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Oxygen-independent stabilization of Hypoxia Inducible Factor (HIF) - 1 during Respiratory Syncytial Virus infection

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Geniale Menschen beginnen große Werke,
fleißige Menschen vollenden sie.

LEONARDO DA VINCI

Geniuses start great works,
but only the diligent complete them.

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2 Abbrevations

AMP	Adenosine Monophosphate
AP-1	Activator Protein 1
ATP	Adenosine Triphosphate
B	Bartonella
BGA	Blood Gas Analysis
CD73	Ecto-5'-nucleotidase
COX-2	Cyclooxygenase 2
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBV	Epstein Barr Virus
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
ES	Embryonic Stem
EPO	Erythropoietin
FN-1	Fibronectin 1
HBV	Hepatitis B Virus
HBx	Hepatitis B Virus x Protein
HHV-8	Human Herpesvirus 8
HIF-1	Hypoxia Inducible Factor 1
HRE	Hypoxia Responsive Element
HRP	Horseradish Peroxidase
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IL	Interleukin
KCl	Kaliumchlorid
KSHV	Kaposi's Sarcoma-Associated Herpesvirus
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
NaCl	Natriumchlorid

NF-IL6	Nuclear-Factor-IL6
NF-κB	Nuclear factor kappa B
O_2	Oxygen
pO_2	Oxygen Partial Pressure
OV	Orthovanadate
PBS	Phosphat Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque-Forming Units
PMSF	Phenylmethylsulfonyl Fluoride
RIPA	Radio-Immuno Precipitation Assay
RNA	Ribonuclein Acid
siRNA	Small Interfering Ribonuclein Acid
RSV	Respiratory Syncytial Virus
RT-PCR	Realtime Polymerase Chain Reaction
TBS	Tris Buffered Saline
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel-Lindau
VSV	Vesicular Stomatitis Virus
Y	Yersinia

3 Introduction

This first chapter gives a general introduction to the thesis's subject. The terms and concepts used throughout this work are explained and put in context. The role of Hypoxia-inducible factor 1 (HIF-1) in cellular regulation mechanisms is illustrated, and its interrelation with inflammation and infection is shown. We also introduce and discuss the target genes regulated by HIF-1.
Finally, we provide a section on Respiratory Syncytial Virus (RSV) where we show the classification and effects of the virus.

3.1 Preface

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that functions as a master regulator of mammalian oxygen homeostasis and also as a transcriptional regulator during inflammation and infection with different pathogens. HIF-1 activity is induced in a variety of cell types and HIF-1 stabilization can occur via oxygen-dependent (69) or oxygen independent (56) pathways. HIF-1 has been found to regulate the transcription of multiple genes that include hypoxia responsive elements (HRE) in their promoter regions in a cell type specific manner.
Whereas HIF-1 was originally discovered as a biologic oxygen sensor that enables the organism to adapt to hypoxia, recent studies have shown that it plays a central role in cellular responses beyond hypoxia. These include not only inflammation and infection but also angiogenesis of tumors, cell proliferation, glucose and iron metabolism (58, 83).

Respiratory syncytial virus (RSV) is the major etiologic agent of severe epidemic lower respiratory tract infections in infancy. Beyond that, it is amongst the most potent biological stimuli to induce an inflammatory milieu. Infection with RSV is rapidly followed by a network of cellular responses. Acute bronchiolitis, a severe clinical manifestation of RSV infection is characterized by wheezing, respiratory distress, and the pathologic findings of peribronchial

cell infiltration and release of inflammatory mediators (100). In addition to the acute morbidity of RSV infection, there are long-term consequences: RSV has been shown to predispose to the development of hyperreactive airway disease and recurrent episodes of wheezing are often precipitated by subsequent RSV infection (54, 127).

Previous studies have shown increased levels of HIF-1α during bacterial, viral and parasital infections. These findings led us to explore the HIF-1 pathway during infection with RSV. We hypothesized a role of HIF-1 as transcriptional regulator during infections with respiratory syncytial virus and pursued HIF-1 activation and gene-transcription during RSV infection.

3.2 Transcription factors

Transcription factors are proteins that bind to regulatory sequences, usually in the 5' upstream promoter region of target genes, to increase (or sometimes decrease) the rate of gene transcription. This may result in increased or decreased protein synthesis and altered cellular function (8).
Transcription factors can be activated by many extracellular influences, but may also be directly activated by ligands. They act as "nuclear messengers" and can convert transient environmental signals at the cell surface into long-term changes in gene transcription (7). In the context of inflammatory diseases, transcription factors activated by inflammatory stimuli (such as cytokines or viruses) switch on inflammatory genes. This leads to increased synthesis of inflammatory proteins. In this way, transcription factors may amplify and perpetuate the inflammatory process.
Several families of transcription factors exist. The members of each family share structural characteristics (103). Whereas many transcription factors are ubiquitous, others are cell specific and determine the phenotypic characteristics of a cell (8).

3.3 Hypoxia inducible factor

A number of elegant studies, exemplified by those defining induction of the erythropoietin (EPO) gene (16, 120), have utilized multidisciplinary approaches to elucidate basic hypoxia-adaptive responses. Today, convincing evidence confirms a central role of hypoxia-inducible factor (HIF)-1 in mammalian oxygen homeostasis (107, 117, 125, 126).
Such studies demonstrated that HIF-1 is a heterodimeric helix-loop-helix transcription factor composed of two subunits: constitutively expressed HIF-1β and oxygen-regulated HIF-1α (147). Under normoxic conditions, HIF-1α is continuously synthesized and subjected to hydroxylation on proline residues (118). The modification is required for the binding of the von Hippel-Lindau (VHL) tumor suppressor protein, the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation. Under hypoxic conditions, hydroxylation is inhibited and the VHL protein does not bind to HIF-1, eventually leading to stabilization of the alpha-subunit, heterodymerization, nuclear translocation and transcription of HIF-dependent genes (88, 150). In the absence of hydroxylation due to hypoxia, HIF-1 translocates to the nucleus and binds to hypoxia responsive elements (HRE) in the regulatory regions of target genes and affects the rate of gene transcription.

In keeping with the complexity of the hypoxic response, three principal isoforms of HIFα exist: HIF-1α, HIF-2α and HIF-3α (150).
The HIF-2α protein is structurally similar to HIF-1α, dimerizes with HIF-1β, and contains proline and asparagine residues that are hydroxylated in an oxygen-dependent manner. Unlike HIF-1α, which is active in all hypoxic cells, HIF-2α only appears to be transcriptionally active in a restricted number of cell types (119). HIF-3α is less closely related and its role is not yet fully understood (150).

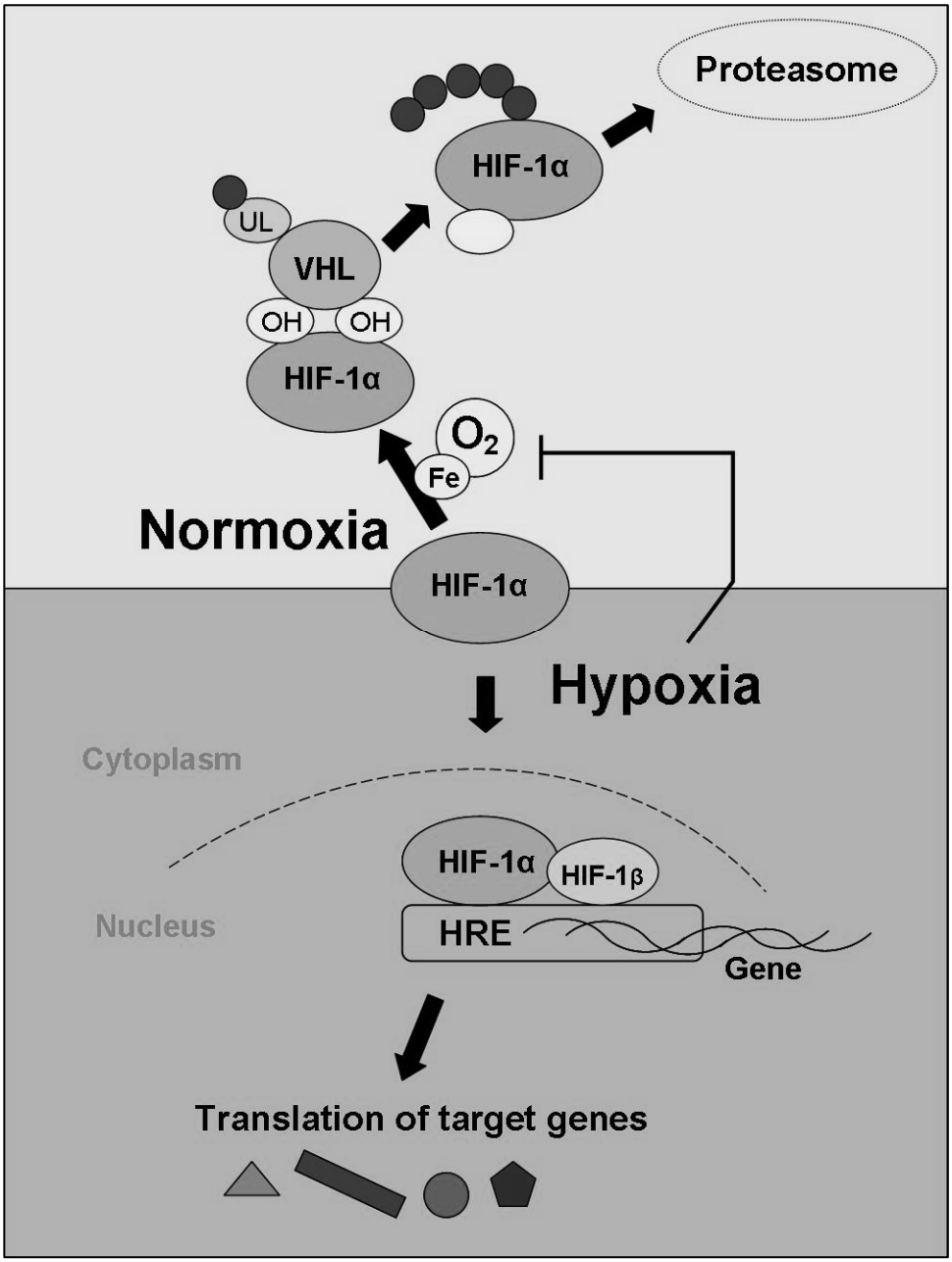
HIF-1 was originally discovered as a biologic oxygen sensor that enables the organism to adapt to hypoxia. Under baseline conditions, normal tissues show

little evidence of HIF-1 activation (132). When oxygen levels fall, HIF-1α is activated by post-transcriptional changes in its stability and transcriptional activity (89). The half-life of HIF-1α is regulated in an oxygen-dependent manner (115) and the posttranslational modifications of HIF-1α are rapidly and precisely modulated according to the cellular oxygen concentration by multiprotein complexes. In non-hypoxic cells (cultured in 20% O_2), there is no detectable HIF-1α protein. When cells are transferred to hypoxic conditions (1% O_2), HIF-1α expression can be detected within 30 minutes (121). Previous studies demonstrated a time- and O_2 concentration-dependent manner of the HIF-1α induction in cells exposed to hypoxia (153).

Up-regulation of HIF-1α was also observed in a broad range of cancers including breast and prostate cancer which was correlated well with increased vascularity and metastatic potential, indicating that the expression of HIF-1α is associated with tumor progression (26, 154). The expression of HIF-1α is of primary importance in angiogenesis, tumor growth, invasion and metastasis. The expression of HIF-1α is high in many carcinoma cells compared to normal tissues and it is an essential transcription factor for different factors and enzymes that are required for the continued growth and survival of tumors (152). This is a result of genetic alterations and intratumoral hypoxia (154).

The HIF system provides a molecular basis for understanding a set of classical physiological responses. It is evident that HIF shapes very extensive aspects of development, physiology and disease, providing cells with a master regulator that coordinates changes in gene transcription.

The following figure gives an illustration of the HIF-1α pathway.



Regulation of HIF-1α protein

Under normoxic conditions, hydroxylation on proline residues allow capture of HIF-1α by the von Hippel Lindau (VHL) protein. An ubiquitin-protein ligase (UL) targets HIF-1α to proteasomal degradation.

In the absence of hydroxylation due to hypoxia, HIF-1α translocates to the nucleus, heterodimerizes with HIF-1β and binds to hypoxia-responsive elements (HRE) in the regulatory regions of target genes.

3.4 Hypoxia

Oxygen is required by the cells of most organisms to produce adequate amounts of Adenosine Triphosphate (ATP) necessary for metabolic activities. Decreased oxygenation causes the tissues to become hypoxic, and this in turn initiates a variety of signals and produces alterations in many physiological parameters. These are well-known and have been extensively documented (89). Oxygen has therefore been identified as a major control element in cellular processes (1). Hypoxia occurs within human tissues and cells due to many different conditions, including disorders of the heart and lungs, anemia, and circulatory problems. Depending on the severity, permanent damage to tissues and cells may occur (114). However, hypoxia also can play an important and beneficial role in human physiology and development (155). Many organisms have evolved adaptive mechanisms for hypoxic conditions. Changing oxygen levels can result in activation or repression of certain homeostatic regulatory genes, allowing for the survival of tissues and cells despite fluctuating environmental conditions. Genes whose activation is prompted by hypoxic conditions, can interact with enzymes and other transcriptional factors in order to control vascularization and tissue growth. While microenvironments surrounding cancerous tumors are extremely hypoxic, proliferation of such masses is often made possible by HIF-1 activation. This leads to increased angiogenesis and, thus, an increased oxygen supply to the area (18).
However, in hypoxic environments, there is a shift to anaerobic metabolism for cellular energy production. HIF-1 is among the principal genes to coordinate this shift, by inducing a variety of glycolytic enzymes and glucose transporters. This helps cells to efficiently produce energy in hypoxic environments (18, 143).

During inflammatory processes, we find significant changes in tissue metabolism. In particular, metabolic shifts during inflammation can result in significant tissue hypoxia. Prior studies assume hypoxia in infected cells due to

increased oxygen consumption. It has been shown that this causes stabilization of HIF-1. However, other studies of HIF-1 during inflammation and infection have found oxygen-independent activation of HIF-1 during infections with human pathogens (56).

3.5 HIF-1 and Inflammation

Active inflammation is characterized by dramatic shifts in tissue metabolism. These changes include diminished availability of oxygen (hypoxia) (45) with subsequent lactate accumulation and resultant metabolic acidosis. A number of studies have implicated hypoxia in mucosal inflammatory diseases such as colitis (57, 77). Other studies in murine models identified the epithelium as the central target of hypoxia during active inflammation (66).

In the meantime, a number of studies have identified HIF-1 as transcriptional regulator of inflammation and infection. For example, HIF-1 is essential for myeloid cell-mediated inflammation, bactericidal capacity of phagocytes (105) and mice with conditional knockouts of HIF-1 show profound impairment of myeloid cell aggregation, motility, invasiveness, and bacterial killing (25). Moreover, studies of HIF-1 during infection with enterobacteriaceae revealed hypoxia-independent activation by bacterial siderophores (56). Similarly, HIF-1 has been identified as key regulator of the inflammatory transcription factor Nuclear factor kappaB (NF-κB) (145). Other studies confirmed several parallels between the transcriptional regulation of hypoxia and inflammation/infection (34, 56, 69, 78, 94). For example, a recent study suggests that NF-κB is a critical transcriptional activator of HIF-1 and that basal NF-κB activity is required for HIF-1 protein accumulation under hypoxia (108). Similarly, studies of human pathogenes have revealed that exposition of host cells to bacteria (e.g. *Bartonella henselae*) results in HIF-1 activation and VEGF secretion *in vivo* and *in vitro* (69). Similar findings were reported when macrophages were infected with group B streptococci (25, 105).

In conclusion it can be said that HIF-1 obviously plays a central role in infections with human pathogens.

This might have important medical implications in terms of the treatment of sepsis. For example, it has been shown that serum VEGF levels (known to be regulated via HIF-1) are dramatically increased in patients suffering from septicemia (142) or meningitis (141).

3.6 HIF-1 target genes

HIF-1 acts as a master regulator of oxygen-regulated gene expression and regulates the transcription of hundreds of genes in a cell type specific manner (88). For example, binding of HIF-1 to consensus domains in the erythropoitin promoter results in the transcriptional induction of HIF-1-bearing gene promoters (116). A series of experiments by Wang and Semenza (147-149) and Maxwell *et al.* (91) has demonstrated that reporter genes containing the erythropoitin enhancer were induced by hypoxia in a variety of cell types that did not normally produce erythropoitin. Subsequently, it was determined that HIF-1 is widely expressed and that consensus HIF-1 binding sequences exist in a number of genes other than that of erythropoitin, and were termed hypoxia responsive elements (HRE (116)). When this sequence was introduced into a heterogous reporter gene and transfected into cultured cells, there was a dramatic increase in reporter gene trenscription when the cells were exposed to hypoxia (121). In particular, HIF-1 has been found to regulate multiple genes that include HRE in their promoter region, including vascular endothelial growth factor (VEGF), insuline-like growth factors (IGFs), their binding proteins [insuline-like growth factor binding proteins (IGFBPs)] and iron supply regulating genes [e.g. transferrin (113)]. HIF-1 binds to the hypoxic responsive elements (HRE) of target gene regulatory sequences, resulting in the transcription of these genes.

The discovery of HIF-1 represented a major advance in the understanding of gene regulation by hypoxia. Different studies on the role of HIF-1 in cellular processes have established the general agreement that induction of HIF-1 responsive genes drives a variety of effects. These include altered cellular metabolism, increased vascular mass and diameter and increased oxygen carrying capacity of the blood. All of these effects are conducive to an adaptive response to diminished oxygen supply (18, 44, 80, 120).
HIF-1 activation is capable of mediating a response that is precisely tailored to the requirements of the cell, tissue and organism.

Table 1 (83) presents a structured overview of the genes that get transcriptionally activated by HIF-1. It also shows the rich variety of functions of the genes regulated by HIF-1.

From the wealth of genes regulated by HIF-1, we have used four genes exemplarily in our study. These four genes will be discussed in detail in the sections following Table 1.

Table 1: Transcriptionally activated genes by HIF-1 (83)

Function	Genes
Cell proliferation	Cyclin G2, IGF2, IGF-BP1, IGF-BP2, IGF-BP3, WAF-1, TGFα, TGFβ3
Cell survival	ADM, EPO, IGF2, IGF-BP 1-3, NOS2, TGFα, VEGF
Apoptosis	NIP3, NIX, RTP801
Motility	ANF/GPI, c-MET, LRP1, TGFα
Cytoskeletal structure	KRT14, KRT18, KRT19, VIM
Cell adhesion	MIC2
Erythropoieseis	EPO
Angiogenesis	EG-VEGF, ENG, LEP, LRP1, TGF-β3, VEGF
Vascular tone	α_{1B}-adrenergic receptor, ADM, ET1, Haem oxygenase-1, NOS2
Transcriptional regulation	DEC1, DEC2, ETS-1, NUR77
pH regulation	Carbonic anhydrase 9
Regulation of HIF-1 activity	P35srj
Epithelial homeostasis	Intestinal trefoil factor
Drug resistance	MDR1
Nucleotide metabolism	Adenylate kinase 3, Ecto-5'-nucleotidase
Iron metabolism	Ceruloplasmin, Transferrin, Transferrin receptor
Glucose metabolism	HK1, HK2, AMF/GPI, ENO1, GLUT1, GAPDH, LDHA, PFKBF3, PFKL, PGK1, PKM, TPI, ALDA, ALDC
Extracellular-matrix metabolism	CATHD, Collagen type V (α1), FN1, MMP2, PAI1, Prolyl-4-hydroxylase α (1), UPAR
Energy metabolism	LEP
Amino-acid metabolism	Transglutaminase 2

ADM	adrenomedullin
ALDA	aldolase A
ALDC	aldolase C
AMF	autocrine motility factor
CATHD	cathepsin D
EG-VEGF	endocrine-gland-derived VEGF
ENG	endoglin
ET1	endothelin1
ENO1	endolase1
EPO	erythropoietin
FN1	fibronectin
GLUT	glucose transporter
GAPDH	glyceraldehyde-3P-dehydrogenase
HK	hexokinase
IGF	insulin-like-growth factor
IGF-BP	IGF-binding protein
KRT	kreatin
LDHA	lactate dehydrogenase
LEP	leptin
LRP1	LDL-receptor-related protein
MDR	mulitdrug resistance
MMP	matrix metalloproteinase
NOS2	nitric oxide synthase
PFK	phosphofructokinase
PGK	phosphoglycerate kinase
PAI	plasminogen-activator inhibitor
PKM	pyruvate kinase M
TGF	transforming growth factor
TPI	triosephosphate isomerase
VEGF	vascular endothelial growth factor
UPAR	urokinase plasminogen activator receptor
VEGFR2	VEGF-receptor2
VIM	vimentin

3.6.1 CD73

Ecto-5'-nucleotidase (CD73) is a membrande-bound glycoprotein that functions to hydrolyze extracellular nucleotides into bioactive nucleoside intermediates (133, 156). Surface-bound CD73 converts adenosine monophosphate (AMP) to adenosine, which, when released, can activate several transmembrane adenosine receptors or can be internalized through dipyridamole-sensitive carriers (30, 131). Extracellular Adenosine Triphosphate (ATP) liberated during hypoxia and inflammation can either signal directly on purinergic receptors or can activate adenosine receptors following phosphohydrolysis to adenosine (35, 36). A number of studies indicated that CD73 is hypoxia-responsive through transcriptional induction and is associated with tissue protection in a variety of physiological settings (32, 85).

In situations characerized by ATP hypercatabolism such as hypoxia, CD73 is implicated, because AMP, produced from ATP release in the extracellular medium, must be converted into adenosine before reuptake (81, 134). Therefore CD73 plays an important role in the defense against hypoxia, particularly in the intravascular space. Surface CD73 likely represents a protective pathway for the maintenance of barrier function in epithelia and vascular endothelia. A HIF-1 binding site in the CD73 gene promoter was identified and different approaches defined a role of HIF-1 in the induction of CD73 (133).

3.6.2 VEGF

Vascular endothelial growth factor (VEGF) is a prime regulator of angiogenesis (24). VEGF is expressed in most types of human cancer, and increased expression in tumors is often associated with a less favorable prognosis. Induction of or an increase in VEGF expression can be caused by numerous environmental factors such as hypoxia (106, 123), mediated through the hypoxia-inducible transcription factors 1α and 2α (70). The binding of VEGF leads to a cascade of different signaling pathways (122), resulting in the up-

regulation of genes involved in mediating the proliferation and migration of endothelial cells and promoting their survival and vascular permeability (70). In vivo VEGF induces angiogenesis as well as permeabilization of blood vessels and plays a central role in the regulation of vasculogenesis (97).
Thus, HIF-1 plays a critical role in angiogenesis by regulating the expression of the key angiogenetic factor: VEGF.

3.6.3 FN-1

Important properties of invasive cancer cells include decreased cell-cell adhesion, cytoskeletal remodeling, increased mortility, increased production of extracellular matrix (ECM) proteases, and syntehsis of new ECM components (76). Fibronectin-1 (FN-1) is one of the major ECM proteins associated with tumor invasion (23, 61). Krishnamachary *et al*. showed that hypoxia or HIF-1α overexpression stimulates matrigel invasion and that FN-1 mRNA expression is induced by hypoxia in wild-type embryonic stem (ES) cells but not in HIF-1α-null cells (76).

3.6.4 COX 2

Cyclooxygenase (COX)-2 is the inducible key enzyme for prostanoid biosynthesis. COX-2, like its isoenzyme COX-1, catalyses the conversion of arachidonic acid to endoperoxide intermediates, which are ultimately converted to prostanoids. It is overexpressed in most colorectal carcinomas and a subset of adenomas (29, 33), and accumulating evidence supports an important role for COX-2 in colorectal tumorigenesis (15). Whereas COX-1 is expressed constitutively in most tissues and appears to be responsible for housekeeping functions, COX-2 is transcriptionally induced and thought to be the isoform responsible for the production of pro-inflammatory prostanoids that play a central role in regulation of inflammation (64). Analyses reveal that COX-2 up-regulation is associated with HIF-1α induction (65).

3.7 Respiratory syncytial virus

Respiratory syncytial virus (RSV) is the major cause of serious lower respiratory disease in infancy and early childhood (42) and worldwide the most frequent cause of bronchiolitis and pneumonia in infants requiring hospitalization (3). Bronchiolitis, the more severe clinical manifestation of RSV infection, is characterized by necrosis and sloughing of the respiratory epithelium and plugging of the small bronchioles with fibrin and mucus (2, 28). These infections can be associated with serious morbidity and mortality, especially in children less than six years of age and children with comorbid chronic illnesses (82). Infants with congenital heart disease infected with RSV can have a significantly more severe illness and a higher mortality rate than other children (87). Thus, RSV is one of the most common and troublesome viruses of infancy (102). RSV is also a significant contributor to illness in adult populations especially in the elderly (39) and is recognized to be a major nosocomial hazard, especially in high-risk patients (51).

RSV is an enveloped single-stranded negative-sense RNA virus of the Paramyxoviridae family (52). Closely related viruses include parainfluenza virus, measles and mumps (17). The genome of the virus is composed of a single strand of ribonucleic acid (RNA) containing only 10 genes. A total of 11 proteins are encoded within this RNA genome. Nine of these are structural proteins and surface glycoproteins that form the viral coat and bring about attachment of the virus to the host cell. The remaining two direct the replication process of the virus once it infects its host cell (12).

Recent molecular studies of RSV identified two envelope glycoproteins – protein G and protein F - of RSV as the major viral antigens. Of the 11 viral proteins encoded by the RSV genome, only the F and G glycoproteins were found to induce neutralizing antibodies. The fusion glycoprotein (protein F) appears to be important for inducing protective immunity and is associated with T helper subset 1 cytokine expression (79, 136). It mediates viral penetration by fusion of the viral envelope to the host cell membrane, resulting

in penetration of the viral RNA into the cell and also mediates cell-cell spread via membrane fusion (60, 99, 146). In this way, a characteristic fusion of human cells in tissue cultures known as the syncytial effect is released (71). The major glycoprotein G seems to be the viral attachment protein and appears to be linked to pathogenesis of RSV disease (99).

Two subtypes of RSV, A and B, have been identified. Subtype B is characterized as the asymptomatic strain of the virus that the majority of the population experiences. The more severe clinical illnesses involve Subtype A strain which tends to predominate in most outbreaks (5).

RSV is a ubiquitous pathogen in all human populations and is transmitted through close contact with a person who has an active infection or direct contact with infectious secretions or environmental surfaces. The incubation period is 2-8 days after initial contact, with the most likely period being 4-6 days. An RSV infection does not produce substantial immunity to subsequent infection; thus, reinfections are common (12).

The epithelium of the respiratory mucosa, which main function is to provide a protective physical barrier against injurious inhaled stimuli, is the main target of RSV. Following inhalation or self-inoculation of the virus into the nasal mucosa and infection of the local respiratory epithelium, RSV spreading along the respiratory tract occurs mainly by cell-to-cell transfer of the virus (53). Infection of respiratory epithelial cells is the first event occurring after RSV inhalation or inoculation. This is rapidly followed by the induction of a network of epithelial cell gene products and a number of molecules are produced by human epithelial cells as a consequence of RSV infection. Among them are potent immunomodulatory and inflammatory mediators (47, 112).
In acute bronchiolitis the main lesion is epithelial necrosis when a dense plug is formed in the bronchiolar lumen leading to trapping air and other mechanical interference with ventilation: in interstitial pneumonia, there is widespread

inflammation and necrosis of lung parenchyma, and severe lesions of the bronchial and bronchiolar mucosa as well (2).
As such, RSV is characterized by a particularly prominent inflammation of the pulmonary mucosa - both in natural and experimental infections (42, 137). In fact, RSV is among the most potent biological stimuli that induce the expression of inflammatory genes, including those encoding chemokines. Studies on mechanisms that control virus-mediated airway inflammation are currently areas of intense investigation (46-50).

To examine the immunopathogenesis of RSV infection – during both the acute and chronic phases of the disease – and to study the dynamics of RSV replication, a mouse model of RSV infection was developed by Mejías *et al.* (92). Using this model, they showed that RSV induces significant inflammatory changes in the lungs which evolve into a chronic phase characterized by persistant airway inflammation and airway hyperresponsiveness, even when the virus is no longer detectable by quantitative culutures. These findings support previous observational studies of children suggesting that RSV is associated with long term airway disease (124, 130). Children who recover from RSV bronchilitis are at increased risk of recurrent wheeze and a significantly higher rate of asthma is diagnosed in later childhood (102). With recent evidence that RSV can persist, it is possible that low-level viral replication might drive chronic inflammation in some individuals with chronic lung diesease (101).

Although it has been clearly shown that the development of a specific immune response to RSV is partly responsible for injury to the lungs and RSV-induced diseases are mediated through active cytokines released during infection, the knowledge of the pathogenetic mechanisms of the RSV disease is still inadequate (101, 102). Severe RSV disease appear to be associated with a misdirected immune response, characterized by enhanced release of mediators and infiltration of a range of monocytes and polymorphonuclear cells (40, 71) but many aspects still remain understood poorly.

3.8 Problem description

Previous studies have shown increased levels of HIF-1 during various infections. These include infections with different viruses like Epstein-Barr (144), Hepatitis B (55) or C (96), bacteria (10, 25, 105, 158) and parasites (4).

However, none of these studies has used RSV as etiologic agent. As discussed in section 3.7, RSV represents a significant hazard, especially to high-risk patients and young children. To explore new therapeutical options, it would be beneficial to know about the interdependencies between RSV infection and HIF-1 stabilization.

Therefore, we have chosen to explore HIF-1 activation and gene-transcription during RSV infection. Beyond that, we have investigated the mechanism used by RSV to induce the HIF-1 pathway.

We hypothesized a role of HIF-1 as transcriptional regulator during infections with respiratory syncytial virus and pursued HIF-1 activation and gene-transcription during RSV infection.

4 Materials

This section lists the materials and equipment used in the experiments executed for the on-hand study.

Equipment

Centrifuge 5417R (Eppedorf, Hamburg, Germany)
Confocal laser scanning Microscope (Leica, Bensheim, Germany).
Electrophorese Unit (Invitrogen, Karlsruhe, Germany)
Hypoxia Chamber (Invivo$_2$ 400) (Ruskinn Technology Limited, Leeds, UK)
iCycler (Bio-Rad Laboratories Inc., Hercules, California, USA)
Incubator Cytoperm2 (Heraeus, Hanau, Germany)
i-STAT Analyzer (Abbott, Wiesbaden, Germany)
Magellan-Reader (Tecan, Crailsheim, Germany)
Megafuge 1.0R (Heraeus, Hanau, Germany)
Microscope (Leica, Bensheim, Germany)
Photometer (Pharmacia, Freiburg, Germany)
Pipetboy (Integra Bioscience, Fernwald, Germany)
Pipetts (Eppendorf AG, Hamburg, Germany)
Power Pac 3000/200 (Bio-Rad Laboratories Inc., Hercules, California, USA)
Refrigerator -20°C (Linde, Siegen, Germany)
Refrigerator -80°C (Labotec, Göttingen, Germany)
Rotator (neoLab, Heidelberg, Germany)
Shaker (Janke&Kunkel IKA-Labortechnik, Staufen, Germany)
Speci-Mix (Bioblock Scientific, Illkirch Cedex, France)
Table Centrifuge (Carl Roth GmbH&Co. KG, Karlsruhe, Germany)
Thermomixer (Eppendorf AG, Hamburg, Germany)
Trans Blot Cell Chamber (Bio-Rad,
UV-Table (Biometra, Göttingen, Germany)
Vortex-Genie2 (Scientific Industries Inc., Bohemia, NY, USA)
Water bath (GFL, Burgwedel, Germany)

Weighing machine (Ohaus, Pine Brook, NJ, USA)

Reagents

Accutase (PAA Laboratories, Pasching, Austria)

Albumin from bovine serum (Sigma-Aldrich, Steinheim, Germany)

Ammoniumpersulfat (Bio-Rad, Hercules, California, USA)

Antibiotic-Antimycotic-Solution (Sigma-Aldrich, Steinheim, Germany)

Bromphenolblau (AppliChem GmbH, Darmstadt, Germany)

Di-Natriumhydrogenphosphat (AppliChem GmbH, Darmstadt, Germany)

Destilled Water (Invitrogen, Karlsruhe, Germany)

EDTA (Sigma-Aldrich, Steinheim, Germany)

Ethanol (Merck, Darmstadt, Germany)

F-12 HAM medium (Invitrogen, Karlsruhe, Germany)

FCS (Invitrogen, Karlsruhe, Germany)

Full range Rainbow RPN800V (Amersham, Buckinghamshire, UK)

Glycerol (Sigma-Aldrich, Steinheim, Germany)

Hepes-KOH (Invitrogen, Karlsruhe, Germany)

Igepal Electrophoresis Reagent, NP40 (Sigma-Aldrich, Steinheim, Germany)

iQ-Sybr Green Supermix (Bio-Rad, Hercules, California, USA)

Kaliumdihydrogenphosphat (Merck, Darmstadt, Germany)

Kaliumchlorid (Merck, Darmstadt, Germany)

L-Glutamin (Sigma-Aldrich, Steinheim, Germany)

Magic Mark (Invitrogen, Karlsruhe, Germany)

Methanol (Merck, Darmstadt, Germany)

$MgCl_2$ (Merck, Darmstadt, Germany)

Natriumchlorid (Sigma-Aldrich, Steinheim, Germany)

Nitrocellulose membrane (Bio-Rad Laboratories, Inc., München, Germany).

Nonfat dried milk powder (AppliChem GmbH, Darmstadt, Germany)

PBS minus (Invitrogen, Karlsruhe, Germany)

PBS plus (Invitrogen, Karlsruhe, Germany)

Perm/Wash-solution (PharMingen, BD-Bioscience, Heidelberg, Germany)

ProLong Gold antifade reagent with DAPI (Invitrogen, Karlsruhe, Germany)

PVDF-Membrane (Bio-Rad Laboratories Inc., München,)
Rainbow Marker(Amersham, Buckinghamshire, UK)
Rotiphorese Gel 30 (Carl Roth GmbH+Co.KG, Karlsruhe, Germany)
SDS (Sigma-Aldrich, Steinheim, Germany)
Temed (Sigma-Aldrich, Steinheim, Germany)
Tris-Base (AppliChem GmbH, Darmstadt, Germany)
Tris/Glycin buffer (Bio-Rad Laboratories GmbH, München, Germany)
Tris/Glycin/SDS buffer (Bio-Rad Laboratories GmbH, München, Germany)
Tris-HCl (Merck, Darmstadt, Germany)
Triton X 100 (Sigma-Aldrich, Steinheim, Germany)
Trypan Blue Solution (0,4%) (Sigma-Aldrich, Steinheim, Germany)
Tween-20 (Applichem, Darmstadt, Germany)
Western Blot Stripping Buffer (Pierce, Bonn, Germany)
Protease-Inhibitors:

- Aprotinin (Appli-Chem, Darmstadt, Germany)
- Leupeptin (Appli-Chem, Darmstadt, Germany)
- Pepstatin (Boehringer, Mannheim, Germany)
- PMSF (Appli-Chem, Darmstadt, Germany)
- OV (Sigma-Aldrich, Steinheim, Germany)
- DTT (Sigma-Aldrich, Steinheim, Germany)

Kits

BCA™ Protein Assay Kit (Pierce, Bonn, Germany)
Chemiluminescent Substrate Kit (Pierce, Bonn, Germany)
Cytofix/Cytoperm (PharMingen, BD-Bioscience, Heidelberg, Germany)
ELISA-Kit for human VEGF (R&D Systems, Minneapolis, MN, USA)
RNA II Kit (Macherey & Nagel, Düren, Germany)
iScript™cDNA Synthesis Kit (Bio-Rad, München, Germany)

Materials

Cell Scraper (Costar, NY, USA)
Combs: 1,5mm; 15well (Invitrogen, Carlsbad, CA, USA)

Eppendorf Cups (Eppendorf AG, Hamburg, Germany)
Eppendorf Tips (Eppendorf AG, Hamburg, Germany)
Gel-Cassettes (Invitrogen, Karlsruhe, Germany)
Glass Slides (NalgeNuc International, Naperville, IL, USA)
Microtiter Plate 96 well (nunc^{TM}, Brand Products, Denmark)
Pipet 5ml/10ml/25ml (BD Bioscience, Erembodegem, Belgium)
Syringe 1ml (Becton Dickinson, Le Pont De Claix, France)
Tissue culture Dishes (Cellstar, Frickenhausen, Germany)
Tissue culture Flasks 75cm^2 (Techno Plastic Products AG, Trasadingen, Switzerland)
Tissue culture Flasks 70ml (Becton Dickinson, Le Pont De Claix, France)
Tubes 50ml/15ml (Becton Dickinson, Le Pont De Claix, France)

Antibodies

Anti-HIF-1α rabbit polyclonal IgG (upstate, Lake Placid, NY)
Anti-HIF-1α mouse Mab (BD-Bioscience, Heidelberg, Germany
Anti-RSV mouse IgG (Acris, Hiddenhausen, Germany)
Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Karlsruhe, Germany)
Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Karlsruhe, Germany)
Beta-Actin antibody (Cell Signaling, Danvers, MA)
COX2 goat polyclonal IgG (Santa Cruz, Heidelberg, Germany)
FN goat polyclonal IgG (Santa Cruz, Heidelberg, Germany)
HRP-labelled secondary antibodies:

- goat anti-rabbit
- donkey anti-goat,
- goat anti-mouse (Santa Cruz, Heidelberg, Germany)

Primers

- CD 73
  - sense primer 5'-AAG GAA GGG GAA GAA CAG GA-3'
  - antisense primer 5'-GGC AGA GCT GAT GGA ATC TC-3'

- COX2
    - sense primer 5'-AAA CCT CAG CTC AGG ACT GC-3'
    - antisense primer 5'-GGC ACT AGC CTC TTT GCA TC-3'
- FN1
    - sense primer 5’-AGG CTC AGC AAA TGG TTC AG-3’
    - antisense primer 5’-TCG GCT TCC TCC ATA ACA A-3’
- Human beta-actin
    - sense primer, 5'-GGT GGC TTT TAG GAT GGC AAG-3'
    - antisense primer, 5'-ACT GGA ACG GTG AAG GTG ACA G-3'
- VEGF
    - sense primer 5’-TTG CCT TGC TGC TCT ACC TC-3’
    - antisense primer 5’-AGC TGC GCT GAT AGA CAT CC-3’
- HIF-1α
    - sense primer 5’-ACC TCG CTG ACC AGT TAT GAT TGT GAT CAA GAG TCA CAA TCA TAA CTG GTC AGC TT-3’
    - antisense primer 5’-CAA AAA GCT GAC CAG TTA TGA TTG TGA CTC TTG ATC ACA ATC ATA ACT GGT CAG CG-3’

Cells

A549 cells (American Type Culture Collection, Wesel, Germany)

Buffer

RIPA-Buffer

1mM Tris-HCl

250mM NaCl

1mM EDTA

Triton X 100 1%

NP40 1x Igepal Electrophoresis Reagent

Transfer buffer:

200ml 10x Tris/Glycin buffer

400ml Methanol

1400ml Aqua dest.

Running buffer:

200ml 10x Tris/Glycin/SDS buffer

1800ml Aqua dest.

Nuclear Proteins:

- Buffer A:

10 mM Hepes-KOH, pH 7.9

1.5 mM $MgCl_2$

10 mM KCl

0.5 mM Dithiothreitol (DTT)

0.2 mM Phenylmethylsulfonyl fluoride (PMSF)

- Buffer B:

20 mM Hepes-KOH, pH 7.9

25% Glycerol

420 mM NaCl

1.5 mM $MgCl_2$

0.2 mM EDTA

0.5 mM DTT

0.2 mM PMSF

- Buffer C

20 mM Hepes-KOH, pH 7.9

25% Glycerol

420 mM NaCl

1.5 mM $MgCl_2$

0.2 mM EDTA

0.5 mM DTT
0.2 mM PMSF
2 mM Benzamidine
5 mg/ml Leupeptin

Upper-Buffer for SDS Page, pH 6,8
30,3g Tris-Base (0,5M)
2g SDS (0,4%)
500ml Aqua dest.

Lower-Buffer for SDS Page, pH 8,8
90,85 Tris-Base (1,5M)
2g SDS (0,4%)
500ml Aqua dest.

Lämmli 4x non-reducing
2,5ml TrisHCl 1M ph 6,8
4,0 ml Glycerol
0,8g SDS
0,01g Bromphenolblau
1ml Aqua dest.

TBS 10x, pH 7,6
24,2g Tris-Base
80g NaCl
in 1l Aqua dest.

PBS 10x, pH 7,2-7,4

8g NaCl (137mM)

0,2g KCl (2,7mM)

1,44g Na_2HPO_4 (8,1mM)

0,2g KH_2PO_4 (1,5mM)

in 1l Aqua dest.

Software

Microsoft Excel, Microsoft Office Professional Edition 2003

Microsoft Word, Micorsoft Office Professional Edition 2003

GraphPad Software, Prism 4 for Windows Version 4.03

JMP statistical software (SAS Institute Inc.)

EndNote 8, Thomson Reuters

5 Methods

The following sections describe the experiments executed for this study, including cell culture, infection with RSV and subsequent testings with samples extracted from infected cells.
Other sections describe the immunohistochemical staining, blood gas analysis and experiments with cells following siRNA repression of HIF-1.

We also describe the setup for the in vivo experiments. The mice were housed and infected at the animal research facility of the University Texas Medical Branch (UTMB), Galveston, Texas. While the extraction of lung nuclear proteins was also performed there, the protein analyses were conducted in the context of the on hand thesis (Department of Anaesthesiology and Intensive Care Medicine, University of Tuebingen, Tuebingen, Germany).

The last section is dedicated to the statistical analysis that have been performed.

5.1 Culture of epithelial cells

A549 cells (American Type Culture Collection, Wesel, Germany) were cultured as described previously (46). In short, A549 were grown in F-12 HAM medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany) and 1% Antibiotic-Antimycotic-Solution (Sigma-Aldrich, Steinheim, Germany). L-Glutamin was adjusted to a total quantity of 2mM.

The cells were maintained under standard-conditions of 37°C in 20% O2 and 5% CO2 and subcultivated with a ratio of 1:3 to 1:5 two to three times per week.

5.2 Infection with RSV

Human RSV was purified by polyethylene glycol precipitation, followed by centrifugation on 35 to 65% discontinuous sucrose gradients as described previously (100, 139). The virus divided into aliquots and stored at -80°C until use. Virus titers were determined by a methylcellulose plaque assay (73).
For experiments with inactivated RSV, the virus was exposed to an UV light source for 20 minutes as described previously for the inactivation of RSV (100).
A549 cells were infected when they reached 70-80% confluence, using different multiplicities of infection (MOI). The virus was added immediately after removal of the culture medium in a small amount of serum-free medium for 1 h. Additional media was added and the infection was continued for indicated time periods (46).

5.3 Protein extraction

Supernatants were discarded and 200µl Lysis-Buffer (1mM Tris-HCl, 250mM NaCl, 1mM EDTA, Triton X 100 1%, NP40 1x Igepal Electrophoresis Reagent, Aprotinin 1µg/ml, Leupeptin 1µg/ml, Pepstatin 1µg/ml, PMSF 1mM and OV 1mM) was added. After scraping and collecting into tubes, the cell-lysate was incubated at 4°C for 20 minutes on a rotator. Cell debris was removed by centrifugation at 13000g for 15 minutes and discharged.
Protein concentrations were determined using the BCA™ Protein Assay Kit (Pierce, Bonn, Germany) according to the manufacturer's instructions.

5.4 Nuclear protein extraction

Nuclear Proteins were isolated from A549 cells using a modification of methods previously described (14).
In short, cells were lysed in 500µl cold buffer A (10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM Dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)), scraped and collected into tubes and

incubated for 15 min on ice. After adding 7,5µl solution containing 10% NP40, vortexing for 30 seconds and incubation for three minutes on ice, the tubes were centrifuged for 2 minutes at 6000g at 4°C. The cytoplasmic proteins in the supernatant were collected and flash-frozen. Next, the pellet was resuspended in 100µl of cold buffer B (20 mM Hepes-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). After centrifugation (12000g at 4°C for 30 minutes), the supernatant was discarded and the pellet was resuspended in 50µl of buffer C (25% glycerol, 20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2 mM benzamidine, 5 mg/ml leupeptin) and incubated for 45 minutes at 4°C. Cellular debris was removed by 5 minutes of centrifugation (6000g at 4°C) and the supernatant was flash-frozen at -80°C.

Protein concentrations were determined using the BCA™ protein assay kit (Pierce, Bonn, Germany) as instructed by the manufacturer.

5.5 Western blotting

Proteins were diluted in radio-immuno precipitation assay (RIPA) buffer to equivalent protein concentrations. After adding 4x Laemmli sample buffer they were immediately heated for 10 minutes at 70°C, separated on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Polyvinylidene Difluoride, Bio-Rad Laboratories, Inc., München, Germany). Rainbow (Amersham, Buckinghamshire, UK) and MagicMark (Invitrogen, Karlsruhe, Germany) were used for size analysis and blotting control. The membranes were blocked overnight at 4°C in TBS containing 0,05% Tween and supplemented with 3% BSA and 3% skimmed milk.
The membranes were then incubated in 1:500 COX2 goat polyclonal IgG (Santa Cruz, Heidelberg, Germany) or 1:500 anti RSV mouse IgG (Acris, Hiddenhausen, Germany) or 1:500 FN goat polyclonal IgG (Santa Cruz,

Heidelberg, Germany) or 1:1000 beta-Actin antibody (Cell Signaling, Danvers, MA) in blocking buffer.
After three washes, membranes were incubated with horse-radish-peroxidase (HRP)-labelled secondary antibodies (goat anti-rabbit or donkey anti-goat or goat anti-mouse 1:1000; Santa Cruz, Heidelberg, Germany) for 45 minutes at room temperature. The wash was repeated and proteins were detected by enhanced chemiluminescence, using the Chemiluminescent Substrate Kit (Pierce, Bonn, Germany).
Western immunoblotting for HIF1-alpha was performed using 1:500 Anti-HIF-1α rabbit polyclonal IgG (upstate, Lake Placid, NY) following the manufacturers protocol without the use of Tween.

5.6 Enzyme-linked immunosorbent assay (ELISA) for determination of chemokines

Total protein samples of infected or non-infected A549 cells were tested for VEGF by use of a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) as instructed by the manufacturer.
In short, samples were added to a 96 well microtiter plate, which was coated with murine monoclonal antibody to VEGF. The unbound protein was removed by washing and an enzyme linked polyclonal antibody specific to VEGF was added. After additional washing, substrate solution was added and incubated for 20 min. The color-reaction was stopped with stop solution and the amount of VEGF was determined by optical density of the samples by comparing the standards at 450 nm using an ELISA reader.

5.7 Immunohistochemistry

A549-cells were cultured on glass slides (NalgeNuc International, Naperville, IL) and infected with RSV at a MOI of 3.
After 24 hours, they were fixed and permeabilized for immunofluorescent staining using Cytofix/Cytoperm (PharMingen, BD-Bioscience, Heidelberg,

Germany). After two washes with Perm/Wash-solution (PharMingen, BD-Bioscience, Heidelberg, Germany) the slides were blocked for 30 minutes with 5% skimmed milk in Perm/Wash-solution. Purified mouse anti-HIF1α Mab (BD Transduction Laboratories, BD-Bioscience, Heidelberg, Germany) and anti-RSV mouse IgG (Acris, Hiddenhausen, Germany) were diluted 1:100 in Perm/Wash-solution and the slides were incubated for 30 minutes. Normal mouse and normal rabbit control IgG in a dilution of 1:200 were used.
After two washes with Perm/Wash-solution, the slides were incubated for 30 minutes with the secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG, Invitrogen, Karlsruhe, Germany) in Perm/Wash. The slides were embedded with a reagent containing DAPI (Invitrogen, Karlsruhe, Germany) for staining of the nuclei.
Fluorescence was visualized with a confocal laser scanning microscope (Leica, Bensheim, Germany).

5.8 Reverse Transcription Polymerase Chain Reaction Analysis

Realtime RT-PCR (iCycler; Bio-Rad Laboratories Inc., Hercules, California, USA), was used to verify COX2, FN-1, VEGF and CD73 transcript levels of RSV-infected A549 cells. After infection with RSV with an infection dose of MOI3 for 24h, total RNA was isolated using the RNA II Kit (Macherey & Nagel, Düren, Germany) and real-time RT-PCR was performed as described previously.
The PCR reaction contained 10 pM for each of the following primers:
Sense primer 5'-AAA CCT CAG CTC AGG ACT GC-3' and the antisense primer 5'-GGC ACT AGC CTC TTT GCA TC-3' for COX2.
Sense primer 5’-AAG GAA GGG GAA GAA CAG GA-3’ and the antisense primer 5’-GGC AGA GCT GAT GGA ATC TC-3’ for CD73.
Sense primer 5’-TTG CCT TGC TGC TCT ACC TC-3’ and the antisense primer 5’-AGC TGC GCT GAT AGA CAT CC-3’ for VEGF.
Sense primer 5’-AGG CTC AGC AAA TGG TTC AG-3’ and the antisense primer 5’-TCG GCT TCC TCC ATA ACA A-3’ for FN1.

The primer set for COX2, FN1 and VEGF was amplified using increasing numbers of cycles of 95°C for 15 sec, 58°C for 30 sec, 72°C for 10 sec, and a final extension of 72°C for 1 minute. The primer set for CD73 was amplified using increasing numbers of cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 10sec, and a final extension of 72°C for 1 minute. Human beta-actin (sense primer, 5'-GGT GGC TTT TAG GAT GGC AAG-3'; and antisense primer, 5'-ACT GGA ACG GTG AAG GTG ACA G-3') was used as control.

5.9 Stable repression of HIF-1α by siRNA

Repression of HIF-1alpha by siRNA was achieved based on a modification of methods previously described (34, 56, 75, 78, 94). In short, a haripin primer with the sequence 5'-ACCTCGCTGACCAGTTATGATTGTGATCAAGAGTCA-CAATCATAACTGGTCAGCTT-3' and 5'-CAAAAAGCTGACCAGTTATGA-TTGTGACTCTTGATCACAATCATAACTGGTCAGCG-3' corresponding to position 2666-2685 of the HIF1α gene was selected. A549-cells were transfected using electroporation, followed by selection with G418 (1mg/ml). The control cell line was transfected with a non-specific control psiRNA-hH1 neoscr plasmid.

5.10 Blood Gas Analysis

Blood gas analysis was performed to assess oxygen partial pressure in supernatants of uninfected or infected A549 cells. The cells were cultured and infected at a MOI of 1 or 5. One hour after infection the cell-culture flasks were filled up with serum free media and were sealed gas-tight. Analysis of the supernatants was performed immediately after removal via the I-STAT Analyzer (Abbott, Wiesbaden, Germany) at different time points as described previously (31, 56).

5.11 Infection of mice with RSV and extraction of lung nuclear proteins

Female, 6- to 8-week-old BALB/c mice were purchased from Harlan (Houston, Texas, USA) and were housed in pathogen-free conditions in the animal research facility of the University Texas Medical Branch (UTMB), Galveston, Texas, in accordance with the National Institutes of Health and UTMB institutional guidelines for animal care. The Institutional Animal Care and Use Committee approved this protocol. Cages, bedding, food, and water were sterilized before use.

Under light anesthesia, female, 6-8 weeks old BALB/c mice were infected intranasally with RSV at 1 x 10^7 plaque-forming units (PFUs), diluted in sterile phosphate-buffered saline (PBS) for a total inoculation volume of 50 µl, as previously described (47). As mock treatment, control mice were inoculated in the same way with an equivalent volume of sucrose diluted in D-PBS (21).
At the indicated time points after infection (12, 24, and 48h) mice were anesthetized with an intraperitoneal injection of ketamine and xylazine before the thoracic cavity was opened (47). Lungs were then removed, quick frozen in liquid nitrogen and stored at -80°C until nuclear protein was isolated.

Nuclear proteins were isolated from the lung tissue using a modified method described by Bohrer and colleagues (14). Lung tissue was homogenized in 5 ml ice-cold Buffer A (10 mM 2-hydroxyethyl-piperazine N'-2-ethanesulfonic acid [Hepes]–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.2 mM phemylmethyl sulfonyl fluoride [PMSF], 0.6% nonident P40 [NP-40]) and centrifuged at 350 x g, 4°C for 30 seconds. The supernatant was kept on ice for 5 minutes and centrifuged for 5 minutes at 6,000 x g at 4°C, and the pellet was resuspended in 200 µl Buffer B (10 mM Hepes–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1.2 M sucrose, 0.5 mM DTT, 0.2 mM PMSF). After centrifugation (13,000 x g, 4°C, 30 minutes), the pellet was resuspended in 100 µl Buffer C (20 mM Hepes–KOH, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM ethylenediamine-tetraacetic acid, 0.5 mM DTT, 0.2 mM PMSF, 2 mM

benzamidine, 5 µg/ml leupeptin, 25% glycerol), incubated on ice for 20 minutes, and centrifuged (6,000 x g, 4°C, 2 minutes). The supernatant was quick frozen in aliquots at –80°C.

Housing and infection of the mice as well as lung nuclear protein extraction took place in the animal research facility of the University Texas Medical Branch (UTMB), Galveston, Texas.

HIF-1α stabilization was determined by Western blot analysis as described previously (94). The protein analyses were conducted in the context of the on hand thesis in the department of Anaesthesiology and Intensive Care Medicine in Tuebingen.

5.12 Statistical Analysis

Data collection and statistical analysis was performed using Microsoft Excel (Microsoft Office Professional Edition 2003) and Graph Pad Prism (GraphPad Software Inc., Prism 4 for Windows Version 4.03) as well as JMP statistical software (SAS Institute Inc.).
All presented values were calculated from at least three separate experiments.

For the analysis of the data of Figure 2 C, 3, 4, 5 C-D and 6 A-D we used an analysis of variance with the fixed factor goups and the random factor number of experiment. The ratios of HIF-1α with respect to β-Actin were transformed to logarithms in order to stabilize the variances.

For the analysis of the data of Fig. 5B and 8B we used an analysis of variance with the fixed factor goups and time together with their interaction and the random factor number of experiment

The geometric means of the ratios are given together with their 95% confidence intervals.

For the analysis of the data in Fig 7A we fitted the following nonlinear model by the method of least squares to the data:

$$b_1 + (b_0 - b_1)\exp(-\alpha t),$$

where b_1 is the asymptotic pO_2, b_0 is the initial pO_2 and α is the rate at which the initial pO_2 approaches the asymptotic pO_2 as a function of time t. We provide the parameter estimates together with their 95% confidence intervals.

6 Results

This chapter presents the results from the study.
Each section is structured to first explain a result and the experiment that delivered it, then show the associated figures and finally provide a figure legend. This system is followed for seven result-sections, including eight figures.

As first step, immunohistochemical staining for immunolocalization of HIF-1α during RSV infection in pulmonary epithelial cells was performed wherein RSV infected cells stained positive for HIF-1α. Western blot analysis confirmed these results and demonstrate HIF-1α protein stabilization during RSV infection. To pursued functional consequences, we measured transcription levels and showed an induction of known HIF-1 target genes following RSV infection. HIF-1α loss-of-function studies revealed significant induction of the tested HIF-1-target genes in control cells, while these responses were abolished in HIF-1 -/- cells. Investigating the effects of UV-inactivation, we showed that only functional RSV virus is capable to cause HIF-1 stabilization and HIF-1-dependent gene induction. In Figure 7 we suggest oxygen independent stabilization of HIF-1α by measurements of oxygen partial pressures in the supernatants of RSV infeced cells.
Finally, we used a mouse model to confirm our in vitro findings and showed that HIF-1α was stabilized during murine RSV pneumonia.

6.1 Immunolocalization of HIF-1α during RSV infection in vitro

Recent evidence revealed that numerous parallels exist between inflammation and hypoxia, including changes in barrier function or inflammatory cell recruitment (37, 38, 67, 74, 75, 125, 126). In addition, recent studies have revealed that during infections with human pathogens HIF-1 is activated (56, 69). Based on previous studies showing that RSV infections are characterized by a particularly prominent inflammation of the pulmonary mucosa - both in natural and experimental infections (42) and the fact that RSV is among the most potent biological stimuli that induce the expression of inflammatory genes, we hypothesized that HIF-1 is stabilized during infections with RSV and may contribute to RSV-associated changes in gene transcription. For our studies, we used A549 cells, a cell line derived from an alveolar cell carcinoma of the lung. As first step, we performed immunohistochemical staining with antibodies for RSV (green) or HIF-1α (red) using confocal laser scanning microscopy (Figure 1A). As counterstaining for the nuclei we used dapi staining (blue). As shown in Figure 1A, RSV infected cells also stained positive for HIF-1α, with localization of HIF-1α both in the cytosole and the nuclei. In contrast, uninfected A549 cells only had a very week signal for HIF-1α (Figure 1B). Isotype controls and staining of infected A549 cells with secondary antibody alone were negative (data not shown). Taken together, these data reveal that during RSV infection, HIF-1α accumulates in the cytosole and the nucleus of infected pulmonary epithelia, suggesting HIF-1 activation during RSV infection in vitro.

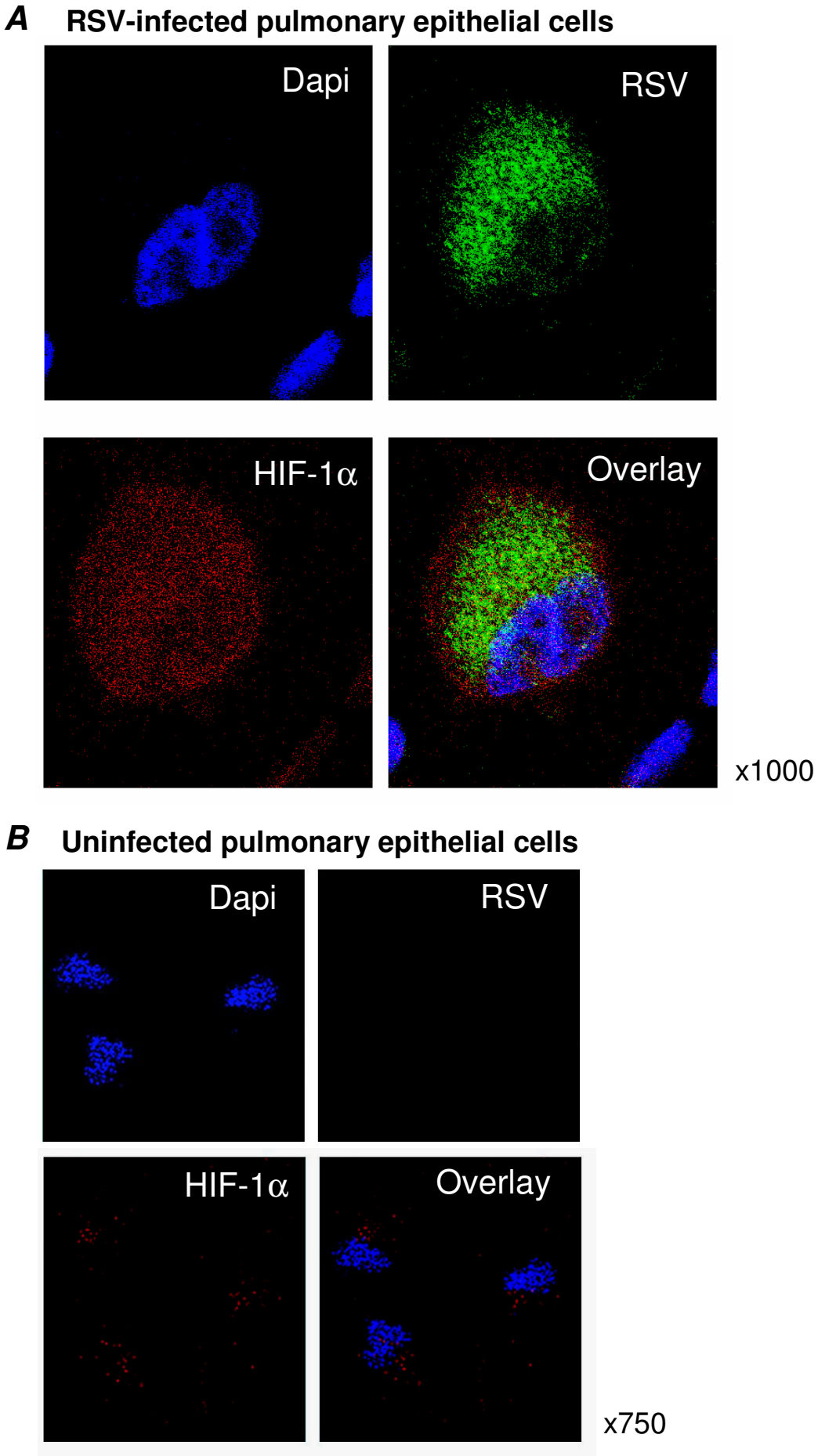

A
RSV-infected pulmonary epithelial cells
Dapi
RSV
HIF-1α
Overlay
x1000
B
Uninfected pulmonary epithelial cells
Dapi
RSV
HIF-1α
Overlay
x750

Figure 1: HIF-1α during RSV infection of pulmonary epithelia.

(A) ~1,5x10^5 A549-cells were seeded on glass slides and infected with RSV (multiplicity of infection, MOI 3). After 24h they were fixed, permeabilized and incubated with anti-HIF1α and anti-RSV IgG as primary antibodies. Alexa Fluor 488 and Alexa Fluor 594 were used for staining. In addition, slides were counter-stained with Dapi. The cells were visualized with confocal laser scanning microscopy. Uninfected cells were used as controls (B).

6.2 HIF-1α protein is stabilized during RSV infection

After having shown by confocal laser scanning microscopy that HIF-1α is stabilized during RSV infection, we next used Western blot analysis to confirm these results with a more quantitative approach. Here, we first confirmed successful infection of A549 cells using different infection doses (MOI1-5). As shown in Figure 2A, we found a close correlation of virus load with RSV G-protein after 24h of infection (Figure 2A). In contrast, RSV pre-exposed to UV light source as previously described for RSV inactivation (100) showed no signal for intracellular RSV G-protein. Uninfected cells were used as negative control. As next step, we measured HIF-1α during RSV infection by Western blot analysis. These studies revealed an 7,15-fold increase in HIF-1α protein compared to non-infected A549 cells (Figure 2B and C, relative to beta-actin, MOI of 3). Interestingly, this HIF-response was completely attenuated when using UV-treated RSV virus (0,84-fold HIF-1α protein relative to beta-actin, Figure 2 B and C). In additional control studies, we exposed A549 cells to ambient hypoxia (2% oxygen over 24h), which was associated with a robust increase in HIF-1α protein (5,70-fold increase in HIF-1α). Taken together, these studies reveal robust stabilization of HIF-1α during infection with life RSV in vitro.

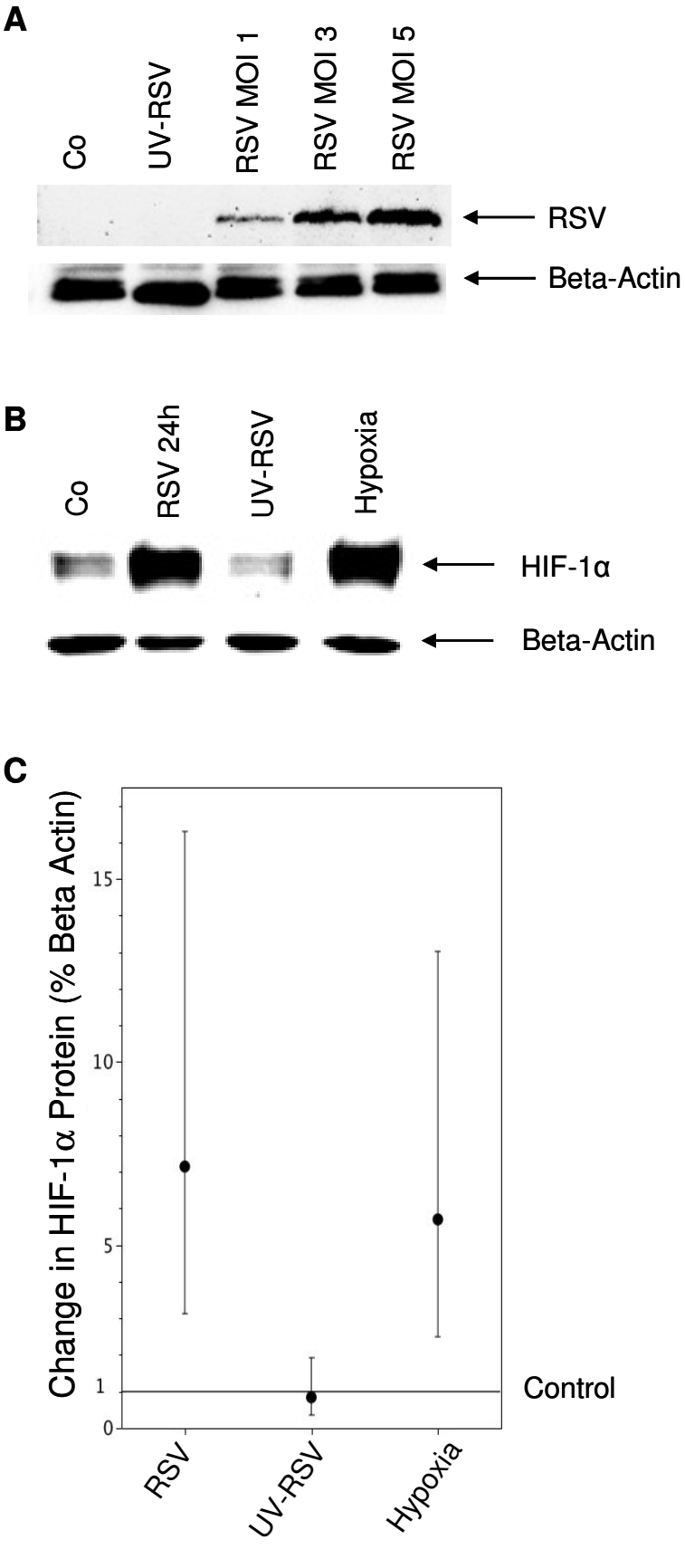

A
Co
UV-RSV
RSV MOI 1
RSV MOI 3
RSV MOI 5
RSV
Beta-Actin
B
Co
RSV 24h
UV-RSV
Hypoxia
HIF-1α
Beta-Actin
C
Change in HIF-1α Protein (% Beta Actin)
15
10
5
1
0
Control
RSV
UV-RSV
Hypoxia

Figure 2: HIF-1α protein measurements during RSV infection in vitro.

(A, B) Cultured pulmonary epithelia (A549) were grown to 80% confluency, infected with intact (multiplicity of infection, MOI 1, 3 or 5) or UV-inactivated RSV (MOI 3). In other studies A549 cells were exposed over 24h to ambient hypoxia (2% oxygen). Cells were lysed and nuclear proteins were isolated, and Western immunoblotting for RSV G-protein (A) or HIF1α was performed. Uninfected cells were used as control (Co). The same blots were probed for β-actin expression as a control for protein loading. A representative blot of 3 is shown, in addition to densitometric analysis of HIF-1α protein levels relative to β-actin (C, n=3). The geometric means of the ratios are given together with their 95% confidence intervals.

6.3 HIF-dependent genes are induced following RSV infection

After having demonstrated HIF-1α protein stabilization during infection with RSV, we next pursued functional consequences of HIF-1α in transcriptional gene induction during RSV infection. For this purpose, we performed expressional studies of known HIF-1-dependent genes during RSV infection. Thus, we measured transcript levels of CD73 (133), VEGF (86), Fibronectin1 (FN1) (76) and COX2 (65) after 24h of RSV infection of A549 pulmonary epithelial cells using different RSV infection doses (MOI1-5). As shown in Figure 3, analysis of transcript levels by real-time RT-PCR revealed induction of all tested HIF-1 target genes. Extensions of these findings at the protein level by Western blot confirmed significant induction of COX2 or FN1 protein levels (Figure 4A and B). Similarly, measurements of VEGF in the supernatants from RSV infected pulmonary epithelial cells revealed significantly elevated levels of VEGF (Figure 4C). Taken together, these studies demonstrate induction of HIF-1-depedendent genes during RSV infection in vitro.

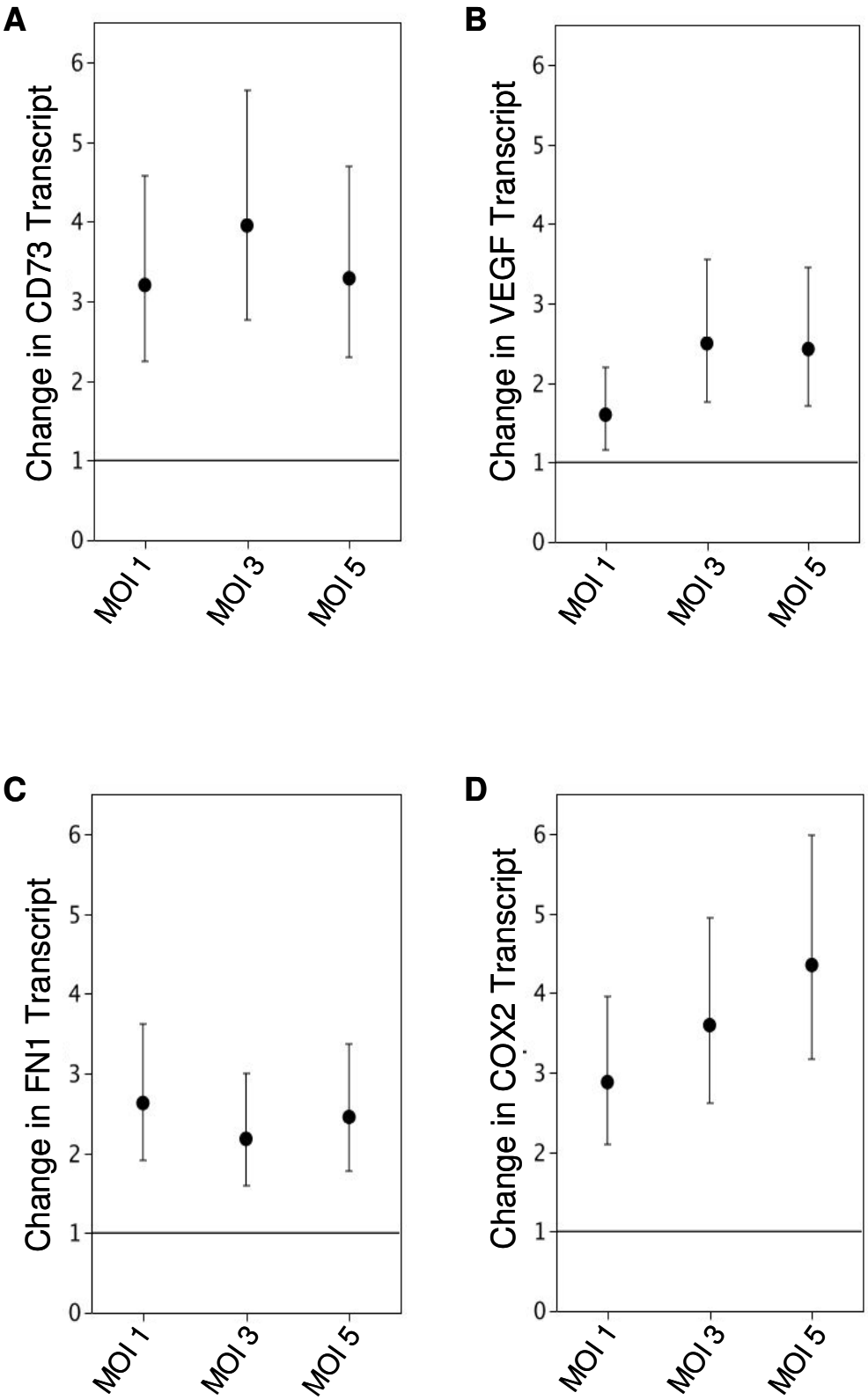
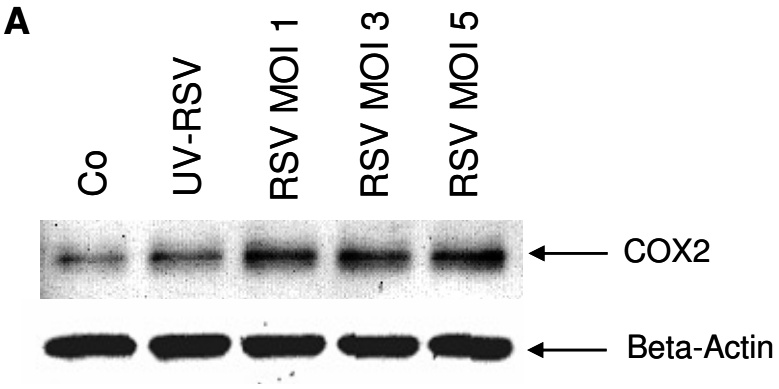
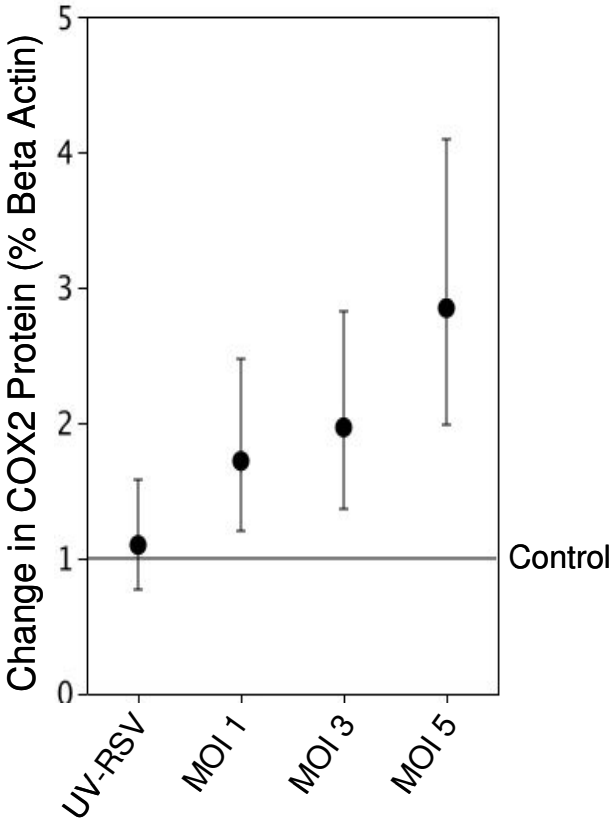
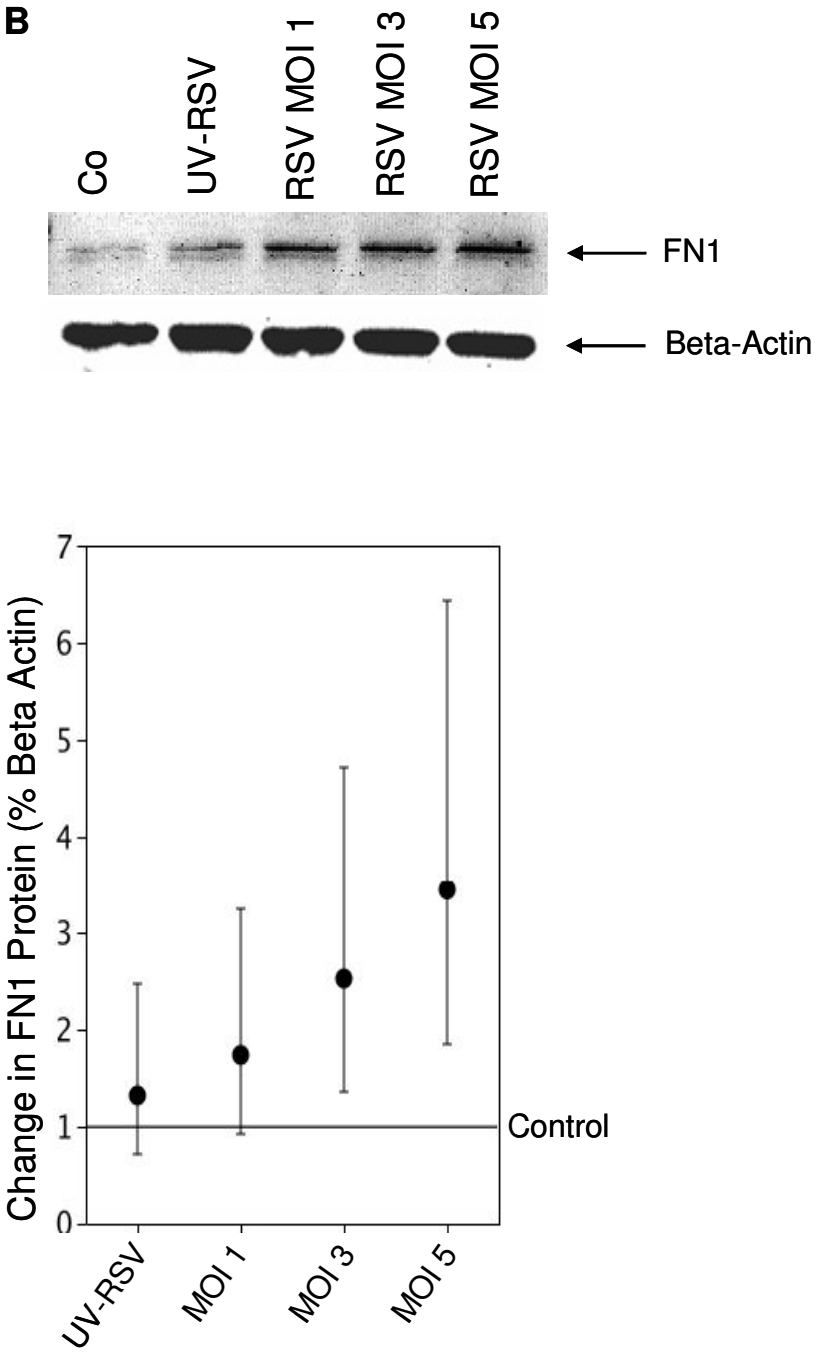

A
Change in CD73 Transcript
0
1
2
3
4
5
6
MOI 1
MOI 3
MOI 5
B
Change in VEGF Transcript
0
1
2
3
4
5
6
MOI 1
MOI 3
MOI 5
C
Change in FN1 Transcript
0
1
2
3
4
5
6
MOI 1
MOI 3
MOI 5
D
Change in COX2 Transcript
0
1
2
3
4
5
6
MOI 1
MOI 3
MOI 5

Figure 3: Transcript levels of HIF-1-dependent genes following RSV infection.

Total RNA was isolated from RSV-infected (multiplicity of infection, MOI 1, 3 and 5) or non-infected A549 cells (control) and (A) CD73, (B) VEGF, (C) FN1, (D) COX2 mRNA levels were determined by real-time RT-PCR. Data were calculated relative to internal housekeeping gene (β-actin) and are expressed as fold increase over uninfected control-cells at each infection dose. The geometric means of the ratios are given together with their 95% confidence intervals.


A
Co
UV-RSV
RSV MOI 1
RSV MOI 3
RSV MOI 5
COX2
Beta-Actin


Change in COX2 Protein (% Beta Actin)
0
1
2
3
4
5
Control
UV-RSV
MOI 1
MOI 3
MOI 5


B
Co
UV-RSV
RSV MOI 1
RSV MOI 3
RSV MOI 5
FN1
Beta-Actin
Change in FN1 Protein (% Beta Actin)
0
1
2
3
4
5
6
7
Control
UV-RSV
MOI 1
MOI 3
MOI 5

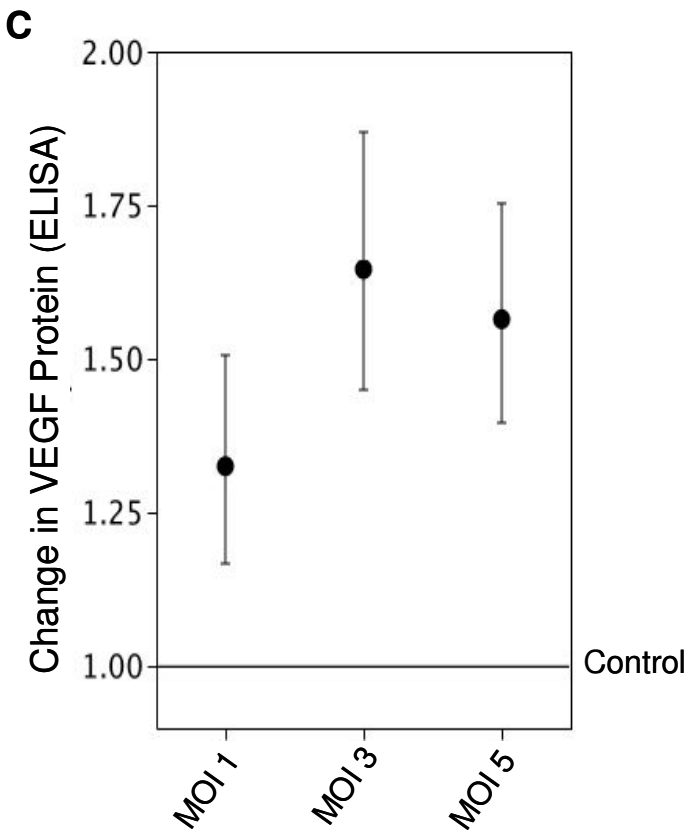
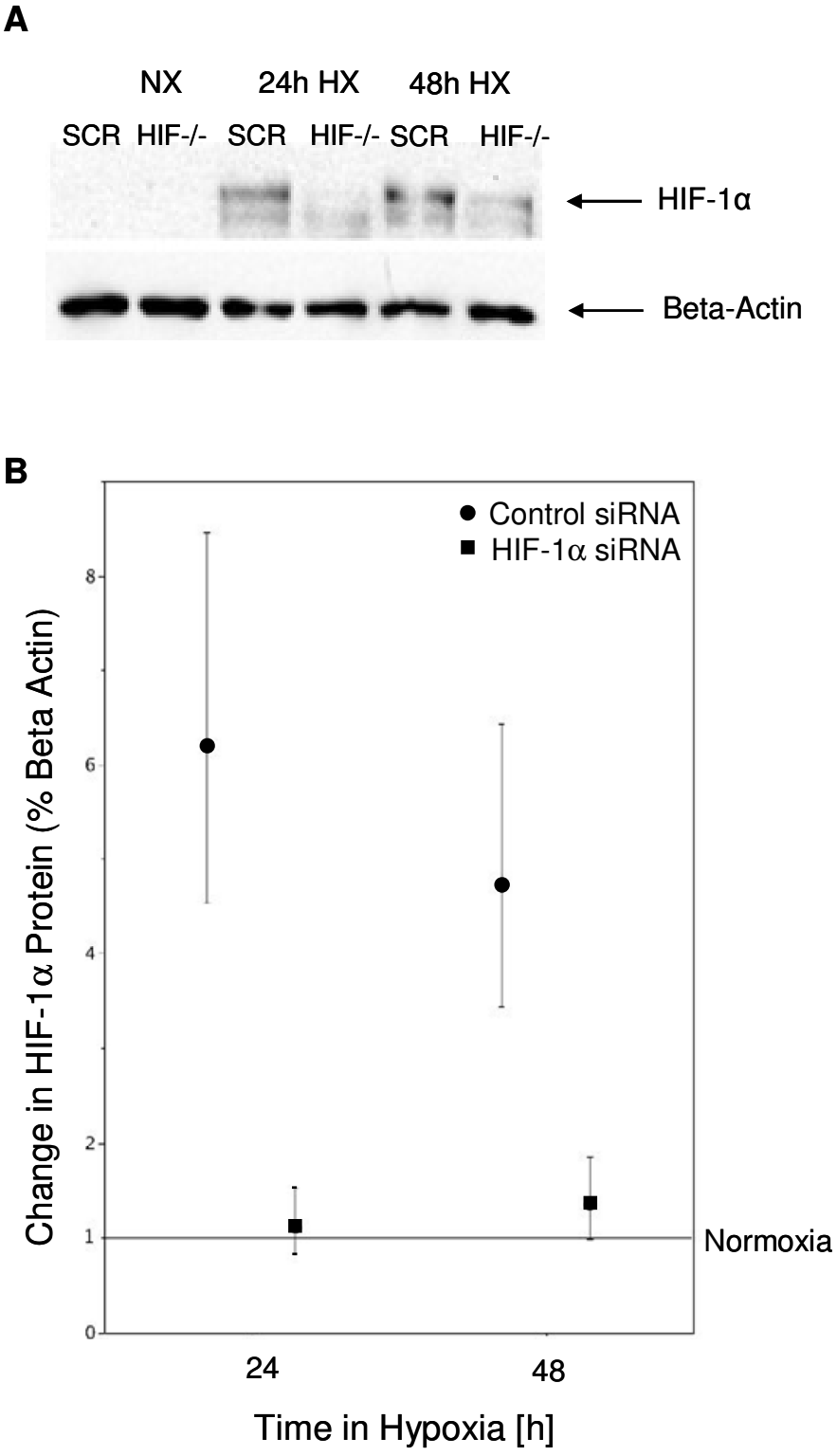

C
2.00
1.75
1.50
1.25
1.00
Change in VEGF Protein (ELISA)
Control
MOI 1
MOI 3
MOI 5

Figure 4: Protein levels of HIF-1-dependent genes following RSV infection.

Total protein was isolated from RSV-infected (multiplicity of infection, MOI 1, 3 and 5) or non-infected A549 cells. Protein levels were determined by Western blot and ELISA. The same blots were probed for β-actin expression as a control for protein loading. In addition, densitometric analysis of protein levels relative to β-actin were performed. Data are expressed as fold increase over uninfected control-cells at each infection dose. (A) COX2; (B) FN1 (C) VEGF. The geometric means of the ratios are given together with their 95% confidence intervals.

6.4 HIF-dependent gene expression during RSV infection in pulmonary epithelial cells following siRNA repression of HIF-1α

To demonstrate a functional role of HIF-1 in the observed induction of HIF-dependent genes induction during RSV infection, we next pursued HIF-1α-loss-of-function studies. For this purpose, we generated a pulmonary epithelial cell line (A549 cells) with stable repression of HIF-1α. This was achieved via hairpin siRNA technique as we have done previously in other cell lines (34, 56, 75, 78, 94). To demonstrate effective repression of HIF-1 in these cell lines, we utilized a model of ambient hypoxia. For this purpose, we exposed these cells to hypoxia over 24 or 48h (2% oxygen), while growth-synchronized control cells were maintained at room air (21% oxygen). These studies revealed significant accumulation of HIF-1α protein in control transfected cells in conjunction with attenuated HIF-1α stabilization in HIF-1α-siRNA-transfected A549 cells (Figure 5A and B). We utilized this model to directly test the functional role of HIF-1 in transcriptional modulation during RSV infection. Here, we measured transcript levels of control transfected or HIF-1α-targeted pulmonary epithelia. These studies revealed significant induction of HIF-1-target genes (VEGF, CD73 or FN1) in control cells, while these responses were abolished in HIF-1α-targeted pulmonary epithelia. Taken together, these studies suggest a functional role of HIF-1α in transcriptional induction of HIF-1-targeted genes during infection with RSV.


A
NX
24h HX
48h HX
SCR
HIF-/-
SCR
HIF-/-
SCR
HIF-/-
HIF-1α
Beta-Actin
B
Control siRNA
HIF-1α siRNA
Change in HIF-1α Protein (% Beta Actin)
0
1
2
4
6
8
Normoxia
24
48
Time in Hypoxia [h]

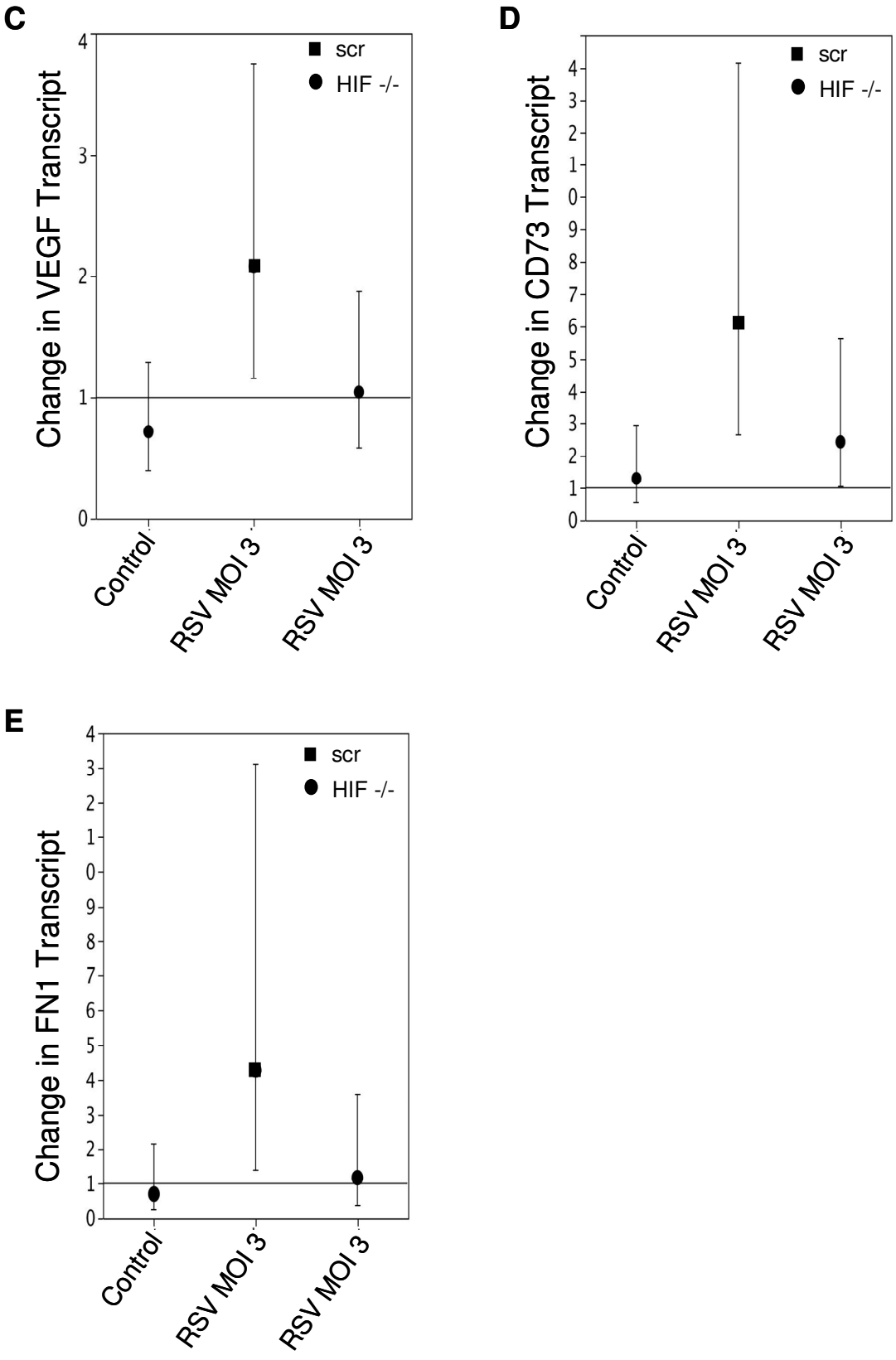

C
Change in VEGF Transcript
scr
HIF -/-
0
1
2
3
4
Control
RSV MOI 3
RSV MOI 3
D
Change in CD73 Transcript
scr
HIF -/-
0
1
2
3
4
5
6
7
8
9
Control
RSV MOI 3
RSV MOI 3
E
Change in FN1 Transcript
scr
HIF -/-
0
1
2
3
4
5
6
7
8
9
Control
RSV MOI 3
RSV MOI 3

Figure 5: Transcript levels of HIF-dependent genes following HIF-1α siRNA repression during RSV infection.

(A) HIF-1α protein levels in A549 cells following hairpin siRNA repression of HIF-1α (HIF-/-; A549 cells transfected with control siRNA:SCR). Cells were grown to 80% confluency and exposed to normoxia or hypoxia (2% oxygen) over indicated time period. Nuclear proteins were isolated and Western Blot analysis was performed for HIF-1α. The same blots were probed for β-actin expression as a control for protein loading. A representative blot of 3 is shown, in addition to densitometric analysis of HIF-1α protein levels relative to β-actin (B). (C, D, E) Total RNA was isolated from RSV-infected (multiplicity of infection, MOI 3) or non-infected A549 following HIF-1α repression (A549 HIF-/-) or transfection with control siRNA (A549 scr). (C) VEGF, (D) CD73, (E) FN1 transcript levels were determined by RT-PCR. Data were calculated relative to internal housekeeping gene (β-actin) and are expressed as fold increase over uninfected control-cells. The geometric means of the ratios are given together with their 95% confidence intervals.

6.5 Influence of UV-inactivation of RSV on HIF-dependent gene induction

In view of the above results, we hypothesized that only intact RSV is capable of HIF-1α stabilization and induction of HIF-1 target genes. Therefore, we next investigated the effects of UV-inactivated virus on the HIF-1α target genes. For this purpose, we measured VEGF, CD73, FN1 and COX2 transcript levels in A549 cells that were infected with intact or with UV-inactivated RSV (Figure 6 A-D). Consistent with our studies above, we found significant induction of HIF-1 target genes in RSV infected A549 cells. In contrast, induction of HIF-1 target genes was completely abolished after similar infection doses with UV-inactivated RSV. Taken together, these studies suggest that only functional RSV virus, capable of intracellular replication – is necessary to cause HIF-1-dependent gene induction.

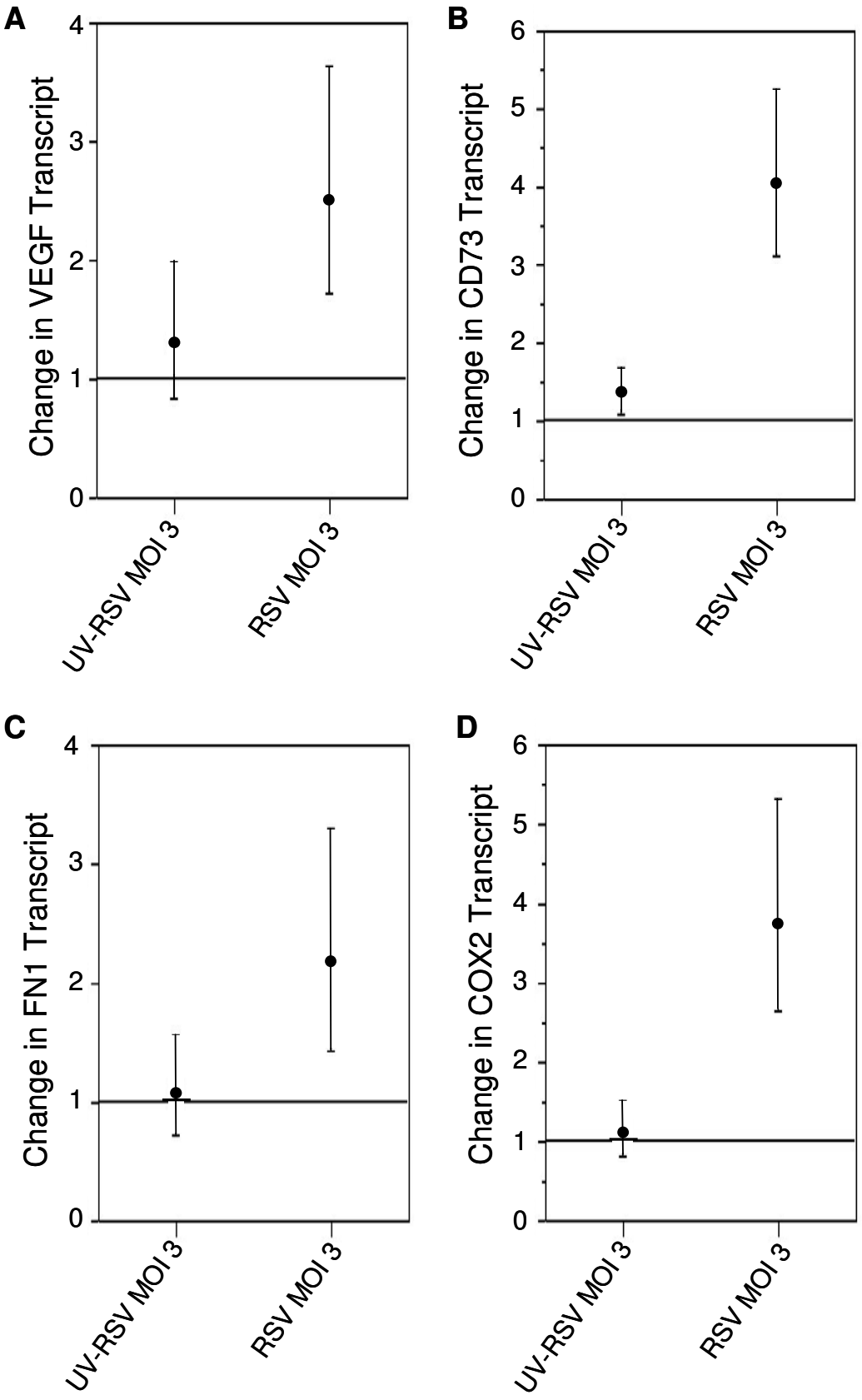
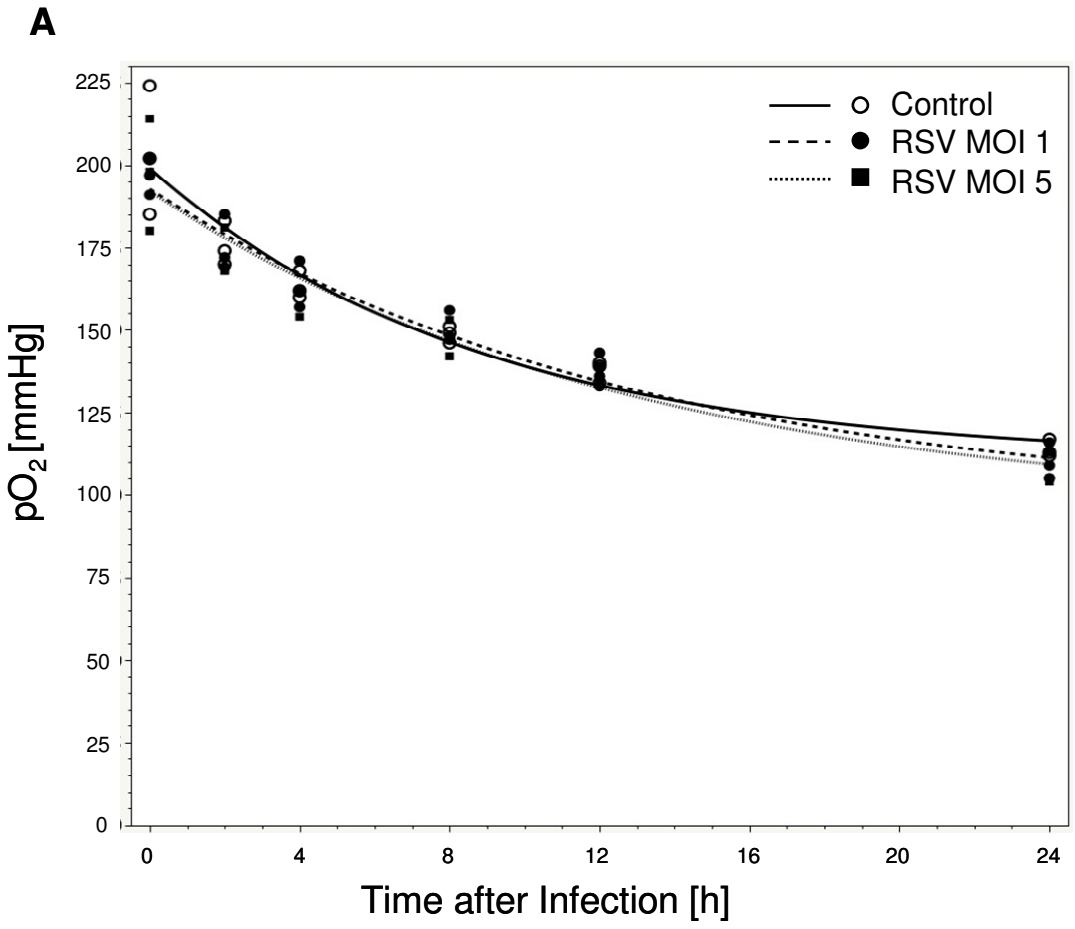

A
Change in VEGF Transcript
0
1
2
3
4
UV-RSV MOI 3
RSV MOI 3
B
Change in CD73 Transcript
0
1
2
3
4
5
6
UV-RSV MOI 3
RSV MOI 3
C
Change in FN1 Transcript
0
1
2
3
4
UV-RSV MOI 3
RSV MOI 3
D
Change in COX2 Transcript
0
1
2
3
4
5
6
UV-RSV MOI 3
RSV MOI 3

Figure 6: Transcript levels of HIF-dependent genes following infection with inactivated RSV.

Total RNA was isolated from uninfected, RSV-infected or UV-inactivated RSV infected A549 cells. (A) VEGF, (B) CD73, (C) FN1, (D) COX2 transcript levels were determined by real-time RT-PCR. Data were calculated relative to internal housekeeping gene (β-actin) and are expressed as fold increase over uninfected control-cells at each infection dose. The geometric means of the ratios are given together with their 95% confidence intervals.

6.6 HIF-1α stabilization after RSV infection occurs independent of hypoxia

Previous studies of infection and inflammation have revealed significant changes in metabolic supply and demand. For example, studies of murine colitis revealed convincing evidence that the mucosal surface is prone to inflammation-associated hypoxia (66, 67). Our above studies showed that intracellular uptake and binding of RSV appears to be necessary for HIF-1-dependent gene induction. Therefore, we hypothesized that changes in metabolic supply and demand ratios during RSV infection may accompany RSF-infection and RSV-associated tissue hypoxia could lead to HIF-1 activation. To address this hypothesis in an experimental setting, we measured oxygen partial pressures (pO_2) values in the supernatants from RSV infected pulmonary epithelia or controls that were maintained in an oxygen impermeable culture system. As shown in Figure 7A, no differences in PO_2 values were observed between experimental groups. In fact, neither supernatant from control nor RSV-infected pulmonary epithelia showed significant degrees of hypoxia. Consistent with these findings, and as shown in Figure 7B, Western blot analysis confirmed that HIF-1α was not stabilized in control cells maintained under the above cell culture conditions. In contrast, we observed significant HIF-1α stabilization in RSV-infected pulmonary epithelia. Taken together, these studies suggest oxygen independent stabilization of HIF-1α during RSV infection.


A
225
200
175
150
125
100
75
50
25
0
pO2 [mmHg]
0
4
8
12
16
20
24
Time after Infection [h]
Control
RSV MOI 1
RSV MOI 5

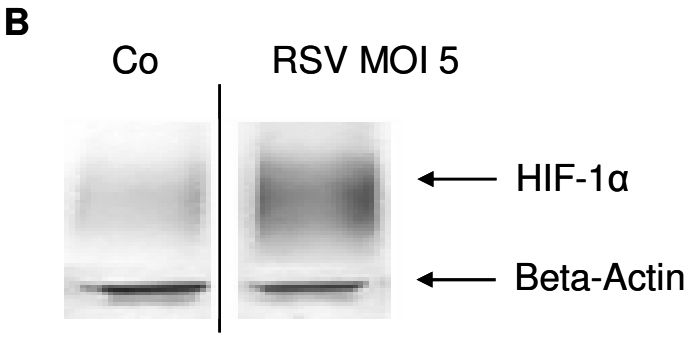

B
Co
RSV MOI 5
HIF-1α
Beta-Actin

Figure 7: Measurements of oxygen partial pressures (pO_2) in the supernatants of RSV infected pulmonary epithelia.

(A) A549 cells were cultured and infected at a multiplicity of infection (MOI) of 1 or 5 in gas-tight sealed flasks. Oxygen partial pressure was measured in the supernatants at indicated time points following infection. (B) The same samples for were assessed for HIF-1α protein levels by Western blot. Blots were probed for β-actin expression as a control for protein loading. We provide the parameter estimates together with their 95% confidence intervals

6.7 HIF-1α is stabilized during murine RSV infection in vivo.

As proof of principle for these concepts in vivo, we compared the influence of RSV infection on pulmonary HIF-1α stabilization using a previously described model (46-48, 50). This mouse model shows close similarity to the pathogenesis of RSV-induced lower airway disease in humans. In fact, recently established that the experimental infection of BALB/c mice with highly purified preparations of RSV A, at a dose of 10^7 PFU, induces a severe inflammatory response in lung tissue as early as 24 h after intranasal inoculation (47). Lung inflammation was characterized by an excess of monocytes/macrophages, lymphocytes, and to a lesser extent, neutrophils surrounding bronchioles and vessels, with evidence of the involvement of alveolar spaces (47). In the present studies, female BALB/c mice were inoculated intranasally with purified RSV. In control experiments, BALB/c mice matched in age, gender and weight were inoculated in the same way with an equivalent volume of vehicle. At the indicated time points after infection, mice were anesthetized, lungs were shockfrozen and HIF-1α was determined by Western blot analysis. As shown in Figure 8A and B, HIF-1α was stabilized with RSV infection at all measurement time points. Taken together, these data confirm our in vitro findings and suggest that during murine RSV pneumonia HIF-1α is stabilized.

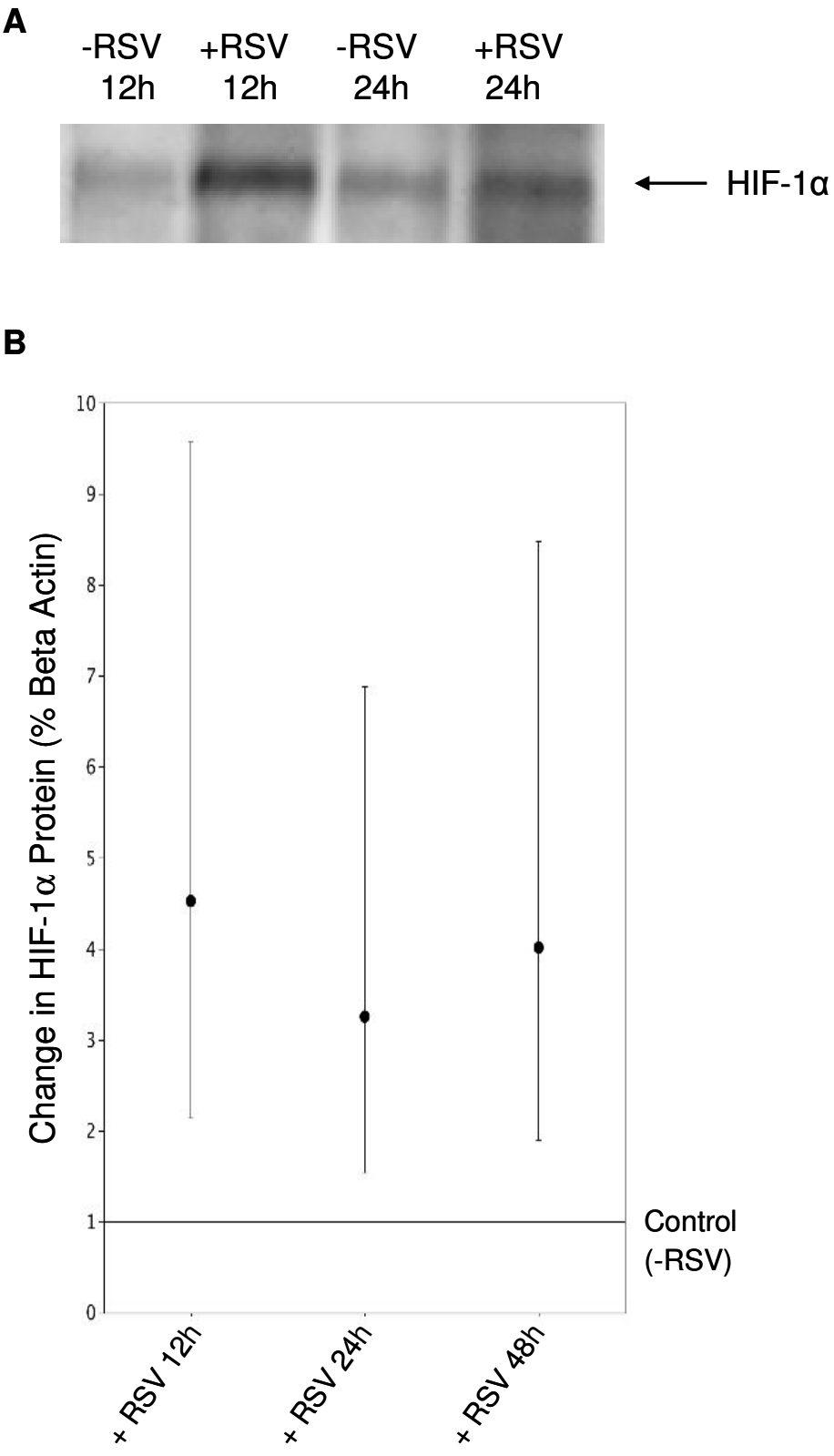

A
-RSV 12h
+RSV 12h
-RSV 24h
+RSV 24h
HIF-1α
B
Change in HIF-1α Protein (% Beta Actin)
10
9
8
7
6
5
4
3
2
1
0
Control (-RSV)
+ RSV 12h
+ RSV 24h
+ RSV 48h

Figure 8: HIF-1α protein during murine RSV pneumonia in vivo.

Female, 4- to 6-week-old BALB/c mice were inoculated intranasally with purified RSV at 1 x 10^7 plaque-forming units (PFUs), diluted in sterile phosphate-buffered saline (PBS) for a total inoculation volume of 50 µl. As mock treatment, control mice were inoculated in the same way with an equivalent volume of sucrose diluted in D-PBS. Lungs were removed at indicated time-points, and HIF-1α protein levels were determined by Western blot analysis (A) or quantified by densitometry, relative to β-actin (B). The geometric means of the ratios are given together with their 95% confidence intervals.

7 Discussion

This chapter discusses the results described in the previous chapter and puts the findings in context with existing studies about the subject topic. While the general role of HIF-1 has been discussed extensively in previous studies, we are concentrating on HIF-1 in the context of inflammation and infection with RSV.

We start by giving an overview of our findings. Then, the respiratory epithelium as primary target of RSV infection is discussed, followed by a section summarizing previous studies about HIF-1 during viral, bacterial and parasital infection. We elaborate on our findings about HIF-1 stabilization during RSV infection, and then discuss the mechanisms of HIF-1 stabilization during infection, particularly with regard to oxygen dependency.
After a glance at other transcription factors, we finally question whether HIF-1 stabilization during RSV infection might be protective or detrimental.

7.1 General findings

Many studies during the last decade have demonstrated a central role of HIF-1 in mammalian oxygen homeostasis (120). HIF-1 activity is induced in a variety of cell types and, more recently many studies have also demonstrated a role of HIF-1 in the transcriptional coordination during inflammation and infection (56, 69, 125, 157). In fact, previous studies have revealed that HIF-1 can be stabilized during infections with human pathogens via oxygen-dependent (69) or oxygen independent (56) pathways.

In the present studies we pursued HIF-1α stabilization and gene-transcription during infection with RSV – one of the most potent biological stimuli to induce an inflammatory milieu (46-48, 50) and the major cause of serious lower respiratory tract infections in infancy and early childhood (42).

Initial studies of cultured pulmonary epithelia infected with RSV revealed stabilization of HIF-1 protein. Moreover, transcription of known HIF-1 target genes (VEGF, CD73, FN-1, COX-2) was induced following RSV infection while siRNA-dependent repression of HIF-1α abolished these responses. Induction of HIF-1 target genes was also completely abolished after infection with UV-inactivated RSV.

However, infection with RSV was not associated with increased oxygen consumption or cellular hypoxia, suggesting that HIF-1α stabilization and HIF-dependent gene induction during RSV infection occurs in an oxygen-independent fashion. Finally, studies of murine RSV pneumonia revealed significant HIF-1α stabilization throughout the course of the disease and suggest that RSV-associated HIF-1 activation also occurs in vivo.

7.2 The respiratory epithelium

In naturally acquired RSV infection, the primary target for viral replication is the respiratory epithelium (63). In bronchiolitis and pneumoniae, RSV antigen can be identified in epithelial cells from throughout the lower respiratory tract (20). Airway epithelial cells represent the principal initiators of pulmonary host defense mechanisms by their ability to synthesize and secret inflammatory mediators upon injury or infection. The immunomodulatory activity of the airway epithelium is of particular relevance to RSV infection, because RSV produces clinical disease through its replication within the airway mucosa (41). This replication is a process that alters epithelial cell chemokine expression, thereby inducing airway inflammation (22). The inflammatory response by infection of respiratory epithelial cells is an essential pathogenic component of RSV disease.

An important role of the alveolar epithelium is to contribute to the alveolocapillary barrier, secret surfactant to lower the surface tension,

and clear edema (62). Epithelial cells are able to produce opsonins such as complement and surfactant proteins responsible for serum-independent phygocytosis of pathogens by neutrophils, monocytes, and macrophages (42). Therefore, respiratory epithelial cells appear to be ideally located and armed to function as initiators of host defense mechanisms by regulating the prototypic cellular elements of the innate immune system.
A549 cells have proven to be excellent models of pulmonary type II epithelial cells for studying the production of several bioactive factors and the mechanisms of chemokine gene regulation (41, 63, 84, 129).
In previous studies, HIF-1α induction was reported by acute hypoxia in A549 cells (138).

HIF-1 may contribute to multiple aspects of pulmonary pathophysiology in patients with chronic lung diesease (119). Immunohistolchemistry revealed expression of HIF-1α in most cell types within the hypoxic lung including bronchial epithelium and smooth muscle, alveolar epithelium and vascular endothelium (153). Reduced oxygen availability induces lung alveolar cell adaption through an activation of genes essential for cell survival mediated by HIF-1 (138).

In this study we investigated the effects of acute RSV infection on pulmonary epithelial cells and revealed concomitant HIF-activation during RSV infection.

7.3 HIF-1 during inflammation

The hypoxia-inducible transcription factor (HIF)-1 is a major regulator of energy homeostasis and cellular adaption to low oxygen stress (157). The expression of HIF-1α plays an important role during inflammation and infection (105) as well as angiogenesis, tumor growth, invasion and metastasis (90). While hypoxia remains the undisputed ubiquitous inducer of HIF-1, other factors can also modulate increases in HIF-1α protein levels.

Activation of HIF-1α pathway during the life cycle of different pathogens has been the subject of increasing investigation, revealing a diversity of functional outcomes in disease progression. A variety of in vitro and in vivo functional assays of acute and chronic inflammation demonstrated a prominent role for the HIF-1α controlled pathway within inflamed tissues.

7.3.1 HIF-1 during viral infections

Previous studies have shown HIF-1 stabilization during infections with different viruses and local hypoxia was identified as a nonspecific host-cell defense against virus infection during the infection with vesicular stomatitis virus (VSV) (95). Epstein-Barr virus (EBV), a human herpesvirus that is associated with several types of malignancies, such as Burkitt's lymphoma, lymphoproliferative disorders, T-cell lymphomas and Hodgkin's disease, is reported to increase the expression of HIF-1α in different cell lines (144).

Chronic infections with the hepatitis B and C viruses are epidemiologically associated with development of hepatocellular carcinoma. Hepatitis B virus (HBV) increases the protein level of HIF-1α by the direct interaction of HIF-1α under both normoxic and hypoxic conditions (55, 93). The cross-talk between a regulatory X protein (HBx) – one of the open reading frames encoded by the HBV genome – and HIF-1α was shown in (152). Furthermore, HBx enhanced transcriptional activity of HIF-1α in the reporter genes encoding hypoxia reponse element (152).

Infection with hepatitis C virus, a single-stranded RNA virus also leads to stabilization of HIF-1α as reported in (96).

In some cases, HIF-1α may help coordinate a host defense program to limit cell damage secondar to viral infection (157).

7.3.2 HIF-1 during bacterial infections

The effetiveness of innate immune defense against bacterial infections reflects a diverse array of highly specialized cellular functions. Biosynthesitic capacity,

metabolic activation and cytoskeletal rearrangements are required to effect microbial killing. Different observations argue for a pronounced dependence of neutrophils and macrophages on the known functions of HIF-1 (104).

Increased levels of HIF-1α have been demonstrated during bacterial infection with a variety of bacterial species, including Streptococcus pyogenes, Streptococcus agalactiae, Staphylococcus aureus, Salmonella typhimurium and Pseudomonas aeruginosa (10, 25, 105, 158). These studies suggested that the HIF-1α response pathway is broadly adaptive in host defense (157). One clear demonstration of HIF-1α induction by a bacterial pathogen was provided in studies of Bartonella (B) henselae, a facultative intracellular bacterium and the causative agent of angiogenic proliferation of capillary vessels in bacillary angiomatosis. Infection with B henselae results in HIF-1 activation in vitro and in vivo and leads to a gene expression pattern typical for the cellular response to hypoxia (69). The activation of HIF-1 was also investigated in a murine yersiniosis model. Infection with Yersinia (Y) enterocolica resulted in even more pronounced HIF-1 activation than during infection with B henselae (56).
In other studies, a strain of the Gram-positive pathogen group A Streptooccus was chosen to provide evidence for increased HIF-1α expression following bacterial exposure. It was also found to represent a more potent stimulus for HIF-1α induction than hypoxia itself (105).

7.3.3 HIF-1 during infections with parasites

HIF-1α also appears to occur in the context of infection with protozoan parasites. Though much less well studied, HIF-1α is shown to be activated during different parasites infections.
For example, cutaneous lesions can be generated in mice by infection with Leishmania amazonensis. HIF-1α induction in the cytoplasm and parasitophorous vacuoles of macrophages recruited to the microenvironmental can be clearly demonstrated in the later stages of infection (4).

Studies with Toxoplasma gondii and Trypanosoma cruza identified these obligate intracellular parasites as activators of HIF-1α induction (128).

7.4 HIF-1 stabilization during RSV infection

Lower respiratory tract infections caused by RSV are characterized by profound cellular inflammation of the airway mucosa, which contributes to disease manifestations, including air flow limitation, lung atelectasis/emphysema, and hypoxemia (111). The molecular mechanisms that regulate airway inflammation in viral respiratory infections are not fully understood and the in vivo signaling and transcriptional pathways that mediate airway mucosal inflammation in RSV infections are still largely unknown (50). It has previously been established that in airway epithelial cell lines a number of molecules are produced as a consequence of RSV infection. Among them are potent immunomodulatory and inflammatory mediators, including cytokines, chemokines, interferons and growth factors (42).
Herein we show HIF-1 activation and gene-transcription during respiratory syncytial virus (RSV) infection.

The requirement of infectious virus for the production of different chemotactic factors, for example the secretion of the proinflammatory C-C chemokines RANTES, MCP-1 and MIP-1α from epithelial cells was reported in previous studies (100). In other studies, the induction of nuclear-factor-IL6 (NF-IL6) was found to be strictly dependent on viral replication and could not be induced by UV-inactivated virus, demonstrating the requirement of viral replication for NF-IL6 snythesis (63). However, UV-inactivation of RSV did not prevent virus-induced EPO release or T-cell proliferation (27).

Our above studies showed that intracellular uptake and replication of RSV appears to be necessary for HIF-1-dependent gene induction. We found significant induction of HIF-1 target genes in RSV infectes A549 cells but only

intact RSV appeared to be able to stabilize HIF-1alpha, whereas the HIF-response was completely attenuated when using UV-treated RSV virus.

7.5 Mechanisms of HIF-1 activation during infection

Inflammatory responses are generally associated with significant changes in tissue metabolism. In particular, metabolic shifts during inflammation can result in significant tissue hypoxia with stabilization of HIF-1α and resultant induction of hypoxia-responsive genes. However, previous studies of HIF-1 during inflammation and infection have found oxygen-independent activation of HIF-1 during infections with human pathogens.

For example, a recent study on molecular mechanisms of how bacteria activate HIF-1 found a role of bacterial siderophores in HIF-1 activation during infection with Enterobacteriaceae (56). Here, the authors studied HIF-1 activation and HIF-1-dependent gene induction in Peyer's patches that were analyzed after orogastric infection with Yersinia (Y) enterocolitica and orogastric Y enterocolitica infection in mice with a conditional deletion of HIF-1α (66) in the intestine. These studies demonstrated that infection of mice with Y enterocolitica led to functional activation of HIF-1 in Peyer's patches. Moreover, mice with conditional deletion of HIF-1α in the intestinal epithelium showed a significantly higher susceptibility to orogastric Y enterocolitica infections, suggesting HIF-1 activation as a host defense mechanism in this model. Additional studies with Y enterocolitica, S enterica subsp enterica, or E aerogenes, and, moreover, application of their siderophores (yersiniabactin, salmochelin, aerobactin) caused a robust, dose-dependent HIF-1 response in human epithelia and endothelia, independent of cellular hypoxia.

Taken together, such studies demonstrate a role for bacterial siderophores in hypoxia-independent activation of HIF-1 during infection with human pathogenic bacteria (56).

Similarly, previous studies on viral infections with RSV have demonstrated induction of HIF-1α in primary human bronchial epithelial cells via a nitric-oxide-dependent pathway (72).

Other studies have identified a crosstalk between viral genes and the HIF-1α pathway during infections with the human herpesvirus 8 (HHV-8) (19, 157). Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) is the etiological agent of Kaposi's sarcoma, a highly vascularized, endothelial-derived tumor. A direct role for KSHV-mediated induction of angiogenesis has been proposed based upon the nature of the neoplasia and various KSHV gene overexpression and infection model systems. These studies revealed that KSHV infection of endothelial cells induces mRNA of HIF-1α and HIF-2α. While HIF-1 is classically activated posttranscriptionally, these studies demonstrate that both alpha-subunits are up-regulated at the transcript level by KSHV infection.
Infections with B henselae in previous studies resulted in increased oxygen consumption and cellular hypoxia in the infected host cells. It was assumed that stabilization of HIF-1α during a B henselae infection occurs similar to that in cells exposed to hypoxia (69).
Other studies observed the phenomen of bacterial induction of HIF-1α under normoxia with different Gram-positive (Staphylococcus aureus) and Gram-negative (Pseudomonas aeruginosa, Salmonella typhimurium) bacterias (105).

Here, the transcriptional activation of HIF-1 leads to a functional increase in HIF-1 activity under normoxic conditions. Infection with RSV was not associated with increased oxygen consumption or cellular hypoxia. The HIF-1α stabilization and HIF-dependent gene induction during RSV infection occured in an oxygen-independent fashion.

7.6 Role of other transcription factors

Transcription factors are proteins that bind to regulatory sequences, usually in the 5’ upstream promoter region of target genes, to influence the rate of gene transcription (8). They play a pivotal role in controlling inflammatory gene expression.

Nuclear factor kappa B (NF-κB) is a transcription factor regulating multiple immune and inflammatory responses and the expression of genes involved in host defence and chronic inflammation (6). A number of highly inducible genes encoding cytokines, chemokines, and acute-phase reactant and adhesion molecules contain NF-κB-binding sites in their proximal promoters (9). Extracellular stimuli initiate a signaling cascade that leads to rapid phosphorylation of cytoplasmatic inhibitors, IκBs (whose members bind and specifically inactivate NF-κB), an event coupled with freeing NF-κB to translocate to the nucleus, where it binds and activates target genes (64, 68).

Whereas activation of HIF-1 is known to prevent ischemic neuronal damage and promote ischemic tolerance, NF-κB seemed to promote inflammation and neuronal death after cerebral ischemia (151). Many NF-κB gene products result in a positive regulatory loop that amplifies and perpetuates the inflammatory response (8). NF-κB activation after focal ischemia was suggested to release detrimental consequences (98). In patients with sepsis, fatal outcome was predicted by an increase in NF-κB binding activity. Nonsurviving septic patients showed increased nuclear binding activity of NF-κB compared with probes of survivors (14).

A recent study however suggests that NF-κB is a critical transcriptional activator of HIF-1 and that basal NF-κB activity is required for HIF-1 protein accumulation under hypoxia (108). These results indicate a role of NF-κB in HIF-1α induction (64) but defining the relationship between NF-κB and HIF-1α has proven elusive. NF-κB and HIF-1α appear to share common regulatory pathways (43). This cross-talk is of major importance for disease states associated with low-oxygen tension as well as for a variety of acute or chronic inflammatory diseases. An improved understanding is challenging and a demanding task.

Previous studies showed, that RSV infection rapidly activates the NF-kappaB activation pathway (22). Actually, RSV is able to induce NF-κB-binding activity

in the lung independently from its ability to replicate (50). In epithelial cells, RSV activates nuclear translocation of NF-κB through proteolysis of its cytoplasmic inhibitor IkappaB (135).

While more evidence accumulates supporting the significance of transcription factors in promoting and curtailing inflammation, the therapeutic targeting of individual transcription factors is still at its infancy.

7.7 HIF-1 activation: protective or detrimental?

From the present studies it remains unclear whether HIF-1 activation during RSV infection represents a host-defence mechanism or is an essential part of the disease pathogenesis enabling virus uptake or replication. While some studies have identified a host-protective role of HIF-1 during inflammation (34, 66, 67, 94, 133) or infections (56), others have found a contribution of HIF-1 activation in growth and survival of human pathogens.
For example, Toxoplasma gondii is an obligate intracellular protozoan pathogen. Recent studies revealed that genes mediating cellular responses to hypoxia were upregulated in Toxoplasma -infected cells but not in cells infected with another intracellular pathogen, Trypanosoma cruzi (13, 140). The inducible expression of these genes is controlled by the HIF-1. Additional studies revealed that Toxoplasma infection rapidly increased the abundance of the HIF-1α and activated HIF-1 reporter gene expression and survival was severely reduced in cells targeted for HIF-1α (128). These studies also suggested that while HIF-1α was not required for parasite invasion, HIF-1 was required for parasite cell division and organelle maintenance, indicating that Toxoplasma activates HIF-1 and requires HIF-1 for growth and survival at physiologically relevant oxygen levels (13, 128, 140).
Rupp *et al.* reported that Chlamydia pneumoniae directly interferes with host cell HIF-1α regulation (110). HIF-1α stabilization was essential for efficient Chlamydia pneumoniae replicaton in hypoxia.

Analyses about changes in tissue barrier function - commonly associated with hypoxia of many origins - revealed mutant HIF1α mice conditionally lacking intestinal epithelial HIF-1α protected from hypoxia-induced increases in intestinal permeability in vivo (109).
Other studies provide insight into tissue microenvironmental changes during model inflammatory bowel diseases (66) and identify HIF-1 as a critical factor for barrier protection during mucosal insult. Studies of colitis in mice revealed that decreased HIF-1 expression correlates with more severe clinical symptoms, while increased HIF levels were protective in the parameters mortality, weight loss and colon length (66).
Another example can be found in the case of human papillomavirus-16, an etiologic agent of cervial intestitial neoplasia that, undetected, can progress to cervial carcinoma. In advanced cervical cancer lesions, many of which are hypoxic, increased HIF-1α levels can be correlated to poor prognosis (11, 59).

The central role of HIF-1α in physiology and pathology makes it an attractive yet intricate target for pharmacological manipulations. Because of its perceived therapeutic potential it is already increasingly studied. As it causes angiogenesis, enhancement of this gene within ischemic patients could promote the vessel proliferation needed for oxygenation. In contrast, as HIF-1 allows for survival and proliferation of cancerous cells due to its angiogenic properties, inhibition potentially could prevent the spread of cancer. With a growing understanding of the HIF-1 pathway, the inhibition and stimulation of its transcriptional activity is now an attractive goal (155).

Understanding the processes of HIF-1 in the oxygen-dependent and oxygen-independent regulation during inflammatory responses and the role they play within inflamed sites may offer new opportunities for therapeutic intervention.

7.8 Conclusion

Taken together, the present studies provide molecular insight into the role of HIF-1 as transcriptional control of gene expression and reveal oxygen-independent stabilization of HIF-1α during RSV infection in vitro and in vivo. Future challenges will include the determination whether such responses elicited during RSV infections are host-protective or host-detrimental. A better understanding of HIF-1α function may provide novel and rational approaches for boosting innate immune function in the therapy of certain complicated infectious disease conditions. Ongoing studies are currently testing HIF-activation or HIF-inhibition in different settings of medical therapy and novel therapeutics will soon become available in patient care to inhibit or to activate the HIF-1 pathway. Such compounds may comprise a novel approach during RSV infections.

8 Summary

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that functions as master regulator of mammalian oxygen homeostasis. In addition, recent studies identified a role for HIF-1 as transcriptional regulator during inflammation or infection. Based on studies showing that respiratory syncytial virus (RSV) is among the most potent biological stimuli to induce an inflammatory milieu, we hypothesized a role of HIF-1 as transcriptional regulator during infections with RSV.

We gained first insight from immunohistochemical studies of RSV-infected human pulmonary epithelia that were stained for HIF-1α. These studies revealed that RSV-positive cells also stained for HIF-1α, suggesting concomitant HIF-activation during RSV infection. Similarly, Western blot analysis confirmed an approximately 7-fold increase in HIF-1α protein 24h after RSV infection. In contrast, HIF-1 activation was abolished utilizing UV-treated RSV. Moreover, HIF-regulated genes (VEGF, CD73, FN-1, COX-2) were induced with RSV infection of wildtype cells. In contrast, HIF-1 dependent gene induction was abolished in pulmonary epithelia following siRNA mediated repression of HIF-1α. Measurements of the partial pressure of oxygen in the supernatants of RSV infected epithelia showed no differences in oxygen content, suggesting that HIF-1 activation is not caused by RSV associated hypoxia. Finally, studies of RSV pneumonitis in mice confirmed HIF-activation in a murine in vivo model.

Taking together, these studies suggest hypoxia-independent activation of HIF-1 during infection with RSV in vitro and in vivo.

9 Data tables

Figure 2 C

	Least Sq Mean	Lower 95%	Upper 95%
RSV	7.15	3.13	16.30
UV-RSV	0.84	0.37	1.92
Hypoxia	5.70	2.50	13.01

Figure 3 A-D

	Least Sq Mean	Lower 95%	Upper 95%
3A, MOI 1	3.20	2.24	4.57
3A, MOI 3	3.95	2.77	5.64
3A, MOI 5	3.29	2.30	4.69
3B, MOI 1	1.60	1.16	2.20
3B, MOI 3	2.50	1.75	3.56
3B, MOI 5	2.42	1.70	3.46
3C, MOI 1	2.63	1.91	3.61
3C, MOI 3	2.18	1.58	2.30
3C, MOI 5	2.45	1.78	3.37
3D, MOI 1	2.88	2.09	3.96
3D, MOI 3	3.59	2.61	4.94
3D, MOI 5	4.35	3.16	5.98

Figure 4 A-C

	Least Sq Mean	Lower 95%	Upper 95%
4A, UV-RSV	1.10	0.77	1.58
4A, MOI 1	1.72	1.20	2.47
4A, MOI 3	1.97	1.37	2.83
4A, MOI 5	2.85	1.98	4.09
4B, UV-RSV	1.33	0.71	2.47
4B, MOI 1	1.74	0.94	3.25
4B, MOI 3	2.53	1.36	4.72
4B, MOI 5	3.45	1.85	6.43
4C, MOI 1	1.33	1.17	1.51
4C, MOI 3	1.65	1.45	1.87
4C, MOI 5	1.56	1.40	1.75

Figure 5 B-E

	Least Sq Mean	Lower 95%	Upper 95%
5B, Control siRNA 24h	6.19	4.53	8.46
5B, HIF-1 α siRNA 24h	1.11	0.81	1.51
5B, Control siRNA 48h	4.71	3.45	6.44
5B, HIF-1 α siRNA 48h	1.35	0.99	1.84
5C, Control HIF-	0.72	0.40	1.29
5C, RSV MOI3 scr	2.08	1.16	3.75
5C, RSV MOI3 HIF-	1.05	0.58	1.88
5D, Control HIF-	1.29	0.57	2.96
5D, RSV MOI 3 scr	6.12	2.65	14.13
5D, RSV MOI 3 HIF-	2.43	1.05	5.62
5E, Control HIF-	0.70	0.23	2.15
5E, RSV MOI 3 scr	4.28	1.40	13.09
5E, RSV MOI 3 HIF-	1.17	0.38	3.58

Figure 6 A-D

	Least Sq Mean	Lower 95%	Upper 95%
6A, UV-RSV MOI 3	1.28	0.83	1.97
6A, RSV MOI 3	2.50	1.72	3.62
6B, UV-RSV MOI 3	1.33	1.06	1.66
6B, RSV MOI 3	4.01	3.09	5.22
6C, UV-RSV MOI 3	1.06	0.72	1.54
6C, RSV MOI 3	2.17	1.43	3.29
6D, UV-RSV MOI 3	1.08	0.79	1.49
6D, RSV MOI 3	3.74	2.63	5.30

Figure 7

	Parameter	Estimate	Lower CL	Upper CL
Control	α	0.11	0.06	0.19
MOI 1	α	0.08	0.04	0.12
MOI 5	α	0.09	0.03	0.14
Control	b_0	199.12	189.33	209.31
MOI 1	b_0	192.80	186.00	199.78
MOI 5	b_0	192.11	182.53	202.06
Control	b_1	110.86	80.49	126.24

MOI 1	b_1	96.28	55.13	113.80
MOI 5	b_1	94.55	8.68	117.14

Figure 8

	Least Sq Mean	Lower 95%	Upper 95%
+ RSV 12h	4.52	2.14	9.56
+ RSV 24h	3.25	1.54	6.88
+ RSV 48h	4.01	1.90	8.48

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