THE MAP KINASE AND TCF-4 SIGNALING PATHWAYS REGULATE HIF-1α TRANSCRIPTIONAL ACTIVITY IN RESPONSE TO HYPOXIA

By

WEI HAO

A Thesis Submitted to the Honors College and the College of Science

In Partial Fulfillment of a Bachelor's of Science with Honors in

Biochemistry and Molecular Biophysics, and Molecular and Cellular Biology

The University of Arizona

May 2009

Approved by:

Dr. John W. Regan

Pharmacology/Toxicology

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Dr. Roger L. Miesfeld

Biochemistry & Molecular Biophysics

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I want to thank Dr. John W. Regan for his patience and guidance throughout this project. I have known him ever since I started working in his laboratory in 2006, and I am fortunate to have learned much from him. Dr. Regan gave me freedom to do my projects, and he provided advice if I was befuddled with a problem; I am forever grateful. I also want to thank Dr. Chih-Ling Chou, who was a post-doctorate in Dr. Regan's lab. She was my mentor from the start and invited me to join the lab. I owe this project's success to her. I also want to thank the other members of the laboratory, Dr. Wei Xu, Dr. Anthony Hutchinson, Dr. Davelene Israel, and Ruyue (Amy) Ji, for constantly teaching and mentoring me, and for putting up with me with my innumerable questions. Without everyone in the lab, I would not have been able to perform independent research. I also want to thank my biochemistry professor, Dr. James T. Hazzard, for his advice in presenting my project for conferences, and my biochemistry thesis advisor, Dr. Roger L. Miesfeld, for his guidance throughout the entire process. Last but not least, I want to thank my family, friends and mentors for keeping me motivated and not straying from my goals.

TABLE OF CONTENTS

Abstract

The mitogen activated protein kinase (MAPK) signaling pathway has been shown to regulate hypoxia inducible factor-1α (HIF-1α), which plays an important role in cancer by helping cells survive under low oxygen conditions through the upregulation of genes that promote angiogenesis. Dysregulation of the β-catenin/T-cell factor (TCF) signaling pathway is also known to occur in several cancers; however, the relationship between up-regulation of HIF-1α and activation of TCF signaling and MAPK pathways has not been previously explored. The present study examines this relationship in a model system using prostate PC-3 adenocarcinoma cells. We hypothesized that dominant negative (DN) mutants of both pathways will decrease the hypoxia induced expression of HIF-1α.

Our laboratory has shown that under *normoxic* conditions, TCF-4 signaling up-regulates HIF-1α expression in a MAPK dependent manner by a mechanism that involves the stabilization of HIF-1α by the transcriptionally mediated up-regulation of the nuclear receptor related 1 protein (NURR-1). This is in contrast to the classic mechanism of HIF-1α stabilization, which involves a decrease in ubiquitin mediated degradation caused by the decreased activity of prolyl hydroxylases. Although the exact mechanism of the stabilization of HIF-1α expression by NURR-1 is not known, our laboratory has shown that under normoxic conditions, a NURR-1 dominant negative mutant will block the MAPK/TCF mediated up-regulation of HIF-1α. Presently, it is unknown if a similar activation of MAPK/TCF signaling and up-regulation of NURR-1 occurs during the hypoxia induced stabilization of HIF-1α. This study tests the hypothesis that the hypoxia

induced stabilization of HIF-1α involves the activation of MAPK/TCF signaling and up-regulation of NURR-1.

To examine this hypothesis, HIF-1α transcriptional activity was assessed using immunoblots and compared to GAPDH as the loading control. Cells were incubated under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) for 2 hr and HIF-1α activity was assessed of either alone or following transient transfection with an empty vector control (pcDNA3) or dominant negative mutants of c-Jun, MEKK-4, Ras, TCF-4, and NURR-1. Dominant negative mutants overexpress mutant signaling proteins that effectively inhibit the signaling activity of their normal endogenous counterpart.

In response to hypoxia, HIF-1α expression in PC-3 cells was increased relative to cells maintained under normoxic conditions in a time dependent manner. In cells pre-treated with each of the dominant negative mutants listed above, HIF-1α expression was decreased in response to hypoxia as compared with cells exposed to hypoxia alone. These findings suggest that the MAPK and TCF signaling pathways are involved in the up-regulation of HIF-1α expression in cells exposed to hypoxia. In addition, the results with the DN-NURR-1 suggest that the activity of NURR-1 is also involved with the hypoxia induced up-regulation of HIF-1α expression.

The relationship between NURR-1 and HIF-1α was further investigated by immunoblot analysis and by measuring mRNA expression levels of NURR-1 and its related proteins in response to hypoxia. In both sets of experiments, the results suggest that NURR-1

expression levels do not change in response to hypoxia, which conflicts with the data obtained with the DN-NURR-1, which suggests that NURR-1 activity is necessary for the hypoxia induced up-regulation of HIF-1α. Thus, future studies are necessary to reconcile these conflicting results.

Chapter 1 Introduction

Hypoxia literally means “low oxygen,” but in a clinical context, it implies that cells are deprived of adequate oxygen supply necessary for normal function and survival. Survival under hypoxic conditions is primarily maintained by the transcription factor hypoxia inducible factor-1α (HIF-1α). HIF-1α is a transcription factor that responds to a decrease in oxygen to promote the expression of downstream genes (Smith et al., 2008), and HIF-1α plays an important role in cancer (Pipinikas et al., 2008).

Under normoxic (normal oxygen) conditions, HIF-1α is hydroxylated by HIF prolyl-hydroxylase (Semenza et al., 2004). This causes HIF-1α to be targeted by the E3 ubiquitin ligase complex, and HIF-1α is then degraded by the proteasome. However, under hypoxic conditions, HIF prolyl-hydroxylase is inhibited by the lack of oxygen as a necessary cosubstrate. As a result, HIF-1α is not degraded and it translocates to the nucleus and forms a complex with HIF-1β, which then binds to hypoxia response elements (HRE) in promoters.

In addition, HIF-1α helps cells survive under hypoxic conditions through the up-regulation of genes that promote angiogenesis (Beniziri et al., 2008), which is the growth of new blood vessels. While angiogenesis is beneficial in normal tissues, for example during would healing, in cancerous tissues, it may favor the expansion and growth of malignant cells (Benjamin et al., 2003).

The β-catenin/T-cell factor (TCF) signaling pathway has been shown to interact with c-Jun, which is also a downstream effector of the MAPK signaling pathway, in regulating intestinal cancer development (Nateri et al., 2005). C-Jun is activated by the JNK pathway and combines with c-Fos to form the transcription factor AP-1. Dysregulation of the TCF signaling pathway is also known to occur in several cancers such as colon cancer (Slattery et al., 2008), endometrial cancer (Domenyuk et al., 2007), and breast cancer (Burwinkel et al., 2006). TCF-4 is a transcription factor that activates Wnt target genes (Jin et al., 2008) as shown in Figure 1. Thus, targeting components of the TCF pathway may provide therapeutic applications such as cancer treatment.

The MAPK signaling pathway has been shown to regulate HIF-1α (Kaidi et al., 2007). Figure 2 shows the MAPK signaling pathway and the points for regulation. The Ras signal transduction proteins are key regulators of several aspects of normal cell growth and malignant transformation. They are aberrant in most human tumors due to activating mutations in the Ras genes themselves or to alterations in upstream or downstream signaling components (Downward, 2003). MEKK-4 is also known as MAPK kinase kinase and it directly regulates the JNK pathways (Ellinger-Ziegelbauer et al., 1997).

Although the TCF and MAPK signaling pathways have been well studied, the relationship between the two and their regulation of HIF-1α needs to be further explored. Our laboratory has shown that under normoxic conditions, TCF-4 regulates HIF-1α in a MAPK dependent manner (Chou et al., 2009). However, the relationship between TCF-4 and HIF-1α in hypoxic conditions has not been investigated. For this study, we

hypothesize that activation of TCF and MAPK signaling is necessary for the expression of HIF-1α.

HIF-1α is classically post-transcriptionally and post-translationally regulated under hypoxic conditions. However, the TCF and MAPK signaling regulate the transcription of downstream genes through transcription factors. Thus, we predict that another regulatory mechanism in the pathway between c-Jun or TCF-4 and HIF-1α is necessary for HIF-1α expression. The nuclear receptor related 1 protein (NURR-1) has been shown to stabilize HIF-1α (Yoo et al., 2004). Our laboratory has also shown that the TCF and MAPK signaling pathways are mediated through the transcriptional regulation of NURR-1 (Chou et al., 2009). However, the same relationship is not well studied under hypoxic conditions. Thus, we hypothesized that NURR-1 is necessary for the hypoxia induced expression of HIF-1α.

In this study, PC-3 prostate adenocarcinoma cell lines were used as a model system to investigate how the TCF signaling and MAPK pathways affect cancer cells and their signaling properties in response to hypoxia.

Chapter 2 Materials and Methods

PC-3. Prostate PC-3 adenocarcinoma cell lines were donated by the Vaillancourt lab (University of Arizona). The cells were cultured in standard RPMI complete medium, 500 mL RPMI-1640 from GibcoBRL 11875-093, 5.0 mL Penicillin (10,000 U)-Streptomycin (10 mg)-L-Glutamine (200 mM) solution from Sigma G-1146, 50 mL of Fetal Calf Serum from HyClone SH30070.03 (heat inactivated for 40 min at 56°C). Usually, 400,000 to 500,000 cells are seeded in 15 ml of RPMI complete medium in regular T75 flasks and incubated at 37°C in 5% CO_2 for 3 to 4 days when the cells are about 80% confluent. Then, the cells are detached from the surface by trypsinization with 1 mL of 0.1% trypsin and 0.04% EDTA (Sigma). The cells are washed once with 1xRPMI and reseeded in a new flask.

Transfection. PC-3 cells were detached by trypsinization with 1 mL of 0.1% trypsin and 0.04% EDTA (Sigma) per flask, seeding 50,000 cells per well on a six well plate (Sigma). The cells were transiently transfected with 5 µL of Lipofectamine 2000 transfection reagent (Invitrogen) and 2 µL of DNA plasmid (pcDNA3, DN-TCF-4, DN-c-Jun, DN-MEKK-4, DN-NURR-1 and DN-Ras) in 1 mL Opti-Mem medium. All plasmids were repeated in triplicates. For the experiments to measure mRNA levels, transfection was not necessary because only endogenous levels of NURR-1, NUR-77, and NOR-1 were measured.

Hypoxia. PC-3 cells were incubated in the hypoxia chamber at 37^{o}C for the following time periods: 0, ½, 1, 2, 3 and 6 hrs under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2).

2 hr of hypoxia was found to be optimal for the induction of HIF-1α expression for PC-3 cells; thus, cells pre-treated with pcDNA3 empty vector plasmids and DN plasmids were incubated in hypoxic conditions for 2 hr only for this study.

Western Blotting. Cells were lysed using lysis buffer (1% PMSF) and harvested. Samples were spun overnight at 4°C and centrifuged at 12,500 RPM for 10 minutes. The supernatant per sample was transferred to measure protein concentrations per sample via Bradford's Assay. BSA was used as the standard and the protein assay solution (BIORAD) was used as the medium. 10 µL of protein ladder (Fermentas) were loaded per gel and equal amounts of protein were loaded alternately of cells in normoxic and hypoxic conditions into wells of 7.5% Bis-Acrylamide (BIORAD) gel and were run for 1 hr at 120 V. The separated proteins of the gel were transferred to a PVDF membrane (BIORAD) for 3 hr at 100 mA per membrane. Ponseau solution was used to stain each membrane to determine loading accuracy and transfer fidelity. Each membrane was washed with ddH_2O (distilled and deionized water) and blocked with 5% non-fat milk in 1x TBST for 30 minutes. For the immunoblot analysis, the membranes were incubated in primary antibodies in 5% non-fat milk in 1x TBST overnight. Each membrane was washed three times with 1x TBST for 5 minutes and incubated in secondary antibodies (1:10000) for 2 hr. Each membrane was washed three times with 1x TBST for 5 minutes and immersed in developing solution (BIORAD). The membranes were exposed to x-ray film (Fischer) and developed.

Real Time PCR. Real time polymerase chain reaction (PCR) was used to quantify the mRNA levels of NURR-1 and its related genes NUR-77, and NOR-1. PC-3 cells were lysed with Qiagen MiniPrep lysis buffer. The mRNA was isolated from the cells and purified using ethanol precipitation. The mRNA concentration and purity were measured using a Nanodrop spectrometer. Reverse transcriptase PCR (RT PCR) was performed to generate cDNA from the purified mRNA. The cDNA was probed with NURR-1, NUR-77, NOR-1, and GAPDH specific probes (Applied Biosystems). TaqMan Gene Expression Arrays (Applied Biosystems) were used to measure mRNA expression levels quantitatively in the spectrometer by Applied Biosystems. GAPDH was used as a control, and nuclease free water was used as a non-template control.

Chapter 3 Results

It has been well established that hypoxia induces HIF-1α expression. Before testing if the TCF and MAPK signaling pathways regulate HIF-1α expression in PC-3 cells, it was necessary to confirm that hypoxia increased HIF-1α expression levels in PC-3 cells. Figure 3 shows the time dependent up-regulation of HIF-1α in PC-3 cells in response to hypoxia using immunoblot analysis with antibodies specific to HIF-1α. The time point at 2 hours was chosen for the following experiments because it showed the highest levels of HIF-1α expression.

Because we have shown that HIF-1α is up-regulated in time dependent manner under hypoxic conditions and because of previous studies showing that TCF signaling up-regulates HIF-1α expression under normoxic conditions, we proceeded to test our hypothesis that TCF signaling up-regulates hypoxia induced HIF-1α expression. Figure 4 shows that after 2 hours of treatment with hypoxia, the HIF-1α expression in PC-3 cells pretreated with DN-TCF-4 was down-regulated using immunoblot analysis with antibodies specific to HIF-1α. GAPDH was used as the loading control to verify that the changes observed in HIF-1α expression were not due to nonspecific changes in the overall protein expression. These results suggest that activation of TCF-4 signaling is necessary for the hypoxia induced expression of HIF-1α.

In addition to the TCF signaling pathway, the MAPK signaling pathway was also tested to see if it was involved in the up-regulation of HIF-1α expression under hypoxic

conditions. Figure 5 shows that after 2 hours of treatment with hypoxia, the HIF-1α expression in PC-3 cells pretreated with DN-c-Jun, DN-MEKK-4, and DN-Ras was down-regulated using immunoblot analysis with antibodies against HIF-1α. GAPDH was used as the loading control to verify that the changes observed in HIF-1α expression were not due to nonspecific changes in the overall expression of the protein. These results suggest that the activation of c-Jun, MEKK-4, and Ras are necessary for the hypoxia induced up-regulation of HIF-1α.

The heterologous expression of NURR-1 in mammalian cells has been shown to induce the expression of HIF-1α in normoxic conditions (Yoo et al., 2004). In addition, unpublished work from our lab has shown that up-regulation of HIF-1α expression under normoxic conditions by the FP prostanoid receptor involves the induction of NURR-1 expression (Chou et al., 2009). Therefore, we hypothesized that the induction of NURR-1 expression is also necessary for the hypoxia induced expression of HIF-1α in PC-3 cells. Figure 6 shows that after 2 hours of treatment with hypoxia, the HIF-1α expression in PC-3 cells pretreated with DN-NURR-1 was down-regulated. GAPDH was used as the loading control to further verify the results. These results suggest that activation of NURR-1 signaling is necessary for the hypoxia induced expression of HIF-1α.

Based on the results of the previous experiment, we hypothesized that the hypoxia induced up-regulation of HIF-1α requires the induction of NURR-1 expression. Therefore, immunoblot analysis was used to measure the expression of NURR-1 following treatment with 2 hours of hypoxia compared to normoxia. Figure 7 shows,

however, that NURR-1 levels did not change in response to hypoxia using immunoblot analysis against NURR-1. GAPDH was used as the loading control to rule out that the negative result was not due to loading error.

Thus, this negative result presents two interpretations. The first interpretation is that the negative result is valid, which may be possible because the dominant negative mutant of NURR-1 is also known to inhibit the activity of the orphan nuclear receptors, NUR77 and NOR-1. The second interpretation is that the negative result is not valid, which needs to be proven through a positive control or other types of experiments to reconcile the diverging interpretations.

We, therefore, hypothesize that the results obtained with the DN-NURR-1 might be explained by the induction of NUR77, NOR-1, as well as NURR-1. Real time PCR was used to examine the mRNA expression of NUR77, NOR-1, and NURR-1 in PC-3 cells following treatment with 2 hours of hypoxia compared to normoxia. Figures 8A, 8B, and 8C show the NUR77, NURR-1, and NOR-1 mRNA expression levels, respectively, measured using real time PCR and normalized against GAPDH. PC-3 cells treated with 2 hours of hypoxia did not have significant fold changes in NUR77, NURR-1, or NOR-1 mRNA expression. These results suggest that NUR77, NURR-1, and NOR-1 are not regulated by hypoxia.

Chapter 4 Discussion

Although the TCF and MAPK signaling pathways have been well studied, the relationship between the two and their regulation of HIF-1α needs to be further explored. Because we have shown that HIF-1α is up-regulated in time dependent manner under hypoxic conditions in Figure 3 and because of previous studies showing that TCF signaling and MAPK signaling up-regulate HIF-1α expression under normoxic conditions, we proceeded to test our hypothesis that TCF signaling and MAPK signaling up-regulates hypoxia induced HIF-1α expression in PC-3 cells.

This study shows in Figures 4 and 5 through immunoblot analysis with antibodies against HIF-1α that dominant negative mutants of components of the TCF signaling pathway, TCF-4, and the MAPK signaling pathway, c-Jun, Ras, and MEKK-4 decreased HIF-1α expression in response to hypoxia. Dominant negative mutants overexpress mutant signaling proteins that effectively inhibit the signaling activity of their normal endogenous counterpart. Thus, these results suggest that the proteins TCF-4, c-Jun, Ras, and MEKK-4 are necessary for the hypoxia induced expression of HIF-1α. Future studies should aim to regulate these components as an indirect means to regulate HIF-1α expression, which may be important in dealing with the expression of downstream genes such as VEGF, which is involved in angiogenesis.

HIF-1α is classically post-translationally regulated through HIF prolyl hydroxylase. However, the TCF and MAPK signaling pathways use transcription factors to regulate

gene expression. Thus, another mechanism that post-translationally regulates HIF-1α is a necessary mediator between the TCF signaling and MAPK signaling pathways and HIF-1α expression.

Our laboratory has also shown that the TCF and MAPK signaling pathways are mediated through the transcriptional regulation of NURR-1 under normoxic conditions (Chou et al., 2009). Thus, we hypothesized that the activity of NURR-1 is also necessary for the hypoxia induced expression of HIF-1α. Figure 6 shows that after 2 hours of treatment with hypoxia, the HIF-1α expression in PC-3 cells pretreated with DN-NURR-1 was down-regulated. These results suggest that the activity of NURR-1 is necessary for the hypoxia induced expression of HIF-1α, although it does not reveal the specific mechanism of this effect.

To further investigate the mechanism that NURR-1 uses for the hypoxia induced expression of HIF-1α, we hypothesized that hypoxia increases NURR-1 expression in order to indirectly increase the hypoxia induced expression of HIF-1α. However, Figure 7 shows that NURR-1 levels did not change in response to hypoxia using immunoblot analysis against NURR-1. GAPDH was used as the loading control to rule out that the negative result was not due to loading error.

Nonetheless, this negative result presents two interpretations. The first interpretation is that the negative result is valid, which may be possible because the dominant negative mutant of NURR-1 can also inhibit the activity of the other orphan nuclear receptors

NUR77 and NOR-1. Dominant negative mutants overexpress mutant signaling proteins that effectively inhibit the signaling activity of their normal endogenous counterpart, which may or may not be the same protein.

The second interpretation is that the negative result is not valid, which needs to be proven through a positive control or other types of experiments to reconcile the diverging interpretations. A positive control experiment would be to repeat a well established experiment that clearly shows the up-regulation of NURR-1. Thus, if the positive control experiment failed to reproduce what was previously well established, then that means that the negative result from this experiment was not valid.

Thus, to address the question if other members of the orphan nuclear receptor family might be involved, real time PCR was used to quantitatively measure mRNA expression levels. By measuring mRNA expression levels, we could determine which of the three orphan nuclear receptors, NUR77, NURR-1, or NOR-1 might be involved in the up-regulation of the hypoxia induced expression of HIF-1α. This is important because dominant negative mutants can inhibit the signaling activity of related proteins in addition to their normal endogenous counterpart. Thus, although DN-NURR-1 decreased HIF-1α expression levels, it does not prove that NURR-1 itself is responsible for the up-regulation of the hypoxic induced expression of HIF-1α. If the results of Figure 7 can be considered valid, i.e. that NURR-1 expression is not induced by hypoxia, then it can be hypothesized that either NUR77 or NOR-1 may be responsible for the up-regulation of HIF-1α expression by hypoxia.

Figures 8A, 8B, and 8C shows the NUR77, NURR-1, and NOR-1 mRNA expression levels, respectively, measured using real time PCR and normalized against GAPDH. PC-3 cells treated with 2 hours of hypoxia did not have significant fold changes in NUR77, NURR-1, or NOR-1 mRNA expression. These results suggest that NUR77, NURR-1, and NOR-1 are not regulated by hypoxia. This further suggests that these orphan nuclear receptors do not regulate the hypoxia induced expression of HIF-1α in PC-3 cells, and that the results of Figure 7 are also valid.

However, the results from the experiments depicted in Figures 7, 8A, 8B, and 8C still conflict with the data shown in Figure 6. In order to reconcile this difference, we must consider new hypotheses and experiments. Although a positive control was not performed in this experiment, it should be done in the future to show a change in the expression levels of any of the orphan nuclear receptors. If a change is observed, then that means that the negative results of the real time PCR experiments above are valid, and that hypoxia does not regulate the expression of orphan nuclear receptors.

Another method is to change the parameters of the real time PCR experiments. Originally, cells were treated for 2 hours with hypoxia. However, 2 hours of treatment with hypoxia was optimal for HIF-1α expression. Because NURR-1 is an upstream effector of HIF-1α, it can be assumed that NURR-1 expression levels peak before HIF-1α does. Furthermore, the mRNA levels of NURR-1 must peak even earlier than the protein expression of NURR-1. Thus, the parameters for the experiments should be changed to measure mRNA

levels for the following time periods of treatment with hypoxia: 0, 15, 30, and 60 minutes. If the results yield an increase in orphan nuclear receptor expression levels, then the results would suggest that the results of 8A, 8B, and 8C are valid, because the mRNA expressed earlier could have been degraded to normoxic levels.

Another possibility may be that the TCF and MAPK signaling pathways regulate the hypoxia induced HIF-1α expression through other mechanisms. For instance, this study considered only the effect that the TCF and MAPK signaling pathways had on downstream genes through transcription factors. However, the MAPK pathways involve kinases that may not necessarily regulate transcriptionally, but they could be phosphorylating HIF-1α or some other protein that regulates HIF-1α. Thus, in order to test for this, it will be necessary to use global inhibitors of transcription, such as actinomycin D (Radhakrishnan et al., 2006), and/or global inhibitors of translation, such as RelE (Christensen et al, 2001). If the inhibitors of transcription, such as actinomycin D, or translation, such as RelE, prevent HIF-1α expression in response to hypoxia, a DNA microarray could be performed to identify which genes are important for regulating the hypoxia induced expression of HIF-1α. If inhibitors of transcription or translation do not regulate the hypoxia induced expression of HIF-1α, then another unknown mechanism must exist.

The functional relationship between the MAPK and TCF signaling pathways in the hypoxia-induced up-regulation of HIF-1α should be explored in future studies. Thus, it's necessary to investigate different mechanisms for their interaction. The thioredoxin

(TRX)-related redox-regulating protein nucleoredoxin (NRX) has been shown to inhibit Wnt-β-catenin signaling through Disheveled (Funato et al., 2008). Disheveled transduces signals from the Wnt receptor, Frizzled, to downstream components that stabilize β-catenin and subsequent activation of the TCF and/or lymphoid enhancer factor (LEF). Thus, there are numerous other points for regulation to indirectly affect HIF-1α expression.

Downstream target genes of HIF-1α under hypoxic conditions should also be explored, such as those that may promote angiogenesis which may lead to malignant tumor growth. Thus, it may be possible to understand certain mechanisms of cancer signaling, which may further lead to clinical applications in cancer therapy. One downstream target may be TWIST. Direct regulation of TWIST, a transcription factor that regulates gastrulation and mesoderm-specification, by HIF-1α has been shown to promote metastasis (Yang et al., 2008). Thus, future studies may also investigate whether upstream genes of HIF-1α can regulate TWIST and other downstream genes.

In summary, TCF-4, c-Jun, Ras, MEKK-4, and NURR-1 have been individually shown to up-regulate HIF-1α expression in hypoxic conditions. However, conflicting results show that NURR-1 expression does not change in response to hypoxia. Thus, for future studies, the proper positive controls should be performed to reconcile those differences. In addition, if the positive controls support the possibility that NURR-1 and its family of orphan nuclear receptors do not have increased expression levels in response to hypoxia,

then more experiments should be performed to test other mechanisms for the hypoxia induced up-regulation of HIF-1α.

Figures

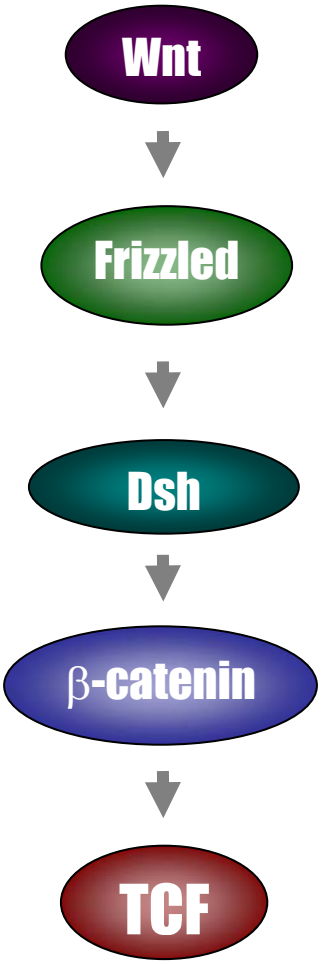

Figure 1. TCF-4 signaling pathway.

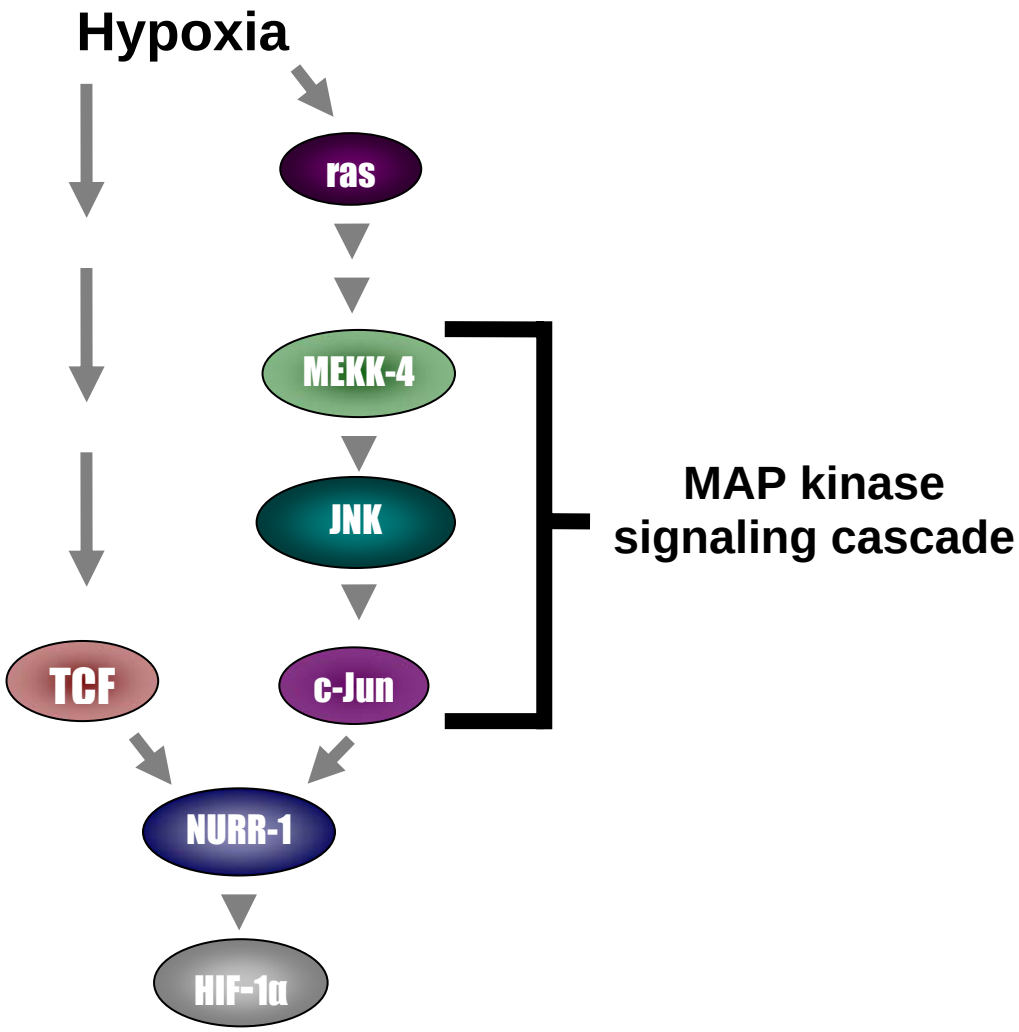

Figure 2. The TCF and MAPK signaling pathways.

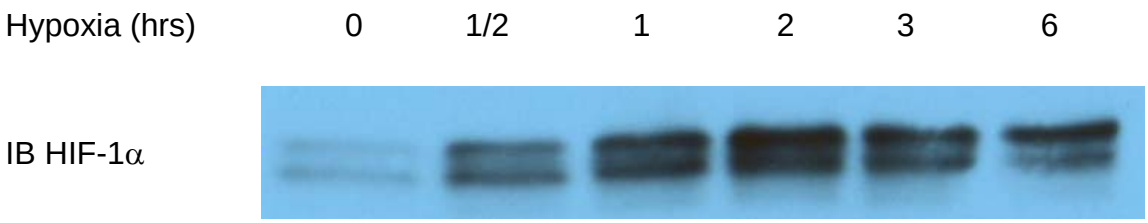

Figure 3. Time dependent up-regulation of HIF-1α was observed. The time point at 2 hours was chosen for the following experiments.

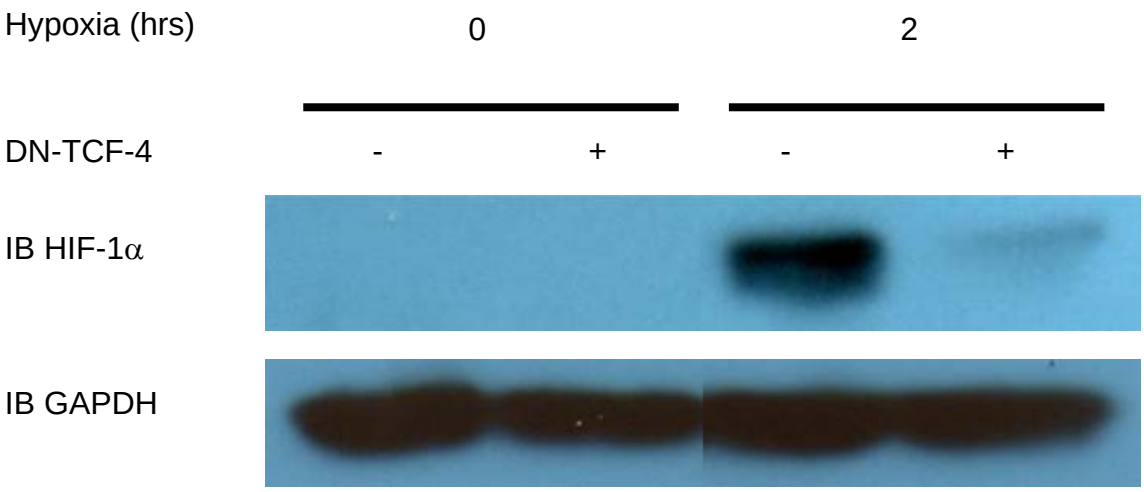

Figure 4. Immunoblot for HIF-1α and GAPDH for PC-3 cells that were either mocked transfected with empty vector (control) or transfected with DN-TCF-4 and then exposed to 2 hours of normoxia (-) or hypoxia (+). GAPDH was used as the loading control. Data are from a representative experiment that was repeated twice.

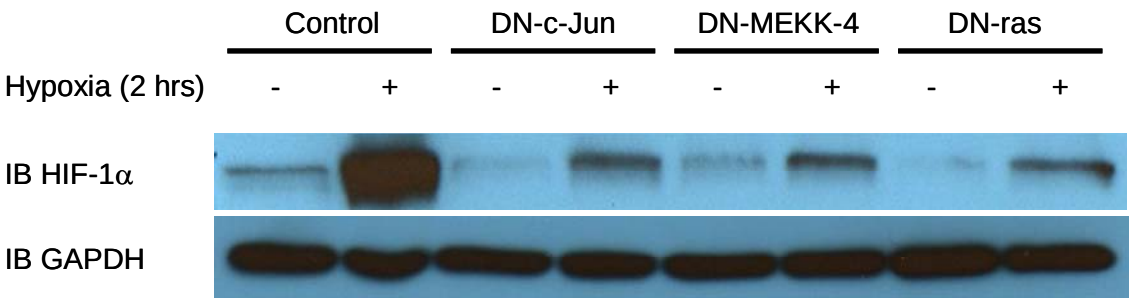

Figure 5. Immunoblot for HIF-1α and GAPDH for PC-3 cells that were either mocked transfected with empty vector (control) or transfected with DN-c-Jun, DN-MEKK-4, or DN-Ras, and then exposed to 2 hours of normoxia (-) or hypoxia (+). GAPDH was used as the loading control. Data are from a representative experiment that was repeated twice.

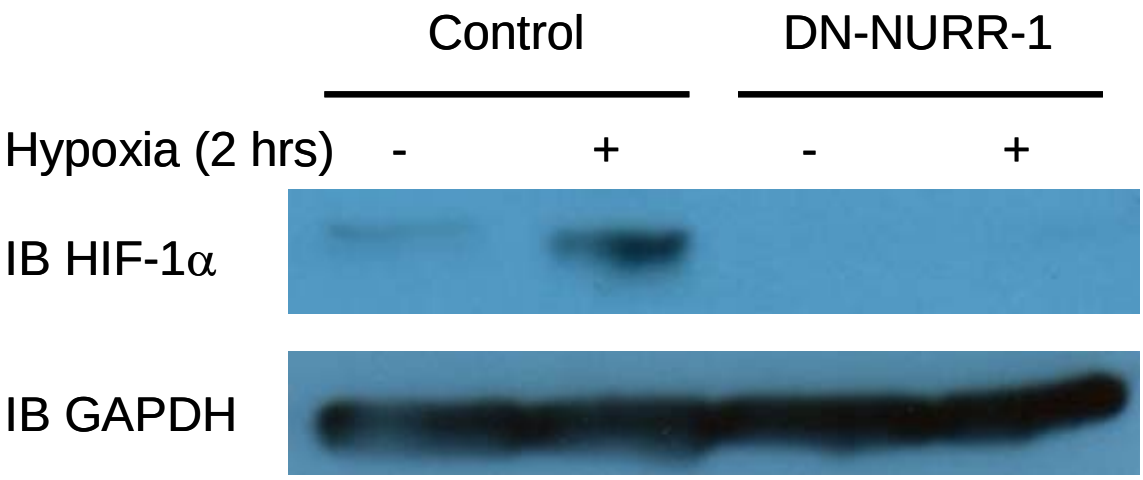

Figure 6. Immunoblot for HIF-1α and GAPDH for PC-3 cells that were either mocked transfected with empty vector (control) or transfected with DN-NURR-1 and then exposed to 2 hours of normoxia (-) or hypoxia (+). GAPDH was used as the loading control. Data are from a representative experiment that was repeated twice.

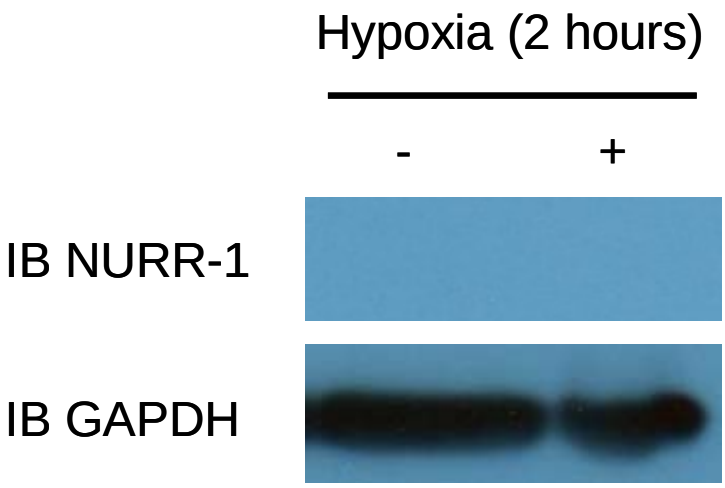

Figure 7. Immunoblot for NURR-1 and GAPDH following exposure of PC-3 cells to normoxic (-) or 2 hours of hypoxia (+). GAPDH was used as the loading control. Data are from a representative experiment that was repeated twice.

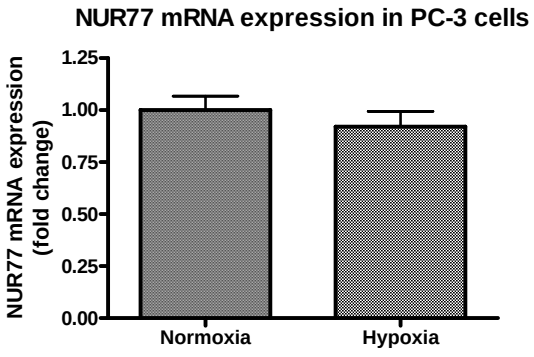

Figure 8A.

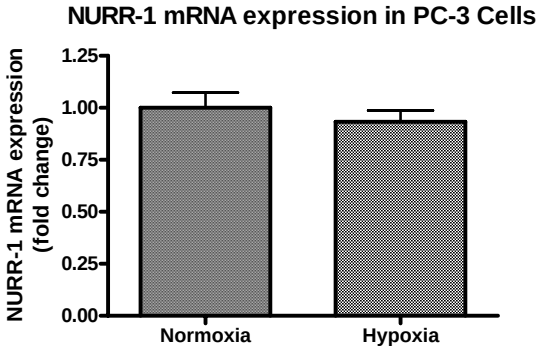

Figure 8B.

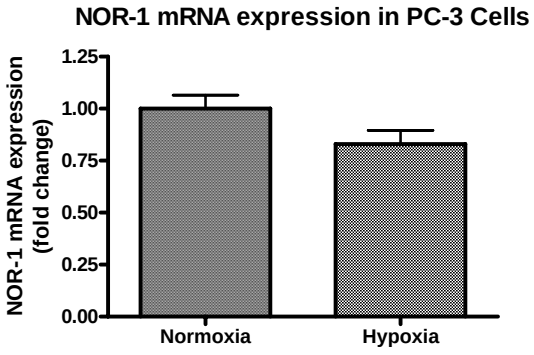

Figure 8C.

Figures 8A, 8B, and 8C. NUR77, NURR-1, and NOR-1 mRNA expression levels, respectively, measured using real time PCR and normalized against GAPDH in PC-3 cells under normoxic conditions or following 2 hours of hypoxia. Data represent the average standard deviation of 3 independent experiments each done in duplicate. Data were analyzed by paired t-tests and there were no significant differences ($p < 0.05$).

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