MICRORNA RESPONSE TO HYPOXIC STRESS IN SARCOMA CELL LINES – HSA-MIR-485-5P, HSA-MIR-210 AND HIF3A
microRNA response to hypoxic stress in sarcoma cell lines – hsa-miR-485-5p, hsa-miR-210 and HIF3A

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Cancer is the first leading cause of death in the Netherlands and the second leading cause of death in the United States of America. It is of great importance that proper research is done into the origins of this disease to unravel the molecular processes that are involved in the development and progression of all types of tumors, including the rare ones like sarcomas.

Hypoxia is one of the key features of quickly progressing tumors, as they may outgrow their blood supply resulting in low oxygen tension. Hypoxia is of great clinical importance because, for one, it promotes malignant progression by increasing genetic instability, angiogenesis, local invasion and distant metastases. In addition, tumors with extensive low oxygen tension tend to exhibit resistance to conventional therapy and consequently poor prognosis for the patient.

MicroRNAs are small RNAs of approximately 18-22 nucleotides in length that are involved in various molecular processes by inhibiting translation or degrading mRNA. The expression pattern of miRNAs is tissue specific and can be altered under stressful conditions. Because miRNAs are closely involved in many processes in the cell, i.e. cell proliferation, differentiation and apoptosis, perturbed function or altered expression of miRNAs may disorganize these processes and eventually contribute to diseases, such as cancer.

Several miRNAs have already been identified to be differentially expressed under hypoxic stress in sarcomas, i.e. miRNA-210. A pilot study which has been performed previous to this report, has identified several other miRNAs to be upregulated in sarcoma cell lines when exposed to hypoxic conditions, i.e. miRNA-485-5p, miRNA 216a and miRNA-185*. MiRNA-625 has been found to be severely downregulated under hypoxic stress in sarcoma cell lines. The focus in this report will be on the expression kinetics of one of the possible target genes of one of the identified miRNAs, hsa-miR-485-5p, namely HIF3A. It is hypothesised that this protein is posttranscriptionally regulated by hsa-miR-485-5p under hypoxic conditions.

In chapter 1 an introduction is given regarding this research project. In the next chapter the materials and methods used are discussed. In chapter 3 the results are presented and these are discussed in chapter 4.
Hypoxia is one of the key features of quickly progressing tumors, as they may outgrow their blood supply resulting in low oxygen tension. A major hypoxic stress response is the upregulation of Hypoxia-Inducible Factor 1A (HIF1A). This gene is a master transcription factor which orchestrates the activation of pathways that are important for the adaption of the cell to low oxygen like angiogenesis and glucose metabolism. Hypoxia-Inducible Factor 3A (HIF3A) is a family member of HIF1A, however not much is known about its regulation and function. It has multiple transcription variants, but only one of them, namely transcription variant 201, has a long 3'UTR containing several binding sites for miRNAs that have been found to be upregulated under hypoxic stress, i.e. 11 binding sites for hsa-miR-485-5p and one binding site for hsa-miR-210.

MicroRNAs (miRNAs) are a specific class of non-protein coding RNAs and are approximately 18-22 nucleotides long. Although they are not protein coding, they seem to be genetically active. They have important roles in normal physiology and can be deregulated in or contribute to diseases such as cancer.

Several miRNAs have already been identified to be differentially expressed under hypoxic stress in sarcomas, i.e. miRNA-210. A pilot study has identified several other miRNAs to be expressed in sarcoma cell lines when exposed to hypoxic conditions, i.e. miRNA-485-5p, miRNA 216a and miRNA-185*. MiRNA-625 has been found to be severely down-regulated under hypoxic stress in sarcoma cell lines. The focus in this report will be on one of the possible target genes of hsa-miR-485-5p and hsa-miR-210, namely the HIF3A transcription variant 201. It is hypothesised that this protein is posttranscriptionally regulated by hsa-miR-485-5p and hsa-miR-210 under hypoxic conditions.

In this report we show that the upregulation of HIF1A is an early response to hypoxic stress as it is already upregulated after the cells have been cultured under hypoxic conditions for 6 hours and its expression is decreasing at 24 and 48 hours of hypoxic stress.

The HIF3A mRNA shows an upregulation as time elapses, but its protein is peaking after 24 hours of hypoxic stress and is decreasing after 48 hours.

In this report HIF3A is validated as a target gene for hsa-miR210 and hsa-miR-485-5p. In this report it is also shown that hypoxia has an effect on the 3'UTR of HIF3A transcription variant 201 and that transfection of mimics of hsa-miR-210 and hsa-miR-485-5p has an effect on the hypoxia response pathway.
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1 INTRODUCTION

Cancer is a collective name for a class of different diseases with one thing in common, namely uncontrolled cell growth. It is the second leading cause of death in the United States of America with over 500,000 deaths every year [Dillhoff et al. (2009)].

1.1 SARCOMAS

A sarcoma is a cancer that arises from transformed cells in tissues that develop from embryonic mesoderm. Sarcomas comprise a heterogeneous group of mesenchymal neoplasms and there are over 100 distinct diagnostic entities. Sarcomas can be divided into two subgroups, namely soft tissue sarcomas (STS) and bone-associated sarcoma. Sarcomas are quite rare in adults as only 1% of all adult cancers are sarcomas and they are usually discovered late, because they often lay deep into the body. Consequently, they are large before brought to medical attention. Sarcomas initially don't cause pain, are not visible and other symptoms are quite general, which means they can point into the direction of numerous other diseases. This means that by time a patient finally reaches an oncologist, the tumor is already large and in a farther staged, often metastasized and this lowers survival chances dramatically [Skubitz and D’amo (2007)].

Possible causes of sarcomas are ionizing radiation used for previous treatment for other tumors, chronic lymph oedema, chemicals such as vinyl chloride (PVC production), human herpes virus 8 (Kaposi sarcoma) and genetic syndromes like von Recklinghausen disease, which causes neurofibromatosis 1 and 2, and Gardner syndrome, which causes retinoblastoma [Skubitz and D’amo (2007)].

Staging of STS is done by looking at tumor grade, size and location. Light microscopy and hematoxylin-eosin (HE) staining are used to view the tumor’s pathological features. Alternately, gene expression can be used to identify and stage the tumor. Because sarcomas are fairly rare it is required that identification and staging of STS is done by an experienced pathologist and even then it is very difficult to identify and stage the tumor correctly [Skubitz and D’amo (2007)].
1.2 HYPOXIA

Several environmental conditions are required for cells to keep dividing and consequently for a tumor to keep growing. Cells for instance need glucose to keep up their energy levels and in addition they need oxygen to efficiently process the glucose into ATP. As mentioned before, one thing all different entities of cancer have in common is uncontrolled cell growth. This uncontrolled cell growth takes place at a higher rate than the cell division in healthy tissue, which eventually causes a tumor to grow quicker than angiogenesis can keep up with resulting in a poor blood supply. A poor blood supply means also a poor oxygen supply and thus the tumor will endure hypoxic stress (figure 1) [Maxwell (2005)].

![Figure 1: The tumor has outgrown its blood supply and angiogenesis has been speeded up, resulting in less carefully constructed blood vessels, through which blood can’t flow efficiently, resulting in hypoxic conditions [Brown and Giaccia (1998)].](image)

In contrast to normoxia, where there is a normal oxygen concentration of 21% (pO2 = 160 mmHg) as it is in the air that we breathe, under hypoxic condition cells are deprived from oxygen, i.e. when the pO2 levels go below 160 mmHg. In the human body however, oxygen concentrations vary in each organ. Normoxia in tissue is defined as the normal oxygen tension in a specific tissue. For example, the pO2 in the upper airway is 150 mmHg, whereas the pO2 in the retina is only 5 mmHg. When the oxygen tension decrease below the normal levels for a specific tissue, it is defined as hypoxia. The golden standard for hypoxia is when the pO2 levels in the arterial blood are below 40 mmHg (5%) [Koh, M.Y., et al. (2008)].

Hypoxia not only occurs as a global consequence of low oxygen tension, but also at sites of inflammation, tissue ischemia and injury and in solid tumor growth. Hypoxic
regions in solid tumors often have oxygen levels below 0.7% (<5 mmHg) [Koh, M.Y., et al. (2008)].

Hypoxia is of great clinical importance because it promotes malignant progression by increasing genetic instability, angiogenesis, local invasion and distant metastases. In addition, tumors with extensive low oxygen tension tend to exhibit poor prognosis and resistance to conventional therapy [Kulshreshta et al. (2007)]. This resistance can for most therapies be caused by the poor blood supply since most conventional treatments rely on the vascular system for the agents to be transported to the target, as is the case for chemotherapy. As for radiation therapy, the resistance is indeed caused by the lack of oxygen, because oxygen is needed to create the free-radical intermediates needed to attack the tumor cells aside from the direct ionizing attacks.

A key role in the cellular hypoxia response is played by the hypoxia inducible factor (HIF) family [Kulshreshta et al. (2007)].

1.2.1 HYPOXIA-INDUCIBLE FACTOR

The hypoxia-inducible factor (HIF) is the oxygen-sensitive master transcription factor which orchestrates the expression of a wide variety of pathways thought to be critical for adaption to low oxygen levels, like angiogenesis, glucose metabolism, migration, survival and death (figure 2) [Kulshreshta et al. (2007); Loscalzo (2010)].

Three different HIFs have been identified, namely: HIF-1, HIF-2 and HIF-3. Whereas HIF-1 and HIF-2 have a similar targets, not much is known about HIF-3, but it is thought to be a regulatory factor of HIF-1 [Lisy and Peet (2008)].

FIGURE 2: HYPOXIA PATHWAY SCHEMATIC [UNIVERSITY OF ZÜRICH (2010)]
Over the years several regulators of HIF1A have been identified gaining a deeper understanding of the complicated pathway of HIF-mediated gene expression. One of the regulators of HIF1A is the Von Hippel-Lindau tumor suppressor proteins, HIF inhibiting factor (figure 3) and recently also HIF3A has been thought to regulate HIF1A.

**1.2.1.1 VON HIPPEL-LINDAU (VHL) TUMOR SUPPRESSOR PROTEINS**

HIF is an active heterodimer assembled of an α- and β-subunit, of which the β-subunit is constitutively expressed, whereas the α-subunit is closely regulated by oxygen dependent mechanisms. One of the regulators of the HIF-α subunits is the von Hippel-Lindau tumor suppressor gene. Under normoxic conditions HIF1A is hydroxylated by prolyl hydroxylases (HIF-PH). The hydroxylated HIF1A subunit is recognised by the Von Hippel-Lindau tumor suppressor. This E3 ubiquitin-protein ligase which will then target HIF-1α for proteosomal degradation (figure 4). This mechanism is dependent on the hydroxylation of Pro 564 in HIF-1α via an enzymatic process which requires O2, as well as iron [Mahon et al. (2001)].

Under hypoxic condition, however, no oxygen is available for the hydroxylation process, which means the VHL protein will not be able to recognize the HIF-1α subunits and thus will not ubiquinate them. Subsequently, the α-subunits will not be degraded, can bind to the β-subunits and assemble the active heterodimer HIF.
1.2.1.1.2  HIF INHIBITING FACTOR (HIF1AN)

Another regulator of HIF-1α is the HIF inhibiting factor, HIF1AN (also known as Factor Inhibiting HIF (FIH)). HIF1AN hydroxylates an asparagine in the C-terminal trans-activation domain (CAD) of the HIF-α subunits. In order to do this it needs oxygen, iron, 2-oxoglutarate and ascorbate. The resulting product prevents the binding of the transcriptional co-activator CBP/p300 to the HIF-α subunit, thus preventing transcriptional activation [Wilkins et al. (2009)]. Under hypoxic conditions HIF1AN will not be able to hydroxylate HIF-1α due to the lack of oxygen, which means the co-activators are free to bind and genes will be activated.

1.2.1.1.3  HIF-3A

As mentioned above, it is hypothesized that HIF-3α is a regulator of HIF-1α on either the translational or the posttranslational level [Jang et al. (2005)]. Not much is known about this protein’s regulation or function. 21 splice variants have been found for HIF3α. Whereas some of the splice variants are similar to each other, others have a completely different primary structure. The splice variants of HIF3α can be found in figure 5. It is unknown how abundant all these splice variants are and whether all variants are expressed in the same cell.
According to the study of Jang et al., one of the different splice variants of HIF3A functions as a negative regulator of HIF-mediated gene expression in mice. In humans a similar splice variant occurs which inhibits the transactivating function of HIF1A under hypoxic conditions by forming a complex with HIF1A proteins, resulting in failure of HIF-1α binding to hypoxia response elements of its target genes. In this study it was concluded that this splice variant of HIF-3α is the recently identified regulator inhibitoR PAS (Per/Arnt/Sim) domain protein (IPAS). The PAS domain of the splice variant of HIF-3α is believed to be crucial for HIF1A regulation [Jang et al. (2005); Koh et al. (2008)].

In addition to its inhibitory function for HIF-1α, the human IPAS protein (one of the splice variants of HIF3A) also blocks the hypoxia-induced VEGF expression and consequently inhibits angiogenesis [Jang et al. (2005); Makino et al. (2001)].
In this study the focus was on HIF3α transcription variant 201, which has a long 3’Untranslated Region (UTR). This 3’UTR contains 11 bindingsites for microRNA (miRNA) hsa-miR-485-5p, which has been found to be upregulated after 24 hours of hypoxic stress in multiple sarcoma cell lines when analysed on a microarray platform (figure 6). The 3’UTR also contains a bindingsite for hsa-miR-210, which is a known hypoxia inducible miRNA (figure 6). These microRNAs (miRNAs) may regulate the gene expression of HIF3α transcription variant 201 on a post transcriptional level.

1.3 MicroRNAs

MicroRNAs (miRNAs) are a specific class of non-protein coding RNAs. Although they are not protein coding, they seem to be genetically active and have important roles in normal physiology and can be deregulated in disease [Wiemer (2007)]. MiRNAs are approximately 18-22 nucleotides in length and have critical functions in cell proliferation, apoptosis and differentiation [Dillhoff et al. (2009)]. It is believed that there are more than 1000 miRNAs in the human genome that may regulate more than one third of all mRNA transcripts. MiRNAs negatively regulate gene transcription by hybridizing to the 3’ untranslated region (UTR) of specific mRNA targets to repress translation, induce mRNA degradation or both [Loscalzo (2010)].

Their regulatory effect is based upon the degree of complementarity between the 3’UTR region of the mRNA and the seed sequence, which is composed of nucleotide 2-8 of the miRNA. A near perfect complementarity will result in mRNA degradation, which was thought to occur only in plants, but it has recently been discovered to occur in mammals as well, even though more rarely than in plants. However, miRNAs do not have
to display a perfect complementary to their target sequence to inhibit mRNA translation. If the miRNA is only partially complementary to the target it will induce transcriptional inhibition instead of mRNA degradation [Wiemer (2007); Dillhoff et al. (2009)]. The flexibility in binding allows a single miRNA to potentially bind with and prevent hundreds of mRNA messages from being translated. To make target prediction even more complicated, a single mRNA may bind more than one miRNA as well [Wiemer (2007)]. Although miRNAs are primarily negatively regulating gene expression, in few cases they may actually activate gene expression [Großhans and Filipowicz (2008)]. miRNAs use the RNA interference pathway (RNAi) to inhibit mRNA translation. This is a posttranscriptional silencing mechanism, where the exposure to double stranded RNA (ds-RNA) will induce sequence specific degradation of homologous mRNA [Wiemer (2007)].

The first miRNA, lin-4, was discovered in the worm Caenorhabditis elegans in 1993 by Ambros and coworkers. They found that a small RNA, instead of coding for a protein, interacted with the 3’UTR of the lin-14 mRNA to inhibit its expression [Wiemer (2007); Großhans and Filipowicz (2008); Dillhoff et al. (2009)].

MiRNAs can occur as single genes, but mostly they occur in gene clusters. They either occur intergenic, in which case they have their own promoter, or in defined transcription units, in which case they are being co-transcribed with the gene in which they reside.

1.3.1 BIOGENESIS OF MICRORNAS AND RNA INDUCED SILENCING COMPLEX (RISC)

As stated, miRNAs are often found in clusters and are consequently transcribed as polycistrons. miRNAs are formed from longer transcripts by two sequential processing steps mediated by two RNase III endonucleases, namely Drosha, which is a nuclear endonuclease and Dicer, which is a cytoplasmic endonuclease [Wiemer (2007)].

First of all a gene is transcribed by RNA polymerase II and folded into a double stranded hairpin (stem loop), which is sized from several hundred to over a thousand nucleotides. This hairpin is called the primary transcript (pri-miRNA). The stemloop is then modified by polyadenylation. The pri-miRNA forms a specific hairpin structure cleaved by Drosha along with its cofactor Di George syndrome critical region 8 (DGCR8) or Pasha in vertebrates to form the precursor miRNA (pre-miRNA) which is 60-70 nucleotides in length with a 5’ phosphate and a 3’ two nucleotide overhang. Exportin 5 recognizes the 3’ two nucleotide overhang, which will then transport the pre-miRNA by this protein out of the nucleus into the cytoplasm. Dicer will then process the miRNA further into a double-stranded mature miRNA of which one or both strands can function
as mature miRNA [Dillhoff et al. (2009)]. Dicer does this together with his ds-RNA binding partner transactivating response RNA binding protein (TRBP). The mature strand is then being incorporated in the RNA induced silencing complex (RISC), where TRBP recruits the hAgo2 to the complex, which is formed with other proteins such as gemin 4 and the DEAD-box RNA helicase gemin 3, TNRC6B and other proteins (figure 7). The precise order of RISC assembly, proteins and factors involved and function of additional proteins in the complex remains unclear to date [Wiemer (2007)].

![FIGURE 7: BIOGENESIS OF MIRNAS IN A CELL. SCHEMATIC IMAGE OF THE PRODUCTION OF MIRNA AND DIFFERENT WAYS OF REGULATING TARGET GENES [ESQUELA-KERSCHER AND SLACK (2006)].](image)

### 1.3.2 MICRORNAS AND CANCER

The expression pattern of miRNAs is tissue specific and can be regulated under stress such as low oxygen or pH disturbances. Because miRNAs are closely involved in delicate processes in the cell, such as cell proliferation, differentiation and apoptosis, dysregulation of the miRNAs may disorganize these processes and eventually contribute to diseases, such as cancer. More than 50% of miRNAs are frequently found at cancer associated regions and fragile sites, which are deleted, amplified or mutated in cancer.
cells. Not only may changes in miRNA expression promote carcinogenesis, miRNA expression can also be altered by changes induced by tumor growth. MiRNA expression patterns may be used to differentiate the different types of tumors where conventional techniques have failed [Dillhoff et al (2009)].

The miRNAs involved in carcinogenesis are called oncomirs. They can either act as an oncogene or as a tumor suppressor gene. If a miRNA acts as an oncogene and is upregulated due to dysregulation, it may inhibit the translation of mRNA of a tumor suppressor gene, thus contributing to the development and progression of a cancer cell. If a miRNA acts as a tumor suppressor gene the opposite will be the case. When this miRNA is upregulated it may inhibit the translation of mRNA of an oncogene.

### 1.3.3 HYPOXIA-REGULATED MICRONAS

Hypoxia-regulated miRNAs or hypoxamirs are miRNAs that are induced or downregulated by hypoxia and comprise three groups, namely those induced by HIFs under hypoxic conditions, which are called HIF-dependent hypoxamirs, the miRNAs that are induced by hypoxia and then affect HIF regulation and those affecting HIF expression, but are not dependent on hypoxic stress [Loscalzo (2010)]. Some of these hypoxamirs affect apoptotic signaling and target genes of critical importance for tumor biology. Their overexpression or downregulation in human cancers suggests a role in tumorigenesis.

#### 1.3.3.1 HSA-MIR-210

One of the most well-known hypoxamirs in humans is hsa-miR-210. This hypoxamir is generally upregulated in both regular cells and cancerous cells under hypoxic stress, however, its upregulation is not dependent of hypoxia, but of the upregulation of HIF-1, which means it is a HIF-dependent hypoxamir.

In breast cancer a correlation has been found between decreasing oxygen tension and the upregulation of hsa-miR-210. Several other stressful factors like osmotic stress with respect to the upregulation of hsa-miR-210 have been studied, but none have triggered the upregulation of this hypoxamir [Fasanaro, P. et al. (2008)].

When cells endure hypoxic stress several cellular changes have to take place in order to survive (paragraph 1.2). One of these changes is the shift from oxidative phosphorylation to anaerobe dissimilation. Hsa-miR-210 has been found to play a role in...
this shift by downregulating several steps of the mitochondrial metabolism including the electron transport chain complexes [Chan, S. Y. et al. (2009); Chen et al. (2010)].

Under hypoxic stress angiogenesis is induced so the blood supply and thus the oxygen supply in cells will increase. Hsa-miR-210 has also been found to play a role in this process. Overexpression of this miRNA has been found to stimulate the formation of capillary structures and the VEGF-driven cell migration. It was found that these processes were inhibited when hsa-miR-210 was blocked [Fasanaro, P. et al. (2008)].

### 1.4 PURPOSE AND HYPOTHESIS

The purpose of this study is gaining a deeper understanding of the role miRNAs fulfil in the hypoxia response pathway in sarcoma cell lines.

Several miRNAs have already been identified to be differentially expressed under hypoxic stress in sarcomas, i.e. miRNA-210. A pilot study, which has been performed previous to this study, has identified several other miRNAs to be upregulated in multiple sarcoma cell lines when exposed to hypoxic conditions, i.e. miRNA-485-5p, miRNA 216a and miRNA-185*. MiRNA-625 has been found to be severely down-regulated under hypoxic stress in sarcoma cell lines. The function of the miRNAs and the exact role they play in the hypoxia response pathway is not yet known. We hypothesize that hsa-miR-485-5p and hsa-miR-210 function in regulating the HIF3A transcription variant 201.
2 MATERIALS AND METHODS

Several experiments have been performed to gain a deeper understanding of the mechanisms involved in the cellular response to hypoxic stress and the role the identified miRNAs play in this response pathway. HIF3A transcription variant 201 has been identified as a possible target of hsa-miR-485-5p and hsa-miR-210, which will be validated. Also, experiments will be done regarding the effects of the miRNAs on the hypoxia response pathway and the effect of hypoxia on HIF3A transcription variant 201.

2.1 STUDYING THE EXPRESSION PROFILE OF HIF1A UNDER NORMOXIA AND HYPOXIA

The expression of HIF1A is described to be an early hypoxic stress response mechanism and has been used to validate the hypoxic conditions used in this project. The expression profile of HIF1A under normoxic (21% O₂) and hypoxic (1% O₂) conditions was studied at the protein level as it is post-translationally regulated and thus the mRNA levels are expected to show similar results under both normoxic and hypoxic conditions. The protein levels, however, are expected to be overexpressed under hypoxic stress quickly after the cells are exposed to low oxygen tension.

To study the protein levels of HIF1A, multiple sarcoma cell lines were cultured under both normoxic and hypoxic conditions. Protein lysates were prepared and SDS-PAGE and western blotting were performed to detect the expression of HIF1A.

2.1.1 CELL CULTURING

Three sarcoma cell lines of several subtypes have been cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) with 10% Fetal Calf's Serum (FCS) under either normoxic or hypoxic conditions (table 1).

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW-872</td>
<td>Liposarcoma</td>
<td>36,666 cells/ml</td>
</tr>
<tr>
<td>SK-UT-1</td>
<td>Leiomyosarcoma</td>
<td>20,000 cells/ml</td>
</tr>
<tr>
<td>A204</td>
<td>Rhabdomyosarcoma</td>
<td>26,500 cells/ml</td>
</tr>
</tbody>
</table>

TABLE 1: SARCOMA CELL LINES USED IN HYPOXIA EXPERIMENTS.
Cells were seeded at $T = -2$ days in 24 well plates and transferred to hypoxic conditions at $T=0$. 5% CO$_2$, 21% O$_2$ and 37°C were used for the normoxic conditions. For the hypoxic conditions only the oxygen percentage was changed to 1% O$_2$. Samples for RNA and protein isolation were harvested at $T = 0, 6, 24$ and 48 hours of culturing.

### 2.1.2 PROTEIN LYSATES

Protein lysates were made by scraping the cells in 0.5mL MCB+ lysisbuffer (10mL MCB buffer (50mM Tris HCl pH 7.5, 50mM NaCl, 10% glycerol, 1% NP-40, 0.5% Na-deoxycholate, 20mM NaF), 100 μl 100mM PEFA block, 100 μl 100mM orthovanadate, 1 tablet Complete Mini (Roche Diagnostics, Lot. 11714800)) per 10cm plate after which the cell lysate was stored at -80°C. To isolate proteins from the cells the samples were subjected to two freeze – thaw cycles in liquid nitrogen and thorough vortexing, after which insoluble cell debris was spun down at 14000xg for 1 minute and supernatant saved and stored at -80°C until further analysis.

### 2.1.3 BRADFORD ASSAY

The concentration of the protein samples was determined with a Bradford protein assay (Bio-Rad). A standard series of 0 ; 0.2 ; 0.4 ; 0.6 ; 0.8 and 1 mg/ml bovine serum albumin (BSA) diluted in Ultra Pure Water was used as reference. Sample dilutions of 5μl and 10μl were prepared in Ultra Pure Water in a final volume of 50μl. The Bradford solution was mixed 1:5 with Ultra Pure Water and 2.5mL of the diluted solution was added to each sample, after which they were vortexed and incubated for 2 minutes at room temperature, after which the absorbance at 595nm was determined with a spectrometer. The absorbance of the standard series was used to make a calibration curve to calculate the concentration of the protein samples.

### 2.1.4 SDS-PAGE AND WESTERN BLOTTING

A 10% polyacrylamide running gel was prepared and poured in between two glass plates. It was left to polymerize for 45-60 minutes after which a 5% polyacrylamide stacking gel prepared and poured on top of the running gel in between the two glass plates. Loading buffer (250 mM Tris-Cl pH 6.8, 8% SDS, 40% Glycerol, 0.02 gram bromophenol blue, 1/5 parts 1M dithiothreitol (added just before use) in 10ml) was added 1:1 (2x loading buffer) or 1:5 (5x loading buffer) to the protein samples. The samples were heated to 95°C for 5 minutes and equally loaded (20μg protein) onto the
The proteins were separated by SDS-PAGE in 1x running buffer (0.025M Tris-Cl, 0.192M Glycine, 0.1% SDS) for 2 hours at 100V.

Proteins were transferred to a PVDF Hybond P-membrane by semi-dry electroblotting. In brief: a PVDF Hybond-P membrane was soaked in 100% methanol for 1 minute after which it was transferred to milliQ for 1 minute. A blotting mask was placed over the anode. A blotting stack was set up consisting of three whatman papers soaked in 1x blotting buffer (0.025M Tris-Cl, 0.192M Glycine, 1/5 parts 100% methanol), the Hybond-P membrane and the gel soaked in 1x blotting buffer, three whatman papers soaked in 1x blotting buffer and the cathode. Blotting was performed for 1 hour at 40mA per gel. The membrane was washed once in PBS-Tween 20 (PBS (phosphate buffer saline), 0.05% Tween 20) after which it was blocked in 20ml block buffer (5% non-fat dry milk in PBS-Tween 20) for 1 hour at room temperature. The membrane was incubated with a primary antibody (Table 2) solution (diluted in 5ml block buffer) and was incubated overnight at 4°C. The membrane was washed 5 times 5 minutes in PBS-Tween 20. It was then incubated for 1 hour at room temperature in secondary antibody (Table 2) solution (diluted in 20ml block buffer).

**TABLE 2: ANTIBODIES USED FOR DETECTION OF THE HIF1A AND HIF3A PROTEINS. B-ACTIN WAS USED FOR NORMALIZATION.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Dilution</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1A</td>
<td>Purified mouse anti-human HIF1A, BDbiosciences, cat#610958</td>
<td>1:1000</td>
<td>Goat-anti-mouse IgG-HRP, Santa Cruz, cat#sc-2005</td>
<td>1:10000</td>
<td>120kDa</td>
</tr>
<tr>
<td>HIF3A</td>
<td>Rabbit Polyclonal to HIF3A, Abcam, cat#ab10134</td>
<td>1:500</td>
<td>Goat-anti-rabbit-HRP, Jackson, cat#111-035-144</td>
<td>1:50000</td>
<td>72kDa; 68kDa; 50kDa; 42kDa</td>
</tr>
<tr>
<td>B-Actin</td>
<td>Monoclonal anti-B-Actin (mouse), Sigma Aldridge, cat#A5441</td>
<td>1:5000</td>
<td>Goat-anti-mouse IgG-HRP, Santa Cruz, cat#sc-2005</td>
<td>1:10000</td>
<td>42kDa</td>
</tr>
</tbody>
</table>
The membrane was washed 3 times 10 minutes in PBS-Tween 20 and 2 times 10 minutes in regular PBS. 4ml ECL reagent I with 4ml ECL reagent II (Pierce) was mixed and the membrane was incubated for 3 minutes at room temperature in this solution after which it was transferred to a photo-cassette. Amersham Hyperfilm™ ECL (cat# 45-001-505) was exposed to the membrane and developed.

To strip the antibodies off the membrane it was washed in PBS and transferred to stripping buffer (Thermo Scientific, cat# 21059) and incubated for 30 minutes at 37°C. The membrane was washed again 3 times 10 minutes in PBS-Tween 20 and was then ready for incubation with another primary antibody.

2.2 STUDYING THE EXPRESSION KINETICS OF HIF3A UNDER NORMOXIA AND HYPOXIA

HIF3A protein was detected in a similar way as described for HIF1A. The expression profile of HIF3A, in contrast to HIF1A was also studied on RNA level.

2.2.1 RNA ISOLATION

The cells for RNA isolation are harvested by suspending the cells in 1mL RNA Bee lysis buffer (Bioconnect, tel-test, inc) per 10cm plate. 200µl 100% chloroform was added and the suspension was vortexed for 15 seconds, after which it was incubated for 5 minutes on ice. Phase separation was done by centrifuging the samples at 4°C and 13900xg for 15 minutes. The upper water phase was added to 500µl 100% isopropanol. The samples were mixed and precipitated for 60 minutes at -80°C and then pelleted by centrifuging at 4°C, 14000xg for 15 minutes. The supernatant was discarded and the pellet was washed twice with 500µl 70% EtOH (in Ultra Pure Water). The pellet was air dried and dissolved in 30µl Ultra Pure Water. RNA concentration was determined with the Nanodrop ND1000 and stored at -80°C until further analysis.

2.2.2 CDNA SYNTHESIS

The RNA samples were diluted to a concentration of 100ng/µl in Ultra Pure Water. 10µl of these dilutions was added to 10µl of the mastermix (1x RT Buffer, 1x random primers, 1x dNTP mix, Reverse Transcriptase, RNase inhibitor (Applied Biosystems,
The following PCR programme was used: 10 min at 25°C; 120 min at 37°C; 5 sec at 85°C. The concentration of the end product cDNA was approximately 50ng/µl.

**2.2.3 ENDPOINT PCR**

Two primer pairs were used to detect the longest transcript of HIF3A, transcript 201 [Ensembl (2012)]. The first primer pair (primer pair 5 (FW05; RV05)) was located near the beginning of the 3'UTR, whereas the other primer pair (primer pair 6 (FW06; RV06)) was located further on, near the end of the 3'UTR (figure 8).

![FIGURE 8: LOCATION OF PRIMER PAIRS USED FOR THE IDENTIFICATION OF HIF3A TRANSCRIPT 201 AND TO STUDY ITS EXPRESSION KINETICS UNDER HYPOXIC CONDITIONS.](image)

The first primer pair generates a product of 212bp and the second primer pair generates a product of 311bp. HPRT was used for normalization. The primer pair used are listed in table 3.

**TABLE 3: PRIMERS USED FOR END-POINT PCRS ON 3'UTR OF HIF3A TRANSCRIPT 201.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Name</th>
<th>Sequence</th>
<th>Fragment length</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer pair 5</strong></td>
<td>hif3a-fw05</td>
<td>5'-AGA ACA ATG ATC CAC GGG T-3'</td>
<td>212bp</td>
</tr>
<tr>
<td></td>
<td>hif3a-rv05</td>
<td>5'-GCC TCA ATC GGA AGT CAC-3'</td>
<td></td>
</tr>
<tr>
<td><strong>Primer pair 6</strong></td>
<td>hif3a-fw06</td>
<td>5'-GCC TCA CAG CTT CCA ACT-3'</td>
<td>311bp</td>
</tr>
<tr>
<td></td>
<td>hif3a-rv06</td>
<td>5'-TGG GGC ACA GAG ATT GTA G-3'</td>
<td></td>
</tr>
<tr>
<td><strong>HPRT</strong></td>
<td>hprt fw</td>
<td>5'-ATG GGA GGC CAT CAC ATT G-3'</td>
<td>336bp</td>
</tr>
<tr>
<td></td>
<td>hprt rv</td>
<td>5'-GGT CT TTT CAC CAG CAA G-3'</td>
<td></td>
</tr>
</tbody>
</table>
5µl sample (10ng/µl) was added to 45µl mastermix (1x Go Taq Flex buffer, 0.025U Go Taq Flex enzyme, 0.2mM of each dNTP, 1.5mM MgCl₂ and 300nM of the forward and reverse primer in milliQ). The PCR program was as follows: 2 min at 95°C, 30-40 cycles (45 sec at 95°C, 45 sec at 60-65°C, 45 sec at 72°C), 5 min at 72°C. The results were analysed on a 1.5% agarose gel in 0.5x TBE buffer with 0.5µg/ml Ethidium Bromide. The gel was run in 0.5x TBE buffer for 1 hour at 100V.

Samples used for the endpoint PCRs came from two individual experiments. The first set of samples came from a previous independent experiment which covered thirteen sarcoma cell lines which can be found in the supplementary data (table S1). The second set of samples have been described in paragraph 2.1.1.

2.3 STUDYING THE EFFECT OF HYPOXIA ON THE 3’UTR OF HIF3A TRANSCRIPTION VARIANT 201

2.3.1 CLONING OF THE 3’UTR OF HIF3A INTO THE PSI-CHECK2 VECTOR

Two constructs were created by cloning (a part of) the 3’UTR of HIF3A transcription variant 201 into the Psi-Check2 vector. The fragments to be cloned into the vector were first amplified using a Phusion DNA polymerase which generates blunt ends and has proofreading activity.

2.3.1.1 PROOFREADING PCR

Two fragments of the 3’UTR of the HIF3A transcription variant 201 were amplified using proofreading PCR. The PCR mixture contained 1x High Fidelity buffer; 0,2mM dNTPs; 0,3µM FW primer; 0,3µM RV primer; 0.5U (unit) High Fidelity polymerase phusion (Finnzymes, cat# F-530S); 250ng human genomic DNA (Promega, cat# G1471) in a total volume of 50µl for both fragments. The primers used contain restriction sites for the enzymes XhoI (forward primer) and NotI (reverse primers) and the sequences are presented in table 4.
### TABLE 4: THE PRIMERS USED FOR THE AMPLIFICATION OF THE FRAGMENTS CONTAIN RESTRICTION SITES FOR THE ENZYMES NOTI AND XHOI.

<table>
<thead>
<tr>
<th>Construct short</th>
<th>Construct long</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primer</strong></td>
<td><strong>Reverse primer</strong></td>
</tr>
<tr>
<td>5’- GTC/TCGAGCCGGCTCTCTCCCATCTG-3’</td>
<td>5’- GTC/TCGAGCCGGCTCTCTCCCATCTG-3’</td>
</tr>
<tr>
<td>5’- GAGC/GGCCGCAGACCACATTGAGGTTG-3’</td>
<td>5’- GAGC/GGCCGCAGACCACATTGAGGTTG-3’</td>
</tr>
</tbody>
</table>

The forward primer for both fragments was the same and was located at the very beginning of the 3’UTR of HIF3A transcription variant 201. The reverse primer to generate the short amplification product was located after 800bp so that the fragment contained the binding site for hsa-miR-210 and three binding sites for hsa-miR-485-5p. The reverse primer to create the longer amplification product was placed 4 bases from the end of the 3’UTR (figure 9). This fragment contains all binding sites for hsa-miR-485-5p and the binding site for hsa-miR-210.

**FIGURE 9: HIF3A TRANSCRIPTION VARIANT 201 3’UTR CONTAINING 1 BINDING SITE FOR HSA-MIR-210 AND 11 BINDING SITES FOR HSA-MIR-485-5P. PRIMERS INDICATED HAVE BEEN USED FOR CLONING INTO THE PSI-CHECK2 VECTOR.**

For each product a different PCR program was used. The PCR program to generate the shorter amplification product was as follows: 2 min at 98°C, 40 cycles (10 sec at 98°C, 30 sec at 63°C, 30 sec at 72°C), 10 min at 72°C. The PCR program to generate the longer amplification product was as follows: 2 min at 98°C, 40 cycles (10 sec at 98°C, 30 sec at 68°C, 2 min at 72°C), 10 min at 72°C.

The samples were subjected to agarose electrophoresis and subsequently the fragments were isolated from a 0.7% agarose gel.
2.3.1.2 DNA ISOLATION FROM AGAROSE GEL

The amplification products were isolated from gel with the QIAquick Gel Extraction kit (Qiagen, cat# 28704), following manufacturer’s protocol. In short: fragments were sliced out if the gel and dissolved in buffer GC (QIAquick Gel Extraction kit). To dissolve the gel in this buffer, the sample was incubated 10 minutes at 50°C. The DNA was then brought onto a QIAquick spin column, washed with EtOH and eluted in 30-50µl nuclease free water. The DNA concentration and the integrity of the DNA fragments was estimated with a low mass ladder on an agarose gel and fragments were ligated into a PCR blunt vector.

2.3.1.3 LIGATION INTO PCR BLUNT VECTOR

To ligate an insert into the PCR blunt vector a ratio of 10:1 insert:vector is required. The ligation mixture contained the following: 1x T4 ligation buffer, 4 units T4 ligase, 25ng vector linearized blunt PCR blunt and 250ng amplification product in a total volume of 10µl (Zero Blunt PCR Cloning Kit, Invitrogen, Part no. 44-0302). The samples were mixed and centrifuged shortly. The ligation was performed for 1 hour at 16°C. The vectors were then transformed into E. coli Top 10 competent cells (One Shot TOP10 Chemically Competent E. coli (Invitrogen, cat# C4040-06)).

2.3.1.4 TRANSFORMATION INTO TOP 10 COMPETENT CELLS

TOP10 competent cells (One Shot TOP10 Chemically Competent E. coli (Invitrogen, cat# C4040-06)) were gently defrosted on ice, after which 2µl 0.5M B-mercaptoethanol and 2µl of the ligation mixture were added and the mixture was gently stirred. The cells were incubated on ice for 30 minutes. The cells were then exposed to a heat shock of 42°C for 45 seconds and then immediately incubated on ice for 2:30 minutes. 250µl of SOC medium (One Shot TOP10 Chemically Competent E. coli (Invitrogen, cat# C4040-06)) was added to the mixture and the cells were incubated at 37°C for 1 hour. The cells were transferred to agar plates with 50µg/ml kanamycin. When enough single bacteria colonies were observed, one colony was transferred to 4ml LB medium with 50µg/ml kanamycin (mini culture).

2.3.1.5 PLASMID DNA ISOLATION FROM MINI CULTURES

1.5ml of the bacteria culture was transferred to a 1.5ml tube and the bacteria were spun down for 10 sec at 13900xg. The majority of the supernatant was removed by decanting and the bacteria pellet was resuspended in the remainder supernatant left in
the tube. 300µl TENS buffer (0.2N NaOH, 1% SDS, 0.02M Tris-Cl pH 7.5, 2mM EDTA pH 8) was added to the bacteria suspension. After thorough vortexing 150µl 3.0M NaAc pH 5.2 was added. The suspension was thoroughly vortexed and centrifuged for 2 minutes at 13900xg. The supernatant was transferred to a new 1.5ml tube. 900µl 100% cold EtOH was added and the samples were then incubated at -20°C for 30 minutes after which they were centrifuged for 2 minutes at 13900xg. The supernatant was discarded and the pellet was washed twice with cold 75% EtOH. The pellet was air dried and dissolved in 20µl nuclease free water with a RNase cocktail (20ng RNase A; 400U RNase T1 in nuclease free water). The samples were incubated 30 minutes at 37°C and subjected to an agarose gel and used in subsequent cloning steps.

2.3.1.6 RESTRICTION WITH NOTI AND XHOI

The amplified HIF3A 3’UTR fragments were cut out of the PCR Blunt vector using the restriction enzymes NotI (Promega, cat#R6431) and XhoI (Promega, cat#R6161). 1x buffer D (Promega, cat#R6431), 1U Not I, 1U XhoI and 1 µl for the short product and 5µl sample for the long product was added to a total volume of 20µl. The fragments were separated on a 0.7% agarose gel and isolated according to the protocol described in paragraph 2.3.1.2.

2.3.1.7 LIGATION INTO PSI-CHECK2 VECTOR

A vector:insert ratio of 1:3 and 1:6 was used to ligate the amplified products into the psiCHECK-2 Vector (Promega, cat#C8021). 48ng of the psiCHECK-2 vector (6273bp), digested with NotI and XhoI, and 150-300ng of the amplified products (short – 816bp; long – 3807bp) were used for ligation. 1x T4 ligation buffer and 4 units T4 ligase were added to the ligation mix in a total volume of 10-15µl. The samples were mixed and centrifuged shortly. The ligation was performed overnight at 16°C. The constructs were then transformed into E. coli Top 10 competent cells (paragraph 2.3.1.4).

2.3.1.8 PLASMID DNA ISOLATION FROM MAXI’S

A maxiprep of 200ml LB medium with 50µg/ml ampicillin was inoculated with the remainder of the mini culture, after which the bacteria were cultured overnight. The suspension was divided over four 50ml tubes and the bacteria were centrifuged at 4°C and 2880xg for 25 minutes. The supernatant was removed and the pellets were suspended in buffer P1 (Plasmid Maxi Kit). The DNA was isolated with the Plasmid Maxi Kit (QIAGEN, cat#12163) according to manufacturer’s protocol.
2.3.2 LUCIFERASE ASSAY

SW872 and SK-UT-1 cells were seeded at T=-2 days in 24 well cell culturing plates. 24 hours after seeding the cells were transfected with two constructs, namely the psi-CHECK2 vector with the short HIF3A construct and with the long HIF3A construct as described in paragraph 2.3.1.1. 24 hours after transfection the cells were cultured under either normoxic (21% O₂) or hypoxic (1% O₂) conditions. Cells were harvested for the luciferase assay at T=0, 6, 24 and 48 hours (figure 10).

![Diagram of cell culture process](image)

**FIGURE 10**: CELLS OF TWO SARCOMA CELL LINES, NAMELY SW872 AND SK-UT-1 WERE SEEDED AT T=-2 DAYS. THESE CELLS WERE TRANSFECTED WITH TWO PSI-CHECK2-HIF3A 3’UTR CONSTRUCTS AT T=-1 DAYS AND 24 HOURS AFTER TRANSFECTION THESE CELLS WERE CULTURED UNDER EITHER NORMOXIC OR HYPOXIC CONDITIONS. FOR THE LUCIFERASE DUAL REPORTER ASSAY CELLS WERE HARVESTED AT T=0, 6, 24 AND 48 HOURS.

2.3.2.1 CONSTRUCT TRANSFECTION

For the transfection of the psi-CHECK2 vector with the HIF3A 3’UTR fragments, FuGENE 6 transfection reagent (Promega, cat#E2691) was diluted into serum free medium (Roswell Park Memorial Institute 1640 medium (RPMI-1640)) in a ratio of 8:1 (8µl FuGENE to 1µg DNA). This solution was incubated for 5 minutes at room temperature after which the DNA (psi-CHECK2 vector with HIF3A insert short/long) was added. This was then incubated at room temperature for 15 minutes and then 25µl solution was added dropwise to each well. For each construct, two wells per cell line were transfected in order to have biological replicates.

2.3.2.2 DUAL-LUCIFERASE REPORTER ASSAY

For the luciferase assay cells were lysed at T=0, 6, 24 and 48 hours. To make the lysates, the lysisbuffer of the Dual-Luciferase Reporter Assay System kit (Promega, cat#E1910) was used which were diluted 5x in nuclease free water. 100µl of the diluted lysisbuffer (1x lysisbuffer) was added to each well, after which the 24-well plate was placed on a shaker platform for 30 minutes. The cell lysates were stored at -20°C until all cells were lysed.
For the Dual-Luciferase reporter assay 20µl of each cell lysate was added to a 96-well plate. 50µl of the LARII solution (Dual-Luciferase Reporter Assay System kit (Promega, cat#E1910)) was added to the samples and the firefly luciferase activity was measured at 595nm. The Dual Stop&Glo solution (Dual-Luciferase Reporter Assay System kit (Promega, cat#E1910)) was diluted 50 times in the corresponding buffer and 50µl was added to each well and the Renilla Luciferase activity was measured at 595nm as well.

2.4 VALIDATION OF HIF3A TRANSCRIPTION VARIANT 201 AS A TARGET OF HSA-MIR-210 AND HSA-MIR-485-5P

To validate the hypothesis that HIF3A transcription variant 201 is a target of both hsa-miR-210 and hsa-miR-485-5p a luciferase assay was performed using the two constructs that were created by cloning (a part of) the 3’UTR of HIF3A transcription variant 201 into the psi-CHECK2 vector and mimics for hsa-miR-210, hsa-miR-485-5p and a scrambled mimic.

At T= -2 days SW872 cells were seeded in 24-wells plates. At T= -1 days the mimics were transfected into the cells (paragraph 2.4.1.1) and 24 hours later the constructs were transfected as well (paragraph 2.3.2.1). Cells were harvested for the luciferase assay at 24 and 48 hours after transfection of the constructs (figure 11). The luciferase assay was performed as described in paragraph 2.3.2.2.

2.4.1.1 MIMIC TRANSFECTION

For the transfections cell line SW872 was seeded in 24-wells plates. Mimics for hsa-miR-485, hsa-miR-210 and a scrambled mimic were diluted 1:20 in serum free medium (Roswell Park Memorial Institute 1640 medium (RPMI-1640)). DharmaFECT 1 (Thermo Scientific, cat# T-2001) was used as transfection reagent and diluted 1:80 in serum free medium. The diluted mimic was added 1:1 to the DharmaFECT solution and after mixing properly the reagents were incubated for 20 minutes at room temperature. 50µl of the solutions was added dropwise to each well.

2.5 STUDYING THE EFFECT OF HSA-MIR-485 AND HSA-MIR-210 ON THE HYPOXIA RESPONSE PATHWAY

When the effect of the miRNAs on the 3’UTR of human HIF3A transcription variant 201 was determined the next question was regarding the effect of the miRNAs on the hypoxic response pathway. It was hypothesized that the endogenous HIF3A levels were lower in cells transfected with both hsa-miR-210 and hsa-miR-485-5p when compared to cells transfected with a scrambled mimic. Another hypothesis was that HIF1A was controlled by HIF3A, and thus it was hypothesized that the HIF1A levels in cells transfected with both miRNA mimics were lower as well when compared to the levels in cells transfected with a scrambled mimic.

Like previous experiments, SW872 cells were seeded at T=-2 days in 24-wells plates and mimics were transfected 24 hours after seeding as described in paragraph 2.4.1.1. At T=0, 48 hours after seeding, cells were cultured under both normoxic and hypoxic conditions and cells were harvested at T=0, 6, 24 and 48 hours for further analysis using western blotting to determine the protein levels of human HIF1A and HIF3A as described in paragraphs 2.1.2, 2.1.3 and 2.1.4 (figure 12).

FIGURE 12: SW872 CELLS WERE SEEDED AT T=-2 IN 24-WELLS PLATES AND MIMICS FOR HSA-MIR-210, HSA-MIR-485-5P AND A SCRAMBLED MIMIC WERE TRANSFECTED INTO THE CELLS 24 HOURS AFTER SEEDING. CELLS WERE CULTURED UNDER BOTH NORMOXIC AND HYPOXIC CONDITIONS. CELLS WERE HARVESTED AT T=0, 6, 24 AND 48 HOURS FOR FURTHER ANALYSIS OF THE ENDOGENOUS HUMAN HIF1A AND HIF3A LEVELS.
Three sarcoma cell lines representing different sarcoma subtypes have been subjected to either normoxic or hypoxic (1% O₂) conditions, after which both RNA and proteins were analysed at T=0, 6, 24 and 48h.

### 3.1 UPREGULATION OF HIF1A AFTER CULTURING 6 HOURS UNDER HYPOXIC CONDITIONS

In three sarcoma cell lines, namely SW872, SK-UT-1 and A204 HIF1A protein levels were determined by western blotting. A strong upregulation of HIF1A was observed after 6 hours of hypoxic stress in all three of the sarcoma cell lines. At 24 and 48 hours of hypoxia HIF1A still appeared to be upregulated compared to normoxia control incubations, but in time HIF1A quantities are decreasing despite continuing hypoxic conditions. Figure 13 shows HIF1A levels in SW872(A). The results in figure 13A have been normalised to for B-actin which served as an equal loading control (B) and the quantified and normalised results are shown in figure 13C.

![SW872: HIF1A protein expression](image)

**FIGURE 13:** THE HIF1A PROTEIN IS UPREGULATED IN ALL THREE CELL LINES AFTER THEY WERE CULTURED UNDER HYPOXIC CONDITIONS FOR 6 HOURS. AFTER 24 AND 48 HOURS THE HIF1A PROTEIN IS STILL UPREGULATED, BUT IT IS LESS PRONOUNCED (A). THESE RESULTS HAVE BEEN CORRECTED FOR HUMAN B-ACTIN (B) AND THE QUANTIFIED (C) AND (B).
3.2 UPREGULATION OF HIF3A AFTER CULTURING 24 HOURS UNDER HYPOXIC CONDITIONS

To study the kinetics of HIF3A at the protein and mRNA level under normoxic and hypoxic conditions an endpoint PCR was performed in parallel to the western blots. Two primer pairs (5 and 6) that hybridize to the 3’UTR of HIF3A transcription variant 201 were used [Ensembl (2012)]. Primer pair 5 is complementary to the beginning of the HIF3A 3’UTR, whereas primer pair 6 hybridizes to the mRNA sequence near the end of the 3’UTR.

The endpoint PCR results for cell line SW872 are presented in figure 14. Endpoint PCRs on the 3’UTR of HIF3A transcription variant 201 have been performed on RNA from a previous experiment in which thirteen different sarcoma cell lines were used. These cell lines and the results for these cell lines can be found in the supplementary data. The majority of the cell lines showed a similar pattern for HIF3A regulation during 48 hours of 1% oxygen.

HIF3A transcription variant 201 is slightly downregulated after 6 hours of 1% oxygen, but after 24 hours an upregulation can be clearly observed which is even more pronounced after 48 hours at 1% oxygen. Both primer pairs 5 (A) and 6 (B) showed a similar trend. HPRT (C) was used for normalization.

FIGURE 14: THE mRNA OF HIF3A TRANSCRIPTION VARIANT 201 IS UPREGULATED AFTER BEING CULTURED FOR 24 HOURS UNDER HYPOXIC CONDITIONS. THIS EXPRESSION PATTERN WAS FOUND WITH BOTH PRIMER PAIR 5 (A) AND PRIMER PAIR 6 (B). HPRT WAS USED FOR NORMALIZATION (C).

The results obtained for sarcoma cell line SW872 have been quantified and normalized to the housekeeping gene HPRT (figure 14C). These results are presented in figure 15.
FIGURE 15: THE MRNA OF HIF3A TRANSCRIPTION VARIANT 201 IS UPREGULATED AFTER BEING CULTURED FOR 24 HOURS UNDER HYPOXIC CONDITIONS. THIS EXPRESSION PATTERN WAS FOUND WITH BOTH PRIMER PAIR 5 (A) AND PRIMER PAIR 6 (B). THE RESULTS OF HAVE BEEN NORMALIZED USING HPRT EXPRESSION LEVELS.

For all three cell lines examined, western blotting was performed to determine HIF3A protein levels. Several protein bands of various size were observed perhaps representing various HIF3A isoforms or alternatively unrelated cross-reactive proteins. Cell lines SK-UT-1 and A204 showed a similar expression pattern of HIF3A proteins, while cell line SW872 showed a different HIF3A profile. Figure 16 depicts the western blots of both SW872 and A204.

The human HIF3A gene has at least 21 different transcription variants according to ensembl [Ensembl (2012)]. It is not known whether all variants are expressed in all cells and whether they all code for proteins. Some variants may be cell type specific or specifically expressed under certain conditions.

FIGURE 16: MULTIPLE TRANSCRIPTS OF THE HIF3A PROTEIN WERE PICKED UP BY THE PRIMARY ANTIBODY TARGETING HIF3A USED. CELL LINES A204 AND SK-UT-1 SHOWED A SIMILAR EXPRESSION PROFILE, WHILE CELL LINE SW872 PRESENTED A DIFFERENT EXPRESSION PATTERN.

A protein of approximately 55kDa was the only protein that was observed in all three cell lines. Deducting from the end point PCR results, transcriptions variant 201 is expressed in all three cell lines. The calculated molecular weight for the protein encoded
by the HIF3A transcription variant 201 was approximately 58kDa. For these reason we consider the 55kDa protein to represent the HIF3A isoform we are interested in.

The human HIF3A protein was found slightly upregulated in sarcoma cell line SW872 up to 24 hours of hypoxic stress, after which its expression appeared to be decreasing (figure 17A). The obtained results have been corrected for B-actin (B) and quantified (C).

Cell lines A204 and SK-UT-1 show a different induction of HIF3A compared to cell line SW872. After 24 hours of hypoxic stress a HIF3A peak was observed in both cell lines. However after 48 hours of hypoxic stress the expression of HIF3A already dramatically decreased. Another difference with cell line SW872 is the almost complete lack of expression under normoxic conditions (figure 18A). These results have been normalized for B-Actin expression (B) and quantified (C).
The next step was to determine whether HIF3A expression is controlled under hypoxic conditions through binding of miRNAs to its 3’UTR. In order to determine this, SW872 cells were transfected with the two constructs. In the first construct (short), the first 816bp of the 3’UTR of HIF3A transcription variant 201 was cloned behind the Renilla luciferase gene in the Psi-ChECK2 Vector. In the second construct (long) the entire 3’UTR of HIF3A transcription variant 201 was cloned behind the Renilla luciferase gene. The results were normalized using the Firefly luciferase gene in the PsiCheck2 Vector. This luciferase gene is not regulated by the 3’UTR of HIF3A and is constitutively expressed.

Since hsa-miR-210 and hsa-miR-485-5p have been found to be upregulated under hypoxic conditions, it is hypothesized that the Renilla luciferase activity decreases under hypoxic conditions when compared to normoxic samples.

After 24 hours of hypoxic stress, the Renilla luciferase shows indeed a decrease in activity when compared to normoxia in cells transfected with both constructs (figure 19). Under normoxic conditions a decrease in Renilla luciferase activity is observed as well, however, this decrease in activity is slightly less prominent than under hypoxia (figure 20).

**FIGURE 19: AFTER 24 HOURS HYPOXIA THE LUCIFERASE ACTIVITY DECREASES FOR BOTH CONSTRUCTS WHEN COMPARED TO NORMOXIA. THE DECREASE IN LUCIFERASE ACTIVITY IS MORE PRONOUNCED IN CELLS TRANSFECTED WITH THE LONG CONSTRUCT.**

**FIGURE 20: AFTER 24 HOURS OF HYPOXIC STRESS THE RENILLA LUCIFERASE ACTIVITY DECREASES UNDER BOTH NORMOXIA AND HYPOXIA, HOWEVER THE DECREASE UNDER HYPOXIA IS A LITTLE MORE PROMINENT. A: RELATIVE LUCIFERASE ACTIVITY IN CELLS TRANSFECTED WITH THE SHORT CONSTRUCT; B: RELATIVE LUCIFERASE ACTIVITY IN CELLS TRANSFECTED WITH THE LONG CONSTRUCT.**
3.4 HIF3A TRANSCRIPTION VARIANT 201 IS REGULATED BY BOTH HSA-MIR-210 AND HSA-MIR-485-5P THROUGH ITS 3’UTR

To determine whether hsa-miR-210 and hsa-miR-485-5p target the 3’UTR of HIF3A transcription variant 201 and were indeed responsible for the 3’UTR mediated decrease of luciferase activity after 24 hours, another luciferase assay was performed. The activity of the Renilla luciferase in cells transfected with mimics for hsa-miR-210 and hsa-miR-485-5p were compared to the Renilla luciferase activity in cells transfected with a scrambled mimic. This mimic will not bind to the 3’UTR of HIF3A transcription variant 201 and will therefore not control Renilla luciferase expression.

The Renilla luciferase activity in cells transfected with a mimic for hsa-miR-210 was decreased to approximately 25% for short construct and 30% for the long construct at 24 hours after transfection of the constructs into SW872 cells when compared to cells transfected with a scrambled mimic (figure 21).

The activity of the Renilla luciferase in cells transfected with hsa-miR-485-5p also showed a decrease when compared to the activity of the Renilla luciferase in cells transfected with a scrambled mimic. The activity was approximately 55% for the short construct and 45% for the long construct at 24 hours after transfection (figure 21).
3.5 THE TRANSFECTION OF MIMICS FOR HSA-MIR-210 AND HSA-MIR-485-5P HAS A NEGATIVE EFFECT ON HIF1A AND HIF3A PROTEIN LEVELS

The next question raised was what the effect of the miRNAs was on the hypoxic response pathway in sarcoma cell lines. In order to determine this, mimics for hsa-miR-210 and hsa-miR-485-5p were transfected into sarcoma cell line SW872. These cells were cultured under either normoxic or hypoxic conditions and were harvested at several time points during the culturing. The protein levels of both HIF1A and HIF3A were analyzed at the different time points. The most pronounced effect was observed after 24 hours of culturing under hypoxia. Both HIF1A and HIF3A protein levels were clearly downregulated in cells transfected with the miRNA mimics when compared to cells transfected with a scrambled mimic. The effect of hsa-miR-210 is more pronounced than the effect of hsa-miR-485-5p for both HIF1A and HIF3A. The results were normalized for B-actin expression and quantified (figure 22).

4 DISCUSSION

In a pilot study prior to this research project, several miRNAs have been identified to be differentially expressed in sarcoma cell lines when they were exposed to hypoxic stress. Several experiments have been performed to determine the function of two of these miRNAs namely has-miR-210 and has-miR-485-5p under hypoxic stress in the sarcoma cell line SW872. These experiments showed that human HIF3A transcription variant 201 is a direct target of both has-miR-210 and has-miR-485-5p and that these miRNA have an effect on the hypoxic response pathway. Prior to these functional experiments, the kinetics of the hypoxia response pathway have been studied by investigating both HIF1A and HIF3A induction upon hypoxia at both protein and mRNA levels.

4.1 HIF1A UPREGULATION AS AN EARLY RESPONSE MARKER TO HYPOXIA

The expression kinetics of HIF1A upon hypoxic stress has been studied at protein level. The stabilization of transcription factor HIF1A has been described as an early hypoxic response maker. It has a transactivating function and with this it is an important key regulator in the hypoxic response pathway [Lisy and Peet, (2008); Maxwell (2005); Wilkins, Hyvärinen, et al. (2009)]. The results in this report show a strong upregulation of the HIF1 protein during hypoxia and thus confirm its involvement in the early response to hypoxia described in the literature. After 24 and 48 hours of hypoxic stress, the results still show an upregulation of HIF1A protein, but its quantity seems to be decreasing, which also confirms it is an early response mechanism.

4.2 HIF1A AS A REGULATOR OF HIF3A

Not much is known about the activation, transcription, translation or function of one of the other members of the HIF-family, HIF3A. Whereas HIF2A is described to have a similar function to HIF1A [Maxwell, P.H. (2005)], HIF3A has a different protein structure and most likely different function. HIF1A and HIF2A proteins, under normoxic conditions, are hydroxylated and subjected to degradation. This is not the case for HIF3A [Pasanen, A. et al. (2010)]. Instead it is thought to be under control of HIF1A [Jang et al. (2005); Pasanen, A. et al. (2010)]. Our results obtained with both the western blots (protein) and endpoint PCRs (mRNA) for HIF3A show an increased expression of HIF3A after 24 and 48
hours of hypoxic stress. When observing the expression kinetics of HIF1A during hypoxia, an upregulation of this protein is already seen after 6 hours of hypoxia. If HIF1A is a regulator of HIF3A, this expression pattern could be expected, because HIF1A needs to be upregulated prior to HIF3A in order to regulate HIF3A. Although this is not conclusive evidence that HIF1A regulates the expression of HIF3A under hypoxic stress, it does seem to be possible. The endpoint PCRs for HIF3A on the mRNA of an individual experiment (supplementary data) show an upregulation of HIF3A under normoxic conditions as well, however there was a minimal to no upregulation of HIF1A detected under normoxic conditions. This contradicts the hypothesis that HIF3A is regulated by HIF1A, however it does not exclude this hypothesis. It is possible that other regulating mechanisms are at work under normoxic conditions, whereas HIF1A regulated HIF3A under hypoxic conditions.

These results could also be indicating that HIF3A is not only responsive to severe hypoxic stress which leads to HIF1A stabilization, but could also be sensitive to small hypoxic stress e.g. due to the increasing cellular confluency during the experiments.

4.3 HIF3A AS A NEGATIVE REGULATOR OF HIF1A

One of the hypotheses was that HIF3A could be upregulated under hypoxic stress to negatively regulate HIF1A. HIF3A has 21 transcription variants according to ensembl [Ensembl (2012)]. It is not known whether all variants are expressed in all cells. Perhaps HIF3A mRNA variants are cell type specific or specific to certain conditions, which could explain the western blot results of HIF3A, where different proteins were observed in different cell lines used in this project.

One of the transcription variants of HIF3A is described to be a negative regulator of HIF1A [Jang et al. (2005)]. This protein contains the inhibiting Per/Arnt/Sim (IPAS) domain, which HIF1A and HIF2A lack.

In this project transcription variant 201 is of interest, because of its large 3'Untranslated Region (3'UTR). If transcription variant 201 codes for a HIF3A isoform capable of regulating HIF1A, one expects an upregulation of HIF3A around the same time the expression of HIF1A decreases. Our results on the HIF3A mRNA kinetics upon hypoxia suggest that HIF3A could indeed be a regulator of HIF1A. This is believed because the HIF3A protein is upregulated after 24 and 48 hours of hypoxia, which is at the same time point where the HIF1A levels start to decrease.
4.4 HSA-MIR-485-5P AS A REGULATOR OF HIF3A TRANSCRIPTION VARIANT 201 UNDER HYPOXIA

The 3’UTR of HIF3A transcript 201 contains 11 binding sites for hsa-miR-485-5p, a miRNA that has been found to be upregulated after 24 hours of hypoxic stress in several sarcoma cell lines on a microarray platform in a pilot study. It is believed that this miRNA could be a regulator of HIF3A transcript 201 and may indirectly be a regulator of HIF1A under hypoxic conditions.

The results with luciferase reporter constructs showed a decreased HIF3A-3’UTR regulated luciferase signal in cells transfected with a mimic for hsa-miR-485-5p when compared to cells transfected with a scrambled mimic. This means the miRNA hsa-miR-485-5p binds to the 3’UTR of HIF3A transcription variant 201 causing translation inhibition and/or degradation of, in this case, the mRNA of the luciferase gene and thus, in the normal situation, the mRNA of HIF3A transcription variant 201. These results confirm the hypothesis that HIF3A transcription variant 201 is regulated by hsa-miR-485-5p.

In order to determine the effect of hsa-miR-485-5p on HIF1A, a mimic for this miRNA was transfected into cells which were then cultured under either hypoxia or normoxia. These results show a small but distinct effect of this miRNA on the endogenous levels of HIF1A which were found to decrease. This leads to the conclusion that hsa-miR-485-5p may regulate HIF1A either directly or indirectly. It is unclear at the moment how exactly the effects on HIF1A levels can be mechanistically explained.

This same experiment was performed to determine the effect of hsa-miR-485-5p on HIF3A. These results show a slight downregulation of the HIF3A endogenous levels in sarcoma cell line SW872, which was expected if hsa-miR-485-5p indeed targets the HIF3A protein.

4.5 HSA-MIR-210 AS A REGULATOR OF HIF3A TRANSCRIPTION VARIANT 201 AND HIF1A UNDER HYPOXIA

The 3’UTR of human HIF3A transcription variant 201 also contains a single preserved binding site among mammals for hsa-miR-210. This miRNA is a known hypoxamir which is controlled by HIF-1 [Fasanaro, P. et al. (2008)]. A mimic for this miRNA was transfected into cells that were also transfected with luciferase reporter constructs containing the 3’UTR of HIF3A transcription variant 201. The results show a negative
The effect of hsa-miR-210 on the luciferase activity when compared to cells transfected with a scrambled mimic i.e. has-miR-210 expression clearly reduced luciferase activity.

A mimic for hsa-miR-210 was also transfected into cells which were then cultured under both normoxic and hypoxic conditions. The results of this experiment showed that has-miR-210 also has a negative effect on endogenous HIF1A and HIF3A protein levels after 24 hours of culturing under hypoxic conditions. This contradicts a report by Puissegur et al., where it was observed that overexpression of hsa-miR-210 leads to the maintenance of high levels of the HIF-1 protein under hypoxia [Puissegur, M. P., et al. (2010)]. However, when we consider the effect of this miRNA after 48 hour of culturing under hypoxia, it was observed that the HIF1A protein levels had not decreased as much as in cells transfected with a scrambled mimic. This observation may confirm the maintenance of the HIF1A levels in time by hsa-miR-210. The HIF1A levels in the SW872 cells, however, are much lower at 24 hours than they are in cells transfected with a scrambled mimic.
In a pilot study performed previous to this study, several miRNAs have been identified to be regulated in sarcoma cell lines when they were exposed to low oxygen tension (hypoxia). Experiments to functionally characterize one of these newly identified miRNAs, hsa-miR-485-5p and an already known hypoxamir, hsa-miR-210, have been performed. Taken together, the following conclusions can be drawn from the results of these experiments.

HIF1A protein levels increased thus validating the hypoxia conditions HIF1A is an early response factor to hypoxic stress as it is already upregulated after the cells have only been under hypoxia for 6 hours. HIF1A protein expression is decreasing in time at 24 and 48 hours of hypoxic stress.

HIF3A is present under both normoxic and hypoxic conditions. The results for HIF3A show that the upregulation of its mRNA increases as time elapses, but its protein is peaking after 24 hours of hypoxic stress and is decreasing after 48 hours. Whether the decrease of the protein at 48 hours of hypoxic stress is caused by pre- or posttranslational mechanisms cannot be deduced from the results. Further research regarding this subject is required.

From the results it can be concluded that HIF3A transcription variant 201 is indeed a direct target for hsa-miR-210 and hsa-miR-485-5p.

Transfection of mimics of both hsa-miR-210 and has-miR-485-5p have a reducing effect on the protein levels of both human HIF1A and HIF3A under hypoxic conditions. These results provide evidence that these two miRNAs do play a role in the hypoxia response pathway.
6 RECOMMENDATIONS

Several experiments have been performed to functionally characterize hsa-miR-210 and hsa-miR-485-5p, however, to fully understand their role in the hypoxia response pathway further research is necessary. In this part of the report some recommendations are proposed to gain a deeper understanding of the role and importance of these miRNAs in the hypoxia response in sarcoma cell lines.

6.1 VALIDATION OF MICROARRAY RESULTS

In a pilot study several miRNAs have been found to be upregulated in sarcoma cell lines when exposed to 1% oxygen for 24 hours using a microarray platform. Validation of these microarray results has been unsuccessful so far. Taqman probes were used to detect these miRNAs. This method relies on the sequences of the miRNAs in mirBase, however, the 3’ end of the miRNAs have already been found to be different among different experiments. Also, multiple variants could be found among one single experiment. This could be the case for hsa-miR-485-5p. On a microarray platform, multiple variant with the same seed sequence could be picked up, while a Taqman assay is only able to detect one single variant. In order to be able to detect this single miRNA with a Taqman assay, knowing the exact sequence is crucial. This can be done by deep sequencing.

Another attempt to validate the microarray results was done by Northern blotting. Hsa-miR-210, being a validated hypoxamir, was used as a positive control for this experiment. In the literature hsa-miR-210 is severely upregulated under hypoxic conditions in both normal and transformed cells. This miRNA was picked up using Northern blotting, but the signal was weak. Hsa-miR-485-5p has been identified on a microarray platform, but it’s expression under hypoxia was not very upregulated when compared to normoxia, only a factor of 1.5 higher. The Northern blotting conditions may not have been optimal and/or sensitive enough to validate these results. Optimization of this technique may be necessary for validation of the microarray results.
6.2 DETERMINING WHICH BINDING SITE FOR HSA-MIR-485-5P IS IMPORTANT FOR ITS REGULATION OF HIF3A TRANSCRIPTION VARIANT 201

One of the miRNAs that was identified on a microarray platform in a pilot study performed previous to these experiments, namely hsa-miR-485-5p, has 11 binding sites on the 3’UTR of human HIF3A transcription variant 201. This miRNA was found to be upregulated in sarcoma cell lines that were incubated 24 hours under hypoxic conditions.

In this study we cloned two amplification products of the 3’UTR of human HIF3A transcription variant 201 into the Psi-CHECK2 vector, one with only three binding sites for hsa-miR-485-5p and one that contained the entire 3’UTR. Both constructs showed to be inhibited by hsa-miR-485-5p, which leads to the conclusion that at least one if not all of the first three binding sites are of importance for the regulation of human HIF3A transcription variant 201. The more pronounced decrease of activity of the HIF3A-3’UTR regulated luciferase indicates that at least one of the remaining nine binding sites for hsa-miR-485-5p is important for its regulation as well. When further research is done it may be of interest to determine which of the binding sites for hsa-miR-485-5p are important for this regulation mechanism by mutating sites in the 3’UTR of HIF3A transcription variant 201 and cloning it behind a luciferase gene.

6.3 DETERMINING THE EFFECT OF HSA-MIR-210 AND HSA-MIR-485-5P ON THE HYPOXIA RESPONSE PATHWAY

Several experiments to determine the effect of hsa-miR210 and hsa-miR-485-5p on the hypoxia response pathway have been done in this study. In these experiments these miRNAs have been overexpressed in sarcoma cell line SW872. In this study we only looked at the effect of the miRNAs on the HIF1A and HIF3A protein levels. In future research projects other important players in the hypoxia response pathway can be looked at as well, like HIF-1 targets such as VEGF and CA-IX. The mRNA levels of HIF3A could be analysed as well, since only the protein levels were analysed in this study.

Another experiment that may be recommended is knocking the miRNAs down and then evaluate the effect this has on the protein and/or mRNA levels of HIF1A, HIF3A and other important players in the hypoxia response pathway.
6.4 DISCOVERING THE CONNECTION BETWEEN HIF3A TRANSCRIPTION VARIANT 201 AND HIF1A

One of the hypothesis during this study was that HIF3A is a regulator of HIF1A, however, no experiments to gain direct evidence to support this hypothesis were performed. An experiment that can be performed to gain a deeper understanding into the function of HIF3A and that may provide evidence to support the hypothesis that HIF3A is a regulator of HIF1A is a knock-down experiment of HIF3A (transcription variant 201). If HIF3A is knocked down with a siRNA and it is indeed a regulator of HIF1A, the HIF1A protein levels are expected to remain upregulated after 24 and 48 hours, where these levels would normally decrease to its normal levels. If HIF1A is a regulator of HIF3A and HIF3A is being knocked down, it could be hypothesised that HIF1A levels increase in an attempt to increase the HIF3A levels.
REFERENCES


19. University of Zürich, Institute of Pharmacology and Toxicology – Molecular Imaging and Functional Pharmacology (2010), *Projects to the Animal Imaging Center (AIC)*.
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APPENDIX

A. SUPPLEMENTARY DATA

SARCOMA CELL LINES USED IN THIS STUDY

13 adherent sarcoma cell lines of several subtypes will be cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) with 10% Fetal Calf’s Serum (FCS) and 10% penicillin (100 U/ml)–streptomycin (100 mg/ml).

The 13 different cell lines used in this study are displayed in table 1.

TABLE S1: CELL LINES USED IN PREVIOUS EXPERIMENTS. ENDPOINT PCRS ON THE 3'UTR OF HIF3A TRANSCRIPTION VARIANT 201 HAVE BEEN PERFORMED ON ALL OF THESE CELL LINES.

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Name</th>
<th>Sort of sarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CNIO BG</td>
<td>Myxoid fibrosarcoma</td>
</tr>
<tr>
<td>2</td>
<td>HT-1080</td>
<td>Fibrosarcoma</td>
</tr>
<tr>
<td>3</td>
<td>SW-684</td>
<td>Fibrosarcoma</td>
</tr>
<tr>
<td>4</td>
<td>SW-872</td>
<td>Liposarcoma</td>
</tr>
<tr>
<td>5</td>
<td>SW-672</td>
<td>Liposarcoma</td>
</tr>
<tr>
<td>6</td>
<td>SK-UT-1</td>
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<tr>
<td>7</td>
<td>SK-LMS-1</td>
<td>Leiomyosarcoma</td>
</tr>
<tr>
<td>8</td>
<td>A204</td>
<td>Rhabdomyosarcoma</td>
</tr>
<tr>
<td>9</td>
<td>A673</td>
<td>Rhabdomyosarcoma</td>
</tr>
<tr>
<td>10</td>
<td>SW-982</td>
<td>Synovialsarcoma</td>
</tr>
<tr>
<td>11</td>
<td>RD</td>
<td>Rhabdomyosarcoma</td>
</tr>
<tr>
<td>12</td>
<td>SJCRH-30</td>
<td>Rhabdomyosarcoma</td>
</tr>
<tr>
<td>13</td>
<td>RH-30</td>
<td>Rhabdomyosarcoma</td>
</tr>
</tbody>
</table>
All cell lines showed above will be seeded at day \( t = -2 \) at a cell density that leads to a 50% confluence at day \( t = 0 \). At \( t = 0 \) half of the cells are transferred to a hypoxic incubator (1% oxygen), the other half will be cultured under normoxic conditions. At \( t = 0 \) only normoxic cells and at \( t = 6h, t = 24h \) and \( t = 48h \) normoxic and hypoxic cultured cells will be harvested for protein and RNA isolation.
FIGURE S1: ENDPOINT PCR RESULTS OF CELL LINES USED IN A PREVIOUS INDEPENDENT EXPERIMENT. TWO PRIMER PAIRS WERE USED, NAMELY PRIMER PAIR 05 AND 06. PRIMER PAIR 05 HYBRIDIZED NEAR THE BEGINNING OF THE 3'UTR, WHILE PRIMER PAIR 06 HYBRIDIZED NEAR THE END OF THE 3'UTR OF HIF3A TRANSCRIPTION VARIANT 201. IN THE MAJORITY OF THE CELL LINES AN UPREGULATION OF HIF3A TRANSCRIPTION VARIANT 201 AFTER 24 AND 48 HOURS OF HYPOXIC CONDITIONS IS OBSERVED.