Mol. Cells *27*, 243-250, February 28, 2009
DOI/10.1007/s10059-009-0030-2

Molecules and Cells



# HIF-1α-Dependent Gene Expression Program During the Nucleic Acid-Triggered Antiviral Innate Immune Responses

**Sun Woo Hong[1,3], Jae Wook Yoo[1,3], Hye Suk Kang[1], Soyoun Kim[2,*], and Dong-ki Lee[1,*]**

**Recent studies suggest a novel role of HIF-1α under non-hypoxic conditions, including antibacterial and antiviral innate immune responses. However, the identity of the pathogen-associated molecular pattern which triggers HIF-1α activation during the antiviral response remains to be identified. Here, we demonstrate that cellular administration of double-stranded nucleic acids, the molecular mimics of viral genomes, results in the induction of HIF-1α protein level as well as the increase in HIF-1α target gene expression. Whole-genome DNA microarray analysis revealed that double-stranded nucleic acid treatment triggers induction of a number of hypoxia-inducible genes, and induction of these genes are compromised upon siRNA-mediated HIF-1α knock-down. Interestingly, HIF-1α knock-down also resulted in down-regulation of a number of genes involved in antiviral innate immune responses. Our study demonstrates that HIF-1α activation upon nucleic acid-triggered antiviral innate immune responses plays an important role in regulation of genes involved in not only hypoxic response, but also immune response.**

## INTRODUCTION

Hypoxia-inducible factor 1 (HIF-1) is a well known transcriptional regulator of hypoxic genes in low oxygen conditions (Wang and Semenza, 1993). HIF-1 is a heterodimer composed of an oxygen-regulated HIF-1α subunit, and a common HIF-1β subunit (also known as ARNT) (Wang et al., 1995). In normoxic conditions, HIF-1α subunit is rapidly degraded via post-translational modification of HIF-1α oxygen-dependent degradation (ODD) domain (Masson et al., 2001). However, when a cell undergoes hypoxic stress, HIF-1α is stabilized and translocates into the nucleus to form a complex with HIF-1β, and the resulting HIF-1 heterodimer activates target genes.

In addition to this canonical, oxygen-dependent mechanism, a number of recent studies have reported that HIF-1α can be also activated by non-hypoxic stimuli (reviewed in Dery et al., 2005), suggesting that the biological function of HIF-1α is more diverse than originally thought. One of the hypoxia-independent roles of HIF-1α is a transcriptional regulator during the innate immune response (Zinkernagel et al., 2007). Innate immune response is the first line of defense against pathogenic invasion (Akira et al., 2006), and during this response, exogenously introduced pathogen-associated molecular patterns (PAMPs) are sensed by germline-encoded pattern recognition receptors (PRRs), triggering signal transduction cascades and activating a number of immune-related genes (Lee and Kim, 2007). Several studies demonstrated the role of HIF-1α in antibacterial innate immune responses. HIF-1α is transcriptionally induced by lipopolysaccharide (LPS) treatment, a component of Gram-negative bacterial cell wall, and induces a number of target genes (Zinkernagel et al., 2007). It has also been shown that HIF-1α is responsible for the LPS-induced sepsis by transcriptional induction of the expression of inflammatory cytokines such as TNF-α (Peyssonnaux et al., 2007).

The role of HIF-1α in antiviral innate immune response has recently been suggested (Zinkernagel et al., 2007). It has been shown that respiratory syncytial virus (RSV) causes HIF-1α stabilization in primary bronchial epithelial cells (Kilani et al., 2004). Another study reported that hepatitis C virus (HCV) infection can stabilize HIF-1α and stimulate the synthesis of vascular endothelial growth factor (VEGF) (Nasimuzzaman et al., 2007). Hwang et al. (2006) showed that forced activation of HIF-1α by mutation of von Hippel-Lindau (VHL) protein, a negative regulator of HIF-1α, confers resistance to vesicular stomatitis virus (VSV). Thus, collectively the evidence suggests that HIF-1α might play a distinct role in antiviral innate immune responses. However, the exact role of HIF-1α in antiviral innate immune responses is still elusive. Also, the viral PAMP molecule that triggers HIF-1α activation has yet to be defined.

In this report, we studied the role of HIF-1α in nucleic acid-triggered antiviral innate immune responses. First, we demonstrate that HIF-1α protein level is induced upon antiviral responses triggered by poly(I:C) and poly(dAdT), dsRNA and dsDNA viral PAMPs, respectively. Using the HIF-1α specific siRNAs, we then show that the expression of GLUT1, a known HIF-1α target gene (Ebert et al., 1995), as well as the expression of IFIT1, an interferon-inducible antiviral gene (Hwang et al., 2006), is regulated by HIF-1α upon double-stranded nucleic

[1]Department of Chemistry and Brain Korea 21 School of Chemical Materials Science, Sungkyunkwan University, Suwon 440-746, Korea, [2]Department of Biomedical Engineeering, Dongguk University, Seoul 100-715, Korea, [3]These authors contributed equally to this work.
*Correspondence: skim@dongguk.edu (SK), dklee@skku.edu (DL)

Received November 24, 2008; revised December 4, 2008; accepted December 6, 2008; published online February 20, 2009

**Keywords:** Antiviral response, DNA microarray, dsDNA, dsRNA, HIF-1α, nnate immunity

acid treatment. Genome-wide microarray experiments further confirm that HIF-1α regulates not only hypoxia-inducible genes but also innate immune-related genes during the antiviral response triggered by poly(I:C) and poly(dAdT). Taken together, these results suggest a pivotal role for HIF-1α as a key regulator in nucleic acid-triggered antiviral innate immune responses.

## MATERIALS AND METHODS

### Cell culture

T98G, a human glioblastoma cell line, was obtained from ATCC and maintained at 37°C in DMEM (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were routinely subcultured to maintain exponential growth.

### Nuclear fractionation

For nuclear fractionation, 0.5 ml of Buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05 % NP40 pH 7.9) with protease inhibitor cocktail was added to the cultured cells in 100 mm dish on ice. Cells were scraped thoroughly and left on ice for 10 min. Cells were collected by centrifugation at 4°C, 3,000 rpm for 10 min and supernatant was removed. The cell pellet was resuspended in 93.5 μl of Buffer B [5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), pH 7.9] with 6.5 μl of 4.6 M NaCl. Further the cell membrane was disrupted by homogenization with 20 full strokes in a Dounce homogenizer (Sigma). For nuclear protein extraction, samples were centrifuged at 24,000 × *g* for 20 min at 4°C and the supernatant was collected.

### Western blot analysis

Cells were lysed in 2× Laemmli sample buffer. Protein concentration was determined using a BCA assay. Nucleic extracts (10 μg) were resolved in sodium dodecyl sulfate-polyacrylamide gels (10%) and electrophoretically transferred onto nitrocellulose membrane (Protran®, Whatman). Proteins of interest were detected with specific antibodies as indicated (1:2000 dilution), and then visualized with an enhanced chemiluminescence (ECL plus) reagent (Amersham Bioscience). Mouse monoclonal anti-HIF-1α antibody (NB100-105) was purchased from Novous Biologicals. Rabbit anti-DREAM, used as an internal control, was purchased from Koma Biotechnology through custom production service.

### Transfection of siRNAs and nucleic acids

siRNA target sequences of HIF-1α, NF-κB (p65), and IRF3 were selected from published data (An et al., 2005; Franovic et al., 2007; Liu et al., 2006; Samanta et al., 2006). As a control siRNA, we used equimolar concentration of a set of six siRNAs targeting luciferase (Ui-Tei et al., 2004). siRNAs were chemically synthesized by Bioneer. The sequence information of each siRNA is provided in Supplementary Table 1. siRNAs were transfected using DharmaFECT 2 (Dharmacon) as manufacturer's instruction. Final concentration of siRNA transfected was 10 nM. Poly(I:C) and poly(dAdT) were purchased from Sigma and MP Biomedicals, respectively. For poly(I:C) and poly(dAdT) transfection, Lipofectamine 2000 (Invitrogen) reagent was used.

### Quantitative real-time PCR

Cells were harvested and total RNA was extracted using the TRI Reagent® (Ambion) according to the instructions of the manufacturer. Reverse transcription and quantitative real-time RT-PCR were performed as previously described (Yoo et al., 2006). PCR primers used to amplify each gene were chosen by using the Primer3 (http://frodo.wi.mit.edu/primer3/input.htm) program and their sequence information is provided in Supplementary Table 2.

### NF-κB and ISRE activity test

One day before transfection, T98G cells were plated in 24-well plate and grown to 50% confluency in complete medium without antibiotics. 10 nM of siRNA was transfected and 24 h later, second transfection was performed with either NF-κB or ISRE reporter plasmid (100 ng, pNF-κB-Luc or pISRE-Luc, Stratagene). *Renilla* luciferase vector (1 ng, pRL-SV40, Promega) was co-transfected for normalization. In case of plasmid transfection, Lipofectamine and Plus reagent (Invitrogen) was used according to manufacturer's instruction. The 24 h later the second transfection of poly(I:C) and poly(dAdT) was performed for 16 h and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). Results were quantified with the 20/20$^n$ Luminometer (Turner Biosystems).

### ODD degradation assay

pSV-Luc-ODD plasmid, which expresses luciferase-HIF-1α ODD domain fusion protein, was a gift from M. H. Lee (Kim et al., 2007a). The 400 ng of pSV-Luc-ODD plasmid was transfected into T98G cells as described above. *Renilla* luciferase vector (1 ng, pRL-SV40, Promega) was co-transfected for normalization. 48 h later, cells were treated with poly(I:C) and poly(dAdT) for 16 h and luciferase assay was performed.

### DNA microarray experiment and data analysis

DNA microarray experiment and analysis were performed as described (Hong et al., 2008). Total RNA was extracted using the TRI Reagent® (Ambion) and an RNeasy® mini kit (Qiagen) and 15 μg of total RNA was used for each double-stranded cDNA (dscDNA) synthesis, employing a commercial kit (Invitrogen). Reactions were stopped with EDTA and RNase A was treated. Samples were ethanol-precipitated and rehydrated to 250 ng/μl. The 1 μg of dscDNA was used for labeling by Klenow fragment (NEB) using Cy3-labeled random 9mer (TriLink Biotechnologies) and labeled samples were precipitated using isopropanol. The 4 μg of Cy3-labeled DNA (containing sample tracking control and alignment oligo) was hybridized to Nimblegen 385K 4-plex human microarray for 18 h at 42°C using the Nimblegen Hybridization system (Nimblegen). Arrays were washed and array images were obtained using a GenePix 4000B scanner (Axon Instruments). Scanned images were imported into NimbleScan software (Nimblegen). Expression data was normalized through quantile normalization (Bolstad et al., 2003) and Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003). Gene ontology (GO) analysis was performed by using web-based tool provided in Database for Annotation, Visualization, and Integrated Discovery (DAVID) website (http://niaid.abcc.ncifcrf.gov/home.jsp) (Dennis et al., 2003). Selected gene list was uploaded and whole genes were used as background. For promoter analysis, DiRE tool was used (Gotea and Ovcharenko, 2008). Gene list was uploaded and processed with default setting.

## RESULTS AND DISCUSSION

### HIF-1α protein level is up-regulated in response to poly(I:C) and poly(dAdT) treatments

As a first step towards the understanding of the role of HIF-1α in antiviral innate immune responses, we tested whether trans-

**Table. 1.** HIF-1α regulated genes with HIF-1α consensus binding sequence

| | Poly(dAdT) | | Poly(I:C) | | Induction by poly(dAdT) ($log_2$) | Induction by poly(I:C) ($log_2$) |
|---|---|---|---|---|---|---|
| | $log_2$ (h-01/cont) | $log_2$ (h-02/cont) | $log_2$ (h-01/cont) | $log_2$ (h-02/cont) | | |
| PPP1R3C | -1.247 | -1.494 | -0.942 | -1.154 | -1.130 | -0.973 |
| SEMA6D | -0.724 | -1.419 | -1.073 | -1.009 | 1.100 | 1.099 |
| KCTD11 | -0.686 | -0.846 | -0.640 | -0.819 | 0.580 | 1.130 |
| FAM59A | -1.879 | -1.102 | -0.883 | -0.899 | 2.278 | 2.304 |
| POMC | -1.321 | -0.822 | -0.623 | -0.813 | 1.028 | 1.150 |
| TMEM45A | -0.654 | -1.050 | -1.146 | -1.024 | 0.711 | 0.573 |
| PAM | -0.689 | -1.138 | -0.633 | -0.774 | 0.802 | 0.562 |
| CA9 | -3.419 | -3.448 | -0.884 | -1.923 | 3.263 | 2.842 |
| PAX5 | -0.857 | -0.772 | -0.670 | -1.068 | 2.226 | 2.156 |

Genes with HIF-1α binding sequence on the promoter were identified through DiRE analysis. Level of induction by poly(I:C) or poly(dAdT) treatment, and the expression level change upon HIF-1α siRNA treatment are presented.

fection of double-stranded nucleic acids, the viral nucleic acids mimics, into cells affects HIF-1α protein level. We transfected T98G cells, a well-studied, dsRNA sensitive, human glioblastoma cell line (Zamanian-Daryoush et al., 1999), with either poly(I:C), a synthetic long dsRNA, or poly(dAdT), a synthetic long dsDNA, respectively. The effect of these nucleic acid mimics on HIF-1α protein level was measured at different time points by Western blotting with a HIF-1α specific antibody. As a positive control, cells were treated with $CoCl_2$, a chemical inducer of hypoxia (Kim et al., 2006). Basal HIF-1α protein level was barely detectable but, as expected, $CoCl_2$ treatment strongly induced HIF-1α protein level (Fig. 1A). Interestingly, transfection of synthetic, double-stranded nucleic acids also showed a significant increase in the HIF-1α protein level (Fig. 1A). The kinetics of HIF-1α induction observed with poly(I:C) and poly(dAdT) treatments were found to be quite similar. HIF-1α level began to increase 8 h following nucleic acids transfection, and reached to maximum between 12 h and 16 h. To our knowledge, this is the first demonstration that long dsRNA and dsDNA, which are the mimics of the viral nucleic acid PAMPs, can up-regulate HIF-1α protein level.

**Down-regulation of HIF-1α attenuates induction of GLUT1 and IFIT1 during the nucleic acid-triggered antiviral responses**

To test whether the activation of HIF-1α by nucleic acid PAMPs plays a role in transcription of genes during the antiviral innate immune responses, we performed RNAi experiments. First, the expression of HIF-1α in T98G cells was reduced by two independent HIF-1α-specific siRNAs (named as H-01 and H-02, respectively) (Franovic et al., 2007; Liu et al., 2006) (Fig. 1B). The cells were then transfected with either poly(I:C) or poly(dAdT) and incubated for 12 h. First we examined the mRNA levels of GLUT1 which is a well-known HIF-1α target gene (Ebert et al., 1995) and found that down-regulation of HIF-1α by both siRNAs reduced GLUT1 mRNA level (Fig. 1C). We also observed moderate up-regulation of GLUT1 gene expression upon either poly(I:C) (1.5 fold) and polydAdT (1.7 fold) treatment. This result confirms that during the antiviral innate immune responses triggered by poly(I:C) or poly(dAdT) treatment, GLUT1 gene expression is controlled by HIF-1α transcription factor activity.

A previous study reported that forced HIF-1α stabilization by the loss of VHL reinforced the induction of several ISGs after VSV infection, including IFIT1 gene, which is one of the interferon-stimulated genes (ISGs) (Hwang et al., 2006). We found that, upon poly(I:C) and poly(dAdT) treatments, the mRNA level of IFIT1 increased more than 200 fold in both condition and this induction was repressed by siRNAs targeting HIF-1α (Fig. 1D). This result suggest that HIF-1α might play a role in expression of not only hypoxia-related genes, but also immune response genes, during the antiviral responses triggered by poly(I:C) or poly(dAdT) treatment.

**Mechanistic study of HIF-1α accumulation triggered by double-stranded nucleic acids**

We then investigated the mechanism of HIF-1α induction during the antiviral innate immune responses triggered by double-stranded nucleic acids. First we examined the possibility that poly(I:C) or poly(dAdT) might stabilize HIF-1α protein by a similar mechanism executed during the hypoxic stimulus. To test this, we utilized an oxygen-dependent degradation domain-luciferase fusion construct (designated as ODD-luc) (Kim et al., 2007a). As expected, as a result of luciferase-ODD protein stabilization, increased luciferase activity, was observed by $CoCl_2$ treatment (Fig. 2A). However, treatment of neither poly(I:C) nor poly(dAdT) resulted in significant increase in luciferase activity (Fig. 2A). This data suggests that ODD-dependent HIF-1α stabilization does not play a major role in HIF-1α induction during the double-stranded nucleic acid-triggered antiviral responses.

Treatment with LPS, a bacterial PAMP, also triggers HIF-1α accumulation. It is reported that the LPS-induced HIF-1α accumulation is a result of transcriptional induction of HIF-1α mRNA (Frede et al., 2006; Kim et al., 2007b; Peyssonnaux et al., 2007). In addition, a recent study on LPS treated murine macrophages demonstrated repression of PHD2 gene expression, a negative regulator of HIF-1α (Peyssonnaux et al., 2007). However, we did not observe induction of HIF-1α mRNA level upon poly(I:C) or poly(dAdT) treatment (Fig. 2B). Upon poly(I:C) treatment, HIF-1α mRNA level decreased at early time point. With further incubation, the transcript level began to increase but was still lesser than the original mRNA level. In the case of poly(dAdT) treatment, HIF-1α mRNA level did not change (Fig. 2B). Therefore, induction of HIF-1α protein level upon nucleic acid transfection cannot be explained by transcriptional induction of HIF-1α gene, as observed with LPS treatment. We also analyzed the PHD2 mRNA levels, but did not observe any reduction (Fig. 2C). Combined together, these results indicate that mechanism of HIF-1α accumulation upon poly(I:C) or poly(dAdT) treatment is distinct from that upon LPS treatment.

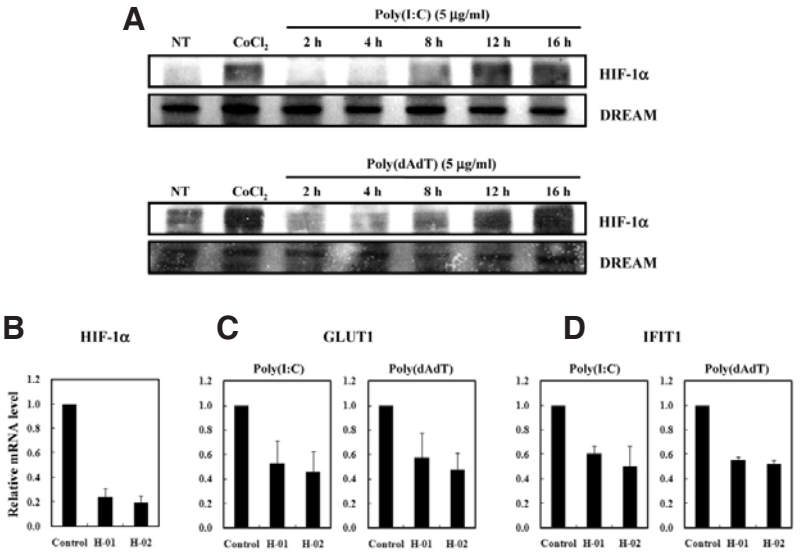


**Fig. 1.** Poly(I:C) and poly(dAdT) increase HIF-1α protein level. (A) Accumulation of HIF-1α protein by poly(I:C) and poly(dAdT) treatment. T98G cells were transfected with poly(I:C) and poly(dAdT) and incubated for indicated time. NT: no treatment. $CoCl_2$: cells treated with 200 μM $CoCl_2$ for 16 h. Nuclear extracts were resolved by 8% or 10% SDS-PAGE and immunoblotted with anti-HIF-1α, and anti-DREAM antibodies. (B) HIF-1α mRNA level Two siRNAs targeting HIF-1α were transfected into T98G cells and resulting mRNA level was analyzed by quantitative real-time PCR. (C, D) mRNA level of GLUT1 and IFIT1 upon poly(I:C) and poly(dAdT) treatment. Control or HIF-1α siRNA pretreated cells were treated with poly(I:C) or poly(dAdT). After nucleic acid transfection, T98G cells were harvested and mRNA expression level was determined by quantitative real time RT- PCR. In mRNA quantitative analysis, at least three independent experiments were performed and relative expression level to control condition was calculated. Mean values are presented with + SD.

### HIF-1α knock-down does not affect NF-κB and IRF3 activity during the nucleic acid-triggered antiviral responses

Walmsley et al. (2005) demonstrated that hypoxia-induced NF-κB activity is mediated through a HIF-1α dependent pathway in neutrophils. Another study (An and Rettig, 2005) also suggested that HIF-1α activation by VHL mutation results in increased NF-κB activity. In contrast, Peyssonnaux et al. recently reported that HIF-1α did not affect NF-κB pathway in LPS challenged murine macrophage (Peyssonnaux et al., 2007). Thus, we tested whether HIF-1α modulates NF-κB activity in nucleic acid-triggered antiviral responses. In addition, we also examined the activity of IRF3, another key transcription factor in antiviral responses (Akira et al., 2006). We confirmed the activation of NF-κB and IRF3 activity upon either poly(I:C) and poly(dAdT) treatment (Fig. 3). However, we did not observe differences in the NF-κB and IRF3 activity between siHIF-1α siRNA- and control siRNA-treated cells (Fig. 3). This result suggests that HIF-1α affects neither NF-κB nor IRF3 activity in the dsRNA and dsDNA-triggered innate immune responses. Rather, HIF-1α might directly affect gene expression upon nucleic acid stimulation (Kim et al., 2006; Peyssonnaux et al., 2007).

### HIF-1α is responsible for the expression of hypoxia-related genes during the nucleic acid-triggered antiviral responses

Toward the comprehensive analysis of the role of HIF-1α in the gene expression program during the nucleic acid-triggered antiviral responses, we performed whole-genome DNA microarray experiments upon HIF-1α knock-down by siRNAs, followed by poly(I:C) and poly(dAdT) stimulation. 10 nM of HIF-1α-targeting siRNAs or control siRNA were transfected into T98G cells, and 48 h later, poly(I:C) or poly(dAdT) was transfected and cells were incubated for additional 12 h. We also included a mock-transfected cell sample as a control for poly(I:C) and poly(dAdT) transfection. Total RNAs were isolated from all samples and microarray analysis was performed by using the Nimblegen homo sapiens 4-plex array platform.

Through DNA microarray data analysis, Chi et al. (2006) identified common hypoxia-related genes, which were induced by hypoxia in various human cell lines. Those genes have been previously identified as key players in hypoxic response, and

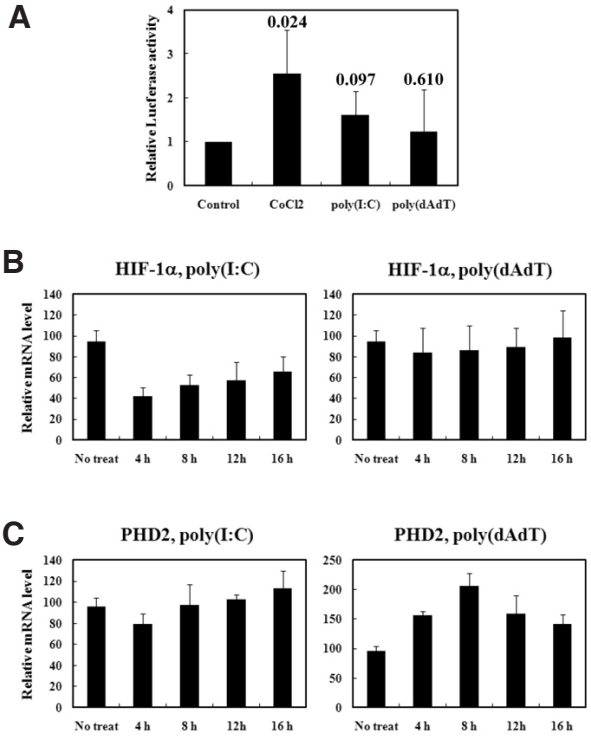


**Fig. 2.** Probing the mechanism of nucleic acid-triggered HIF-1α accumulation. (A) ODD-luciferase construct was transfected into T98G cells. The 48 h later, poly(I:C) and poly(dAdT) was transfected and cells were harvested 16 h after second transfection. Cell lysates were assayed for luciferase activity. As a positive control, 200 μM $CoCl_2$ was treated for 16 h. Results are means + SD for at least three independent experiments. Significance to control was tested and resulting p-values are presented. (B, C) T98G cells were transfected with poly(I:C) and poly(dAdT) and mRNA level of HIF-1α (B) and PHD2 (C) was quantified by quantitative real-time RT-PCR. At least three independent experiments were performed and relative expression level to no treatment control was calculated. Mean values were presented with + SD.

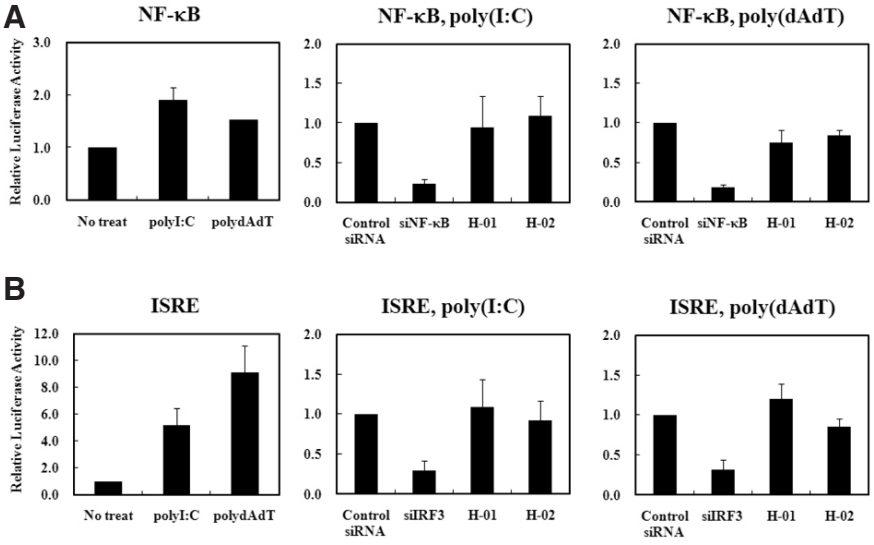


**Fig. 3.** HIF-1α does not affect transcriptional activity of NF-κB or IRF3 upon nucleic acid triggered antiviral innate immune response. Either NF-κB (A) or ISRE (B) reporters were co-transfected with pRL-SV40 plasmid into T98G cells. Then poly(I:C) or poly(dAdT) was transfected, and 16 h later, promoter activity of each reporter was measured by luciferase assay. For middle and right panel, indicated siRNAs were pre-treated. Ratio of firefly luciferase activity over Renilla luciferase activity was determined and normalized with control sample. Data presented are average + SD of at least three independent experiments.

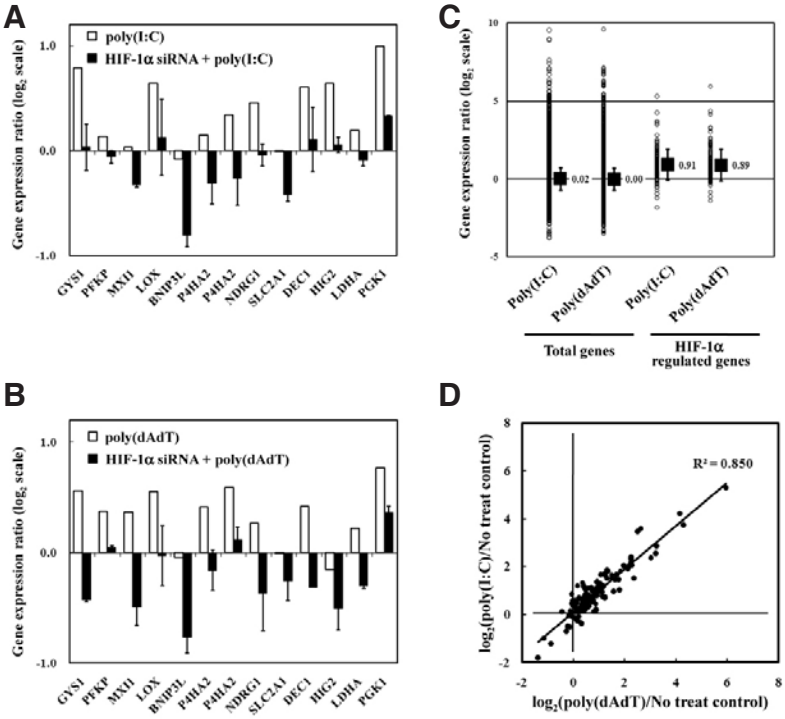


**Fig. 4.** DNA microarray analysis. (A, B) Gene expression ratio of hypoxia genes upon double-stranded nucleic acids treatment. Open bar indicates $\log_2$ scale-transformed gene expression change upon poly(I:C) (A) or poly(dAdT) (B) treatment. Black bar indicates $\log_2$ scale-transformed gene expression change caused by poly(I:C) (A) or poly(dAdT) (B) treatment following HIF-1α siRNA transfection. Values are presented as average of two different siRNA array data with SD. (C) Expression ratios of whole genes and HIF-1α-regulated genes were plotted as scatter plot graph (open circle) and averages of expression ratios in each conditions were presented as box plot with ± SD. Significance test between total genes and HIF-1α regulated genes was performed (p-values less than 0.001 in both condition). (D) Expression ratios of HIF-1α-regulated genes in poly(I:C) and poly(dAdT) treatments were shown as a scatter plot.

include genes encoding proteins with roles in glucose transport, cell proliferation and apoptosis, and other biological processes. We inspected expression levels of the common hypoxia genes in T98G cells stimulated with double-stranded nucleic acids. Out of 12 genes examined, 5 genes (GYS1, LOX, DEC1, HIG2, PGK1) were up-regulated more than 1.5 fold by poly(I:C) or poly(dAdT) treatment (Figs. 4A and 4B). On the other hand, none of these genes were significantly repressed. We also observed that transcriptional responses of those genes were similar in both poly(I:C) and poly(dAdT) conditions. Furthermore, knock-down of HIF-1α with specific siRNAs revealed that expression of these genes were HIF-1α dependent (Figs. 4A and 4B). Taken together, these data support the idea that HIF-1α is a functionally active transcription factor and regulates hypoxia-related target genes during the nucleic acid-triggered antiviral responses.

**HIF-1α is responsible for the expression of genes involved in viral nucleic acid-triggered cellular responses**

To test whether HIF-1α is also involved in expression of genes other than hypoxic response, we analyzed microarray data to obtain the comprehensive list of HIF-1α regulated genes upon poly(I:C) and poly(dAdT) stimuli in T98G cells. To identify HIF-1α regulated genes, we selected genes whose expression levels were repressed by HIF-1α siRNA treatment in both poly(I:C) and poly(dAdT) microarray data. To eliminate false positives, we only selected genes which were down-regulated more than 1.5 fold by siHIF-1α treatment in all four conditions tested [poly(I:C) and poly(dAdT) treatment, two HIF-1α siRNAs]. As a result, a list of 131 HIF-1α target genes was generated

**Table. 2.** Gene ontology analysis of HIF-1α regulated genes

| GO Term (biological process) | Count | % | P-Value |
|---|---|---|---|
| response to stimulus | 34 | 27 | 1.30E-04 |
| innate immune response | 6 | 4.8 | 3.30E-04 |
| immune effector process | 6 | 4.8 | 5.00E-04 |
| cellular polysaccharide metabolic process | 5 | 4 | 5.80E-04 |
| polysaccharide biosynthetic process | 4 | 3.2 | 6.20E-04 |
| cellular polysaccharide biosynthetic process | 4 | 3.2 | 6.20E-04 |
| multicellular organismal process | 37 | 29.4 | 6.20E-04 |
| polysaccharide metabolic process | 5 | 4 | 6.50E-04 |
| G-protein coupled receptor protein signaling pathway | 17 | 13.5 | 8.90E-04 |
| response to other organism | 7 | 5.6 | 1.10E-03 |

Gene ontology analysis of HIF-1α regulated genes were performed by using DAVID platform. Top 10 enriched GO terms (Biological process) are presented with gene numbers, percentage, and p-values.

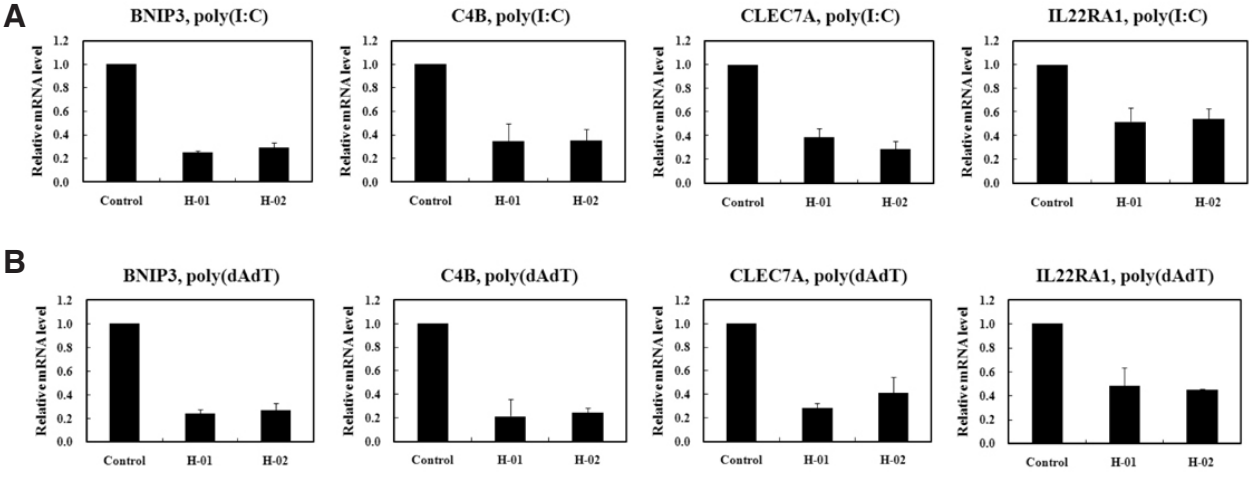


**Fig. 5.** mRNA expression level analysis of individual HIF-1α-regulated genes. From the HIF-1α-regulated gene list, four genes were selected and tested. Control siRNA, or two different siRNAs targeting HIF-1α (H-01 and H-02) were transfected into T98G cells. The 48 h following transfection, poly(I:C) or poly(dAdT) was transfected and cells were incubated for another 12 h. mRNA levels of each genes were quantified by quantitative real-time RT-PCR. mRNA levels were normalized by TBP mRNA level and relative mRNA level to control sample was calculated. At least three independent experiments were performed and mean values were presented with + SD.

(Supplementary Table 3).

First we inspected the gene expression level of HIF-1α target genes upon double-stranded nucleic acids stimuli by calculating the logarithm of fold changes in mRNA level upon nucleic acid treatment. As shown in Fig. 4C, most of HIF-1α target genes were up-regulated by both poly(I:C) and poly(dAdT) treatment. Average values of fold changes (in logarithms) also validate induction of HIF-1α target gene expression upon nucleic acid-triggered antiviral responses. We also found a close correlation of HIF-1α target gene expression level between poly(I:C) and poly(dAdT) stimuli which suggests that HIF-1α similarly regulates target gene expression in both condition (Fig. 4D).

Promoter analysis of HIF-1α target genes using web-based tool DiRE (Gotea and Ovcharenko, 2008) reported 9 genes with HIF-1α binding sequence (Table 1). Expression of all genes was down-regulated by siRNAs targeting HIF-1α, and except PPP1R3C, expression of all genes was activated by double-stranded nucleic acids. In addition, six genes (PPP1RC, KCTD11, POMC, TMEM45A, PAM, and CA9) listed in Table 1 were previously identified as hypoxia-inducible genes (Choi et al., 2004; Gross et al., 2008; Manalo et al., 2005; Sonna et al., 2003; Wykoff et al., 2000), further supporting the idea that these genes are direct HIF-1α targets during the double-stranded nucleic acid triggered antiviral responses.

To understand the cellular function of HIF-1α target genes, we performed gene ontology analysis by using DAVID platform and presented 10 most enriched GO terms with p-values (Table 2). As shown in Table 2, 'response to stimulus' term was most highly enriched and 34 HIF-1α target genes were categorized within this process. In addition, we observed enrichment of immune-related terms such as 'innate immune response', 'immune effector process', and 'response to other organism'. The list of immune-related HIF-1α target genes is shown in Table 3. This list includes cytokine receptors (IL23R, IL22RA1, and XCR1), pro-apoptotic molecules (BNIP3 and BNIP3L), and transcription factor (CEBPE). We also confirmed the expression level of individual genes using quantitative real-time RT-PCR. The genes tested (BNIP3, C4B, CLEC7A, and IL22RA1) showed increased mRNA level upon either poly(I:C) and poly(dAdT) treatment (Supplementary Fig. 1) and we also found that those inductions are dependent on HIF-1α activity (Fig. 5). Collectively, these data demonstrate that HIF-1α is responsible for the

**Table. 3.** List of immune-related HIF-1α-regulated genes

| Gene | Description |
|---|---|
| BNIP3 | bcl2/adenovirus e1b 19kda interacting protein 3 |
| MASP1 | mannan-binding lectin serine peptidase 1 (c4/c2 activating component of ra-reactive factor) |
| CLEC7A | c-type lectin domain family 7, member a |
| BNIP3L | bcl2/adenovirus e1b 19kda interacting protein 3-like |
| CD1D | cd1d antigen, d polypeptide |
| DMBT1 | deleted in malignant brain tumors 1 |
| XCR1 | chemokine (c motif) receptor 1 |
| CEBPE | ccaat/enhancer binding protein (c/ebp), epsilon |
| ORM1 | orosomucoid 1 |
| IL23R | interleukin 23 receptor |
| C4B | complement component 4a (rodgers blood group) |
| RAG2 | recombination activating gene 2 |
| FCN2 | ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin) |
| CHIA | chitinase, acidic |
| CEBPE | ccaat/enhancer binding protein (c/ebp), epsilon |
| IL22RA1 | interleukin 22 receptor, alpha 1 |

From GO analysis data, genes related to immune response are collected and presented.

expression of a subset of immune-related genes during the nucleic acid-triggered antiviral innate immune responses.

In this study, we demonstrated the induction of HIF-1α protein and gene expression program dependent upon HIF-1α during the nucleic acid-triggered antiviral innate immune responses. While previous studies have suggested the role of HIF-1α during antiviral responses (Kilani et al., 2004; Nasimuzzaman et al., 2007), our study is the first to demonstrate that both long dsRNA and long dsDNA, which are mimics of viral genomes, are sufficient to trigger HIF-1α activation. In addition, by using HIF-1α specific siRNAs and DNA microarray, we demonstrate that HIF-1α-dependent gene expression program encompasses the expression of not only hypoxia-related genes, but also immune response-related genes. Our result is in agreement with the recent report showing that hypoxia induced a number of immune-related genes in monocytes (Bosco et al., 2006), and another study which showed the forced activation of HIF-1α confers resistance to vesicular stomatitis virus (VSV) by activating expression of genes involved in antiviral responses (Hwang et al., 2006).

The mechanism responsible for HIF-1α induction upon double-stranded nucleic acids stimuli is still elusive. Mechanisms previously demonstrated to induce HIF-1α during hypoxia or upon LPS stimulus do not seem to play a major role in HIF-1α induction upon antiviral stimuli. In addition, the signaling pathway that senses double-stranded nucleic acids and relays the signal to activate HIF-1α remains to be identified. Future study should focus on identification of the novel HIF-1α induction mechanism during the nucleic acid-triggered antiviral responses.

In conclusion, our study demonstrates that HIF-1α plays a pivotal role in antiviral innate immune responses, by acting as a downstream transcriptional regulator of viral nucleic acid sensing pathway to regulate expression of both hypoxia-related and immune-related genes. Uncovering of novel innate immunity transcriptional regulators and the mechanism of their activation should aid in understanding the complex mechanism of innate immune responses against pathogenic infections (Hong et al., 2008).

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

## ACKNOWLEDGMENTS

We thank Dr. Min Hyung Lee for providing the Luc-ODD construct, and Pooja Dua for critical reading of the manuscript. This work was supported by the grant from the Korea Research Foundation (grant 2007-313-C00468) and National Research Laboratory grant.

## REFERENCES

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783-801.

An, J., and Rettig, M.B. (2005). Mechanism of von Hippel-Lindau protein-mediated suppression of nuclear factor kappa B activity. Mol. Cell. Biol. *25*, 7546-7556.

An, J., Fisher, M., and Rettig, M.B. (2005). VHL expression in renal cell carcinoma sensitizes to bortezomib (PS-341). through an NF-kappaB-dependent mechanism. Oncogene *24*, 1563-1570.

Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics *19*, 185-193.

Bosco, M.C., Puppo, M., Santangelo, C., Anfosso, L., Pfeffer, U., Fardin, P., Battaglia, F., and Varesio, L. (2006). Hypoxia modifies the transcriptome of primary human monocytes: modulation of novel immune-related genes and identification of CC-chemokine ligand 20 as a new hypoxia-inducible gene. J. Immunol. *177*, 1941-1955.

Chi, J.T., Wang, Z., Nuyten, D.S., Rodriguez, E.H., Schaner, M.E., Salim, A., Wang, Y., Kristensen, G.B., Helland, A., Borresen-Dale, A.L., et al. (2006). Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. PLoS Med. *3*, e47.

Choi, J.W., Park, S.C., Kang, G.H., Liu, J.O., and Youn, H.D. (2004). Nur77 activated by hypoxia-inducible factor-1alpha overproduces proopiomelanocortin in von Hippel-Lindau-mutated renal cell carcinoma. Cancer Res. *64*, 35-39.

Dennis, G., Jr., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C., and Lempicki, R.A. (2003). DAVID: database for annotation, visualization, and integrated discovery. Genome Biol. *4*, P3.

Dery, M.A., Michaud, M.D., and Richard, D.E. (2005). Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators. Int. J. Biochem. Cell Biol. *37*, 535-540.

Ebert, B.L., Firth, J.D., and Ratcliffe, P.J. (1995). Hypoxia and mito-

chondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. J. Biol. Chem. *270*, 29083-29089.

Franovic, A., Gunaratnam, L., Smith, K., Robert, I., Patten, D., and Lee, S. (2007). Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. Proc. Natl. Acad. Sci. USA *104*, 13092-13097.

Frede, S., Stockmann, C., Freitag, P., and Fandrey, J. (2006). Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes via p44/42 MAPK and NF-kappaB. Biochem. J. *396*, 517-527.

Gotea, V., and Ovcharenko, I. (2008). DiRE: identifying distant regulatory elements of co-expressed genes. Nucleic Acids Res. *36*, W133-139.

Gross, C., Dubois-Pot, H., and Wasylyk, B. (2008). The ternary complex factor Net/Elk-3 participates in the transcriptional response to hypoxia and regulates HIF-1 alpha. Oncogene *27*, 1333-1341.

Hong, S.W., Kim, S., and Lee, D.K. (2008). The role of Bach2 in nucleic acid-triggered antiviral innate immune responses. Biochem. Biophys. Res. Commun. *365*, 426-432.

Hwang, I.I., Watson, I.R., Der, S.D., and Ohh, M. (2006). Loss of VHL confers hypoxia-inducible factor (HIF).-dependent resistance to vesicular stomatitis virus: role of HIF in antiviral response. J. Virol. *80*, 10712-10723.

Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., and Speed, T.P. (2003). Summaries of affymetrix genechip probe level data. Nucleic Acids Res. *31*, e15.

Kilani, M.M., Mohammed, K.A., Nasreen, N., Tepper, R.S., and Antony, V.B. (2004). RSV causes HIF-1alpha stabilization via NO release in primary bronchial epithelial cells. Inflammation *28*, 245-251.

Kim, K.S., Rajagopal, V., Gonsalves, C., Johnson, C., and Kalra, V.K. (2006). A novel role of hypoxia-inducible factor in cobalt chloride- and hypoxia-mediated expression of IL-8 chemokine in human endothelial cells. J. Immunol. *177*, 7211-7224.

Kim, H.A., Kim, K., Kim, S.W., and Lee, M. (2007a). Transcriptional and post-translational regulatory system for hypoxia specific gene expression using the erythropoietin enhancer and the oxygen-dependent degradation domain. J. Control. Release *121*, 218-224.

Kim, H.Y., Kim, Y.H., Nam, B.H., Kong, H.J., Kim, H.H., Kim, Y.J., An, W.G., and Cheong, J. (2007b). HIF-1alpha expression in response to lipopolysaccaride mediates induction of hepatic inflammatory cytokine TNFalpha. Exp. Cell Res. *313*, 1866-1876.

Lee, M.S., and Kim, Y.J. (2007). Pattern-recognition receptor signaling initiated from extracellular, membrane, and cytoplasmic space. Mol. Cells *23*, 1-10.

Liu, X.H., Yu, E.Z., Li, Y.Y., and Kagan, E. (2006). HIF-1alpha has an anti-apoptotic effect in human airway epithelium that is mediated via Mcl-1 gene expression. J. Cell. Biochem. *97*, 755-765.

Manalo, D.J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B.D., Ye, S.Q., Garcia, J.G., and Semenza, G.L. (2005). Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood *105*, 659-669.

Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J. *20*, 5197-5206.

Nasimuzzaman, M., Waris, G., Mikolon, D., Stupack, D.G., and Siddiqui, A. (2007). Hepatitis C virus stabilizes hypoxia-inducible factor 1alpha and stimulates the synthesis of vascular endothelial growth factor. J. Virol. *81*, 10249-10257.

Peyssonnaux, C., Cejudo-Martin, P., Doedens, A., Zinkernagel, A.S., Johnson, R.S., and Nizet, V. (2007). Cutting edge: Essential role of hypoxia inducible factor-1alpha in development of lipopolysaccharide-induced sepsis. J. Immunol. *178*, 7516-7519.

Samanta, M., Iwakiri, D., Kanda, T., Imaizumi, T., and Takada, K. (2006). EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. EMBO J. *25*, 4207-4214.

Sonna, L.A., Cullivan, M.L., Sheldon, H.K., Pratt, R.E., and Lilly, C.M. (2003). Effect of hypoxia on gene expression by human hepatocytes (HepG2). Physiol. Genomics *12*, 195-207.

Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R., and Saigo, K. (2004). Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. *32*, 936-948.

van Uden, P., Kenneth, N.S., and Rocha, S. (2008). Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. Biochemistry *412*, 477-484.

Walmsley, S.R., Print, C., Farahi, N., Peyssonnaux, C., Johnson, R.S., Cramer, T., Sobolewski, A., Condliffe, A.M., Cowburn, A.S., Johnson, N., et al. (2005). Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. J. Exp. Med. *201*, 105-115.

Wang, G.L., and Semenza, G.L. (1993). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc. Natl. Acad. Sci. USA *90*, 4304-4308.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc. Natl. Acad. Sci. USA *92*, 5510-5514.

Wykoff, C.C., Beasley, N.J., Watson, P.H., Turner, K.J., Pastorek, J., Sibtain, A., Wilson, G.D., Turley, H., Talks, K.L., Maxwell, P.H., et al. (2000). Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res. *60*, 7075-7083.

Yoo, J.W., Hong, S.W., Kim, S., and Lee, D.K. (2006). Inflammatory cytokine induction by siRNAs is cell type- and transfection reagent-specific. Biochem. Biophys. Res. Commun. *347*, 1053-1058.

Zamanian-Daryoush, M., Der, S.D., and Williams, B.R. (1999). Cell cycle regulation of the double stranded RNA activated protein kinase, PKR. Oncogene *18*, 315-326.

Zhou, J., Schmid, T., and Brune, B. (2003). Tumor necrosis factor-alpha causes accumulation of a ubiquitinated form of hypoxia inducible factor-1alpha through a nuclear factor-kappaB-dependent pathway. Mol. Biol. Cell *14*, 2216-2225.

Zinkernagel, A.S., Johnson, R.S., and Nizet, V. (2007). Hypoxia inducible factor (HIF). function in innate immunity and infection. J. Mol. Med. *85*, 1339-1346.