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Regulation of HIF Expression in Human Fibroblasts

A Major Qualifying Project

Of the

Worcester Polytechnic Institute

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

By:

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Approved by:

Dr. Tanja Dominko

Abstract

It has been found that hypoxia (low oxygen conditions) is an advantageous condition for the culture of pluripotent stem cells. Hypoxia-inducible factors (HIFs), are transcription factors involved in the mediation of a hypoxic response. Among other effects, some of their downstream gene targets have implications in pluripotency. While HIFs have been studied extensively in human embryonic stem cells, the current research attempts to characterize their activity in human adult fibroblasts. Specifically, it aims to determine if HIFs are responsive to hypoxia in fibroblasts and whether their regulation is affected at transcriptional, translational or post-translational levels. Further, HIF localization was examined as an implication of protein function. It was found that HIFs are expressed in fibroblasts, but that the hypoxic response is different than observed in human embryonic stem cells.

Introduction

Low Oxygen Concentrations

Hypoxia is a term used to describe low oxygen concentrations that typically range from 1–5% O_2. In laboratory experiments, it is standard practice to culture cells in atmospheric (20%) O_2; however, oxygen levels are significantly lower inside of the human body (Ivanovic, 2009). In the reproductive tract the oxygen concentration is 1.5 – 8% (Forristal, et al., 2010), while in bone marrow, oxygen concentrations range from 0-5%, and in the brain oxygen levels are between 0.5 – 7 % (Ivanovic, 2009). To more closely mirror *in vivo* conditions, research was performed on human embryonic stem cells (hES cells) in low oxygen conditions. Results have shown that under hypoxic conditions less spontaneous differentiation, increased cell proliferation, as well as enhanced formation of embryoid bodies are observed than when grown at 20% $0_{2.}$ (Ezashi, et al., 2005) Therefore, it can be concluded that hypoxic conditions are beneficial for the successful culture of hES cells (Forristal, et al., 2010), and its use is increasing as a standard culture practice in laboratories (Ezashi, et al., 2005).

In cell culture practices of mammalian tissues, stressful microenvironments can trigger spontaneous cell differentiation. An example of a stressful environment could be human cells that are accustomed to low oxygen concentrations in vivo that are subjected to laboratory culture conditions of normoxia. It has been shown in primary adult dermal fibroblasts that low oxygen culture conditions coupled with FGF2 lead to a significant increase in cell proliferations as well as leading to the expression of stem cell genes. (Page, et al., 2009) Additionally, it has been experimentally determined that varying oxygen concentrations as a sole independent variable has a significant effect on cell culture health such that lower oxygen concentrations produced higher

population doublings than normoxic laboratory conditions which yielded early cell aging. (Saito, et al., 1995)

Hypoxia Inducible Factors

It has been found that several hypoxia inducible factors (HIFs) serve as the link between low oxygen and maintenance of pluripotency (Wenger, 2002). Target genes of HIFs include stem cells markers such as SOX2, NANOG, and OCT4 that are necessary for preventnion of differentiation (Forristal, et al., 2010). HIFs are sequence specific DNA-binding protiens represented by four subunits. There are three alpha subunits that are oxygen destructible: HIF1α, HIF2α (also named EPAS), and HIF3α that act as transcription factors in the cell. There is also a beta subunit that is oxygen indestructible (HIF1β) that will not be discussed in this paper because of its differing oxygen response. (Brahimi-Horn & Pouyssegur, 2009)

Under laboratory normoxic conditions, HIFs are post-translationally modified. Prolyl hydoxylation at the 402 or 564 location in HIF1α or the 405 and 531 location in HIF2α causes these HIFs to interact with the von Hippel-Lindau (VHL) complex which targets the protien for proteasomal degradation. Additionally, hyrdoxylation of asparagine at the C-terminus of HIF-alphas activate FIH (factors inhibiting HIF) whereby HIFα downstream activity is blocked. Under laboratory hypoxic conditions, the lack of asparagine hydroxylation causes HIF binding to HREs (Hypoxia ResponsiveElement) which are sequences in the promoter regions of target genes. Under hypoxia, the protien isn’t degraded and can perform its activity as a transcription factor. (Brahimi-Horn & Pouyssegur, 2009)

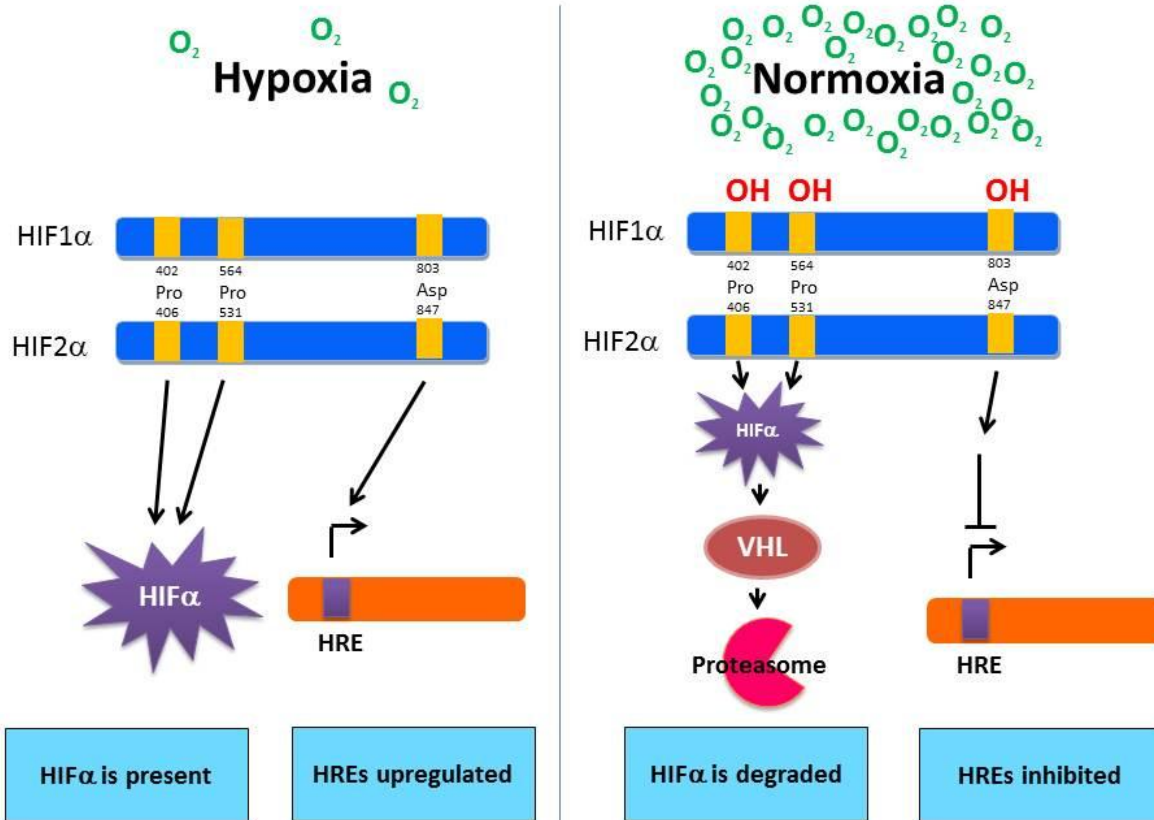

Figure 1: Working Model of HIFα Regulation in hES Cells

HIFs of Interest

In the current research, the focus will be on HIF1α and HIF2α. It has been found that while both HIF1α and HIF2α activate HRE, HIF3α can, under a particular splicing arrangement, acts against HRE gene expression. (Wenger, 2002). While HIF1α and HIF2α are similar, they have found to be "non-redundant" in that they are different in their preference of target genes. Additionally, it has been found that HIF1α and HIF2α are working together in that both are required for the phenotype, and each is not sufficient on their own to create the same phenotype. (Ratcliffe, 2007)

The first research question posed was; are HIFs in fibroblasts responsive to hypoxia? It has been shown that fibroblasts cultured under conditions normal for hES cells show stem cell markers. (Page, et al., 2009) and that fibroblasts grown under low oxygen concentrations alone experience increased proliferation (Saito, et al., 1995). Therefore it was experimentally tested whether the hypoxia inducible factors were present during this response. The presence of HIF1α and HIF2α mRNA was tested by RNA extraction and RT-PCR with primers for each mRNA. It was predicted that under hypoxic conditions, expression of HIFs would be seen in fibroblasts.

More in depth analysis of HIF expression in fibroblasts is imperative for the understanding of the function protein. HIF expression in hES cells has been characterized in both its localization and the level of its expression (Brahimi-Horn & Pouyssegur, 2009), thus the second and third topics of interest are to investigate the same in human fibroblasts. The location of HIFs was experimentally determined using immunocytochemistry and differential protein extractions (nuclear and cytoplasmic extracts) coupled with Western blotting. The level of protein expression was tested through translational blocking with cyclohexamide and subsequent Western blotting. It was predicted that these processes would mirror expression in hES cells in that the proteins would translocate to the nucleus over time which is required for their role as transcription factors. It was also predicted that the HIFs would be post-translationally modified in that the protein would be degraded, and the HREs would be inactive under normoxia.

Materials and Methods

Cell Culture

Primary adult human dermal fibroblasts (CRL-2352) from a 24-year-old Caucasian male from an amputation below the knee were obtained from American Tissue Culture Collection (ATCC: Manassas, VA) at passage 2. Cells were cultured in a medium of DMEM (45%), Ham's F12 (45%), and Fetal Bovine Serum (10%). DMEM was supplemented with 4mM L-glutamine prior to use. In specified conditions, the medium was also supplemented with FGF2 at 4ng/mL. Cultures were carried out in a 37°C incubator in 5% CO_2, 2% O_2 and 90% N_2. Standard control culture was carried out at 5% CO_2 in air (19% O_2). Cells were expanded in plastic T75 flasks at 19% O_2, and were cryogenically frozen with 10% DMSO when necessary.

RNA Extraction

Fibroblasts were cultured on 10cm plates at 19% O_2 until 80% confluency and then differentially incubated in 2% O_2 for 30 minutes, 45 minutes, 1 – 24 hrs, 3 days, and 7 days. A second set of plates used media supplemented with 4 ng/ml FGF2 and sampled at the same time point. Control cells were grown at 19% O_2 for 7 days with and without FGF2. RNA was extracted from each of these samples using the Trizol (Invitrogen) protocol.

Reverse-Transcription Polymerase Chain Reaction

The QuantiTect Reverse Transcription Kit (Qiagen) was used with 1ug RNA from each sample. The resulting cDNA was then prepared for PCR using the GoTaq (Promega) protocol. Three primer sets (HIF1α, HIF2α, and Actin) were used in this experiment. The HIF1α primers were CAC CAC AGG ACA GTA CAG GAT GCT (forward) and GGT ACT TCC TCA AGT

TGC TGG TCA (reverse). The HIF2α primers were GCC GAA GCT GAC CAG CAG ATG G (forward) and CCG TGC AGT GCA AGA CCT TCC A (reverse). The Actin primers were TCT GGC ACC ACA CCT TCT ACA A (forward) and CTT CTC CTT AAT GTC ACG CAC G (reverse). The PCR for Actin was cycled as follows: 95°C for 2:00, 94°C for 0:15, 59°C for 0:30, 72°C for 1:00 all cycled 35 times, 72°C for 10:00, then 4°C forever. For HIF1α and HIF2α and 95°C for 2:00, 94°C for 0:15, 58°C for 0:30, 72°C for 1:00 all cycled 35 times, 72°C for 10:00, then 4°C forever. The PCR products were loaded at 12 uL per well into a 2% agarose gel with 0.5ug/mL ethidium bromide run with 1x TAE buffer and imaged with a Kodak 4000MM Image Station.

Translation Blocking

Fibroblasts were plated into four wells of two six well plates until they reached confluence. Two wells on each plate were then incubated with media with cyclohexamide added at 60ug/mL. The cells were then incubated for 6 hours at either 2 or 19 % O_2. From the first well in each condition, the nuclear and cytoplasmic extracts were taken. Cells were washed twice with PBS and centrifuged. 200uL buffer A (50mM NaCl, 10mM HEPES pH8.0, 500mM sucrose, 1mM EDTA, 0.5mM spermidine, 0.15mM spermine 0.2% TX-100, 7mM beta-mercaptoethanol) + protease inhibitors were added and spun. After incubating on ice for ten minutes, the resulting supernatant was stored as cytoplasmic extract. The pellet was washed with 500uL buffer B (50mM NaCl, 10mM HEPES pH8.0, 25% glycerol, 0.1mM EDTA, 0.5mM spermidine, 0.15mM spermine, 7mM beta-mercaptoethanol) + protease inhibitors, and then with 50uL buffer C (350mM NaCl, 10mM HEPES pH8.0, 25% glycerol, 0.1mM EDTA, 0.5mM spermidine, 0.15mM spermine, 7mM beta-mercaptoethanol) + protease inhibitors. Pellet was incubated on ice for 30

minutes, spun at full speed, and the resulting pellet was the nuclear extract. From the second well in each condition a total cell extract was prepared using RIPA buffer. Cells were detached from adherent surface, pelleted, and resuspended in 5mL ice-cold PBS. After incubation, cells were centrifuged at 500g for 5 minutes and the supernatant was aspirated. Cells were subsequently resuspended in Lysis buffer (40mM Tris pH7.5, 150mM NaCl, 8% glycerol, 0.01252% Triton X-100, 0.005% Tween-20, 0.02% NP-40) at 1mL per $5x10^6$ cells and centrifuged. The supernatant was removed to a separate microcentrifugre tube and kept on ice. 200uL 6x SDS buffer was added, mixed and the result placed in a boiling water bath for 10 minutes and then stored at -20C.

In a second set of experiments, plates were incubated in media with cyclohexamide added at 60ug/mL for incubation for 6 hours in 2% O_2. After 6 hours the media was changed to standard media. Protein extraction was performed on these plates in pairs at 0hrs, 2hrs, and 4hrs. Additionally, two plates were incubated in media with cyclohexamide added at 60ug/mL for incubation for 6 hours in 19% O_2 with protein extraction immediately following. A control condition was conducted without the addition of cyclohexamide and with total incubation at 19% O_2.

Western Blotting

The protein isolated from translation blocking (see above) was separated on 8% SDS-PAGE gels and transferred to PVDF membranes using Towbins transfer buffer. Membranes were blocked with 5% dry milk in Tris buffered saline-Tween (TBST). 1% milk in TBST buffer was used for primary and secondary antibody incubations. The antibody used was HIF2α (Novus Biological) and HRP-conjugated secondary antibody (Abcam) was used. Membranes were

washed between incubations with TBST. Images were taken with a Kodak 4000MM Image Station and exposed for 15 minutes.

Immunocytochemistry

Fibroblasts were grown in 19% O_2 in two wells of four well plates until confluence (with / without incubation in FGF2. To fix the cells, they were washed twice with PBS for 5 minutes, incubated with ice-cold methanol for 10 minutes, and washed two more times for PBS for 5 minutes. For immunocytochemistry, PBS was aspirated, and 0.5mL of 1.5M HCl was added for a 20 minute incubation period. Each well is washed with PBS three times for 5 minutes, blocked with FBS and incubated with the primary antibody for 2.5 hours at 37°C, washed with PBS three times for 5 minutes, which was followed by incubation with the secondary antibody for 30 minutes. Lastly, samples were washed three with PBS for five minutes. The plates were then covered and stored at 4C for imaging.

Results and Discussion

RT-PCR

Cells were cultured on plastic 10cm plates for timepoints of 30mins, 45mins, 1-24 hours, 3 and 7 days spent in 2% O_2 and cultured in DMEM/F12 + 10% FBS. A duplicate set of plates was also cultured all with the addition of FGF2 at 4ng/mL. Control plates were grown in 19% O_2 both with and without FGF2. RNA extraction and reverse transcription reactions, and PCR cycling were performed as described above. Three primer sets (HIF1α, HIF2α, and Actin) were used on sample timepoints with and without FGF2 and run on a DNA gel.

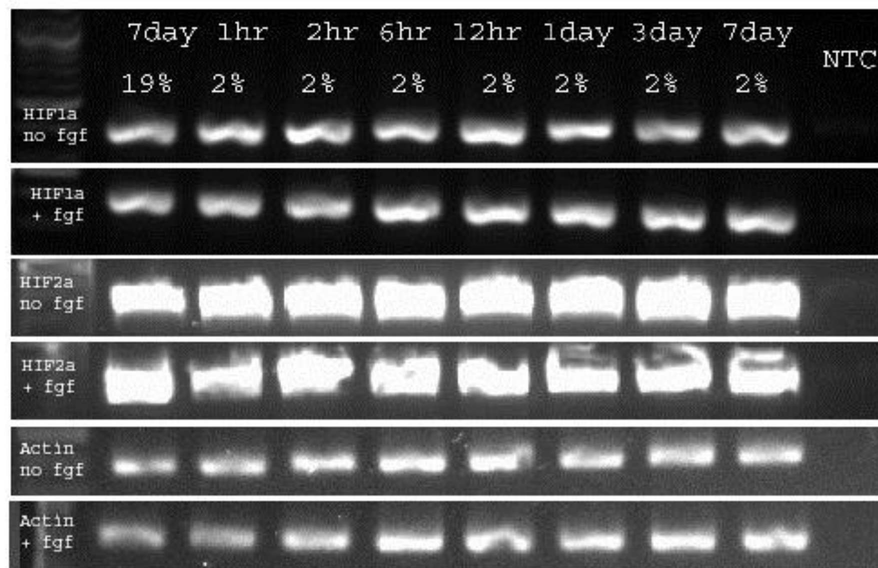

Figure 2: RT-PCR of HIF1α, HIF2α, and Actin With and Without FGF2

The results of the RT-PCR can be seen above in Figure 2. The presence of bands in all lanes except for the NTC (No Template Control) indicate that HIF1α , HIF2α and Actin are all transcriptionally present across oxygen conditions over time and with and without the addition of FGF2. From here, it was concluded that FGF2 had no significant effect on the transcriptional presence of HIFs and it was excluded as a future culture condition.

Immunocytochemistry

In order to investigate the localization of HIF, immunocytochemistry was performed as described in the methods section on cells incubated at the indicated oxygen concentration for two hours. Anti-HIF2α primary antibody and Alexafluor488 conjugated secondary antibody were used to visualize HIF2α presence and localization (green) while Hoechst was used to visualize nuclear staining (blue). Images were taken on a confocal microscope and can be seen in Figure 3 below.

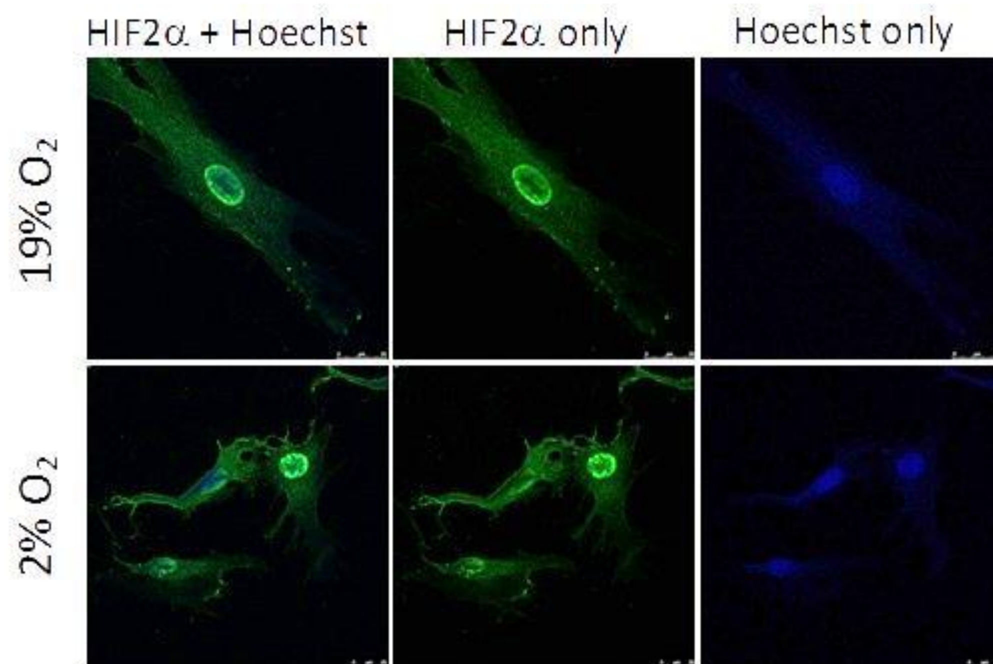

Figure 3: Immunocytochemistry with HIF2α

In Figure 3, we can determine that HIF2α is nuclear due to the high concentration of bright green in the center of the cells. Although the nuclear staining is difficult to see in conjunction with the Alexafluor488, from looking at the full figure it can be seen that the Hoechst nuclear staining is present. This staining was difficult to visual due to the nature of confocal

imagining in that only one layer of an image can be observed at a time and that Hoechst and Alexafluor488 do not always appear.

The nuclear staining in 19% O_2 was unexpected and thus further immunocytochemistry was performed in order to further explore this novel presence of HIF. The subsequent experimental setup investigated both HIF1α and HIF2α over time. Cells were cultured at 19% O_2 and then were incubated for varying timepoints at different oxygen concentrations. When the new incubation was started, the cells were immersed in fresh media that had been previously stored at the desired oxygen concentration in order to quickly assimilate the cells. Timepoints included 15 minutes, 2hours, and 3 and 7 days in order to account for a range of transient to long term location and stability. Anti-HIF1α primary antibody and Alexafluor568 conjugated antibody were used to visualize HIF1α presence and cellular localization (orange) while anti-HIF2α primary antibody and Alexafluor488 conjugated secondary antibody were used to visualize HIF2α presence and localization (green) while Hoechst was used to visualize nuclear staining (blue)

The results of the second immunocytochemistry experiment can be seen on the next page in Figure 4. It was found that HIF2α is stable and nuclear across all timepoints and oxygen concentrations while HIF1α was transiently nuclear up to 2 hours in both oxygen concentrations. The HIF1α results are surprising at 19% O_2 in that all timepoints were grown in normoxia for the same amount of time and thus should all express HIF similarly. The only difference between the timepoints is how long they were in fresh media for, which seems to be having an unexpected effect on HIF expression. While the HIF2α results were consistent, they are not concurrent with the hES cells working model and thus the next step was to confirm these findings.

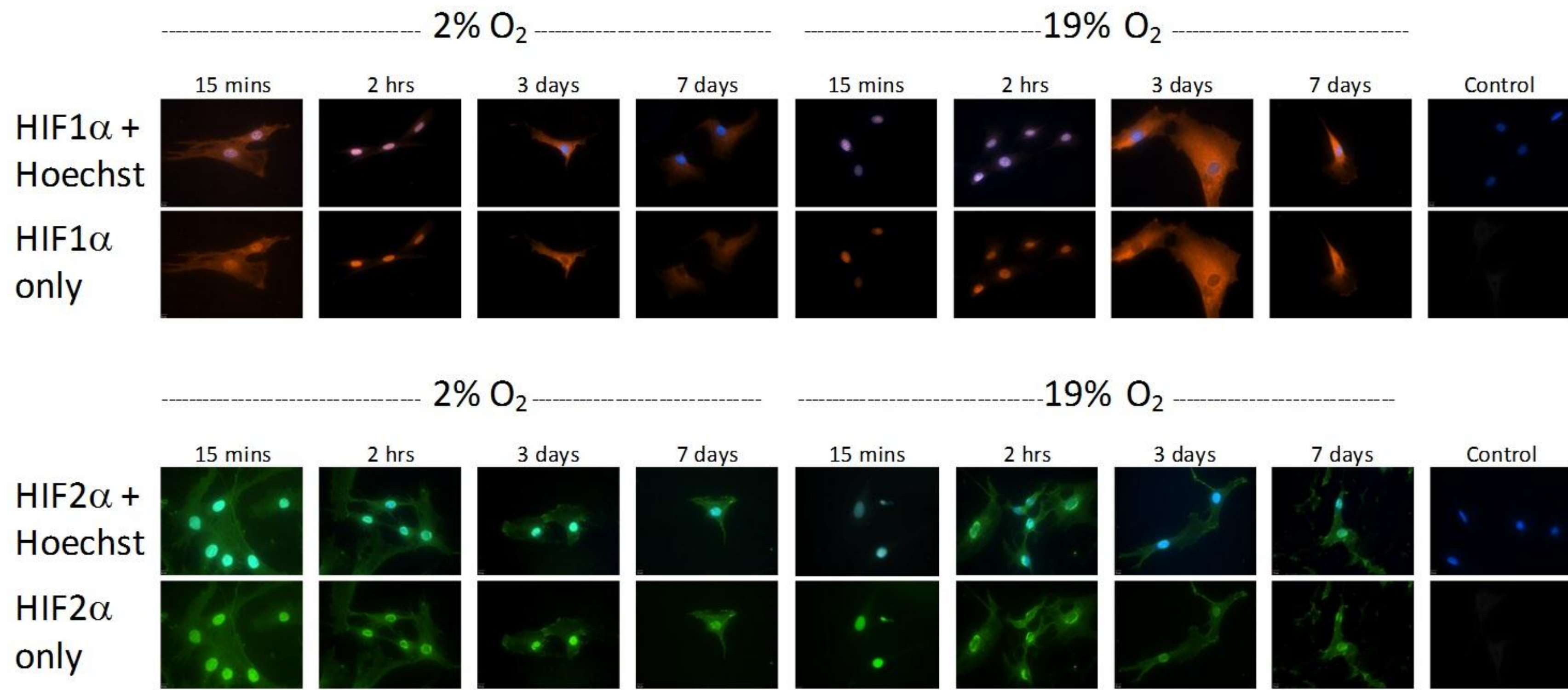

Figure 4: Immunocytochemistry with HIF1α and HIF2α over Time

Characterizing Western Blots

A Western blot was performed in order to confirm the immunocytochemistry findings that HIF2α is stable over time in both hypoxia and normoxia. Cells were grown under 19% O_2 and then moved to 2% O_2 for the indicated timepoints. The control condition was cells grown at 19% O_2 for 7 days, and the nuclear fractions of Cos7 cells grown at 19% O_2 and treated with $CoCl_2$ 16 hours served as a control. Nuclear and cytoplasmic fractions were extracted and separated on a SDS-Page gel. The resulting Western blot can be seen below in Figure 5.

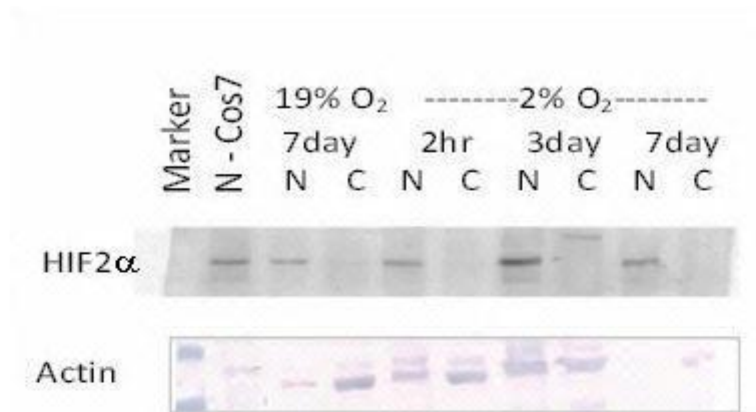

Figure 5: Western Blot Characterizing HIF2α Nuclear and Cytoplasmic Fractions

The results above clearly show that HIF2α is nuclear over time and varying oxygen concentrations which confirms the immunocytochemistry findings. At this point, we conclude that HIF2α is much more stable than originally predicted. In order to make sure that the culture conditions being used properly indicate what occurs in fibroblasts inside the body, various culture conditions were tested via Western blotting. Conditions include cells grown at 2% O_2 in regular media, at 2% O_2 in media with FGF2, and at 2% O_2 in media with $CoCl_2$ (known to stabilize HIF). Cos7 cells grown at 19% O_2 and treated with $CoCl_2$ for 16 hours served as a control. Total cell lysates of each were separated on an SDS-Page gel and the resulting Western blot can be seen below (Figure 6).

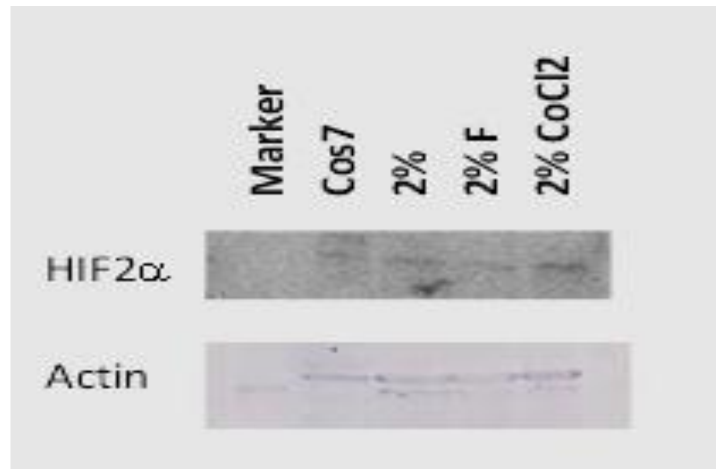

Figure 6: Western Blot of HIF2α Controls

In Figure 6, we see bands across all culture conditions for HIF2α which indicates congruency between conditions. This tells us thus that our findings up to this point could be considered valid across multiple culture conditions.

Western Blotting with Cyclohexamide

In order to observe HIF2α at a strictly post-translational level, all new protein synthesis was inhibited through the use of cyclohexamide. Cells were grown until 80% confluent at 19% O_2 and subsequently transferred to 2 or 19 % O_2 with fresh media and incubated for six hours. One plate under each oxygen concentration was supplemented with 60ng/mL cyclohexamide and the other plate at each condition served as a control. Total cell lysates were separated on an SDS-Page that can be seen in Figure 7.

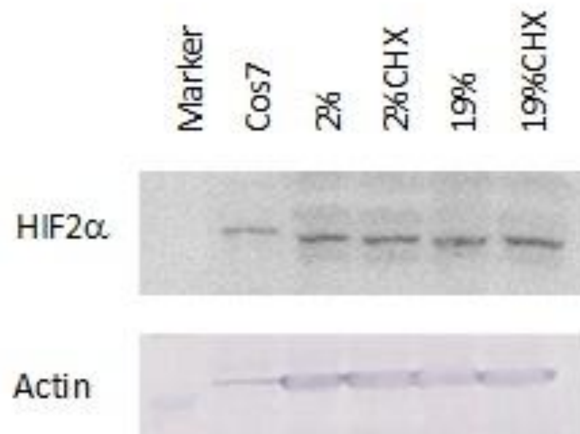

Figure 7: Preliminary Cyclohexamide Western Blot

In Figure 7, bands can be seen in all experimental lanes. This means that at both oxygen concentrations the protein was stable. Even with cyclohexamide treatment where no new protein is being made, HIF2α is still present and stable. This further confirms the results from immunocytochemistry and characterizing Western blots that HIF2α is stable over time and under varying oxygen concentrations. This experiment was repeated with cytoplasmic and nuclear fractions for each condition instead of total cell lysates (see Figure 8) in order to test transcription factor activity.

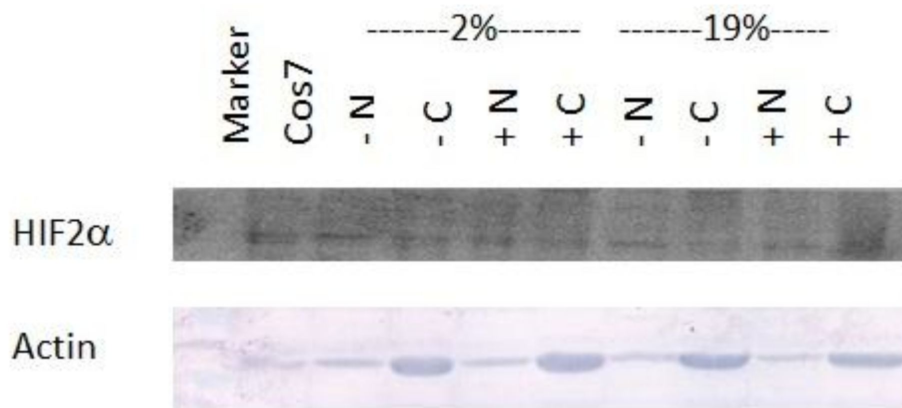

Figure 8: Western Blot with Cyclohexamide Treatment (Nuclear and Cytoplasmic Extractions)

Figure 8 once again shows bands in all experimental lanes, but this is unexpected in that only bands were predicted to be observed in the nuclear fraction as was observed in Figure 5. Taking a closer look at the Actin control, it can be seen that the cytoplasmic bands are much stronger than the nuclear bands. This may point to an overloading of sample in the cytoplasmic columns which could normalize to decreased band presence in those lanes. While we wouldn't even expect to see nuclear bands in the actin control, seeing the bands in both fractions suggest that the extractions were not optimally performed. All in all, this test is inconclusive.

A third cyclohexamide experiment was performed in order to further test the long term stability of HIF2α. Cells were incubated with cyclohexamide for six hours in order to block translation and subsequently re-incubated with fresh media for varying amounts of time from 0 – 4 hours with a control of no cyclohexamide treatment. This experimental setup was carried out under 2% O_2 and 19% O_2 with an additional control of Cos7 cells. Total cell lysates were taken from each plate and separated on an SDS-Page gel (see Figure 9).

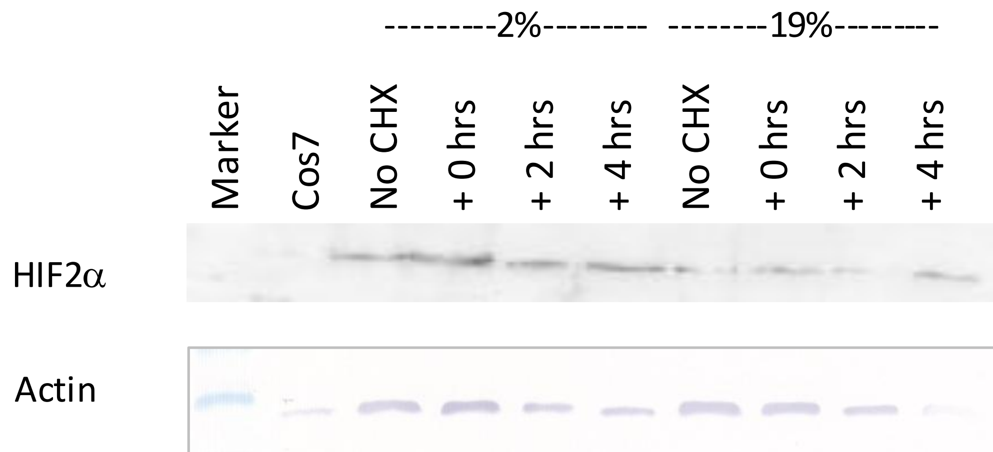

Figure 9: Western Blot with Extended Cyclohexamide Treatment

In Figure 9, the 2% bands and the 19% bands display different amount of protein, but are constant within their group from no cyclohexamide and 0-4 hours. From this lack of change over

time we can conclude that the blocking of protein synthesis had no significant effect on the presence of protein and thus that HIF2α is stable over time.

Conclusions and Future Research

The RT-PCR results show that HIFs are responsive to hypoxia in fibroblasts and that they are present at the transcriptional level. Immunocytochemistry shows that HIF1α is transiently located in the nuclear up to 2 hours in both hypoxia and normoxia. Immunocytochemistry with HIF2α also shows that it is located in the nucleus, but that remains stable and nuclear for all time points tested (up to 7 days) and in both hypoxia and normoxia, which has been confirmed by Western blotting. This unexpected stability of HIF2α was further tested using cyclohexamide to block translation and it was found that in the absence of new protein synthesis HIF2α remained stable over time.

These results do not fit into the working model earlier presented for hES cells. However, research has investigating HIFs in human skeletal muscle found that HIFs were not only stable at 5% O_2, and 19% O_2, but also at 42% O_2 (Kubis, et al., 2005). This suggests that the long term and oxygen independent stability of HIFs observed in fibroblasts may be of close relation to what has been observed in skeletal muscle. At this point it may be appropriate to discard the hES cell working model and to adopt a new working model for fibroblasts. Such a model is incomplete (see figure 10), it better characterizes fibroblasts and points towards future research questions. Figure 10 shows HIFs to be stable at 2% O_2, 19% O_2, and potentially also at 42% O_2 and should be built upon after further characterization of HIFs in human adult fibroblasts has been performed.

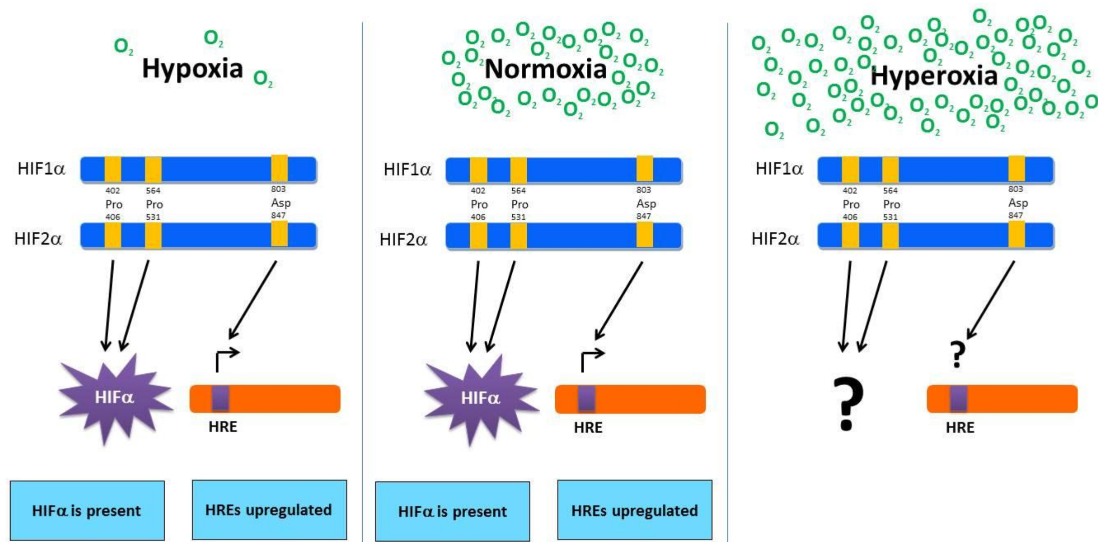

Figure 10: New Working Model for HIF Regulation in Fibroblasts

Future research should attempt to further characterize HIFs by investigating its stability at high oxygen concentrations in order to add to the current working model. Additionally, it should be verified that the stable protein is actually what we're seeing. To do this pulse-chase experiment should be performed in order to track HIF from protein synthesis and to provide a more complete description of its level of regulation. To this effect, further experiments could also be performed using cyclohexamide (to block translation) in combination with MG132 (to inhibit proteasomal degradation) to examine HIF stability.

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