then compared to control cells. Furthermore, the cytostatic drug imatinib (10 µM for 2 h), an agent that has not yet been investigated in this regard, was employed for the analysis of stress granule formation. When performing immunocyto-fluorescent analysis for TIAR and TIA-1, stress granules were detected after treatments with H_2O_2, heat shock and imatinib. Stress granules were not detectable in untreated control cells (Figure 4).

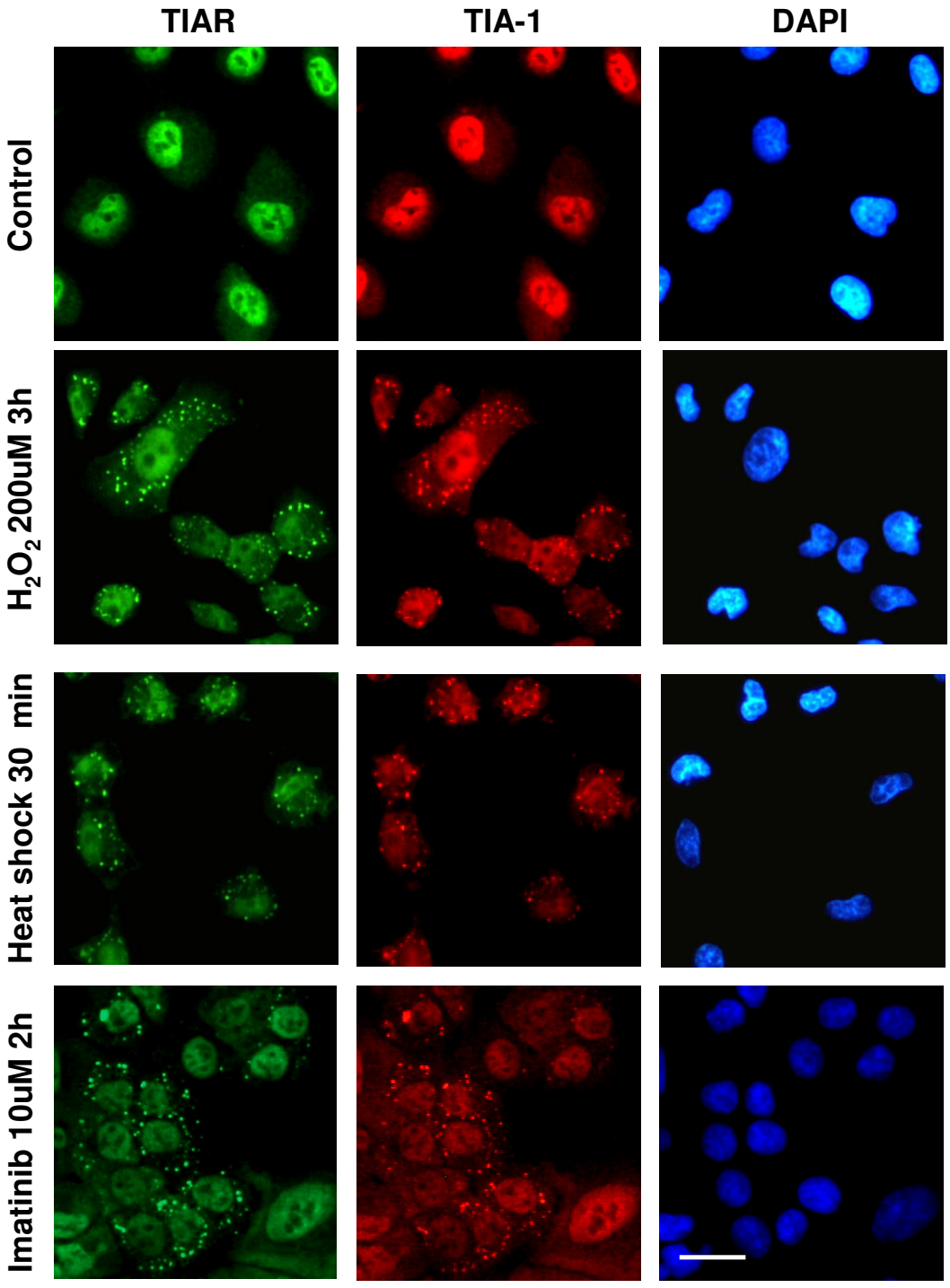


Figure 4. Formation of TIAR/TIA-1 containing stress granules under different stress conditions in A549 cells.
Immunocyto-fluorescent analysis of TIAR (green), TIA-1 (red) and the nuclear stain DAPI (blue) in A549 cells untreated (control), treated with H_2O_2 200 µm for 3 h, cultured under 41 °C for 30 min or treated with imatinib 10 µM for 2 h (scale bar represents 50 µm).

4.2 Analyses of different hypoxic conditions employed for culturing of A549 cells

We investigated TIAR/TIA-1-dependent stress granule formation under different hypoxic conditions in cultured A549 cells compared to normoxic conditions. For this purpose, cells were placed within an incubator that was set to an oxygen concentration of either 1 % O_2 or 0.1 % O_2. Furthermore, we employed oxyrase, an enzyme that consumes oxygen from the medium thus generating low oxygen tensions in the medium. Employing these three different approaches, the oxygen concentrations within the cell supernatants were determined as a function of time. The settings of 1 % O_2 and 0.1 % O_2 resulted in a final level of hypoxia of about 7 mmHg and 0.7 mmHg O_2 partial pressure that was achieved after about 60 min. Employing oxyrase, a final O_2 partial pressure of about 7 mmHg was achieved. It is obvious from the displayed curves that the oxygen partial pressure decline was considerable stronger in case of the addition of oxyrase when compared to the settings of the incubator to either 1 % O_2 or 0.1 % O_2 (Figure 5).

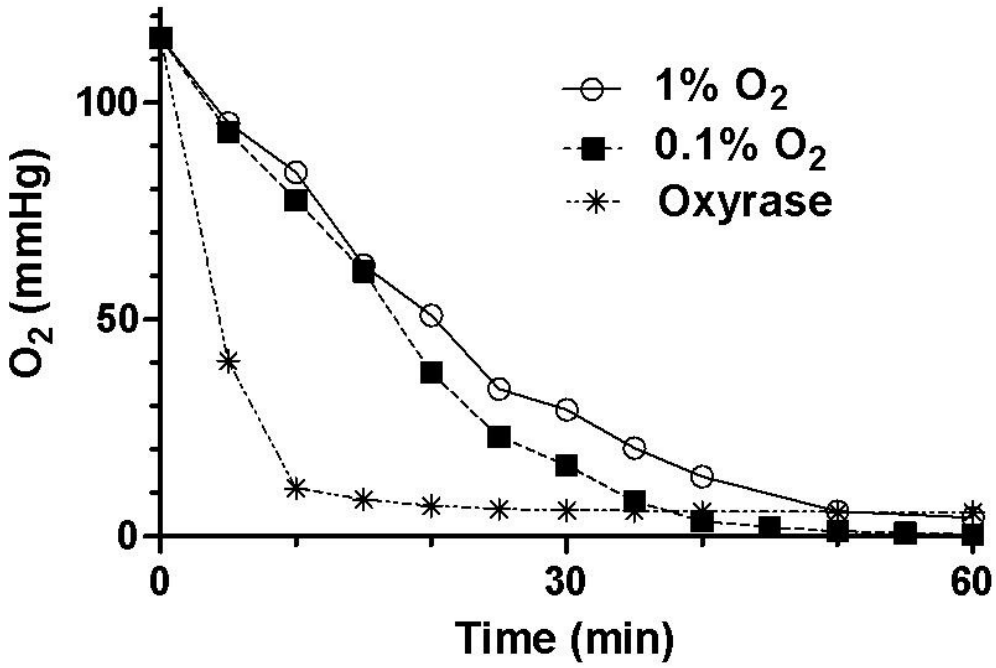


Figure 5. Measurement of the oxygen partial pressure equilibration time in the cell supernatant.
Measurement of the oxygen partial pressure equilibration time in the cell supernatant after setting the hypoxic incubator to 1 % O_2, 0.1 % O_2 or after addition of the enzyme oxyrase (see also text in the Results section). An incubator setting of 1 % O_2 resulted in an O_2-partial pressure of about 7 mmHg, while an incubator setting of 0.1 % resulted in about 0.7 mmHg. The addition of oxyrase caused the strongest decline in O_2-partial pressure, which reached about 7 mmHg.

Furthermore, hypoxia within the A549 cells was validated by employing immunocyto-fluorescence analysis with Hypoxiprobe^{TM}-1 (pimonidazole hydrochloride), a well established hypoxia marker for cells or tissues (70, 71).

The cells cultured under the mentioned hypoxic conditions were positive for the Hypoxiprobe^{TM}-1 staining while those cultured under normoxic conditions were negative for the Hypoxiprobe^{TM}-1 staining (Figure 6).

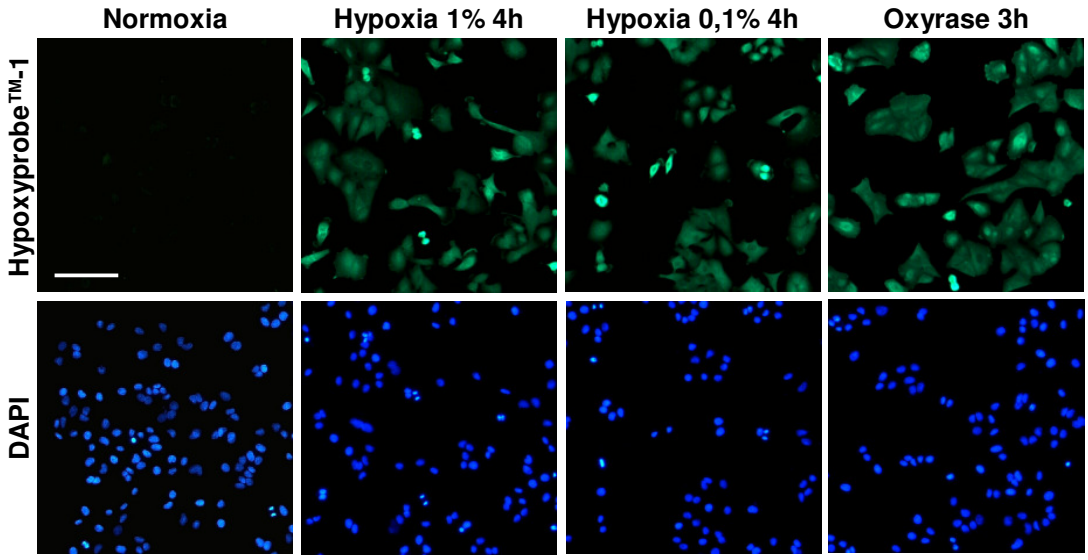


Figure 6. Validation of hypoxia in A549 cells cultured under different hypoxic conditions.
Immunocyto-fluorescent analysis of Hypoxiprobe™-1 (green), a hypoxia marker and the nuclear stain DAPI (blue) in A549 cells cultured under normoxia, in a hypoxic incubator set for 1 % O_2 and respectively 0.1 % O_2 for 4 h or after addition of the enzyme oxyrase in the cell-culture medium for 3 h. Hypoxia (green) within the A549 cells was observed under all 3 hypoxic condition and not under normoxia.

4.3 Formation of TIAR/TIA-1 containing stress granules under different conditions of hypoxia in A549 cells

To analyse the stress granule formation under hypoxic conditions, immunocyto-fluorescence analysis for TIAR and TIA-1 was performed in A549 cells cultured under normoxic or hypoxic conditions.

Stress granules were detected under conditions of the addition of oxyrase or an incubator setting of 0.1 % O_2 whereas stress granules were not detected at the setting of 1 % O_2 or in normoxia. The stress granules were observed in about 10 % of the cells under oxyrase and about 20 % of the cells under 0.1 % O_2. Thus, the absolute hypoxic degree and the gradient of the oxygen decline contribute to the formation of stress granules in hypoxia. The occurrence of stress granules was transient and disappeared when re-culturing the cells under normoxic conditions (Figure 7).

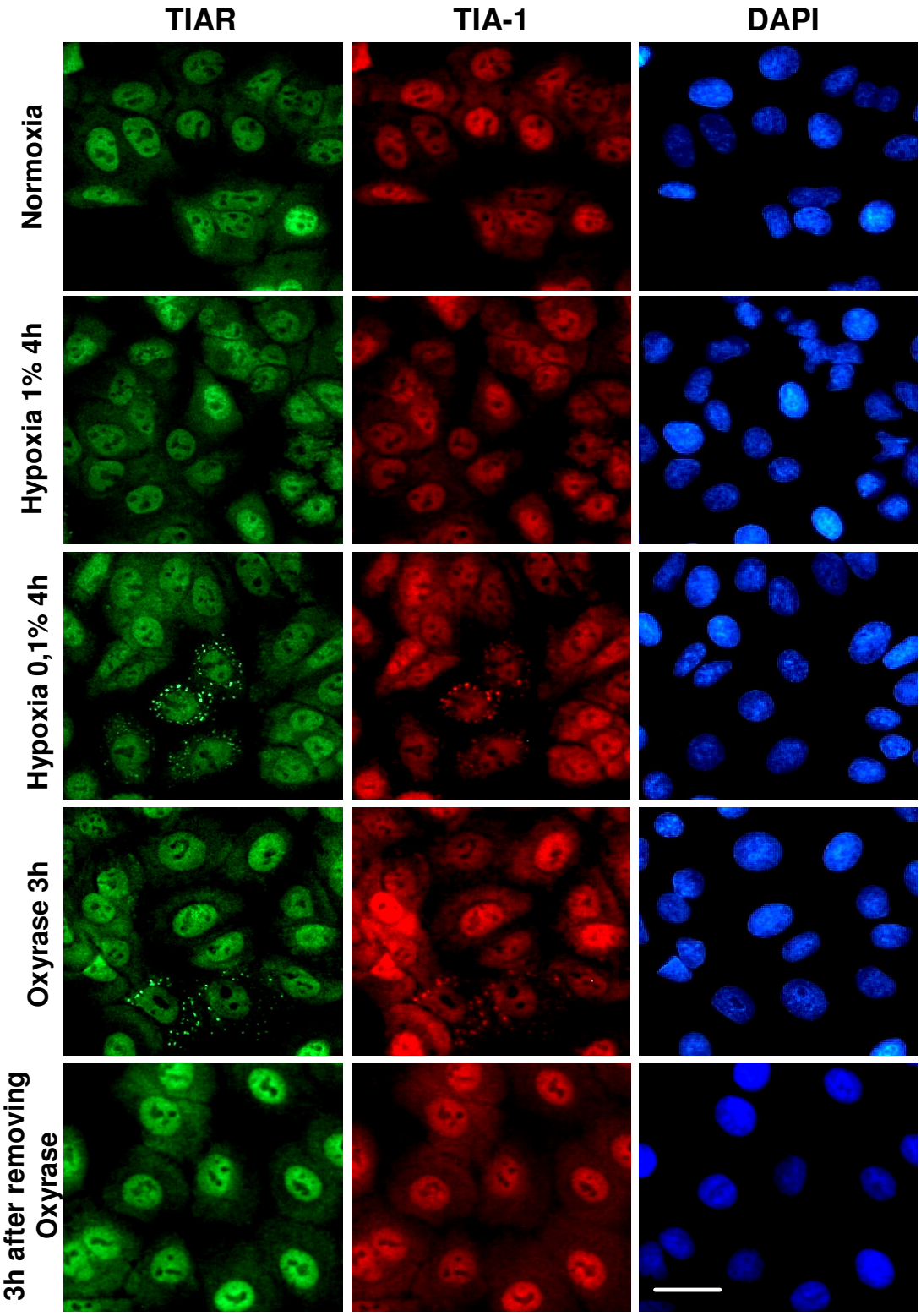


Figure 7. Formation of TIAR/TIA-1 containing stress granules under different conditions of hypoxia in A549 cells.
Immunocyto-fluorescent analysis of TIAR (green), TIA-1 (red) and nuclear stain DAPI (blue) in A549 cells cultured under normoxia, in a hypoxic incubator set to 1 %

O_2 or to 0.1 % O_2 for 4 h, after addition of the enzyme oxyrase in the cell-culture medium for 3 h and additionally 3 h after removing the oxyrase-containing medium. Setting of the incubator to 0.1 % O_2 and addition of oxyrase triggered the aggregation of TIAR/TIA-1 to stress granules whereas in normoxia and a setting of the incubator to 1 % O_2 did not cause the formation of stress granules. The occurrence of stress granules was transient and disappeared when removing the oxyrase-containing medium and re-culturing the cells under normoxic conditions (scale bar represents 50 µm).

4.4 Immunocyto-fluorescent analysis of HIF-1α, c-Myc and lamin B in relation to TIAR and the nuclear stain DAPI in A549 cells cultured in hypoxia.

Since HIF-1α is an important factor that is induced in hypoxia, we further analyzed how TIAR/TIA-1 dependent stress granules affect the accumulation of HIF-1α. Immunocyto-fluorescent costaining of HIF-1α and TIAR in A549 cells cultured under hypoxia 0.1 % O_2 revealed that cells displaying stress granule were negative for HIF-1α, whereas HIF-1α was expressed in the nucleus of cells without stress granules. In comparison, we also analyzed the transcription factor c-Myc an established target of TIAR (39, 72) and the nuclear protein lamin-b used as a non-target control. Accordingly, c-Myc expression was abolished in cells with stress granules but not in cells without stress granules whereas lamin-b expression was not affected by stress granules (Figure 8).

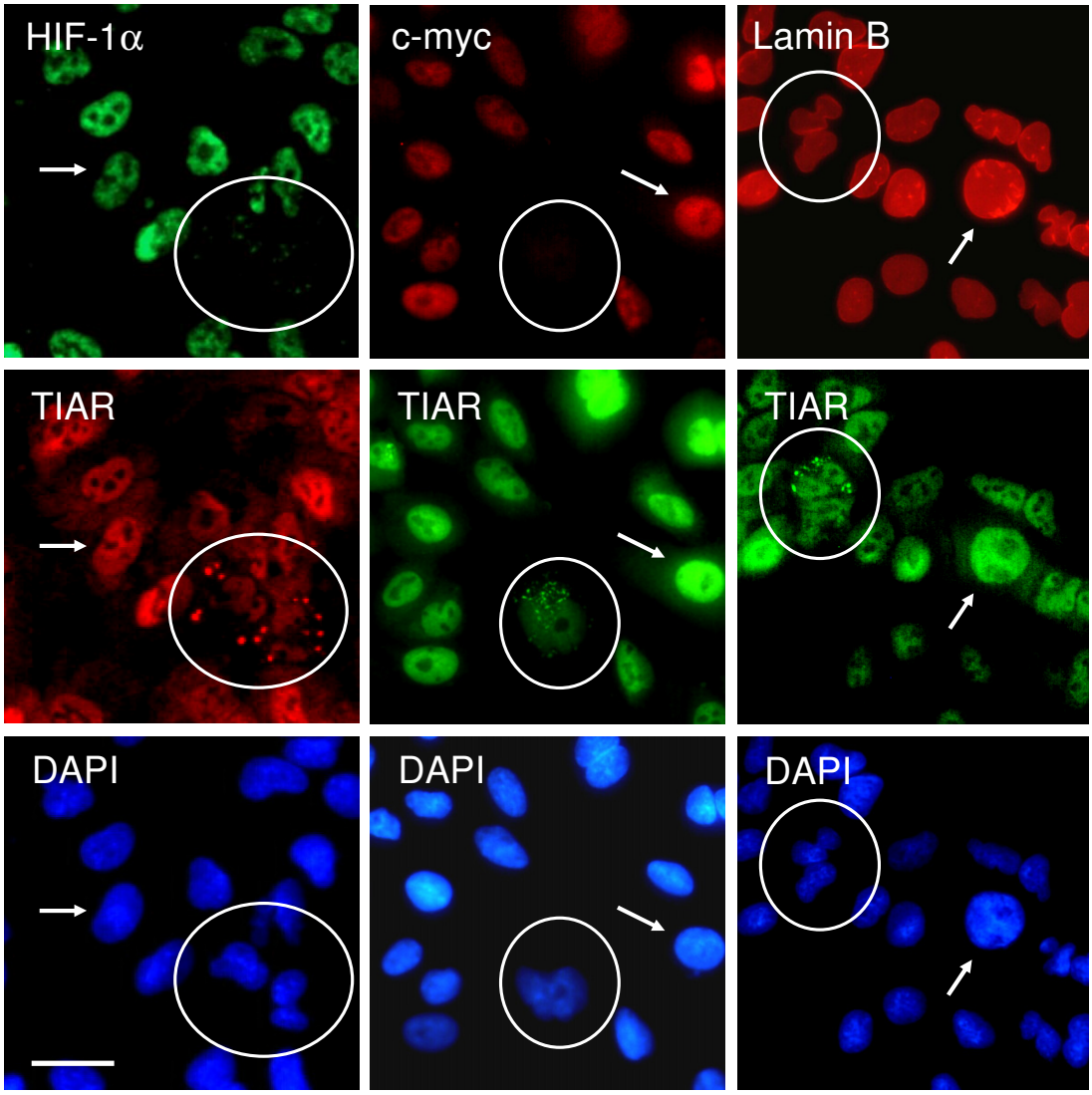


Figure 8. Immunocyto-fluorescent analysis of HIF-1α, c-Myc and lamin B in relation to TIAR and the nuclear stain DAPI in A549 cells cultured in hypoxia.
In cells displaying stress granules (labelled with a white circle) expression of HIF-1α is abrogated. Also, the expression of c-Myc an established target of TIAR is abrogated in cells with stress granules whereas expression of lamin B expression is not affected in cells with stress granules. Arrows indicate some cells without stress granules. In these cells HIF-1α and c-Myc are expressed (scale bar represents 50 μm).

4.5 Inhibition of TIAR and TIA-1 by siRNA (si-TIAR, si-TIA-1) in relation to a random siRNA (si-con) in A549 cells

For further analysis of the dependence of HIF-1 on TIAR and TIA-1, selective inhibition of TIAR and TIA-1 was performed using specific siRNAs (si-TIAR, si-TIA-1). As a control, was employed a siRNA sequence (si-con) that does not target any gene in the human genome and has been tested by micro-array analysis. After 48 h after transfection, the inhibitory effects of the selected siRNA sequences were validated by Western-blot analysis in relation to the loading control β-actin and by immunocytologic-fluorescent analysis in relation to DAPI staining (Figure 9 A and B). Both methods showed that si-TIAR and si-TIA-1 were reducing the level of the targeted protein, respectively. Of note is an upregulation of TIA-1 in cells treated with si-TIAR.

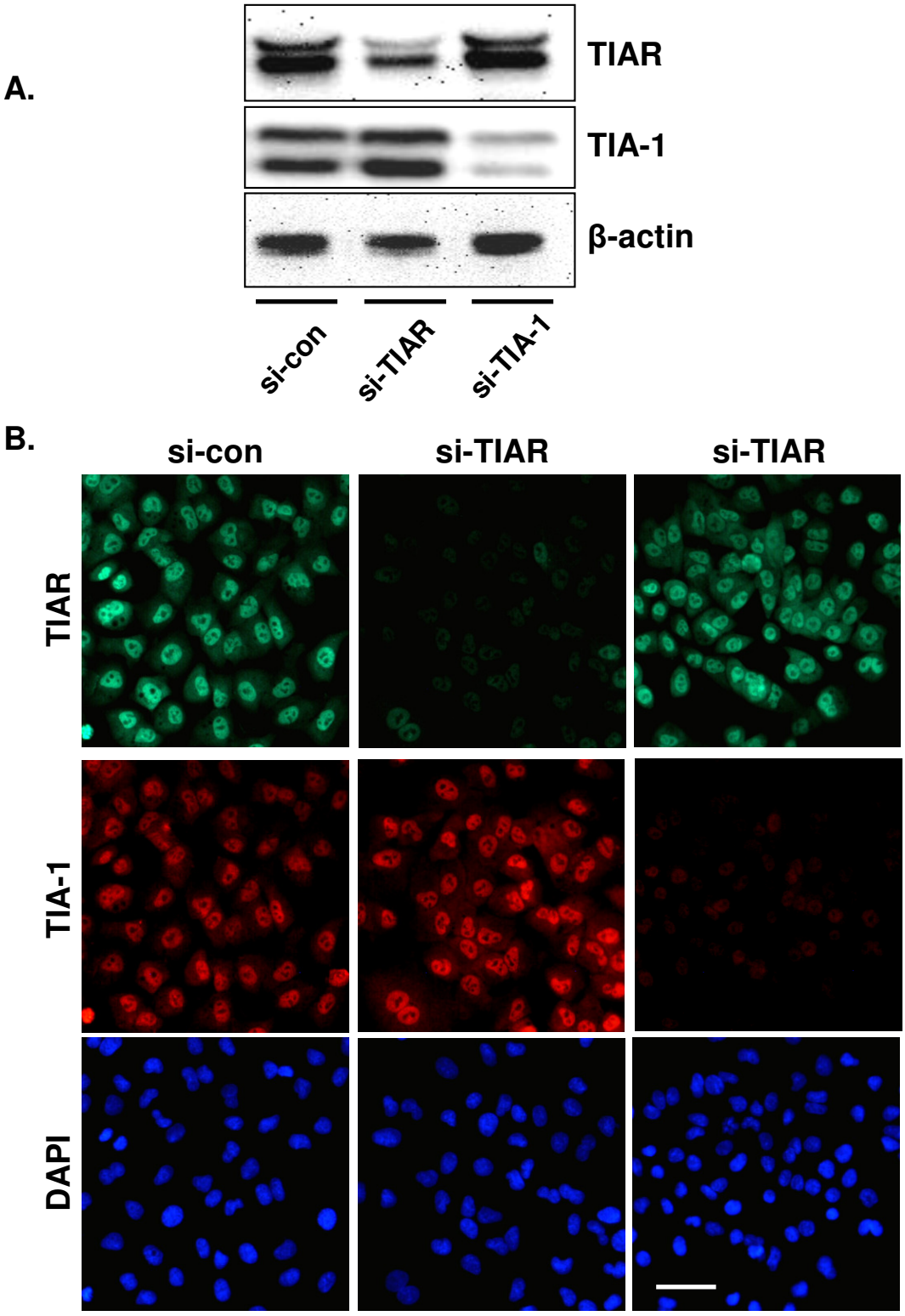


Figure 9. Inhibition of TIAR and TIA-1 by siRNA (si-TIAR, si-TIA-1) in relation to a random siRNA (si-con) in A549 cells.

Validation of the inhibitory effects of si-TIAR and si-TIA-1 treatment in comparison to si-con on TIAR and TIA-1 as measured by A) Western-blot analysis in relation to the loading control β-actin and by B) immunocytologic-fluorescent analysis in relation to DAPI staining. Of note is that the inhibition of TIAR caused an increase of TIA-1 (scale bar represents 100 µm).

4.6 Effects of TIAR and TIA-1 on HIF-1α

A549 cells were transfected with si-TIAR, si-TIA-1 or with si-con employed as a control. 48 h later, the cells were incubated under normoxia or hypoxia (4h). The Western-blot analysis revealed the typical induction of HIF-1α under hypoxia which was enhanced in the cells transfected with si-TIAR of si-TIA-1 when compared to the si-con group. ß-actin was used as a loading control (Figure 10).

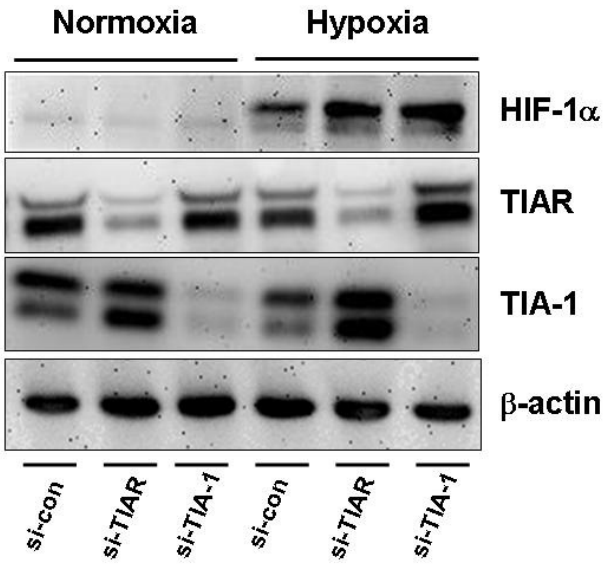


Figure 10. Effects of the treatment of A549 cells by si-TIAR or si-TIA-1 compared to si-con on the expression of HIF-1α analysed by Western-blot.
Western-blot analysis shows the typical increase of HIF-1α in hypoxia and a further increase of HIF-1α after treatment of cells by si-TIAR or si-TIA-1. β-actin was used as a loading control.

Similar results were obtained by immunocyto-fluorescent analysis. The experiments were conducted in a similar manner. Cells were transfected with si-TIAR, si-TIA-1 or si-con and after 48 h cells were cultured under normoxic or hypoxic conditions. Co-staining of HIF-1α and TIA-1 Figure 11 A or of HIF-1α and TIAR Figure 11 B was performed afterwards. The nuclei were stained with DAPI (blue). As expected, the normoxic cells displayed no HIF-1α signal. The hypoxic cells revealed the typical HIF-1α induction and the cells treated by si-TIAR and si-TIA-1 displayed strong enhancement of HIF-1 α signal in hypoxia in comparison with the cells treated with si-con.

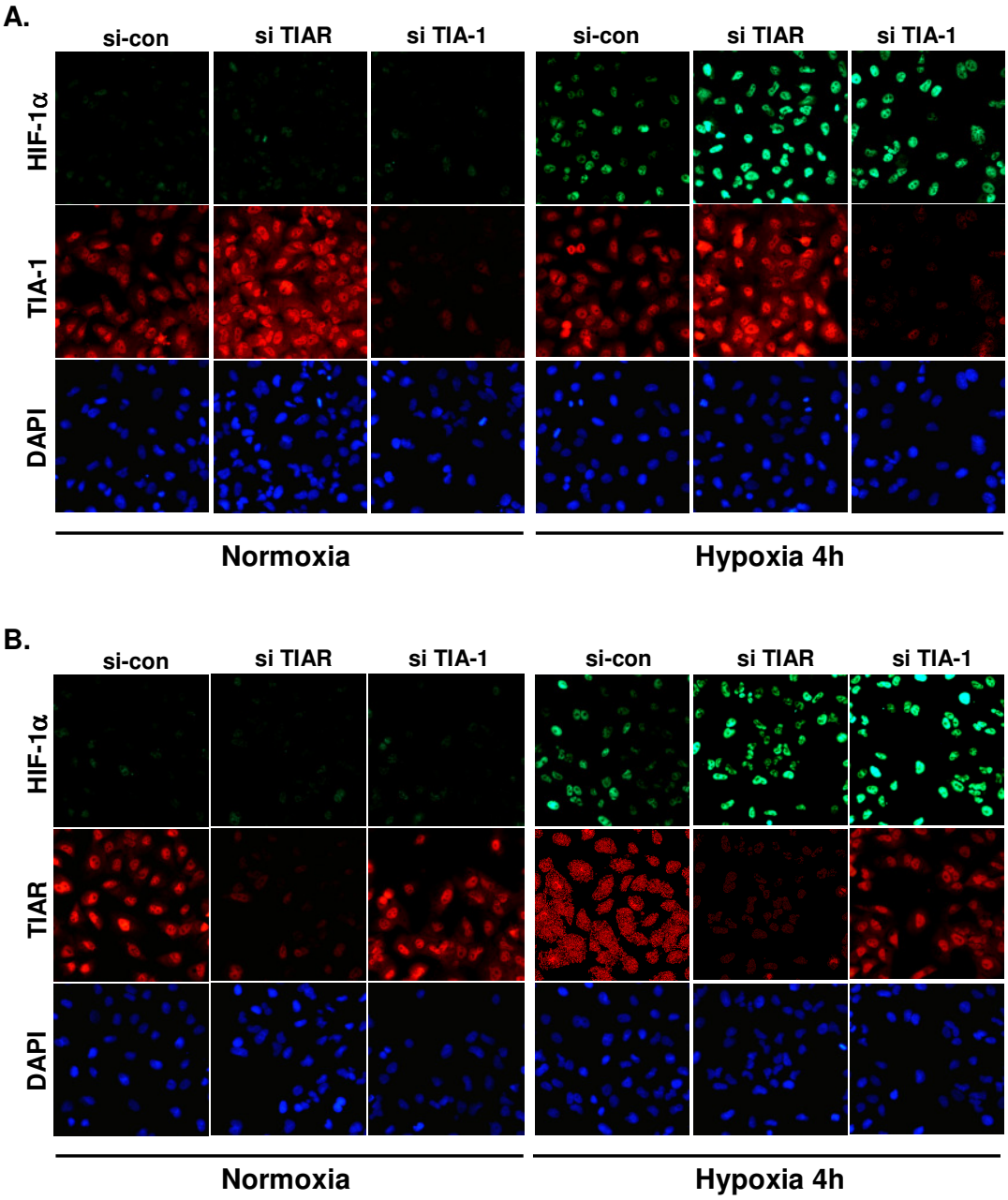


Figure 11. Effects of the treatment of A549 cells by si-TIAR or si-TIA-1 compared to si-con on the expression of HIF-1α, analysed by immunocytologic-fluorescence.

A) Immunocytologic-fluorescent analysis of HIF-1α (green), TIA-1 (red) and nuclear stain DAPI (blue) in A549 cells transfected with si-TIAR, si TIA-1 or si-con. HIF-1α was enhanced after treatment of cells by si-TIAR or by si-TIA-1 in cells not displaying any stress granules. B) Immunocytologic-fluorescent analysis of HIF-1α (green), TIAR (red) and nuclear stain DAPI (blue) in A549 cells transfected with si-TIAR, si TIA-1 or si-con. HIF-1α was enhanced after treatment of cells by si-TIAR or

by si-TIA-1 in cells not displaying any stress granules (scale bar represents 100 µm).

4.6.1 HRE reporter gene assay

Furthermore an HRE reporter gene assay was employed to analyse the effects of TIAR/TIA-1 on HIF-1α dependent target gene expression. The A549 cells were cotransfected with the HRE-plasmid and concomitantly with si-TIAR, si-TIA-1 or si-con. In hypoxia, the expected activation of luciferase activity was noticed. Treatment of cells by si-TIAR or si-TIA-1 resulted in enhanced activation of luciferase activity when compared to si-con further supporting that TIAR/TIA-1 inhibits HIF-1-dependent target gene expression (Figure 12). This effect was more pronounced in cells treated with si-TIAR than in cells treated with si-TIA-1. Of note is that this effect was also obvious under normoxic conditions.

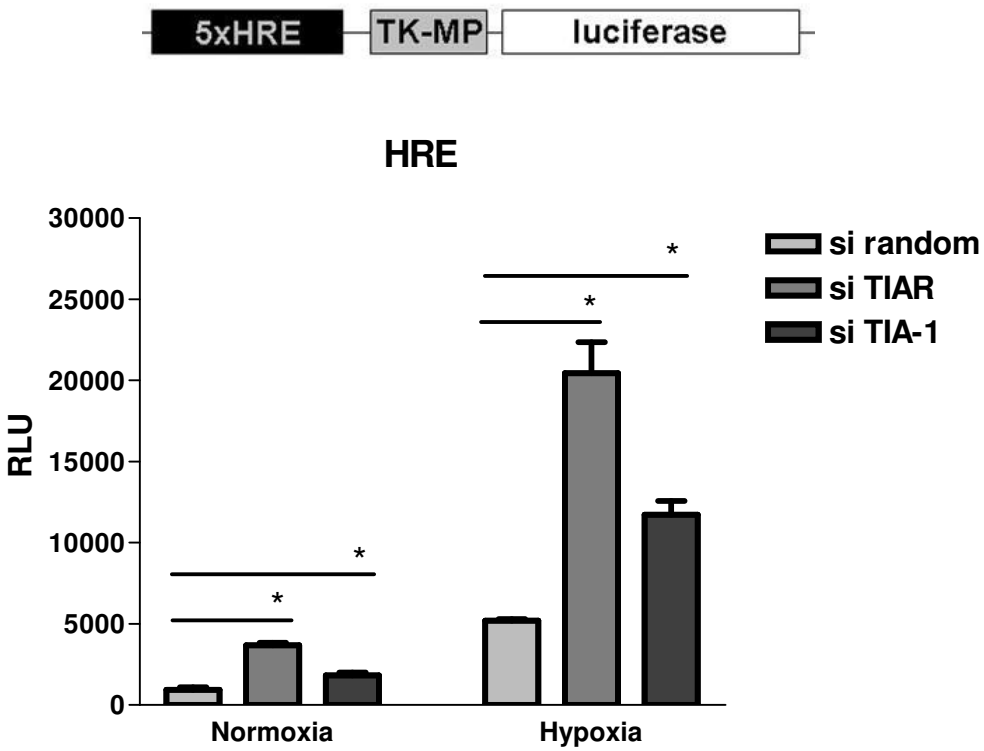


Figure 12. HRE-reporter gene analyse of A549 cells treated by si-TIAR or si-TIA-1 compared to si-con.
Diagram of the HRE-reporter plasmid employed for the experiment as shown below. Treatment by si-TIAR and si-TIA-1 causes an upregulation of HRE-dependent luciferase activity in normoxia and hypoxia compared to si-con. (n=3, SEM, $p<0.05$, unpaired t-test).

4.6.2 AUR reporter gene assay

TIAR and TIA-1 were demonstrated to mediate their specific effects to target mRNAs by AU-rich elements (AUR) located at the untranslated 3'end of the mRNAs. A possible AUR-sequence within the 3' end of the HIF-1α mRNA was identified by *in silico* screening. Coupling of this putative AUR-element to a luciferase reporter gene plasmid resulted in significant downregulation of luciferase expression in transfected A549 cells compared to cells transfected with the control plasmid (consider the si-con bars) (Figure 13). Concomitant si-TIAR transfection resulted in upregulation of luciferase expression in cells

transfected with the AUR-plasmid but not in cells transfected with the control plasmid when compared to si-con. Concomitant si-TIA-1 transfection caused downregulation of luciferase activity in cells transfected with the control-plasmid but a slight upregulation in cells transfected with the AUR-plasmid when compared to si-con. These experiments reveal that TIAR and TIA-1 downregulate the luciferase activity by targeting the AUR element.

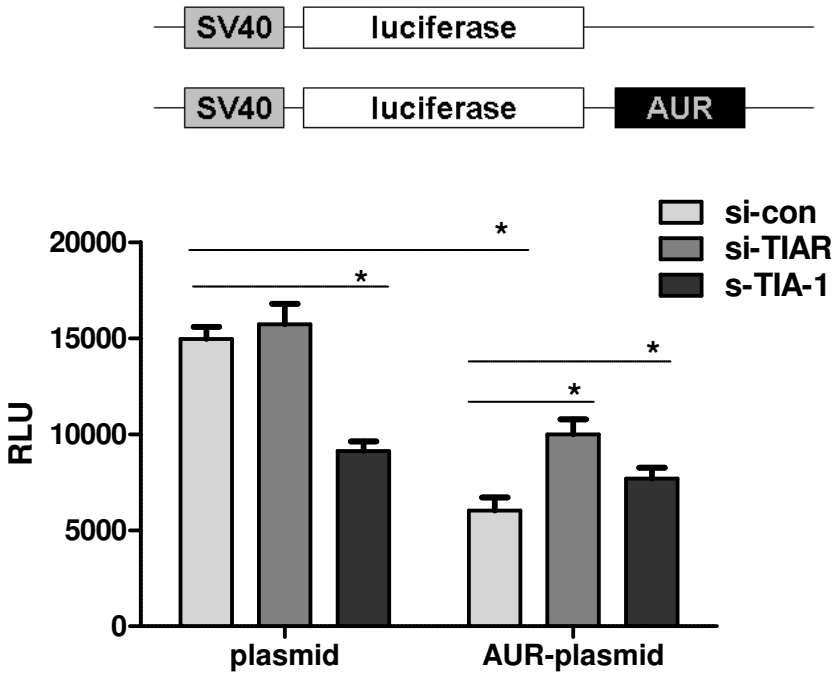


Figure 13. AUR-reporter gene analyse of A549 cells treated by si-TIAR or si-TIA-1 compared to si-con.
Diagram of the AUR-plasmid and control plasmid employed for the experiments shown below. Ligation of the AUR-sequence to the reporter plasmid results in downregulation of the luciferase activity. Concomitant treatment of cells by si-TIAR and si-TIA-1 leads to upregulation of luciferase activity only in cells transfected with the AUR containing plasmid (n=3, SEM, $p<0.05$, unpaired t-test).

4.7 HIF-1α and TIA-1 distribution in A549 tumor xenografts

The distribution of HIF-1α and TIA-1 in vivo was analysed by immunohistologic-fluorescence in sections from A549 tumor xenografts. The first observation was that the distribution of HIF-1α and TIA-1 was not uniform throughout the tumor section, most likely reflecting different oxygenation conditions within the tumor section (Figure 14). In addition, a nearly complementary pattern of HIF-1α and TIA-1 signals was observed reflecting the inhibitory effect of TIA-1 on HIF-1α as demonstrated in the cell culture studies employing si-TIAR and si-TIA-1.

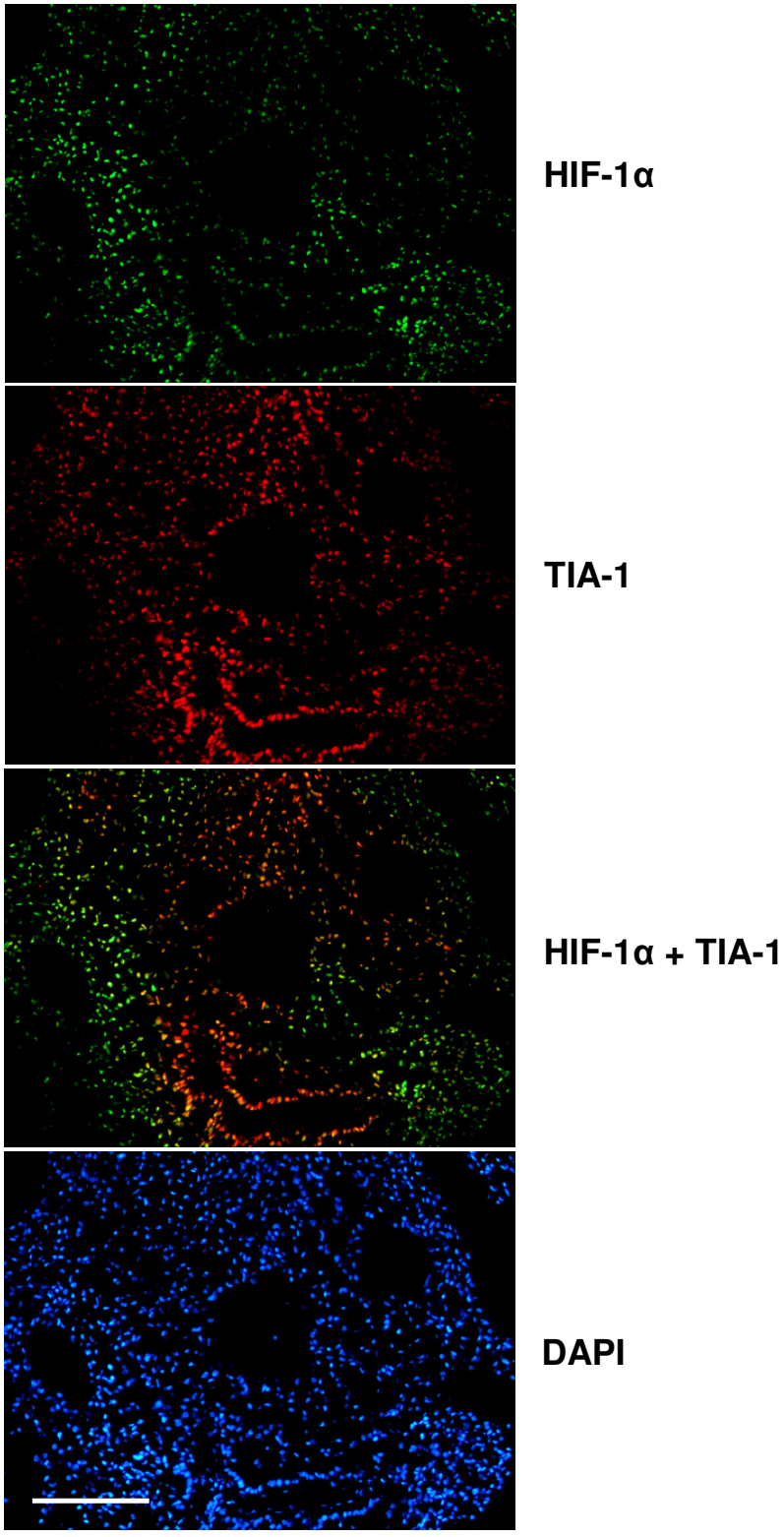


Figure 14. Immunohistologic-fluorescent analysis of HIF-1α (green), TIA-1 (red) and the merged version (HIF-1α + TIA-1) in sections from A549 tumor xenografts in relation to DAPI staining.

Of note is a nearly complementary expression pattern of HIF-1α and TIA-1 (scale bar represents 100 µm).

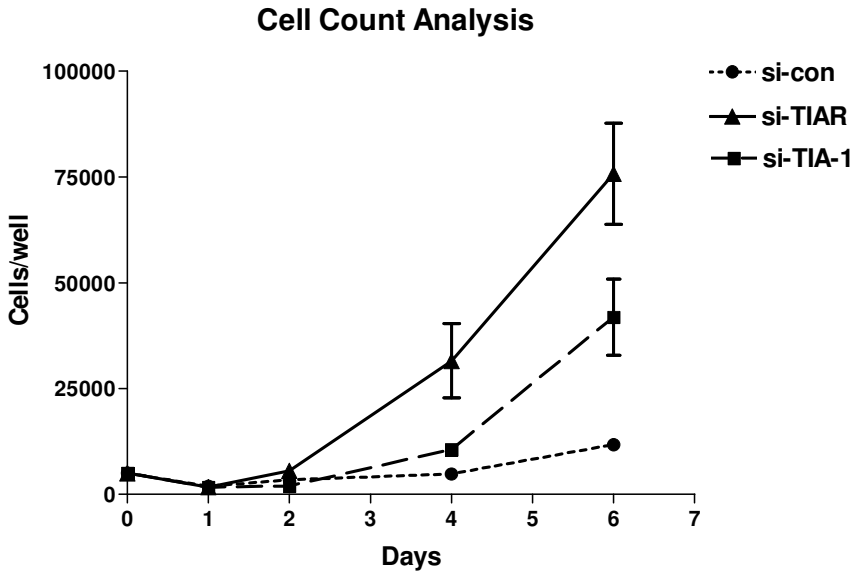
4.8 Effects of TIAR and TIA-1 on proliferation

TIAR and TIA-1 proteins have been described to target various mRNAs with quite different functions. In order to analyze the effects of TIAR and TIA-1 on proliferation, A549 cells were transfected with si-TIAR, si-TIA-1 or si-con and the proliferation was analysed employing two different methods.

The first method used was cell count analysis. The A549 cells were plated in 24-well plates and transfected as indicated. The cells were detached by trypsin treatment and counted at different time points: 1 day, 2 days, 4 days and 6 days after plating. An increase in cell count was observed in the A549 cells transfected with si-TIAR and si-TIA-1 when compared to si-con and this effect was stronger in the si-TIAR group (Figure 15 A).

The second method used was the BrdU incorporation assay for direct measurement of S-phase. For this purpose A549 cells were transfected with si-RNA as indicated and the BrdU incorporation was analysed after 4 days of transfection using a commercially available ELISA-Kit. This assay gave similar results as the cell count analysis, showing a stronger BrdU uptake in the cells transfected with si-TIAR or si-TIA-1 when compared to si-con. Employing this assay, no significant difference between the si-TIAR and si-TIA-1 groups (Figure 15 B) was observed.

A.



B.

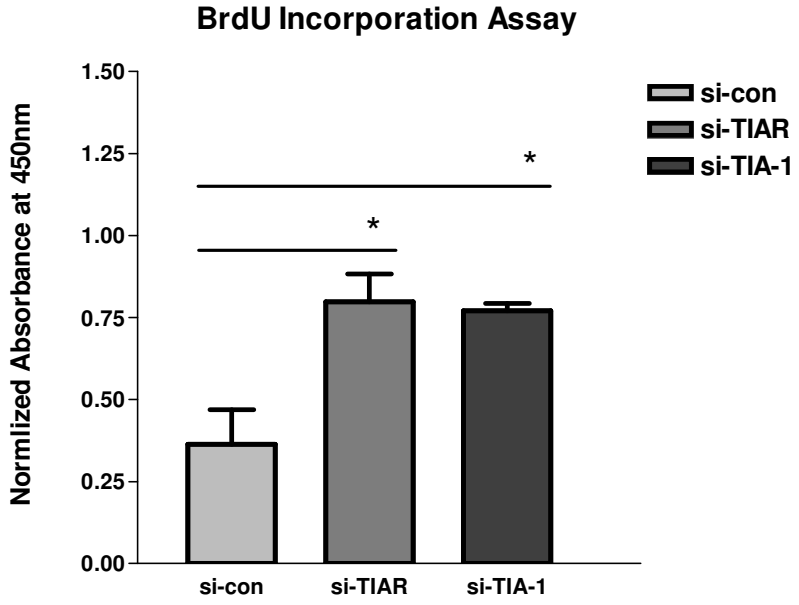


Figure 15. Effects of the treatment of A549 cells by si-TIAR or si-TIA-1 compared to si-con on proliferation

A) Cell count assay showing an increase in the number of the cells/well in the groups treated with si-TIAR or si-TIA-1 when compared to the si-con group. This effect was more pronounced in cells treated with si-TIAR than in cells treated with si-TIA-1 (n=3, SEM, $p<0.05$, unpaired t-test). B) BrdU incorporation assay revealing an increase in BrdU incorporation in A549 cells treated with si-TIAR or si-TIA-1 when compared to the si-con group. (n=3, SEM, $p<0.05$, unpaired t-test).

4.9 Formation of TIAR/TIA-1 containing stress granules under different conditions of hypoxia in HUVEC cells

Beside proliferation, angiogenesis plays an important role in tumor progression, a process that is strongly activated by HIF-1 in hypoxia. Since TIAR/TIA-1 has an inhibitory effect on HIF, we were interested to study the functional effects of TIAR/TIA-1 on angiogenesis employing endothelial cells.

Initially, we analyzed whether stress granule formation is also observed under certain hypoxic conditions in HUVEC cells by immunocyto-fluorescent analysis for TIAR and TIA-1. HUVEC cells were cultured under normoxic conditions, in a hypoxic gas incubator set to 1 % O_2 for 4 h or within a medium to which the enzyme Oxyrase was added for 3 h.

Stress granules were detected under conditions of the addition of oxyrase, whereas stress granules were not detected at the setting of 1 % O_2 or in normoxia. The stress granules were observed in about 10 % of the cells under oxyrase. The occurrence of stress granules was transient and disappeared after removing the oxyrase containing medium and culturing the cells under normoxic conditions for further 3 h (Figure 16).

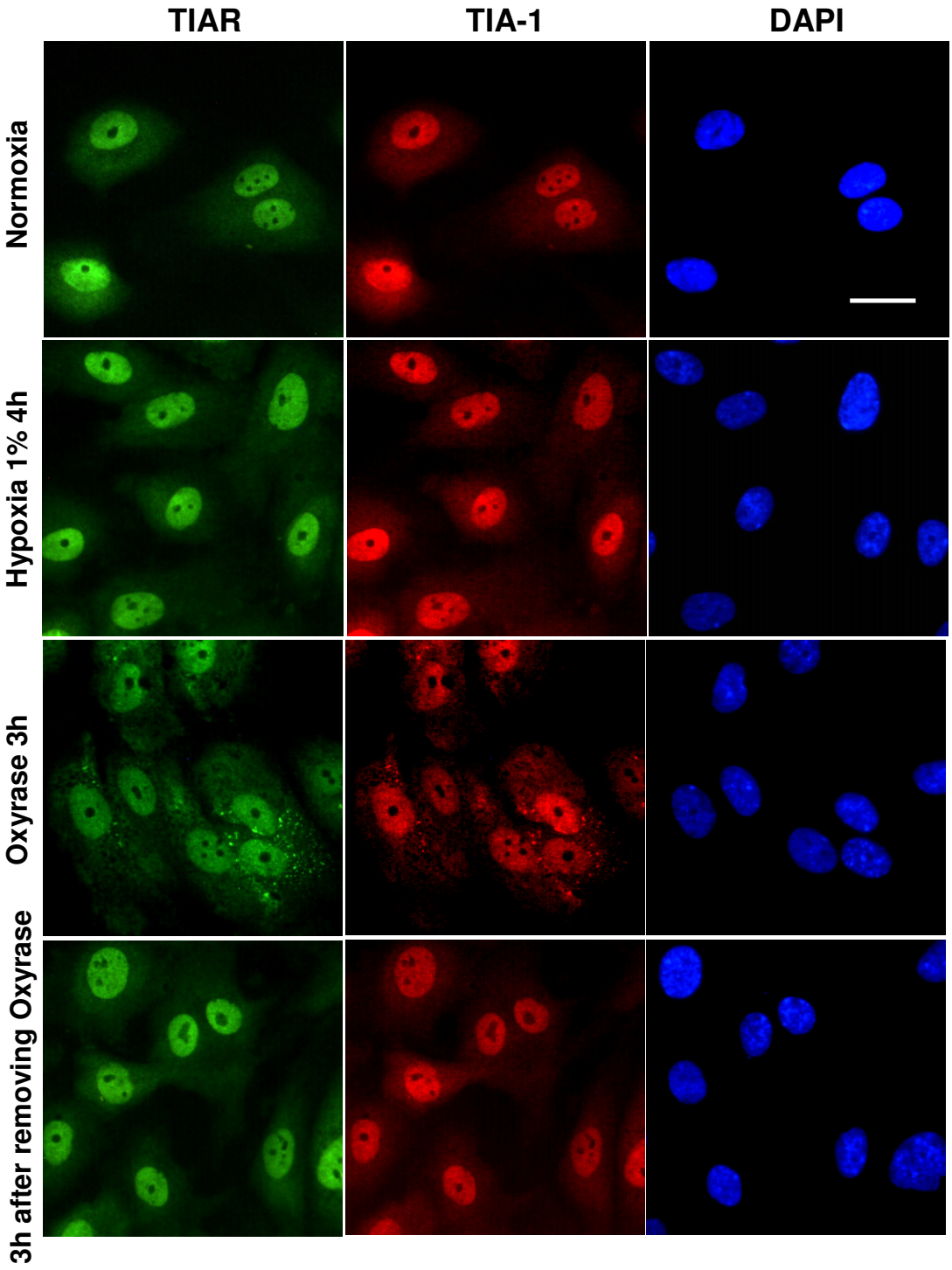


Figure 16. Formation of TIAR/TIA-1 containing stress granules under different conditions of hypoxia in HUVEC cells.
Immunocyto-fluorescent analysis of TIAR (green), TIA-1 (red) and nuclear stain DAPI (blue) in HUVEC cells cultured in normoxia, in a hypoxic incubator set to 1 % O_2 for 4 h, and after addition of the enzyme oxyrase in the cell-culture medium for 3 h and 3 h after removing the oxyrase-containing medium. The addition of oxyrase

triggered the aggregation of TIAR/TIA-1 to stress granules whereas normoxia and a setting of the incubator to 1 % O_2 did not cause the formation of stress granules. The occurrence of stress granules was transient and disappeared when removing the oxyrase-containing medium and re-culturing the cells under normoxic conditions (bar represents 50 µm).

4.10 Inhibition of TIAR and TIA-1 by siRNA in HUVEC cells

For analysis of the dependence of angiogenesis on TIAR and TIA-1, selective silencing of TIAR and TIA-1 was performed in HUVEC cells. 48 h after transfection, the inhibitory effects of the selected siRNA sequences were validated by Western-blot analysis in relation to the loading control β-actin (Figure 17). The results showed that si-TIAR and si-TIA-1 were reducing the level of the targeted protein. Of note is an upregulation of TIA-1 in cells treated with si-TIAR.

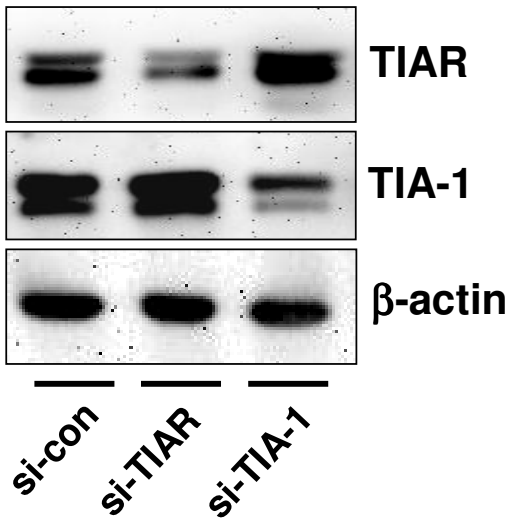


Figure 17. Inhibition of TIAR and TIA-1 by siRNA (si-TIAR, si-TIA-1) in relation to a random siRNA (si-con) in HUVEC cells as measured by Western-blot.
Validation of the inhibitory effects of si-TIAR and si-TIA-1 treatment in comparison to si-con on TIAR and TIA-1 as measured by Western-blot analysis in relation to the loading control β-actin.

4.11 Effects si-TIAR and si-TIA-1 on angiogenesis in endothelial cells

For analysing the effects of TIAR/TIA-1 on angiogenesis, tube formation assay was performed. For this purpose HUVEC cells transfected with si-TIAR, si-TIA-1 or si-con were cultured on Matrigel for 16 h and the total tube length formed within this time interval was measured. The total tube length is taken as an indicator of the angiogenetic potential of the HUVEC cells. Treatment by si-TIAR and si-TIA-1 caused an increase in the total numbers of tubes when compared to si-con (Figure 18).

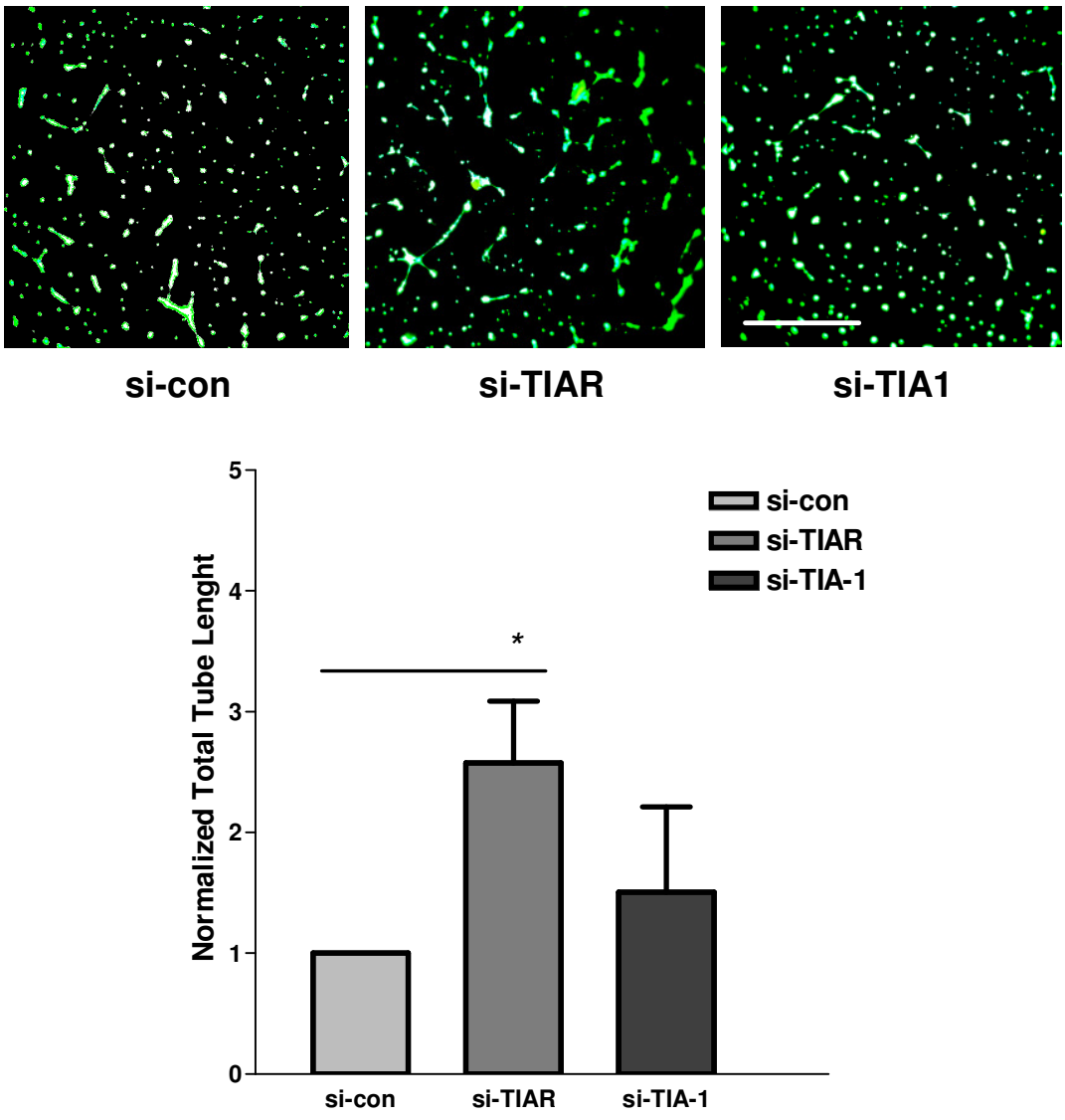


Figure 18. Effects of the treatment of HUVEC cells by si-TIAR or si-TIA-1 compared to si-con on angiogenesis as measured by tube formation assay.

Tube formation assay of HUVEC cells transfected with si-TIAR, si TIA-1 compared to si-con. Treatment by si-TIAR and si-TIA-1 causes an increase in the total tube length formed by HUVEC cultured on the Matrigel when compared to si-con (n=3, SEM, $p<0.05$, unpaired t-test) (scale bar represents 1000 µm).

5 Discussion

Different mechanisms exist which regulate global and specific translation under conditions of hypoxia (31, 32). In this regard, the mRNA-binding proteins TIAR and TIA-1 are suggested to regulate the turnover and stability of specific mRNA targets, thereby affecting translational efficiency (50) with possible impact on gene regulation mediated by HIF-1.

In this study, the formation of TIAR/TIA-1-containing stress granules was analyzed under different oxygen tensions. We observed that the absolute level of hypoxia (O_2 ~ 0.7 mmHg) and the degree of oxygen decline (ΔO_2 > -10 mmHg/min) contributed to the formation of stress granules. Under these conditions, up to 20% of the cells developed stress granules, a ratio that would likely increase under even more severe hypoxic conditions and a more rapid oxygen decline. Formation of stress granules is triggered by the phosphorylation of the eukaryotic initiation factor eIF2α, as was demonstrated in a study where stress granules were induced by heat shock or arsenite employing eIF2α mutants (67). Also, phosphorylation of eIF2α is triggered by hypoxia in a reversible manner (42, 43). Interestingly, the kinetics of eIF2α phosphorylation strongly depends on the severity of hypoxia. This is in accordance with our observation that the formation of stress granules strongly depends on the degree of and kinetics of the drop in O_2 partial pressure. However, other triggers of stress granule formation such as agents that target eIF4A have been described as well and may contribute (73).

The second important observation made in this study was that TIAR/TIA-1 proteins strongly affect HIF-1 signaling. HIF-1α is a transcription factor that is specifically induced by limiting oxygen concentrations (15). HIF-1α expression was blocked in cells bearing TIAR/TIA-1 containing stress granules. This effect was comparable with the inhibition of expression of the c-Myc transcription factor, a known target of TIAR (39, 72), whereas

expression of the non-target nuclear protein lamin B was unaffected. We employed specific inhibition of TIAR and TIA-1 by RNA interference to analyze effects of TIAR and TIA-1 exerted on HIF-1α. Of note is that TIAR inhibition caused an upregulation of expression of TIA-1. In other studies, TIA-1 overexpression led to a drop in TIAR levels also revealing dependence between TIAR and TIA-1 expression (58, 74). Based on their structural similarity, as well as from genetic knockout models, it is suggested that TIAR and TIA-1 are redundant to some extent and can compensate for each other.

Inhibition of TIAR and TIA-1 also upregulated HIF-1α expression in cells without TIAR/TIA-1 containing stress granules, as demonstrated by immunocytochemical and western blot experiments with TIAR, TIA-1 and HIF-1α. Inhibition of TIAR and TIA-1 also caused upregulation of HIF-1 target gene expression, as analyzed by HRE reporter gene assay. Inhibition of TIAR caused a stronger induction of HIF-1α than did inhibition of TIA-1. Both TIAR and TIA-1 are suggested to mediate their specific effects on mRNAs by sequences of the ARE type that are located at the 3′ untranslated mRNA end. TIAR- and TIA-1-specific target mRNAs, as well as common targets of both, have been described (39, 40, 53, 54, 58, 75). These target mRNAs are suggested to share similar motifs however, a unique consensus sequence could not be identified.

A putative AUR sequence from the HIF-1α mRNA sequence was identified by an *in silico* search employed for the cloning experiments. This sequence was characterized by three AUUUA motifs, and thus represents the class I AUR-type that is defined by the occurrence of up to five copies of this motif (76). The class I type motif is the most common type observed in the mRNA sequences of transcription factors. Coupling of this sequence to a reporter gene diminished its expression. A functional link between this AUR-sequence and TIAR/TIA-1 mediated effects on HIF-1α was supported by the observation that inhibition of TIAR and to a lesser extent inhibition of TIA-1 attenuated the AUR-dependent downregulation of the expression the coupled reporter gene. In this regard, one study revealed significant binding of TIAR

to HIF-1α mRNA that was not induced in hypoxia whereas binding of TIA-1 was almost not detectable (77). First, these observations may reflect our finding that the inducible effects exerted on HRE- reporter gene activity were much stronger pronounced after si-TIAR than after si-TIA-1 treatment. Second, in particular the effects of TIAR on HIF-1a could be observed both in normoxia and hypoxia at a different absolute level but with a similar ratio (Figure 13).

The complete blockade of HIF-1α by TIAR/TIA-1-containing stress granules under conditions of extreme hypoxia or by a rapid drop in cellular oxygenation represents an important level of HIF-1α regulation. Triggering of the HIF pathway can result in the induction of about 100 target genes. This process itself consumes ATP (because of *de novo* transcription and translation), but requires some time to become effective. Particularly in the short term, ATP generation is optimized by shifting from mitochondrial respiration towards glycolysis. In the long term, cellular oxygenation is improved by induction of erythropoiesis and angiogenesis. In the case of rapid oxygenation decline and low absolute oxygen concentrations, triggering of the HIF pathway may exceed the actual ATP supply. Thus, transient blockage of the HIF-1 pathway may be of benefit for the cell. Stress granules silence mRNAs encoding HIF-1α and enable rapid and enhanced engagement of the hypoxic gene regulatory program in stages when reoxygenation occurs that cause the depolymerization of stress granules.

This phenomenon was suggested to be relevant in the context of the development of radioresistance in tumors (62, 78). Irradiation caused reoxygenation of tumors that was accompanied by the depolymerization of TIAR-dependent stress granules. In turn, HIF-1 signaling was enhanced leading to augmented translation and secretion of angiogenic factors that enhanced the viability of endothelial cells in the tumor vasculature.

Furthermore, this study analyzed the functional role of TIAR/TIA-1 in cell proliferation and angiogenesis. Inhibition of TIAR and TIA-1 by siRNA led to the stimulation of proliferation in A549 cancer cells and angiogenesis in

HUVEC endothelial cells. This might be caused by enhancement of the translation of TIAR/TIA-1 dependent target mRNAs (39, 79) including those of HIF1α. that affect proliferation and angiogenesis (16, 80). Also, blockage of the pro-apoptotic effects of TIAR/TIA-1 may contribute to enhanced proliferation (56, 57). One recent study investigated the effects of TIAR/TIA-1 on global gene expression by DNA-microarray. In this study, an increase of a gene cluster encoding inflammatory, angiogenic and cell growth related genes was observed after silencing of TIAR/TIA-1 employing Hela cells. In these cells silencing of TIAR/TIA-1 also caused an increase of proliferation and anchorage independent growth (81).

In conclusion, we demonstrated that TIAR/TIA-dependent stress granule formation depends on the degree of hypoxia and the kinetic of oxygen partial pressure decline. Furthermore, we identified HIF-1α as a functional target of TIAR/TIA-1 mRNA binding proteins, likely mediated by AUR sequences of HIF-1α mRNA. In particular, TIAR/TIA-1 inhibited HIF-1α expression most strongly under conditions when TIAR/TIA-1 containing stress granules occurred but also to a lesser extent under conditions where no stress granules are visible. Additionally TIAR/TIA-1 was demonstrated to have inhibitory effects on proliferation. Furthermore, TIAR/TIA-1 displayed inhibitory effects on angiogenesis being likely mediated by its inhibitory effect on HIF-1α.

6 Summary

T-cell intracellular antigen (TIA)-1 and TIA-1 related protein (TIAR) are mRNA-binding proteins that aggregate within stress granules under specific stress conditions. In this study, we analyzed TIAR/TIA-1 aggregation under different hypoxic conditions, and studied the effects on hypoxia-inducible factor (HIF)-1α, as well as on proliferation and angiogenesis. TIAR/TIA-1 formed stress granules under acute and pronounced hypoxic conditions in A549 adenocarcinoma cells. In parallel, HIF-1α expression was blocked in cells displaying TIAR/TIA-1 stress granules. Silencing of TIAR and TIA-1 caused upregulation of HIF-1α expression, as demonstrated by western blot, immunocytochemistry, and HIF-1α-dependent reporter gene expression. TIAR/TIA-1 are suggested to mediate their effects by interaction with certain mRNA regions. Thus, a critical region of the 3′ end of the untranslated HIF-1α cDNA with possible adenosine-uridine rich (AUR) elements was coupled to a reporter gene plasmid. Indeed, this coupling caused downregulation of the reporter gene expression, supporting the relevance of this mRNA region for the effects of TIAR/TIA-1 exerted on HIF-1α. Furthermore, employing this reporter construct inhibition of TIAR by siRNA attenuated the inhibitory *cis*-effect of this AUR sequence. Additionally, immunohistochemical analysis of A549 cell tumor xenografts revealed a nearly complementary expression of HIF-1α and TIAR. This *in vivo* observation may reflect the inhibitory regulation of HIF-1α by TIAR as revealed in the cell culture studies. In sum, rapid and severe hypoxia caused TIAR/TIA-1-dependent formation of stress granules, specifically suppressing HIF-1α expression. Additionally TIAR/TIA-1 was demonstrated to exhibit inhibitory effects on proliferation and angiogenesis.

7 Zusammenfassung

T-cell intracellular antigen (TIA)-1 und *TIA-1 related protein* (TIAR) sind mRNA-bindende Proteine, welche unter bestimmten Stressbedingungen zu Stressgranula aggregieren. In dieser Arbeit wurde die Aggregation von TIAR/TIA-1 unter verschiedenen Hypoxie Bedingungen analysiert. Zudem wurde der Effekt auf den Hypoxie-induzierbaren Faktor (HIF-1α) sowie auf Proliferation und Angiogenese in humanen Adenokarzinomzellen (A549) untersucht.

TIAR/TIA-1 formten Stressgranula unter akuten und extremen Hypoxiebedingungen. In Zellen mit TIAR/TIA-1-haltigen Stressgranula war keine Expression von HIF-1α nachweisbar. Inhibition der Expression von TIAR und TIA-1 mit siRNA verursachte eine Hochregulation von HIF-1α. Diese wurde nachgewiesen durch Western Blot, Immunzytofluoreszenz und HIF-1α-abhängige Reportergen Expression. Die Effekte von TIAR/TIA-1 werden vermutlich durch Interaktion mit bestimmten mRNA Sequenzen vermittelt. Daher wurde eine kritische Region des 3'-Endes der nicht translatierten HIF-1α cDNA mit möglichen Adenosin-Uridin reichen (AUR) Elementen an ein Reportergen Plasmid gekoppelt, was eine Herunterregulation der Expression des Reportergens bewirkte. Diese Beobachtung stützt die Relevanz dieser mRNA Sequenz für die beobachteten Effekte von TIAR/TIA-1 auf HIF-1α. Ferner führte die Inhibition von TIAR mit siRNA beim Einsatz dieses Reporterkonstruktes zur Abschwächung des inhibitorischen cis-Effektes der AUR-Sequenz. Interessanterweise zeigte in weiteren Experimenten, die Analyse von A549 Xenotransplantaten durch Immunhistofluoreszenz eine nahezu komplementäre Expression von HIF-1α und TIAR an. Diese *in vivo* Beobachtung ist stimmig mit der inhibierenden Regulation von HIF-1α durch TIAR, wie sie in den Zellkulturstudien beobachtet wurde.

Zusammenfassend verursacht akute und extreme Hypoxie die TIAR/TIA-1-abhängige Bildung von Stressgranula mit spezifischer Unterdrückung der HIF-1α Expression. Außerdem konnten für TIAR/TIA-1 inhibitorische Effekte auf Proliferation und Angiogenese nachgewiesen werden.

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Declaration

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus-Liebig-University of Giessen in carrying out the investigations described in the dissertation."

Place and Date

Oana Raluca Gottschald

9 Appendix

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Oral presentations

1. Oana R. Domnisoru, Gabriela Krasteva, Friedrich Grimminger, Werner Seeger, Frank Rose, Jörg Hänze. TIAR/TIA-1stress granules formation is induced by rapid oxygen partial pressure decline and supresses HIF pathway**.** *American Thoracic Society (ATS) International Conference Toronto, Canada, 16-21 May 2008*

2. Oana R. Domnisoru. Analysis of RNA binding proteins (TIAR, TIA-1) and their interference with HIF pathway**.** *The 5th MBML annual retreat, Rauischholzhausen, Germany, 9- 11 July 2007*

3. Oana R. Domnisoru. Relation of hypoxia and cellular stress responses for therapy resistance of cancer cells. The 4th *MBML annual retreat, Rauischholzhausen, Germany, 31 July- 2 August 2006*

Poster presentations

1. Oana R. Domnisoru, Gabriela Krasteva, Werner Seeger, Frank Rose, Jörg Hänze. Formation of TIAR/TIA-1-containing stress granules depends on the kinetics of oxygen partial pressure decline, and suppresses the HIF pathway. *Keystone Symposium: Molecular, Cellular, Physiological, and Pathogenic Responses to Hypoxia (J6) , Vancouver, Canada, 15-20 January, 2008*
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3. Oana R. Domnisoru, Gabriela Krasteva, Friedrich Grimminger, Werner Seeger, Frank Rose, Jörg Hänze. Formation of TIAR/TIA-1-containing stress granules depends on the kinetics of oxygen partial pressure decline and may suppress angiogenesis by HIF pathway. *99th American Association for Cancer Research (AACR) Annual Meeting in San Diego, USA, 12-16 April, 2008*
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Conferences attended as a non-presenting delegate

1. 102nd International Meeting of the Anatomische Gesellschaft", Giessen, Hessen, Germany 30 March - 2 April 2007
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