The Role of HIF-1 Alpha in the Localization of Embryonic Stem Cells with Respect to Hypoxia Within Teratomas

by

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ABSTRACT

In embryonic stem (ES) cell tumors, the hypoxia-inducible transcription factor, HIF-1α, has been shown to be a tumor suppressor, and HIF-1α-expressing cells have been shown to localize preferentially in vivo to regions near tumor vasculature. These differences were proposed to be due to increased hypoxia-induced apoptosis and growth arrest of HIF-1α-expressing ES cells. This thesis presents a careful investigation into the localization of ES cells in vitro and in vivo with respect to hypoxia. A sandwich culture system was utilized in which controlled gradients of oxygen and nutrients are developed in the vicinity of the tumor cells. A diffusion-consumption model was utilized to predict the oxygen and glucose concentration profiles within the system. Oxygen and glucose consumption rates were measured and used as inputs into the model, and the concentration profiles were found to depend on a single experimental parameter, the cell density within the system. The optimum cell density was found in which stable, measurable oxygen gradients develop over 2-3 mm. The model demonstrated excellent agreement between the predicted oxygen concentration profiles and experimentally determined oxygen gradients. In vitro, there was no difference in localization with respect to hypoxia between tumor cells expressing or lacking HIF-1α. In addition, there was no difference in apoptosis, proliferation, or migration of the tumor cells in vitro based on HIF-1α expression. Likewise, a quantitative study on localization of tumor cells within tumors in vivo demonstrated no difference between localization of HIF-1α-expressing vs. HIF-1α-lacking ES cells within tumors with respect to blood vessels or hypoxia. These results differ from previous studies, perhaps due to clonal variation of the cell phenotype or the interplay of other complex environmental factors that were not considered in this study. Interestingly, the HIF-1α-lacking cells were found to exhibit increased tumor growth relative to the HIF-1α-expressing cells, perhaps due to a normalization of the blood vessels within the HIF-1α-lacking tumors. These studies reveal the complex role of HIF-1α in tumor growth and tumor cell localization, as well as develop a useful quantitative experimental model for studying the role of the microenvironment in tumors or in embryonic stem cell biology.

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My soul finds rest in God alone; my salvation comes from Him. 
He alone is my rock and my salvation; He is my fortress, I will never be shaken. 
Ps. 62:1-2
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Chapter 1: Original Contributions

Hypoxia has long been recognized to play an important role in cancer, both in the pathophysiology of cancer and in determining the response of cancer to chemotherapeutic and radiation therapies. In recent years, the understanding of the role of hypoxia in tumors has increased dramatically with the discovery of hypoxia-inducible transcription factors (HIFs) that drive important genetic changes in response to a hypoxic environment. In general, tumors develop hypoxia due to the abnormal and inadequate vasculature that supplies the tumor as a result of pathological angiogenesis. Many of the genes upregulated by HIFs in response to hypoxia are active in pathways important to cancer development, such as angiogenesis, survival, apoptosis, and migration. In addition, several key oncogenic mutations have been shown to lead to the dysregulation of HIF activity, so that many tumors express the HIF protein complex even in the presence of normoxia. HIF-1 has been shown to have both tumor-promoter and tumor-suppressor activity depending on the conditions, while the precise role of HIF-2 in tumor biology is yet to be determined. The key function of HIF in multiple pathways in tumor development has led to clinical interest in HIF as a possible therapeutic target.

This thesis makes important contributions toward a quantitative understanding of the role of HIF in tumor development. A teratoma model derived from mouse embryonic stem cells is used as a model system for HIF involvement in tumor growth and progression. These tumors have been extensively studied using genetically modified ES cells that lack HIF-1α expression, which is the subunit of HIF that is regulated by oxygen tension and is responsible for much of the hypoxic response seen in mammalian cells. One key characteristic of these tumors that has been reported, but not well developed, is that in tumors grown from mixed cell populations of HIF-1α+/− and
HIF-1α⁺ cells, the HIF-1α⁺ cells seem to accumulate in regions distant from the vasculature. The mechanism for this accumulation of cells distant from vasculature is not completely understood. By investigating this phenomenon both in vitro and in vivo, this thesis will be invaluable in understanding the role of HIF in these tumor models and provides provocative new insights into the more aggressive phenotype seen in these tumors upon eliminating HIF-1α activity.

In order to study the mechanism for the localization of tumor cells with respect to hypoxia, it was desirable to have an in vitro model that mimics the in vivo tumor microenvironment. I have adapted a previously published sandwich culture technique by combining it with fluorescence time-lapse microscopy to provide a unique method for modeling in vitro the effect of the development of hypoxic gradients on tumor cells. This method uses state-of-the-art techniques to address previously unanswered questions about the response of tumor cells to the development of oxygen gradients.

It was desirable to be able to rationally design the experiments to measure the behavior of ES cells in oxygen gradients using the sandwich culture system. To this end, in Chapter 4, I will develop a mathematical model based on the diffusion and consumption of oxygen and glucose within the sandwich culture system to predict the oxygen and glucose concentration profiles for various experimental conditions. This model is dependent on a single parameter that can be varied experimentally, and relies on the experimental measurement of glucose and oxygen consumption rates by the ES cells. I have developed this model, measured the relevant consumption rates, and determined the optimum conditions for the design of the sandwich
culture experiments. Moreover, I have validated the model experimentally, and the theoretically predicted oxygen profiles match extremely well the experimentally measured oxygen concentration profiles measured by phosphorescence quenching microscopy. This model will be an invaluable design tool for future experiments in which well-controlled, stable, measurable oxygen gradients are desirable.

In Chapter 5, I use the sandwich culture system to investigate more deeply the localization phenomena of these ES tumor cells \textit{in vitro}, including investigating three possible mechanisms of tumor cell localization: 1) differential migration of the two cell types, 2) differential apoptosis, or 3) differential proliferation. I have determined by careful measurement that within the sandwich culture system, there is no difference in cell localization with respect to hypoxia based on HIF-1α status. Moreover, there is no difference in migration, proliferation, or apoptosis between the HIF-1α\(^{+}\) and HIF-1α\(^{-}\) ES cells in the hypoxic environment of the sandwich culture system. These findings were confirmed by more careful studies in a controlled hypoxic environment. This surprising finding of no difference between the two cell types did not validate previous reports in the literature (1) that HIF-1α\(^{+}\) ES cells exhibited increased hypoxia-induced apoptosis and growth arrest compared to HIF-1α\(^{-}\) ES cells.

Because of the controversial findings discovered \textit{in vitro} in Chapter 5, the \textit{in vivo} behavior of these tumor cells was further investigated in Chapter 6. Significantly, the HIF-1α\(^{-}\) ES tumors grew faster when grown subcutaneously than the HIF-1α\(^{+}\) tumors. This confirmed previous reports in the literature; however, combined with the \textit{in vitro} growth studies the \textit{in vivo} results are provocative. These results call into question the proposed mechanism of hypoxia-induced
apoptosis as the reason for increased tumor growth of HIF-1α⁺ ES cells. In actuality, the mechanism is likely much more complex. One intriguing possibility to be explored is that the increased tumor growth despite decreased angiogenesis, as shown in previous studies (1), may be due to the normalization of the blood vessels within the HIF-1α⁻ tumours compared to the HIF-1α⁺ tumours, leading to more efficient delivery of oxygen and nutrients to the tumors (2). The concept of normalization implies that the structure and function of the blood vessels within the tumor may be more like those in normal tissue than the dilated, leaky vessels typically found in tumors (2). This thesis also uses tumors of mixed HIF-1α⁺ and HIF-1α⁻ ES cells to make another important insight into the role of HIF in tumor growth: while the tumor growth rate is inversely proportional to the amount of HIF-1α present, the initiation of tumor growth is delayed by the presence of HIF-1α to a degree that is disproportionate to the amount of HIF-1α present.

The mixed tumors were also used to investigate the localization phenomenon in vivo. Previous studies of these mixed cell tumors have been limited by 1) the inability to visualize precisely the localization of both types of ES cells, and 2) focusing on the localization with respect to vasculature rather than direct correlation with oxygen levels. Previous studies showing the accumulation of HIF-1α⁻ cells distant from vasculature have relied on indirect methods of analyzing the tumors or have only directly visualized the HIF-1α⁻ cells without a clear picture of the location of the HIF-1α⁺ cells. Also, these studies have only related the cell location to vasculature and have inferred a relation to hypoxia by assuming that regions distant from vasculature would be more hypoxic. In this thesis, I have directly visualized for the first time both cell populations within the mixed cell tumors, and have measured the localization of tumor cells with respect to both blood vessels and hypoxia quantitatively in a way that has not been
feasible in previous studies. Interestingly, using these more quantitative and exhaustive methods of analysis, I have shown that there is in fact no difference in localization with respect to blood vessels or hypoxia between the HIF-1α⁺⁺ and HIF-1α⁻⁻ ES cells.

These studies have provided significant contributions to the literature by calling into question key fundamental assumptions about ES cell tumors based on previous studies. In this thesis, these cells have shown no tendency to localize near blood vessels, and no difference in apoptosis or proliferation based on HIF-1α status. All of these findings contradict previous findings using different ES clones from the same source. Thus, these studies raise the potential that there is significant clonal variation in the ES cells that may affect their tumor phenotype, or that unidentified environmental factors may play a crucial role in directing the cell behavior. The findings also raise the possibility of a difference in the functionality of the blood vessels between the tumors due to a normalized vessel phenotype within the HIF-1α⁺⁺ tumors (2). Regardless of the reason for these differences, this thesis makes an important contribution by raising these questions and perhaps giving reason for caution in using these ES cell tumors to generalize to more universal principles regarding tumor growth and phenotype based on the presence or absence of HIF-1α.

The previous demonstration of increased tumor growth of HIF-1α⁺⁺ ES tumors appropriately resulted in an increased level of caution among researchers investigating HIF-1 as a possible target for tumor therapy. The understanding of why HIF-1α behaves as a tumor suppressor in these tumors remains an important goal if successful HIF-targeted therapy is ever to make its way to the clinic. This thesis provides a significant corollary to previous studies by indicating
that the mechanism of increased tumor growth may be more complex than anticipated. Further studies are indeed warranted to investigate the reasons for the discrepancies in the literature. It remains to be seen whether the ambiguous results seen in these ES cell tumor studies can provide insight into a broader array of cancers, but the insights gained from this work will be critical in the further pursuit of this and other unanswered questions in the continuously unfolding story of hypoxia, HIF, and cancer.
Chapter 2: Introduction and Specific Aims

Despite many advances in detection and treatment of malignancies, cancer remains the second leading cause of death in the U.S., exceeded only by heart disease. Recently, the importance of the tumor microenvironment in tumor growth and progression has come to the forefront of cancer research, especially with developments in the understanding of how angiogenesis plays a role in tumor development (3). Angiogenesis is the process by which new blood vessels are formed by the budding of endothelial sprouts, primarily from post-capillary venules, to produce new capillary networks. Because mammalian cells require oxygen and nutrients for growth and survival, tumors cannot grow beyond a critical size (on the order of 100 μm) without the recruitment of blood vessels. The regulation of angiogenesis is primarily regulated by a balance between pro-angiogenic and anti-angiogenic molecules, which can be produced by cancer cells, endothelial cells, stromal cells, blood and the extracellular matrix (4-6).

Multiple signals can lead to the ‘angiogenic switch’ – that is, the tipping of the balance in favor of pro-angiogenic molecules – including metabolic stress, mechanical stress, immune response, inflammation, and genetic mutations (3). The focus of this work is on the specific metabolic stress placed on cancer cells when the cells experience hypoxia – a reduction in the normal tissue oxygen tension. Hypoxia has been found to be a key regulatory factor in the growth of tumors and the induction of angiogenesis (7). A primary means by which hypoxia effects its response in mammalian cells is through the hypoxia-inducible factor (HIF) family of transcription factors. This thesis will attempt to elucidate the role of the primary member of the HIF family, HIF-1, in the localization of tumor cells with respect to hypoxia in tumors.
2.1 Background

2.1.1 The HIF Family of Transcription Factors

The HIF (hypoxia-inducible factor) transcription factors are members of the basic helix-loop-helix (bHLH) superfamily of transcription factors. The HLH domains mediate the dimerization of subunits, while the basic units are involved in DNA binding. A family of these bHLH proteins also contain an auxiliary dimerization interface, the PAS domain, named for the first three proteins discovered with this structure (PER, ARNT, and SIM) (8). All bHLH-PAS proteins are heterodimers composed of a class I subunit and a class II subunit. In the HIF transcription factors, the class I subunit is the aryl hydrocarbon receptor nuclear translocator (ARNT) also known as HIF-1β. The class II subunit is either HIF-1α, HIF-2α (also known as EPAS-1), or HIF-3α.

HIF-1α and HIF-1β mRNA expression is ubiquitous in adult and embryonic human and mouse tissues, having been found in every tissue that has been analyzed (9, 10). HIF-2α, in contrast, is expressed in vascular endothelial cells, catecholamine-producing cells, kidney, and lung during embryogenesis (9, 11-14). HIF-3α has also been identified, although its function is much less understood. It has been shown to be expressed in the adult mouse thymus, lung, brain, heart, and kidney (15), as well as the human kidney (16), where it may play a role in suppressing hypoxia-inducible HIF-mediated gene expression. HIF-1α and HIF-2α have a high degree of sequence homology within the bHLH-PAS and transactivation domains, and thus have similar propensities to dimerize with HIF-1β, bind to DNA, and activate transcription (13, 17). It is not well understood whether HIF-2α and HIF-1α activate distinct target genes in certain cell types, or whether they are somewhat redundant in action. This thesis deals with the role of HIF-1α in an
embryonic stem cell teratoma model (a benign tumor), with the implicit understanding that future work should also focus on the auxiliary role of HIF-2α as well as utilizing more malignant tumor models for broadening the impact of these studies.

2.1.2 Mechanism of HIF Induction by Hypoxia

The primary function of HIF-1 is to regulate mammalian oxygen homeostasis by mediating the physiological and pathophysiological responses of mammalian cells to hypoxia (18, 19). To accomplish this, HIF-1 must be involved in the sensing of decreased oxygen concentration, or hypoxia, by cells. HIF-1 is composed of the HIF-1β subunit, which is constitutively expressed, and HIF-1α, which is the hypoxic response factor (20). For several years after the discovery of HIF-1α, the mechanism for oxygen sensing was unknown, but two groups recently discovered the role of a prolyl hydroxylase enzyme (a tetramer with two hydroxylase units and two protein disulfide isomerase units) in the regulation of HIF-1α expression by oxygen levels (21, 22).

The prolyl hydroxylase responsible for oxygen sensing requires oxygen, ferrous iron, and 2-oxoglutarate for activity, and its mode of action is to covalently modify HIF-1α by hydroxylation of two conserved proline residues at amino acid 402 and 564 of the 826 amino acid HIF-1α subunit (23, 24). These residues lie within the larger region previously known as the oxygen-dependent degradation (ODD) domain of HIF-1α (25). Prolyl hydroxylation is necessary and sufficient for interaction of HIF-1α with the von Hippel-Lindau (VHL) tumor suppressor protein (21, 22, 26, 27). VHL is the recognition component of an E3 ubiquitin ligase that targets HIF-1α for proteasomal degradation (28-32). Under physiological conditions, oxygen is a limiting substrate for the prolyl hydroxylation (24), thus providing the mechanism for the oxygen-dependent decrease in HIF-1α expression (33).
Another mechanism of hypoxic regulation of HIF-1α is through the modulation of transcriptional activation of HIF-1α. Under hypoxic conditions, the C-terminal transactivating domain (C-TAD) of HIF-1α, localized between residues 786-826, interacts with transcriptional co-activators such as P300/CBP to promote transcription of hypoxia-responsive targets (34-36). The mechanism for this hypoxic induction has also been determined to be due to inhibition of hydroxylation of HIF-1α, in this instance at the asparagine residue at amino acid 803 in a conserved region of the C-TAD domain (37). The asparagine hydroxylase responsible for this regulation has been identified as factor inhibiting HIF-1 (FIH-1) (38-41). Thus, oxygen-dependent hydroxylation provides a common method for the interactions of HIF-1α with other proteins to be regulated at multiple levels. The overall result is both increased expression and increased activity of HIF-1α under hypoxic conditions.

2.1.3 Oxygen-Independent Regulation of HIF

Many of the same processes that are necessary for oxygen homeostasis upon exposure to hypoxia are also necessary when cells proliferate, thereby increasing oxygen consumption. To link these two pathways, many of the proliferative and survival signals initiated by growth factor receptors also induce HIF-1α expression. Unlike hypoxia, growth factor activity stimulates HIF-1α synthesis rather than affecting protein degradation. The mechanism for increased synthesis involves activation of the phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathways (42-46). The PI3K/AKT pathway acts through the downstream kinase mammalian target of rapamycin (mTOR), as the stimulation of HIF-1α synthesis can be blocked by treatment with rapamycin (44). The RAF-MEK-ERK kinase pathway not only stimulates HIF-1α protein synthesis, but has also been shown to stimulate the function of the HIF-1α
transactivation domain (47, 48). The oxygen-independent regulation of HIF-1α differs from the universal hypoxic regulation of protein stability in that it is cell-type specific. For example, HER2 (neu) signaling through the PI3K pathway induces HIF-1α synthesis but does not influence transactivation domain function in MCF-7 breast cancer cells (44), whereas rapamycin treatment in PC-3 prostate cancer cells inhibits protein stability and transactivation domain function (49).

HIF-1α activity can also be affected by several oncogenic mutations that activate signal transduction pathways. One of the most dramatic examples is loss of the tumor suppressor protein VHL function in clear-cell renal cell carcinoma (RCC) and cerebellar hemangiomas (30, 50). Because VHL is necessary for the ubiquitylation and proteasomal degradation of HIF-1α, VHL loss of function results in dramatic increases in HIF-1α levels under non-hypoxic conditions. Mutation of several other oncogenes and tumor suppressor genes also leads to increased HIF-1α activity; notable examples are p53 loss of function (51), PTEN loss of function (42, 43), ERBB2 gain of function (44), SRC gain of function (52), ARF loss of function (53), and BCL2 overexpression (54).

HIF-1α can also exhibit autocrine signaling to regulate its own synthesis. Several growth factors are also HIF-1α target genes, such as insulin-like growth factor 2 (IGF2) and transforming growth factor-α (TGF-α) (55, 56). When these growth factors act on the insulin-like growth factor 1 receptor (IGF1R) and epidermal growth factor receptor (EGFR), respectively, the signal transduction pathways described above are activated and HIF-1α synthesis is increased. In this way, HIF-1α can act to increase its own production in an autocrine manner.
2.1.4 Activity of HIF Through its Target Genes

Having established the upregulation of HIF-1α expression and activity under conditions of hypoxia and growth factor stimulation, the obvious question raised is how does HIF-1α mediate responses to these conditions? Much of HIF-1α’s role in oxygen homeostasis can be understood by examining the types of genes that are upregulated by HIF-1α activity. When HIF-1α is stabilized by hypoxic conditions, it translocates to the nucleus, where it can form complexes with HIF-1β, CBP/p300 and the DNA polymerase II (Pol II) complex to bind to hypoxia-responsive elements (HREs) in the promoters of target genes and activate transcription (57).

More than 60 putative HIF-1α target genes have been identified (reviewed in (58)). References for many of these discovered genes can be found in reference (59). Notable protein products of these target genes are involved in many processes that are important for oxygen homeostasis, such as: cell proliferation, cell survival, apoptosis, motility, cell adhesion, erythropoiesis, angiogenesis, vascular tone, transcriptional regulation, pH regulation, glucose metabolism, extracellular matrix metabolism, energy metabolism, and amino acid metabolism.

Expression of some HIF-1α target genes, such as vascular endothelial growth factor (VEGF), a key player in angiogenesis, is induced by hypoxia in most cell types. However, expression of most target genes is induced in a cell-type specific manner. It is the interaction of HIF-1α with other transcription factors in a particular cell that determines the subgroup of target genes that are activated in hypoxia (58). Therefore, there is no universal program for cells to follow under hypoxic conditions. Some may have a robust angiogenic response, some will turn on survival pathways, and others may activate ‘suicide signals’ leading to apoptosis. Thus, the role of HIF-
1α in tumorigenesis, for example, may not be as clear-cut as it seems at first glance. Important insights can be gained, however, by examining the evidence that HIF-1α is involved in the pathogenesis of human cancers.

2.1.5 HIF and Human Cancers

Four groups of HIF-1α target genes that are particularly relevant to cancer progression are: 1) angiogenic factors (VEGF, endoglin, VEGFR2), 2) genes involved in glucose metabolism (aldolase A, enolase 1, glyceraldehyde-3-P-dehydrogenase, glucose transporters, hexokinase, lactate dehydrogenase, and other glycolytic enzymes), 3) survival factors (erythropoietin, endothelin-1, insulin-like growth factors and binding proteins, transforming growth factor-α), and 4) factors involved in invasion and metastasis (autocrine motility factor, cathepsin D, fibronectin 1, matrix metalloproteinase 2, urokinase plasminogen activator receptor, and vimentin) (56). The role of HIF in metastasis is probably the least understood, but two recent discoveries have renewed interest in this connection. It was discovered that HIF-1 activates the Met tyrosine kinase receptor, a key controller of invasive tumor growth, in multiple cell lines, from both normal tissues (breast epithelium and hepatocyte precursors) and tumors (A549, human lung carcinoma; SK-OV-3, human ovarian carcinoma; SiHa, human cervical carcinoma; HepG2, human hepatocarcinoma; U2-OS, human osteosarcoma) (60). Also, it was shown that CXCR4, a chemokine receptor, was activated by HIF-1 in A498 renal cell carcinoma cells, and that this activation causes tumor cells to migrate and home in on specific organs (61).

Given the types of genes activated by HIF-1 mentioned above, it is not surprising that HIF-1α has been extensively studied in multiple cancer phenotypes. Immunohistochemical analysis of tumor tissue compared to normal tissue revealed that many human cancers have higher than
normal levels of HIF-1α protein, including colon, breast, bladder, brain, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas (50, 62). Significant positive correlations between higher than normal HIF-1α expression and patient mortality have been demonstrated in cervical cancer (63, 64), breast cancer (65, 66), oligodendroglioma (cancer of the brain) (67), oropharyngeal squamous cell carcinoma (68), ovarian carcinoma (with p53 mutation) (69), uterine endometrial cancer (70), and gastrointestinal stromal tumors of the stomach (71). There have been contradictory reports on whether there is increased or decreased mortality with higher than normal HIF-1α expression in head and neck cancer (72, 73) and in non-small-cell lung cancer (74, 75). In the ovarian cancer study (69), higher than normal HIF-1α expression alone was correlated with increased apoptosis and increased patient survival. When higher than normal HIF-1α expression was combined with a p53 mutation that led to p53 overexpression, apoptosis levels were low and survival was decreased. In patients with early-stage esophageal cancer, the combination of higher than normal HIF-1α and BCL2 expression was associated with a decreased response to photodynamic therapy (76). It is clear that the effect of higher than normal HIF-1α expression, while exhibiting some similarities across tumor types, is ultimately dependent not only on the type of cancer but also on the local microenvironment as well as the genetic alterations present within the tumor, such as mutations in the apoptosis pathways. Further mechanistic studies of HIF-1α expression in cancer have been performed in xenografts of human tumors in animal models.

2.1.6  *In Vivo* Manipulation of HIF Expression in Tumors

Many xenograft studies in which tumor cells are grown subcutaneously in immunodeficient mice have been performed to study the effect of HIF-1α on tumor growth and progression. In mouse
hepatoma cell lines (Hepal) deficient in HIF-1β, xenografts showed reduced tumor growth and angiogenesis with increased necrosis compared to wild type tumors (52, 77).

HIF-1α deficient mouse embryonic stem (ES) cells have also been studied and these studies form the basis for the studies described in this thesis. Two independently derived HIF-1α⁺ ES cells were used in studies of xenograft tumor growth. Both studies showed markedly impaired vascularization of the HIF-1α⁺ tumors compared to the wild type tumors (1, 78). In one study, HIF-1α⁺ tumors also demonstrated decreased apoptosis and increased cell proliferation, leading to overall increased tumor growth of the HIF-1α⁺ tumors (1). In the other study, however, tumor growth was reported to be decreased in the HIF-1α⁺ tumors due to the decreased angiogenesis (78). It seems in these studies as if the genetic background of either the ES cells or the xenograft recipient mice play a role in determining the effect of HIF-1α deficiency on tumor growth.

The study showing increased tumor growth of HIF-1α deficient embryonic stem cells (1) is provocative because this increased tumor growth occurred in spite of decreased angiogenesis. A follow-up study from our lab demonstrated that the HIF-1α⁺ ES cell tumors expressed significantly less VEGF than WT tumors, and that deleting the HRE element in the VEGF promoter caused an even more dramatic decrease in VEGF expression (79). This indicates that the hypoxia-response element is extremely important in driving VEGF expression in these cells, although only part of this response is due to HIF-1α activity. It is possible that HIF-2α plays a role in the increased VEGF expression of HIF-1α⁺ tumors compared to HRE⁻⁺ tumors, although its role has not been demonstrated experimentally. This paradoxical increase in tumor growth despite decreased VEGF levels and angiogenesis suggests that the remaining vessels within the
HIF-1α+/ tumors may be more efficient in delivering oxygen and nutrients to the tumor. However, this concept of “normalization” of blood vessels (2) within the ES tumors was not investigated, and the difference in tumor growth was attributed to the hypoxia-induced apoptosis and growth arrest in the HIF-1α−/ tumors (1).

A separate group proposed that the paradoxical increase in tumor growth with decreased angiogenesis could be due to a decreased vascular dependence of the cells that lose HIF-1α (80). To investigate, this group developed tumor xenografts from mixtures of HIF-1α+/+ and HIF-1α−/ ES cells and examined the distribution of each cell type relative to perfused blood vessels. Their results suggested that HIF-1α−/+ cells predominantly and preferentially localized to areas immediately surrounding perfused blood vessels. Their technique relied on the uptake of an i.v. injected dye by the cells as well as a cell sorting protocol after sacrifice of the animals. Multiphoton in vivo microscopy of tumors developed from a mixture of green fluorescent protein (GFP)-labeled HIF-1α−/ ES cells and non-fluorescent HIF-1α+/+ ES cells showed a similar preferential location of HIF-1α−/ cells distant from the vasculature, although interpretation of the images is complicated by the inability to visualize the HIF-1α++ cells (see Figure 2.1) (81).
Figure 2.1 a–c. The tumor is derived from wild-type embryonic stem cells and embryonic stem cells that lack the gene for HIF-1α and express EGFP. The tumor vasculature is highlighted by TMR-BSA injected systemically. Distant from the vasculature (red), HIF-1α−/− (EGFP-expressing) cells appear to predominate (green). Each image is spaced 10 μm apart. Scale bars, 50 μm. Adapted from Ref. (81)

One experiment demonstrated decreased tumor growth in xenografts derived from ES cells that expressed HIF-1α under normoxia due to a VHL mutation (82). However, most xenograft tumor studies have shown the opposite effect: a positive correlation between HIF-1α expression and tumor growth. Transformed mouse embryonic fibroblasts that were deficient in HIF-1α demonstrated decreased tumor growth, although tumor angiogenesis was not affected (83). HCT116 colon cancer cells that overexpressed HIF-1α exhibited increased VEGF expression, xenograft growth, and angiogenesis (51). HIF-1α overexpression in PCI-10 pancreatic cancer cells also caused increased xenograft growth without an effect on microvascular density (84). In contrast to the role of HIF-1α in ES cell xenografts (1), HIF-1α conferred on transformed fibroblasts and PCI-10 cells a resistance to apoptosis under adverse conditions (84, 85). The
difference may be due to a difference in p53 status between these cell types. HIF-1α in renal cell carcinoma xenografts will be reviewed separately in the next section.

Inhibition of HIF-1α in tumor cells often has the effect of decreasing xenograft tumor growth. Overexpression of dominant-negative HIF-1α in PCI-43 pancreatic cancer cells led to an increase in apoptosis under hypoxic conditions in vitro and a decreased ability to form tumors in vivo in severe combined immunodeficient (SCID) mice with no effect on angiogenesis (86, 87). A fusion protein designed to inhibit HIF-1-dependent transcription by blocking HIF-1 interaction with coactivators CBP and p300 caused reduced tumor growth when expressed in human breast cancer cells (MDA-MB-435) and HCT116 colon cancer cells (36).

Until recently, the only model that had shown a negative correlation between HIF-1α expression and tumor growth had been in ES cell-derived tumors, which have often been criticized as not reflective of the behavior of cancer cells which have multiple genetic mutations. However, another common factor in all of the experiments showing a positive correlation between HIF-1α and tumor growth has been the ectopic subcutaneous site of tumor growth. A recent study (88) highlighted the importance of tumor microenvironment in the role of HIF-1α as well as demonstrating another example of decreased HIF-1α expression leading to increased tumor growth. Growth of HIF-1α deficient transformed astrocytes in the subcutaneous environment resulted in severe necrosis, reduced growth, and reduced vessel density of the xenografts. In contrast, when the astrocytomas were grown in the brain parenchyma (an orthotopic site), tumors deficient in HIF-1α grew faster and penetrated the brain more rapidly and extensively. Thus, the full story of the role of HIF-1α in tumor growth and progression continues to unfold.
2.1.7 HIF-1α, HIF-2α, and Renal Cell Carcinoma

The effect of HIF-1α and HIF-2α on renal cell carcinoma (RCC) is of particular interest because the defining genetic lesion in RCC is often a loss of VHL function, which results in high levels of the HIF-1α and/or HIF-2α protein constitutively (89-91). In fact, clear cell renal cell carcinoma is one of a variety of tumors commonly seen in the clinical phenotype of von Hippel-Lindau disease, a hereditary syndrome characterized by loss of heterozygosity of the VHL tumor suppressor gene (92). A commonly used experimental renal cell carcinoma, 786-O, only expresses HIF-2α. Expression of VHL in this cell line by introduction of a VHL expression vector caused HIF-2α expression to become regulated by oxygen and xenograft growth was reduced (93). Introduction of a mutated HIF-2α that is not oxygen-regulated into the VHL-expressing 786-O cells led to increased tumor growth even beyond the level seen in the VHL-deficient cells, indicating that HIF-2α inhibition is a necessary component of the tumor suppressor function of VHL (94). A similar study demonstrated that introducing a fusion protein containing the oxygen-dependent domain of HIF-1α blocked HIF-2α substrate recognition by VHL-expressing 786-O cells, leading to normoxic HIF-2α accumulation and increased tumor growth (95). More recent studies have shown that inhibition of HIF-2α by small interfering RNA sequences is sufficient to induce tumor suppression in VHL-deficient 786-O xenografts, suggesting a critical role for HIF degradation in the tumor suppressor function of VHL (96, 97).

Interestingly, while the mutated HIF-2α (constitutively expressed) introduced into VHL-expressing 786-O cells led to increased xenograft growth (94), a similar introduction of mutated HIF-1α (also constitutively expressed) tended toward decreased tumorigenesis in the same cell line (95). This suggests that HIF-1α and HIF-2α could play distinct, non-overlapping roles in
renal cell carcinoma, although the biological significance is unclear because the cell line in question does not naturally express HIF-1α. However, there are data that suggest that these two highly similar transcription factors have somewhat different transcription activation profiles and functions. HIF-2α has preferentially been associated with protection of embryonic stem cells from hypoglycemia, whereas HIF-1α protects cells from both hypoxia and hypoglycemia (98, 99). In transformed mouse embryonic fibroblasts, HIF-1α, but not HIF-2α, was degraded in hypoxic conditions (100). HIF-2α expressed in a mutant CHO cell line was a stronger activator of VEGF than HIF-1α (101). Further, as mentioned earlier, HIF-1α and HIF-2α demonstrate cell-type specificity with little redundancy (9). In breast cancer cells and endothelial cells that expressed both HIF-1α and HIF-2α, hypoxia-inducible responses were critically dependent on HIF-1α, but not HIF-2α (102).

2.2 Thesis Aims

This thesis provides a more thorough investigation of the phenomena of tumor cell localization with respect to hypoxia based on HIF-1α activity, as seen in the embryonic stem cell tumor model. Using multiphoton microscopy, Brown et al. (81) visualized tumors of mixed HIF-1α+/− non-fluorescent cells and HIF-1α+/GFP-labeled cells. Their findings suggested that the HIF-1α+/− cells were preferentially located distant from the vasculature (see Figure 2.1), in presumed areas of hypoxia. In a related study, Yu et al. (80) labeled and isolated tumor cells from mixed HIF-1α+/−/HIF-1α−/− tumors based on their relative proximity to perfused vessels. They hypothesized that the faster growth of HIF-1α−/− tumors was due to a decreased rate of apoptosis in hypoxia, resulting in reduced vascular dependence of these cells. Using an intravital Hoechst 33342 staining technique, they analyzed the 5% of cells closest to blood vessels and the 5% of cells most distal from blood vessels. They found that the 5% perivascular cell population had a
higher proportion of HIF-1α⁺⁺ cells and the 5% distal cell population had a decreased proportion of HIF-1α⁺⁺ cells.

This finding of localization of tumor cells with respect to hypoxia is important in the understanding of the heterogeneity of tumors. Hypoxic areas of tumors are typically difficult to treat due to the decreased accessibility to chemotherapeutic drugs and a higher radioresistance during radiation therapy because of a reduced production of toxic reactive oxygen species within the hypoxic areas. The discovery of a single factor that controls the dependence of the tumor cells on oxygen and blood supply could lead to a better understanding of tumor development and possible therapeutic targets for future study. However, the previous studies mentioned above left key questions unanswered as to the mechanism of tumor cell localization with respect to hypoxia. Given the role of HIF-1 in a variety of cellular adaptations to hypoxia, multiple possible mechanisms could contribute to the developing localization seen in vivo. This thesis aims to study these phenomena more in depth in both in vitro and in vivo systems, using the embryonic stem cell model described above to address these questions. Specifically, the working hypothesis for this thesis is the following.

**Hypothesis:**

**HIF-1α activates multiple pathways in ES cells, leading to:**

i) increased migration of HIF-1α⁺⁺ cells relative to HIF-1α⁺⁺ cells, allowing them to “find” regions of higher oxygen concentration,

ii) decreased proliferation of HIF-1α⁺⁺ cells relative to HIF-1α⁺⁺ cells in hypoxic areas, and/or

iii) increased hypoxia-induced cell death of HIF-1α⁺⁺ cells in hypoxic areas.
The primary question addressed in this thesis is to determine the relative contribution of these three mechanisms to tumor cell localization with respect to hypoxia.

**SPECIFIC AIM 1:** *Develop a method to study the localization of ES cells with respect to hypoxia in a quantitative, dynamic, and direct manner.*

In order to fully explore the mechanisms of tumor cell localization with respect to hypoxia, it is first necessary to develop a method of visualizing the localization in a way that is 1) quantitative such that the degree of localization of the HIF-1α⁺⁺ cells vs. HIF-1α⁺ cells can be accurately measured; 2) dynamic such that the localization can be followed over time during and after the development of hypoxia; and 3) direct in that both cell types are visualized such that the location of the cells can be easily identified. The first aim of the thesis is to develop an *in vitro* method of measurement that attains these features.

**SPECIFIC AIM 1A:** *Create ES cells that can be directly visualized independently based on the presence or absence of HIF-1α in the cells.*

One of the key limitations of previous studies regarding the localization of ES cells with respect to hypoxia based on HIF-1α status has been the inability to directly visualize the location of the cells. In the primary study investigating the preferential location of HIF-1α⁺⁺ cells near the vasculature (80), Yu et al. claim to directly show the preferential localization of HIF-1α⁺⁺ cells proximal to the vasculature. However, their method involves injection of a DNA-binding dye, sacrifice of the animals, and FACS sorting to sort out the 5% of cells that took up the most dye
and the 5% that took up the least. They label these fractions “proximal” and “distal” and analyze them for expression of wild-type or mutant (knock-out) HIF-1α. Because the signal for the wild-type gene is weak or absent in the “distal” population, they claim that this is direct evidence for the localization of HIF-1α<sup>+</sup> cells proximal to the vasculature. The problem with this method is twofold: 1) they only look at 10% of the total cell population in analyzing the tumor cell localization; and 2) there is no quantification of the distance from the vasculature of these “distal” cells. They also attempt to use labeling techniques to label the cells with fluorescent protein or lacZ expression, but they only label the HIF-1α<sup>++</sup> cells. When they demonstrate a localization of lacZ-positive cells around blood vessels, there is no corresponding way to visualize the localization of the HIF-1α<sup>-</sup> cells.

In this thesis, to circumvent these problems and more directly visualize the localization of cells both in vitro and in vivo, the ES cells were fluorescently labeled such that the HIF-1α<sup>++</sup> ES cells expressed CFP (cyan fluorescent protein), and the HIF-1α<sup>-</sup> ES cells expressed YFP (yellow fluorescent protein). It was determined that expression of CFP or YFP did not affect the tumor growth phenotype of either cell type. In this way, fluorescence microscopy could be used to directly visualize the two cell types independently and in the same tumor or in vitro system.

**SPECIFIC AIM 1B:** Develop a method to visualize the cells directly under conditions of developing oxygen gradients in real time so that their behavior can be analyzed during the initial development and maintenance of hypoxic conditions.
In order to further investigate the mechanisms for the localization of tumor cells with respect to hypoxia, it is necessary to be able to visualize directly the behavior of the cells under different oxygen conditions. Ideally, the dynamics of the tumor cell localization would be followed in vivo as a function of tumor growth. However, for the sake of quantification, a controlled system is desired in which the oxygen levels can be readily measured and the dynamics of the cell behavior can be followed over time. Also, because migration is one of the mechanisms being investigated, it is desirable to design a system in which gradients of oxygen can be created and sustained over time to provide the proper stimulus for migration.

The sandwich culture system provides a simple, effective means of meeting the desired objectives. In the sandwich culture system (103), a single layer of cells is subjected to self-created gradients of nutrients and metabolic products, including oxygen. These systems have been likened to multicellular spheroids as tumor analogues, because of the development of a ‘viable rim’ of cells (in this case near the edge of the sandwich culture), as well as a “necrotic center” (the hypoxic interior of the sandwich culture system). This well-controlled microenvironment mimics the conditions experienced in tumors, where greater inter-blood vessel distances and pathological blood flow leads to the development of hypoxic regions distant from the functional vasculature. The advantage of the sandwich culture system is the ability to microscopically view the entire system of cells throughout the experiment, providing a “window” into the behavior of tumor cells during the development of hypoxia. In this thesis, the sandwich culture system will be combined with time-lapse fluorescence microscopy to dynamically view the cells during the development of oxygen gradients.
SPECIFIC AIM 1C: Apply a method of quantitatively measuring oxygen levels by phosphorescence quenching in the real-time visualization system developed in Aim 1B.

The final technique necessary to fully characterize the system developed in Aim 1B is the ability to accurately measure the spatial and temporal variations in oxygen tension within the sandwich culture system. The phosphorescence quenching of porphyrin derivatives by oxygen provides an optical method of measuring oxygen tension at a high resolution (104-106). The ability to accurately and reproducibly measure oxygen levels will be demonstrated and applied to the sandwich culture system using a temperature-controlled chamber to allow for following of oxygen levels over time within the system.

SPECIFIC AIM 2: Determine whether localization with respect to hypoxia occurs in vitro in the sandwich culture system and determine the mechanism by which localization occurs.

The sandwich culture system provides an ideal, well-controlled environment in which to investigate the localization of ES cells with respect to hypoxia based on their HIF-1α status. However, the system must first be validated to determine whether the desired conditions are satisfied and whether the localization seen in vivo occurs in the in vitro system. The aim of this part of the thesis is to demonstrate the localization of ES cells with respect to hypoxia within the sandwich culture system and to determine the mechanism by which the localization occurs.
**SPECIFIC AIM 2A:** Quantify the development of oxygen profiles in the sandwich culture over time after introduction of the sandwich culture conditions.

First, the oxygen profiles within the sandwich culture system will be quantified and followed over time to characterize the system. The goal of the sandwich culture system is to have stable oxygen gradients develop and persist over a period of time sufficient to visualize the cell behavior and determine the mechanism of localization of the cells.

**SPECIFIC AIM 2B:** Mathematically model the development of oxygen and glucose profiles in the sandwich culture system to determine the experimental parameters that can be optimized to better control the oxygen profile.

Once the measurement of oxygen within the sandwich culture system has been measured, it may be necessary to “tune” the system in order to obtain stable gradients that are over a distance great enough to allow adequate observation of the cell behavior microscopically within the gradient. In order to accomplish this in a rigorous, methodical manner, a mathematical model will be developed characterizing the oxygen and glucose concentration profiles within the system. The model will allow for the identification of key parameters within the system that can be experimentally altered to modify the oxygen and glucose profiles. Appropriate measurements of the various parameters within the model will be made. The model proposed for the sandwich culture in this thesis is a set of coupled diffusion-reaction equations in which oxygen and glucose are the primary nutrients analyzed. The oxygen and glucose coupled diffusion equations will be
solved accounting for the diffusion of the nutrients from the outside of the sandwich culture and the consumption of the nutrients within the sandwich culture by cellular metabolism.

**SPECIFIC AIM 2C:** Optimize the sandwich culture system to create suitable oxygen gradients and directly visualize the behavior of HIF-1α+/− cells and HIF-1α−/− cells with respect to hypoxia, quantifying the dynamics of localization using time-lapse microscopy.

Using the model developed in Aim 2B, the key experimental parameters within the sandwich culture system will be varied to obtain a system in which stable, long-term oxygen gradients are developed and maintained. Then, using fluorescence time-lapse microscopy, the fluorescently labeled HIF-1α+/− and HIF-1α−/− cells will be visualized microscopically in the sandwich culture system. In this way, the development of localization of cells with respect to hypoxia will be observed over time. A method of quantifying the number of each cell type in normoxia vs. hypoxia will be developed so that the dynamics of the localization can be characterized objectively.

**SPECIFIC AIM 2D:** Determine the contribution of migration, apoptosis, and proliferation to the localization of tumor cells with respect to hypoxia in the sandwich culture system.

Using the sandwich culture system as described above, the next objective will be to analyze the localization of tumor cells with respect to hypoxia to determine the mechanism by which it occurs. As described previously, the likely effects of hypoxia through HIF-1 that could have an effect on tumor cell localization are the increased migration, increased apoptosis, or decreased
proliferation of HIF-1α<sup>+/+</sup> cells when exposed to hypoxia. The sandwich culture system provides an ideal technique to investigate each of these proposed actions in more detail.

Specifically, the migration of cells under exposure to oxygen gradients will be studied using time-lapse microscopy of the cells within the sandwich culture as the oxygen gradients develop and are maintained over time. Apoptosis will be investigated by staining the cells at specific time points in the sandwich culture for markers of apoptosis, and proliferation will be studied both in the sandwich culture by visualizing the increase in cell density over time and by MTT and cell-counting proliferation assays under normoxia and hypoxia. All of these proposed mechanisms will be compared between HIF-1α<sup>+/+</sup> and HIF-1α<sup>−/−</sup> cells, making use of the fluorescently labeled cells as described in Aim 1A to simultaneously monitor the two cell types.

**SPECIFIC AIM 3:** *Directly visualize localization of HIF-1α<sup>+/+</sup> and HIF-1α<sup>−/−</sup> cells with respect to hypoxia in vivo.*

The findings in the *in vitro* sandwich culture system will finally be validated by looking at *in vivo* tumor models. First, the localization with respect to blood vessels of both HIF-1α<sup>+/+</sup> and HIF-1α<sup>−/−</sup> cells will be analyzed simultaneously. As mentioned above, previous studies have been limited by their ability to only visualize directly one cell type and infer the behavior of the opposing cell type. With this in mind, the more rigorous studies carried out in this thesis will provide more definitive evidence of tumor cell localization based on HIF-1α status. Second, the localization will be investigated *in vivo* with respect to hypoxia rather than focusing solely on blood vessels. It has been shown that oxygen levels in tumors do not necessarily correlate with
distance from blood vessels (106), so the previous studies have not actually shown a hypoxia-dependent localization, only a blood vessel-dependent localization. This thesis will provide a more direct measurement of localization with respect to hypoxic regions of tumors.

**SPECIFIC AIM 3A:** Determine the effect of CFP/YFP transfection on tumor growth in vivo.

The first goal of the *in vivo* studies will be to ensure that the transfection of ES cells with CFP or YFP does not affect their tumor growth behavior. Growth curves for transfected tumors will be compared with growth curves for non-transfected tumors to determine the effect of transfection on tumor growth, if any.

**SPECIFIC AIM 3B:** Measure effect of heterogeneous cell population on ES cell-derived tumor growth.

Next, the effect of mixing tumor cell populations on tumor growth will be investigated. In previous studies in tumor models in which HIF-1α acts as a tumor promoter, it has been shown that a very small fraction of HIF-1α⁺⁺ cells mixed in with HIF-1α⁻⁻ cells can recapitulate the HIF-1α⁺⁺ tumor growth behavior(107). This is believed to be due to the production of soluble factors by the HIF-1α⁺⁺ cells that affect the surrounding microenvironment of the tumors. This thesis will investigate in the ES cell tumor model, in which HIF-1α acts as a tumor suppressor, whether a small fraction of HIF-1α⁺⁺ cells within the tumor can suppress tumor growth to a larger degree than expected. If the mechanism of tumor suppression is hypoxia-mediated apoptosis of HIF-1α⁺⁺ cells, as has been suggested, then the tumor growth should not be
significantly affected by a small fraction of HIF-1α++ cells, as the HIF-1α− cells within the majority of the tumor will still escape this apoptotic fate.

**SPECIFIC AIM 3C: Evaluate quantitatively and by direct visualization the localization of tumor cells with respect to vasculature in dorsal chamber.**

One of the significant contributions of this thesis will be to provide a quantitative measure of tumor cell localization in the dorsal skinfold chamber. In a previous study, it was suggested that HIF-1α− cells in a mixed tumor grown in a dorsal window were preferentially located distant from the tumor vasculature (81). However, this study only visualized HIF-1α− cells and presumed the location of HIF-1α++ cells on the basis of lack of fluorescence. Also, the study only presented one image in one tumor suggesting tumor cell localization. In this study, multiple tumors will be analyzed and the distance of both HIF-1α++ and HIF-1α− cells from vasculature will be quantified. Multiphoton laser scanning fluorescence microscopy will be used to obtain high resolution images for analysis of tumor cell localization with respect to blood vessels.

**SPECIFIC AIM 3D: Evaluate by direct visualization the localization of tumor cells with respect to vasculature and hypoxia in frozen tumor sections from subcutaneous tumors.**

Another previous study (80) demonstrated in subcutaneous tumors of mixed HIF-1α++ and HIF-1α− ES cells the preferential localization of HIF-1α++ cells proximal to blood vessels. However, this study focused on the 5% of cells that were most proximal and most distal to blood vessels, and used an indirect method of collecting the cells. Also, the few images that were
analyzed to look at a broader region of the tumors were limited to only visualizing the HIF-1α⁺⁺⁺ cells. Again, the location of HIF-1α⁻ cells was inferred. This study will directly visualize both HIF-1α⁺⁺⁺ and HIF-1α⁻ cells, and quantify the distance of these cells from blood vessels, using fluorescence microscopy and immunohistochemical staining. In addition, the distance from hypoxic regions will also be assessed by immunohistochemical staining of a hypoxic marker injected into the mice one hour prior to sacrifice.

2.3 Summary

The goal of this thesis is to elucidate the role of HIF-1α in the localization of embryonic stem cells within ES cell tumors with respect to both distance from blood vessels and distance from hypoxic areas. The primary method by which this phenomenon will be investigated is with a sandwich culture system in which gradients of oxygen are maintained over prolonged periods of time. The effect of gradients of oxygen on the migration, proliferation, and apoptosis of ES cells will be investigated for both HIF-1α⁺⁺⁺ cells and HIF-1α⁻⁻ cells. The oxygen profiles within the sandwich culture system will be modeled using a one-dimensional mathematical model accounting for diffusion and consumption of oxygen within the system. These models will be used to optimize the experimental parameters used to investigate the system. Finally, a more quantitative analysis of the in vivo localization of tumors will be performed for tumors grown both subcutaneously and in dorsal skinfold chambers.
Chapter 3: Materials and Methods

This chapter describes the methods of investigation used in the preparation of this thesis. The chapter will be divided into four main sections: 1) Cell Culture Methods, 2) Microscopic Methods, 3) Animal Models, and 4) Immunohistochemical/Immunocytochemical Methods.

3.1 Cell Culture Methods

*Cell Lines and Culture Conditions:* HIF-1α+/+ and HIF-1α−/− mouse embryonic stem (ES) cells were obtained from Dr. Peter Carmeliet (Leuven, Belgium). Their generation has been described previously (1). Briefly, HIF-1α−/− ES cells were generated by homologous recombination with a HIF-1α targeting vector and subsequent selection in G418. HIF-1α+/+ cells were generated from clones containing a randomly integrated HIF-1α gene targeting vector. Cells were maintained at low passage number in Dulbecco’s Modified Eagle Medium (Gibco) with high glucose (4.5 mg/mL) and 20% heat-inactivated fetal calf serum (FCS), supplemented with non-essential amino acids (0.1 mmol/L), sodium pyruvate (1 mmol/L), L-glutamine (6 mmol/L), 2-mercaptoethanol (10 μmol/L), penicillin/streptomycin (100 units/mL, 100 μg/mL), and Leukemia Inhibitory Factor (ESGro, Chemicon International, Inc., 1000 units/mL) to prevent differentiation. For maintenance of ES cells, sandwich culture experiments, and cell growth and proliferation studies, cells were grown on an inactivated primary mouse embryonic fibroblast feeder layer (Specialty Media) at a density of 50,000 fibroblasts/cm². For all other experiments, cells were cultured on gelatin-coated flasks.

*Transfection of ES Cells with Fluorescent Proteins:* Plasmid vectors encoding fluorescent proteins CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) were obtained
from Clontech (pECFP-C1; pEYFP-C1). A retroviral Murine Stem Cell Virus (MSCV) vector with a puromycin selection gene was also obtained from Clontech (pMSCVpuro). Primers directed at sequences surrounding the genes encoding CFP and YFP were used to extract the 735 bp fluorescent protein genes from the respective plasmid vectors by PCR amplification and purification (PfuTurbo HotStart DNA Polymerase and PCR MasterMix – Stratagene; PCR Purification Kit – Qiagen). The CFP and YFP genes were then cloned into the multiple cloning site of the MSCV vector using EcoRI and BglII restriction digests (Fisher BioReagents). The resulting vectors were amplified with a MaxiPrep Kit (Sigma). 293T cells (10^8) were cotransfected with the MSCV-CFP or MSCV-YFP vector (15 μg), a vector encoding gag and pol virus structural genes (7 μg), and a vector encoding the vesicular stomatitis virus envelope protein (VSV-G; 5 μg). Transfections were performed using the Lipofectamine 2000 (40 μL/transfection) standard procedure (Invitrogen). All 293T transfections were performed in 10-cm dishes that were 50-80% confluent at the time of transfection.

After transfection of the 293T cells with the MSCV-CFP or MSCV-YFP vector, the cells begin to produce retroviral vectors encoding the CFP or YFP genes. 8 hour after transfection, the media on the 293T cells was changed to fresh media. Then, the supernatant from the cells was collected from the 293T cells at 24 hr, 48 hr, and 72 hrs after transfection and filtered through a 0.45-μm cellulose acetate filter (Whitman). A 10-cm dish of 80% confluent embryonic stem cells (HIF-1α+/+ for MSCY-CFP and HIF-1α+/ for MSCV-YFP) was trypsinized and passaged at a 1:10 dilution into the 24-hr supernatant from the 293T cells containing the retroviral vectors. The ES cells were cultured for 48 hrs, and the media was changed to fresh viral supernatant every 24 hours. After 48 hours of culturing in the viral supernatant, the ES cells were again trypsinized
and passaged 1:10 into new 10-cm dishes. 24 hours after passaging, the ES cells were examined microscopically to assess the efficiency of transfection with the retroviral CFP and YFP vectors. The results of the transfection are demonstrated in Chapter 5.

**Genotyping of HIF-1α Status in ES cells:** The ES cells were genotyped for expression of wild-type or mutant HIF-1α both before and after transfection with fluorescent protein as described in (80). Briefly, genomic DNA was isolated and purified from cultured ES cells following the Dneasy Tissue Kit protocol (Qiagen). The DNA was amplified by polymerase chain reaction after EcoRI digestion using primers flanking the mutation site for the HIF-1α gene. The following primers were used: HIF-f, 5’-CAAGCATTCTTTAATGTGGAGCTATCT-3’; HIF-r, 5’-TTGTGTGGGGGAGTGACTGGAAGATG-3’; NEO-f, 5’-CGAAGGGGCCACACCC-3’. Amplification of the wild-type allele using HIF-f and HIF-r yielded a predicted product of 270 bp, whereas amplification of the HIF-1α’ allele with primers NEO-f and HIF-r yielded a predicted 340 bp product. The products of the PCR amplification were analyzed by electrophoretic gels to determine the size of the digested product for each cell type.

**Sandwich Culture System:** The ES cells were seeded on a glass microscope slide at a density of $10^5$ cells/cm$^2$. The cells were grown for 24 hours at 37 °C in 5% CO$_2$ in air. A second microscope slide was then placed directly over the first slide, with a 350 µm gap between the two slides as a result of fixed-thickness spacers at either end of the “sandwich” system (see Figure 3.1). A 2-mm thick agarose gel was formed around the sandwich system so that no convection occurred due to inadvertent movement of the culture dish. In this system, substances from the culture medium can only reach the cells within the system by diffusion from the edges of the culture. The
combined processes of diffusion, consumption of oxygen and nutrients, and production of metabolites establish microenvironmental gradients across the width of the culture, similar to those that occur in tumors in vivo (103). The cells in the transparent sandwich culture system were visualized by transillumination and fluorescence microscopy. A temperature and environmentally controlled stage was designed to maintain cells at 37 °C and 5% CO₂ during the collection of time lapse images over a 24 hour period of cells at various locations in the sandwich culture system to visualize proliferation, apoptosis, and migration of the cells within the oxygen gradients. The oxygen tension profile within the sandwich culture system was measured by phosphorescence quenching microscopy (see Section 3.2).

Figure 3.1 Diagram of the Sandwich Culture System. Adapted from Reference (105). The cells are “sandwiched” between two glass slides separated by a spacer of known height. An agarose mold surrounds the system, which allows diffusion of small molecules such as oxygen and nutrients while preventing convective mixing of the fluid in contact with the cells.

Glucose Consumption Rates: ES cells were seeded in 6-cm dishes at a seeding density of 100,000 cells/cm² and 500,000 cells/cm². Separate dishes were used for HIF-1α⁺/⁺ ES cells and HIF-1α⁻/⁻ ES cells. The cells were grown on an inactivated layer of primary mouse embryonic fibroblasts. Separate dishes were also seeded with fibroblasts alone to measure the contribution
of the fibroblasts to glucose consumption. The cells were allowed to grow for 24 hours in normal cell culture conditions. At that time, the cell culture media was changed to fresh media and half of the dishes were placed into a normoxic cell culture incubator, while the other half were placed in a hypoxic chamber supplied with an artificial environment of 1% oxygen and 5% carbon dioxide in nitrogen in a 37°C culture room. Every 6-10 hours for the next 48 hours, one fifth of the cell culture media was removed for analysis and replaced with an equal amount of fresh media to replenish the glucose and nutrients consumed by the cells. In separate 24-well plates, the ES cells were seeded at the same cell densities and cultured under the same conditions. At each time point for which media was removed from the first dishes, two wells of each cell type/condition were trypsinized and counted by hemocytometer. The media taken from the 6-cm dishes was analyzed for glucose concentration (described below) and the hemocytometer counts were used to measure cell density at each time point (cells/cm²).

The glucose concentration in the initial media and in all media samples collected during the 48-hour culture period was measured as follows using a glucose assay kit (Sigma). The principle of the measurement technique is that the glucose within the media is converted to 6-phosphogluconate and NADH by the following reactions:

\[
\text{Glucose + ATP} \rightarrow \text{Glucose - 6 - Phosphate + ADP}
\]
\[
\text{Glucose - 6 - Phosphate + NAD} \rightarrow 6\text{-Phosphogluconate + NADH}
\]

The first reaction is catalyzed by hexokinase, and the second by glucose-6-phosphate dehydrogenase. The increase in absorbance at 340 nm from the conversion of NAD to NADH is measured and is directly proportional to glucose concentration. In practice, 10-200 µL of the media to be tested was combined with 1.0 mL of 1.5mM NAD, 1.0 mM ATP, 1.0 unit/mL of hexokinase, and 1.0 unit/mL of glucose-6-phosphate dehydrogenase. A sample blank and a
reagent blank were prepared by substituting the reagent solution and the sample solution, respectively, with deionized water. The tubes were incubated for 15 minutes at room temperature, and the absorbance ($A$) of the tubes was measured at 340 nm vs. deionized water. The glucose concentration in each sample was calculated by the following equation:

\[
C_G(\text{mg/mL}) = \frac{\Delta A \cdot (TV) \cdot (MW_G) \cdot F}{\varepsilon \cdot d \cdot (SV) \cdot 1000}
\] (3.1)

where $\Delta A = (A_{\text{Test}} - A_{\text{Sample Blank}} - A_{\text{Reagent Blank}})$, $TV$ is the total assay volume (mL), $SV$ is the sample volume (mL), $MW_G$ is the molecular weight of glucose (180.2 g/mol), $F$ is the dilution factor from sample preparation, $\varepsilon$ is the millimolar extinction coefficient for NADH at 340 nm (6.22 mM$^{-1}$ cm$^{-1}$), $d$ is the light path (1 cm), and 1000 is the conversion factor for µg to mg.

The total glucose consumed at each time point was calculated as the initial glucose concentration multiplied by the media volume minus the sample glucose concentration times the media volume, taking into account the replenishment of glucose with each media change. The glucose consumed by the fibroblast layer alone was also subtracted to obtain the actual glucose consumed by the ES cells. The glucose consumed per area of the cell culture dish was plotted vs. time, and a best fit quadratic curve was fitted through the resulting data. The slope of the curve at any time point was calculated to obtain the glucose consumption rate in units of mg glucose/cm$^2$-hr. This slope at each measured time point was divided by the total cell density as measured by the hemocytometer in a separate experiment to give the per-cell glucose consumption rate ($Q_c$) in mg glucose/cell-hr.

**Oxygen Consumption Rate: Stirred-Chamber Method:** The oxygen consumption rate of HIF-1α$^{++}$ and HIF-1α$^{−}$ ES cells in normal culture media (3.5 mg/mL glucose) was measured in
a stirred-chamber reactor as follows. Cells were trypsinized and resuspended in fresh ES media. Cell concentration and viability were determined by fluorescence-based cell counting and membrane integrity tests (Guava Viacount) using a flow cytometer (Guava PCA). The cells were concentrated to a concentration of 5-6x10^6 cells/mL. Cells were suspended in fresh media and sealed in a 200 μL stirred titanium water-jacketed chamber maintained at 37°C (Instech Labs). The resulting chamber is impermeable to oxygen. The time-dependent partial pressure of oxygen \( (p_o) \) within the chamber was recorded with a fluorescence-based oxygen sensor (Ocean Optics), and the decrease in oxygen tension vs. time curve was fit to a straight line. The fiber-optic detection of oxygen tension was accomplished by fluorescence quenching following oxygen binding to a fluorophor in a gel overlaid by a thin silicone rubber film at the tip of the fiber. The actual oxygen tension is calibrated during each experiment both at room air concentrations \( (p_o = 160 \text{ mmHg}) \) and after the oxygen has been fully consumed from the chamber \( (p_o = 0 \text{ mmHg}) \).

The maximal oxygen consumption rate (OCR) was evaluated by the equation \( Q_o = (dp_o/dt)*k_o/C \), where \( k_o \) is the solubility of oxygen in water at 37 °C (1.19 nmol/mmHg-mL) and \( C \) is the concentration of cells in the suspension (cells/mL).

**Oxygen Consumption Rate: Oxygen Biosensing Plate Method:** In order to obtain high-throughput measurements of oxygen consumption rate at various glucose concentrations, an oxygen biosensing plate was used. This 96-well plate method has been described by Guarino et al. (109) and has the advantage of allowing multiple measurements in parallel. While the method has been reported to give relative consumption rates when comparing multiple conditions in a single experiment, the absolute values of oxygen consumption rate predicted by this method consistently underestimate OCR by a constant factor (data not published; personal
communication with Mark Timmins (BD Biosciences) and Clark Colton (Chemical Engineering, Massachusetts Institute of Technology)). Therefore, the plate method is used to calculate relative values of OCR at various glucose concentrations, and then the predicted values are scaled appropriately by a factor determined from the stirred-chamber measurements described above.

The oxygen-sensing microplate used in these experiments was the BD Oxygen Biosensor (OBS) system (BD Biosciences, Bedford, MA). These plates incorporate an oxygen-sensitive ruthenium-based fluorophore in a silicon rubber matrix in the bottom of each well of a standard 96-well microplate. The ES cells were seeded (HIF-1α++ and HIF-1α+ in separate wells) at a density of 25,000 cells/well in five different glucose concentrations (3.5 mg/mL, 2.6 mg/mL, 1.6 mg/mL, 1.0 mg/mL, and 0 mg/mL glucose). The media was prepared for each condition by mixing high-glucose ES media (3.5 mg/mL) with ES media prepared in DMEM containing no glucose at appropriate proportions. HEPES buffer was added to the media at 25 mM. The cells were seeded in quadruplicate at each condition. The microplates were incubated at 37 °C in a temperature controlled microplate fluorometer (Fluroskan Ascent, Thermo Electron Corporation) for 24 hrs, and the fluorescence from the wells were measured every 4 hours (Excitation: 485 nm, Emission: 620 nm).

The fluorescence values were normalized at each time point by a two-step normalization procedure. The fluorescence reading was first divided by the “preblank” reading taken from the identical well before the beginning of the experiment to correct for machine drift over time. Then, the value was normalized by the average fluorescence values of 6 wells at the same time.
point containing only media to correct for fluorophor concentration differences, giving a final normalized relative fluorescence (NRF) for each well.

The fluorescence values (NRF) are converted to oxygen concentration by using the Stern-Volmer equation relating oxygen concentration to the ratio of fluorescence intensity at zero oxygen concentration ($I_0$) to the intensity at the oxygen concentration of interest ($I$):

$$
\frac{I_0}{I} = 1 + K_{sv} \cdot [O_2] 
$$

(3.2)

The Stern-Volmer constant ($K_{sv}$) depends on the fluorophore system and can be calculated experimentally. The normalized fluorescence intensity (NRF) is equivalent to $I/I_A$, where $I_A$ is the intensity at ambient oxygen concentration. The NRF can be related to oxygen concentration by using Equation 3.2:

$$
NRF = \frac{I}{I_A} = \frac{I_0/I_A}{I_0/I} = \frac{NRF_0}{1 + K_{sv} \cdot [O_2]} 
$$

(3.3)

$NRF_0$ is the NRF measured in wells at zero oxygen concentration. Experimentally, this was determined by adding 100mM sodium sulfite, which consumes the oxygen within the well, to six wells and averaging the NRF for these wells after allowing the consumption reaction to proceed to completion. Therefore, rearrangement of Equation 3.3 allows for the calculation of the oxygen concentration at the bottom of the well given the normalized relative fluorescence value:

$$
[O_2] = \frac{(NRF_0 / NRF) - 1}{K_{sv}} 
$$

(3.4)

$K_{sv}$ can be calculated from Equation 3.2 solved for ambient oxygen concentration ([O$_2$]$_A$):

$$
K_{sv} = \frac{I_0 / I_A - 1}{[O_2]_A} = \frac{NRF_0 - 1}{[O_2]_A} 
$$

(3.5)
The oxygen consumption rate of the cells in the bottom of the well can be estimated by assuming steady-state conditions have been established, in which case the rate of oxygen diffusion through the media will equal the rate of oxygen consumption by the cells. The steady-state solution to Fick’s Law of diffusion, adapted from Reference (110) was used to calculate the total oxygen consumption rate (OCR):

\[ \text{OCR (fmol/min)} = \frac{D_o \times S \times L \times \Delta p}{h} \]  

(3.6)

where \( D_o \) is the diffusion constant of oxygen \((3 \times 10^{-5} \text{ cm}^2/\text{sec})\), \( S \) is the surface area of the media exposed to the atmosphere \((0.31 \text{ cm}^2)\), \( L \) is a units conversion factor \((6.0 \times 10^7 \text{ s*L*fmol*min}^{-1}\text{*mL}^{-1}\text{*μmol}^{-1})\), \( \Delta p \) is the difference in oxygen concentration between the air/media interface and the media/cell interface \((μM)\), and \( h \) is the diffusion path length through the media \((0.65 \text{ cm for a 96-well plate with 200μL media})\). The total OCR was plotted versus time for each well of the 96-well plate. The curve for OCR was extrapolated back to time zero to determine the OCR\(_o\) for the 25,000 cells initially seeded in each well. Then, the per-cell oxygen consumption rate, \( Q_o \) \((\text{nmol/10}^5 \text{ cells-hr})\), was calculated by dividing the OCR\(_o\) by the starting cell number \((25,000 \text{ cells})\) with the appropriate units conversion factor.

**MTT Proliferation Assay:** HIF-1\(α^{+/-}\) and HIF-1\(α^{+}\) ES cells were seeded in quadruplicate wells of a 96-well plate at 10000 cells per well. Six total plates were prepared in this manner. Three plates were cultured in normoxia and three plates were cultured under hypoxic conditions (1% oxygen and 5% carbon dioxide in nitrogen). At 0 hrs, 24 hrs, and 48 hrs after the beginning of the experiment, a normoxic plate and hypoxic plate were each analyzed by MTT assay. MTT (Promega; 50 μL of a 2 mg/mL solution) was added to each well. The cells were returned to the cell culture incubator at 37 °C for 1 hour to allow the MTT to form crystals in the viable cells.

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within the well. The media was removed from each well, and 100 μL of DMSO was added to each well to solubilize the MTT crystals. The plates were incubated at room temperature in the dark for 15 minutes to allow the crystals to dissolve. The absorbance in each well at 570 nm was read using a microplate reader. To determine the linear range of absorbance vs. cell number, the same procedure was performed on serial dilutions of cells from 10,000 to 100,000 cells per well, and the absorbance plotted vs. cell density.

**Proliferation Assay – Cell Counting:** HIF-1α+/+ and HIF-1α−/− ES cells were seeded separately in duplicate wells of 12 12-well plates each at two seeding densities (100,000 cells/cm² and 500,000 cells/cm²) on an inactivated layer of primary mouse embryonic fibroblasts. Separate wells containing only fibroblasts were also analyzed for background subtraction. Twenty-four hours after seeding and culturing under normoxic conditions, the cells were placed in either normoxia (6 plates) or hypoxia (6 plates; 1% O₂) for 42 hours, with normoxic and hypoxic plates removed at 6 time points for cell counting (0 hrs, 6 hrs, 16 hrs, 24 hrs, 30 hrs, and 42 hrs). At each time point, the cells were trypsinized and viable cells were counted in a hemocytometer by trypan blue exclusion. At each time point at which a plate was removed, one fifth of the media in the remaining plates was removed and replaced with fresh media to duplicate the conditions of the experiment used to measure glucose consumption rates. The results of the experiment were plotted as cell count vs. time, with the cell count for the wells with fibroblasts alone subtracted from each measurement to ensure that only the ES cells were being counted.
3.2 Microscopic Methods

**Fluorescence Microscopy:** The sandwich cultures were visualized microscopically using an Olympus IX70 fluorescent microscope equipped with excitation and emission filter wheels. Light from a mercury lamp entered the microscope and was deflected to the objective lens by a dichroic mirror appropriate for the excitation and emission wavelength of the fluorescent molecule to be imaged. Fluorescent light was collected by the same objective, passed through the dichroic mirror and an appropriate bandpass filter, and was collected by a CCD camera. Images were taken with a 10X objective, and the resulting images were of fields of view 860 μm across in the x-direction. Brightfield images were obtained using transillumination, and fluorescent images were obtained using filters appropriate for CFP and YFP: CFP – 436/20 nm bandpass excitation, 480/40 nm bandpass emission; YFP – 501/16 nm bandpass excitation, 535/30 bandpass emission. The images were captured using OpenLab software (Improvision) and analyzed in Image J (NIH). For measurement of localization in Chapter 5, the fluorescent images are generated in pseudocolor (CFP in blue channel; YFP in green channel) and the blue and green channels are binarized to determine the area of the image covered by HIF-1α+/+ or HIF-1α−/− cells, respectively.

*Migration Studies within Sandwich Culture System:* For migration studies, CFP-labeled HIF-1α+/+ and YFP-labeled HIF-1α−/− ES cells were seeded in a 50:50 mixture at a total cell density of 100,000 cells/cm² onto a glass slide. Twenty-four hours after seeding, the top slide was placed onto the cells to create the sandwich culture system as described above. A temperature-controlled microscope stage was used to take time-lapse fluorescent images of 29 locations covering the entire width of the sandwich culture every hour for 24 hours. To measure
the migration of a particular group of cells, the cells were outlined at each time point and the
centroid of the outlined area was measured using Image J software (NIH). The x and y
coordinates of the centroid were recorded at each time point, and the total distance traveled was
calculated by summing the distances between the centroids of the same clump of cells at
consecutive time points from 0-24 hours. The tendency of the cells to travel in a particular
direction of increasing or decreasing oxygen was measured by calculating the distance between
the x-location (x is the direction of the oxygen gradient) of the centroid at each time point and
the x-location of the centroid at the initial starting point (time = 0 hr). In this way, the movement
of the cellular clumps was followed over time with respect to its initial starting location.

**Phosphorescence Quenching Microscopy:** Oxygen tension measurements were made *in vitro*
using a phosphorescence quenching microscopic technique described in Reference (106). The
exponential decay of phosphorescence from albumin-bound palladium meso-tetra-
(4-carboxyphenyl) porphyrin (OxyPhor R0; Harvard Apparatus) after a pulse excitation is
oxygen-dependent (104, 111). The porphyrin probe was bound to albumin as follows. Albumin
(8.6 g) and sodium chloride (0.8 g) were added to approximately 150 mL of water and stirred
until dissolved. The pH was adjusted to 7.8 with 1N NaOH. The Pd-porphyrin was dissolved in
1N NaOH and added dropwise to the albumin solution. The pH was maintained between 7.8 and
8.4 at all times. When the porphyrin was completely added, the solution was left stirring for 30
minutes, and the final pH was adjusted to 7.5 with 1N HCl. The final albumin-bound porphyrin
solution was diluted to 5 mg/mL.
The porphyrin solution was mixed 1:10 with ES media with HEPES buffer added (25mM). The porphyrin-containing media was added to the sandwich culture system shortly before oxygen measurements were made. The oxygen measurements were made at 1-mm intervals across the sandwich culture system or a control slide with no sandwich by placing the culture system within a temperature controlled microscope stage designed to maintain the temperature at 37 °C. The stage was placed on an automated x-y stage control, and the stage control was set to automatically move the stage in 1-mm increments from the edge of the sandwich culture to the center, and repeat these movements indefinitely. The phosphorescence signal of the oxygen-dependent phosphor was detected at >630 nm with a photomultiplier tube after a 540-nm flashlamp excitation. The signal was averaged on a digital oscilloscope before computer storage. 

*In vitro* decay signals were fitted with an exponential function and the exponential decay time constants were converted to $p_o$ values according to the Stern-Volmer equation:

\[
\frac{1}{\tau} = \frac{1}{\tau_o} + k_q(p_o) \tag{3.7}
\]

where $\tau$ is the time constant of decay, $\tau_o$ is the time constant of decay in the absence of oxygen, and $k_q$ is a quenching constant.

The calibration constants $\tau_o$ and $k_q$ in Equation 3.7 were obtained in the following manner. Fluid solutions at various gas concentrations were equilibrated at 37 °C for 30 minutes by bubbling gas with known $p_o$ values through the solutions. A zero oxygen concentration solution was obtained by using a solution of 0.3% glucose, 75 μg/mL glucose oxidase, and 12.5 μg/mL catalase. The equilibrated solutions were drawn into capillary tubes and sealed. The time constant of
phosphorescence decay was measured for the sealed tubes using the same experimental setup as was used in the in vitro sandwich culture experiments. The values of $1/\tau$ were plotted against oxygen partial pressure ($p_o$) and the calibration curve is shown in Figure 3.2. The zero oxygen time constant ($\tau_0 = 648$ μs) agrees well with literature values for the phosphor used in these experiments, although the quenching constant ($k_q = 48$ mmHg$^{-1}$ s$^{-1}$) is an order of magnitude lower than the literature values (112). This discrepancy in quenching constant is likely due to some artifact of the flashlamp excitation and optical system used in the current experiments. Because the values predicted were reproducible with the experimental setup used for these experiments, and the same setup was used for all experiments as was used for the calibration, the calibration curve was used as predicted in Figure 3.2 for all oxygen concentration measurements.

**Figure 3.2** Calibration curve for phosphorescence quenching microscopy measurement of oxygen tension. The slope of the curve gives the quenching constant, $k_q$, and the intercept gives the inverse of the time constant for decay of the unquenched phosphor ($1/\tau_0$).

*Intravital Multiphoton Laser Scanning Microscopy:* The construction of the multiphoton laser scanning microscope has been described previously (81). A tunable femtosecond mode-locked
Ti-Sapphire laser was directed into the microscope by a galvanometer-driven x-y scanner. A Glan-Thomson polarizer adjusted the power of the incident laser. The laser light entered the back of the microscope (Zeiss Inc.) and was deflected into the objective lens by a short pass dichroic mirror (Chroma Technology Inc.). A piezo-driven stepper motor that was controlled by the data acquisition computer drove the axial position. The fluorescence emission was collected by the same objective, passed through the dichroic mirror and was focused on the photomultiplier tube (PMT) for detection. The signal is pre-conditioned with a low noise pre-amplifier and a photon discriminator (Advanced Research Instrument Co.). The digital signal was synchronized to the x-y scanner movement by software in order to reconstruct a 2-D image that was then stored on the data acquisition computer. Appropriate dichroic mirrors, excitation filters and emission filters were used to collect CFP fluorescence (HIF-1α+ cells), YFP fluorescence (HIF-1α− cells), and rhodamine fluorescence (perfused blood vessels). The 2-dimensional images were reconstructed into 3-dimensional multifluorescent stacks of images offline using ImageJ analysis routines (NIH). The 3-dimensional stacks were used to create maximum intensity projections of 50-150 μm deep sets of images displaying the red, yellow, and blue fluorescence in separate channels.

3.3 Animal Models and In vivo Methods

All methods involving the experimental use of animals have been approved by the Massachusetts General Hospital Institutional Review Board Subcommittee on Research Animal Care (MGH SRAC protocol # 2001N000077 and 2004N000050). The experiments were continuously monitored by the MGH veterinary staff. Male severe combined immunodeficient (SCID) mice (8 weeks old) were used for all experiments.
**Subcutaneous Injection of ES Cell Tumors:** The mice were anesthetized by an intramuscular injection of ketamine/xylazine (100/10 mg/kg). A syringe with a single cell suspension of $2 \times 10^6$ cells/0.1 mL Hank’s Balanced Salt Solution (Sigma) and a 30-1/2 gauge needle were used for the injection. The hair on the skin of the right flank of the mice was shaved prior to injection. The needle was used to puncture the skin and inject 0.1 mL of the single cell suspension into the subcutaneous space on the right flank of the mice. The resulting tumor volume was measured every 3-5 days after approximately 7 days to allow the tumor to grow large enough to visualize. For each measurement, calipers were used to measure two dimensions of the tumor (length = long axis of tumor, width = short axis), and the volume was calculated by the standard formula: $V = \left(\frac{\pi}{6}\right) \text{(length)} \times \text{(width)}^2$. Animals were monitored daily, and animals which had a tumor size greater than 13 mm x 13 mm were removed from the cage and euthanized according to the procedure outlined below for tissue collection.

**Tumor Collection for Immunohistochemistry:** When the subcutaneous tumors reached a size larger than 13 mm x 13 mm, the tumors were collected for immunohistochemistry for detection of hypoxia and blood vessels. The mice were anesthetized as described above for tumor implantation. Hypoxyprobe-1 (pimonidazole hydrochloride; Chemicon International) is a hypoxic marker that forms adducts with thiol groups in proteins within tissues. Hypoxia ($p_o < 10$ mmHg) is required for the adduct formation, making the molecule an ideal marker for hypoxic areas within tumors. Hypoxyprobe-1 was injected intravenously into the tail vein of the anesthetized mice at a dose of 60 mg/kg in 0.9% sterile saline solution. Approximately 1 hour after injection, the mice were sacrificed by perfusion fixation in order to maintain the fluorescence of CFP and YFP within the tumor.
The mice were pinned to a dissection block, the skin over the thorax removed, and an incision made superior to the diaphragm (between the 3rd and 4th rib). The excision was extended between the axillary midline on each side. The rib cage was pulled up and held using hemostats to expose the beating heart. The heart was held with forceps while a 2 mm incision was made in the apex of the heart. A cannula was inserted into the incision and the incision was held tight around the cannula using forceps. 4% paraformaldehyde was perfused through the cannula into the systemic vasculature under a pressure of 80-120 mmHg. The right atrial appendage was nicked in order for the circulating fixative to exit the mouse. The fixative was allowed to circulate for 5 minutes until the mouse showed visible signs of tissue rigidity. The subcutaneous tumors were immediately removed and placed in 4% paraformaldehyde for 2-3 more hours at 4 °C to achieve complete fixation. The fixed tissue was then placed in 30% sucrose overnight at 4 °C for cryoprotection. The tumors were finally cut with a scalpel and placed into tissue blocks with OCT freezing solution and frozen rapidly on dry ice before storing at -80 °C until cryosectioning.

**Dorsal Skinfold Chamber Preparation:** The dorsal skinfold chambers used for multiphoton intravital microscopy were prepared as described previously (113). Briefly, mice were anesthetized by an intramuscular injection of ketamine/xylazine (100/10 mg/kg). The entire back of the animals were shaved and depilated, and two symmetrical titanium frames were implanted so as to sandwich the extended double layer of skin. One layer of the skin was removed in a circular area of approximately 15 mm in diameter, and the remaining layer, consisting of epidermis, subcutaneous tissue, and striated skin muscle, was covered with a cover slip incorporated into the frames (see Figure 3.3). All surgical procedures were performed under
aseptic conditions in a specific germ-free environment. During surgery the body temperature of the animals was kept constant at 36-37 °C by means of a heating pad.

For implantation of tumor cells into the dorsal chamber, the coverslip of the chamber was removed and 2 μl of a dense tumor cell suspension from a cell pellet of the ES cells was inoculated onto the upper tissue layer of the chamber. The coverslip was then replaced, and the tumors were monitored by intravital microscopy. The mice were sacrificed within four weeks after chamber implantation.

![Dorsal Skinfold Chamber](image)

**Figure 3.3** Dorsal Skinfold Chamber. The titanium chamber sandwiches the skinfold so that window access to the tumor allows for intravital microscopic imaging.

**Dorsal Chamber Tumor Imaging – Angiography:** Prior to imaging, the mice were anesthetized and given a 0.1 mL intravenous injection of 2.5% 2×10^6 molecular weight tetramethylrhodamine-dextran (Molecular Probes) by tail vein cannulation. The animal was fixed to a metal plate designed to stabilize the dorsal skinfold chamber for intravital observation. The tumor and vasculature images were taken with the multiphoton laser scanning microscope as described above.
3.4 Immunohistochemical/Immunocytochemical Methods

**Apoptosis in Sandwich Culture – Activated Caspase-3:** To measure apoptosis in the sandwich culture system, non-fluorescent HIF-1α^{+/−} and HIF-1α^{−/−} ES cells were seeded at 100,000 cells/cm² on separate glass slides. Twenty-four hours after seeding, the top slide was placed onto the sandwich culture system and cultured in the cell culture incubator for 24 additional hours. At the end of the sandwich culture experiment, the top slide of the sandwich culture was carefully removed and the cells washed once with room temperature phosphate-buffered saline (PBS). The PBS was aspirated off of the cells, and the cells were immersed in 4% paraformaldehyde at room temperature for 10 minutes. The cells were then washed with PBS and permeabilized by the addition of 0.2% Triton X-100 for 5 minutes at room temperature. The cells were washed three times for 5 minutes each with PBS at room temperature.

The slides were blocked with 3% bovine serum albumin (BSA) in PBS at room temperature for 60 minutes, and washed once with PBS. The cleaved Caspase-3 Rabbit monoclonal antibody (Cell Signaling Technology #9664) was diluted 1:100 in 3% BSA in PBS. The diluted antibody was added to the slides and incubated overnight at 4 °C. The slides were then washed three times for 5 minutes each with PBS. The slides were incubated with anti-rabbit secondary antibody (1:200 in 3% BSA in PBS) conjugated to fluorescein isothiocyanate (FITC) for 1 hour at room temperature in the dark. The slides were washed three times for 5 minutes each with PBS. ToPro-3 nuclear stain (Molecular Probes) was added at 1:10000 dilution in PBS to the slides for 15 seconds, and the slides were then rinsed in PBS and mounted with Vectashield Mounting Medium (Vector Laboratories). The ToPro-3 stain binds to DNA and produces a nuclear stain that is detectable using an excitation of 650 nm and an emission of 710 nm. Images were taken.
using a confocal microscope at various locations across the slide. Green fluorescent images were taken to visualize the cleaved caspase-3 stain, and far red fluorescent images were taken to visualize the ToPro-3 nuclear stain. The nuclei and apoptotic cells (cleaved caspase-3) were counted in each image to obtain a measure of the fraction of apoptotic cells at various distances from the edge of the sandwich culture. A positive control slide of cells induced to undergo apoptosis by TNF-α was used to ensure that the activated caspase-3 was an adequate marker of apoptosis.

**Apoptosis in Cell Culture Slides – TUNEL staining:** For measurement of apoptosis in normoxia vs. hypoxia, HIF-1α−/− and HIF-1α+/− were seeded separately in triplicate on gelatinized glass coverslips in 24-well plates. The cells were seeded at 20,000 cells/well and cultured for 24 hrs in normal ES media with either 20% fetal calf serum (FCS) or 5% FCS in the media. After 24 hours, the cells were cultured in 1 of 8 conditions: 1) normoxia (5% CO₂ in room air), normoglycemia (3.5 mg/mL glucose), and 20% FCS; 2) normoxia, normoglycemia, and 5% FCS; 3) normoxia, hypoglycemia (0 mg/mL glucose), and 20% FCS; 4) normoxia, hypoglycemia, and 5% FCS; 5) hypoxia (1% oxygen and 5% CO₂ in nitrogen), normoglycemia, and 20% FCS; 6) hypoxia, normoglycemia, and 5% FCS; 7) hypoxia, hypoglycemia, and 20% FCS; or 8) hypoxia, hypoglycemia, and 5% FCS.

After 24 hours of culturing the cells in the above conditions, the slides were treated with the DeadEnd™ Fluorometric TUNEL System (Promega) according to the manufacturer’s protocol. The slides were washed twice with PBS. The cells were fixed by immersing the slides in 4% paraformaldehyde for 25 minutes at 4°C. The slides were washed twice in PBS for 5 minutes at
room temperature. The cells were then permeabilized in 0.2% Triton X-100 for 5 minutes, followed by washing in PBS twice for 5 minutes each. The cells were covered with Equilibration Buffer for 10 minutes, followed by the addition of rTdT (recombinant terminal deoxynucleotidyl transferase) incubation buffer. The cells were covered with plastic coverslips and incubated in a humidified chamber at 37 °C for 60 minutes. The entire plate was covered with aluminum foil to protect from direct light.

After the rTdT incubation, the slides were immersed in 2X SSC for 15 minutes at room temperature to terminate the reaction. The slides were washed three times in PBS for 5 minutes each, and mounted onto slides using Vectashield Mounting Medium with DAPI (Vector Laboratories). The DAPI nuclear stain is a blue nuclear counterstain that can be detected by excitation at 360 nm and emission at 480 nm. The rTdT incubation buffer contains fluorescein-12-dUTP which is incorporated into TUNEL-positive apoptotic cells. The slides were analyzed under a fluorescent microscope using a standard fluorescein filter set to detect the apoptotic cells and a DAPI filter set to detect the total nuclei. The total nuclei and apoptotic cells were counted to calculate the fraction of apoptotic cells under each of the 8 conditions tested.

**CD31 Immunohistochemistry:** The frozen tumor blocks were sectioned with a cryostat, and for each tumor, three 5-µm thick sections at least 300 µm apart were cut onto glass slides. The slides were allowed to air-dry for 15 minutes, and then immersed in acetone for 5 minutes. They were then washed 3 times for 3 minutes each in PBS. The slides were blocked in 3% BSA in PBS for 1 hour, followed by incubation with rat anti-mouse CD31 antibody (DAKO) diluted 1:100 in 3% BSA in PBS overnight at 4 °C. The slides were washed 3 times for 3 minutes each in PBS and
incubated with sheep anti-rat secondary antibody conjugated to AlexaFluor670 (far red fluorescence), diluted 1:200 in PBS, for 1 hour at room temperature. The slides were then washed with PBS 3 times for 3 minutes each, and mounted with coverslips using Vectashield Mounting Medium (Vector Laboratories). The slides were analyzed by fluorescence microscopy as described in Chapter 6.

**Hypoxyprobe-1 Immunohistochemistry:** The frozen tumor blocks were sectioned with a cryostat, and for each tumor, three 5-μm thick sections at least 300 μm apart were cut onto glass slides. The slides were allowed to air dry for 15 minutes, and then immersed in acetone for 5 minutes. They were then washed 3 times for 3 minutes each in PBS. The Mouse on Mouse antibody kit (M.O.M.; Vector Laboratories) kit was used to detect the Hypoxyprobe adducts. The slides were blocked in M.O.M. Blocking Reagent for 1 hour and washed 3 times for 3 minutes each with PBS. The slides were then incubated in M.O.M. diluent for 5 minutes, followed by incubation with mouse anti-Hypoxyprobe-1 monoclonal antibody (Chemicon International) diluted 1:100 in M.O.M. diluent overnight at 4 °C. The slides were washed 3 times for 3 minutes each in PBS and incubated with rabbit anti-mouse secondary antibody conjugated to AlexaFluor670 (far red fluorescence), diluted 1:200 in PBS, for 1 hour at room temperature. The slides were then washed with PBS 3 times for 3 minutes each, and mounted with coverslips using Vectashield Mounting Medium (Vector Laboratories). The slides were analyzed by fluorescence microscopy as described in Chapter 6.
3.5 Statistical Analysis

Results are presented as mean ± SEM. Two sample Student’s t-tests for independent samples of equal variances were performed to compare sample means between groups. Statistical significance was based on $p$ values smaller than 5%.
Chapter 4: Development of Diffusion-Reaction Model of Sandwich Culture System

4.1 Introduction

This chapter describes a mathematical model of the temporal and spatial development of oxygen and glucose gradients within the sandwich culture experimental system described in Chapter 3. In order to address the question of the mechanism for the localization of tumor cells with respect to hypoxia, it was necessary to use an experimental system that allowed for the development and maintenance of areas of high and low oxygen concentration over time. The sandwich culture system developed by Hlatky and Alpen (103) is an ideal system for these purposes.

The sandwich culture system was initially described as an in vitro tumor analog, analogous to multicellular spheroids in mimicking the gradients of oxygen, nutrients, and waste products that develop within a growing tumor. The advantages to using the sandwich culture system over the spheroid system are that the entire gradient can be viewed microscopically without fixation and sectioning and that the viable region of the sandwich culture has been shown to be an order of magnitude larger than the viable rim of a spheroid, which is often only a few cell layers thick (103). The disadvantage of the sandwich culture system is that like any monolayer in vitro system, there is no three-dimensional cell-cell contact. However, for the purposes of monitoring the localization of tumor cells with respect to oxygen concentration, the sandwich culture system provides the necessary requirements of 1) a stable oxygen gradient, 2) a region of interest large enough to monitor the activity of numerous cells, and 3) the ability to monitor the system microscopically in real time.
An important aspect of the sandwich culture system in addressing the mechanism of localization is point 2 above: the developing oxygen and nutrient gradients must be shallow enough that they extend over a long enough distance to observe the behavior of the cells over time. If the gradients are too shallow, hypoxia will not develop and the behavior in question will not be elicited. If the gradients are too steep, all cells will experience hypoxia, and the difference in behavior from well-oxygenated to hypoxic conditions will not be observed. Fortunately, the gradients within the sandwich culture system are somewhat “tunable”, in that the steepness of the gradient can be modulated by varying the cell density of the sandwich culture and the height of the gap between the two glass slides. It is feasible to experimentally determine the optimum conditions for the sandwich culture system. However, the performance of multiple experiments within the sandwich culture and the measurement of oxygen gradients under each set of conditions is time- and labor-intensive. The use of a mathematical model describing the system provides an alternative predictive method of determining the optimum conditions for the sandwich culture experiments. In this chapter, such a mathematical model is described and validated experimentally.

4.2 Diffusion-Reaction Model

Diffusion-reaction models for the prediction of oxygen profiles within three-dimensional spheroids have been developed extensively and microelectrode measurements used to measure oxygen profiles within spheroids (114-119). For example, one comprehensive model by Casciari et al. (114) modeled the growth of spheroids based on gradients of oxygen, glucose, lactate ion, carbon dioxide, bicarbonate ion, chlorine ion, and hydrogen ion within the spheroids. Oxygen and glucose consumption rates as well as glucose diffusivities and cell growth rates were
measured in the EMT6/Ro mouse mammary sarcoma cell line, and the spheroid growth rates, viable rim thickness, and nutrient consumption rates were predicted from the model. In their model, steady-state was assumed so that only the time-independent diffusion-consumption equations were solved. In the realm of spheroids, the utility of these models has typically been their ability to predict spheroid growth rates and determine the factors that contribute to the growth of spheroids.

Because of the advantages listed above of the sandwich culture system as a tumor analogue, models of the sandwich culture system are also useful in predicting and controlling the microenvironment of the system. The sandwich culture can also be modeled using standard diffusion equations with a consumption term for the depletion of oxygen and nutrients by the cells in the sandwich system (103, 120). The model used was originally applied to the sandwich culture system by Hlatky et al. (120) to predict the viable border width of cells within the sandwich culture system. The methods used to measure consumption rates were similar to those described herein. The previous study assumed that necrosis occurred if ATP production dropped to a certain value, determined empirically. The difference in the current study is that the predicted oxygen concentration profiles are directly validated by measurement of oxygen concentrations within the sandwich culture as originally described by Helmlinger et al (105). Another advantage of the current model is that unsteady-state equations are used such that it also predicts the time course of the development of oxygen and glucose concentration profiles.

The sandwich culture system is illustrated in Figure 4.1. As oxygen and nutrients diffuse into the sandwich culture system from the edge of the culture (x = 0 mm), they are consumed by the cells
which are seeded on the bottom slide of the system. A steady-state is established when the diffusion into the system is balanced by cellular consumption within the system. The following assumptions are made for the development of the model of this system:

1) Oxygen and glucose are the only components of interest within the model. This assumption could theoretically be relaxed and the model expanded to include other components of interest. However, oxygen is the primary determinant of HIF-1α activity, which is being studied in this thesis. It is well known that per-cell consumption of either oxygen or glucose depends on the concentration of both substances[121], so glucose is incorporated into the model to account for its effects on oxygen consumption.

2) The cell number is assumed to be spatially and temporally constant over the 24-hour period of interest. In reality, the cells are dividing and growing during the time of interest, and likely at different rates in different locations due to the dependence of cell proliferation on oxygen concentration. Thus, the model is primarily valid at early time points before the cell density changes significantly. To get a sense of how dramatically the steady-state profile would change over time, the model will be evaluated at densities representing the starting and ending cell densities of the system.

3) The per-cell consumption rates of oxygen and glucose do not depend explicitly on time. In reality, as cells are exposed to low concentrations of oxygen or glucose, there may be a time lag in the adjustment of their consumption rate. These time lags are ignored, and the consumption rates at any instantaneous oxygen/glucose concentration are assumed to be their final steady-state values.
4) The oxygen and glucose concentrations are assumed to be independent of location in the y-direction (see Figure 4.1). This assumes the absence of significant edge effects, as measurements are made in the center of the sandwich culture (approximately 3 cm from the edge of the culture in the y-direction).

5) The oxygen and glucose concentrations are independent of z-direction (above the cells). The validity of this assumption will be assessed within this chapter for the chosen experimental conditions.

---

**Figure 4.1** A) Diagram of the Sandwich Culture System. Adapted from Reference (105). The cells are “sandwiched” between two glass slides separated by a spacer of known height. Oxygen and nutrients from the media can only reach the central region of the sandwich culture by diffusion, setting up oxygen gradients within the system as the cells consume oxygen. An agarose mold surrounds the system, which allows diffusion of small molecules such as oxygen and nutrients while preventing convective mixing of the fluid in contact with the cells. B) A schematic of the sandwich culture demonstrating the directions of interest. The x-direction is the direction of diffusion of oxygen and gradients, the y-direction is parallel to the edge of the sandwich culture, and the z-direction is perpendicular to the surface on which the cells are grown.
For these assumptions, the diffusion equations reduce to two coupled, one-dimensional unsteady-state partial differential equations:

\[
\frac{\partial p_o(x,t)}{\partial t} = D_o \frac{\partial^2 p_o(x,t)}{\partial x^2} - Q_o(p_o, C_G) \frac{N}{Z_G} \tag{4.1}
\]

\[
\frac{\partial C_G(x,t)}{\partial t} = D_G \frac{\partial^2 C_G(x,t)}{\partial x^2} - Q_G(p_o, C_G) \frac{N}{Z_G} \tag{4.2}
\]

where \(p_o(x,t)\) and \(C_G(x,t)\) are the location- and time-dependent partial pressure of oxygen and concentration of glucose, respectively; \(D_o\) and \(D_G\) are the diffusional constants of oxygen and glucose in water at 37°C (the temperature at which all experiments are performed); \(N\) is the number density of cells in the sandwich culture system (cells/cm^2); \(Z_G\) is the gap height of the sandwich culture system; and \(Q_o(p_o, C_G)\) and \(Q_G(p_o, C_G)\) are the per-cell consumption rates of oxygen and glucose that are potentially dependent on oxygen and glucose concentration. The quantity \(N/Z_G\) is a valid measure of the volume density of cells within the sandwich culture as long as the assumption of z-independence of nutrient concentration holds (103, 120). The diffusion constants of oxygen and glucose at 37°C are: \(D_o = 3 \times 10^{-5} \text{ cm}^2/\text{sec}\) (122, 123) and \(D_G = 9 \times 10^{-6} \text{ cm}^2/\text{sec}\) (124).

The appropriate initial and boundary conditions for the sandwich culture condition arise from the ambient culture conditions at either edge of the sandwich culture (width = 2.5 cm) and across the entire culture at the initial time point when the top slide is put in place:
\[ p_o(x = 0 \text{ cm, } t) = p_o(x = 2.5 \text{ cm, } t) = p_o(x, t = 0) = 160 \text{ mmHg} \quad (4.3) \]

\[ C_g(x = 0 \text{ cm, } t) = C_g(x = 2.5 \text{ cm, } t) = C_g(x, t = 0) = 3.5 \text{ mg/mL} \quad (4.4) \]

Equations 4.1-4.4 can be solved if the per-cell consumption rates of oxygen and glucose are known for the cellular system of interest and if the cell density and gap height within the sandwich culture system are specified. The experimental determination of the oxygen and glucose consumption rates as functions of oxygen and glucose concentrations is described in the next section. To optimize the sandwich culture system for maintaining stable, measurable oxygen gradients, the effects of varying cell density and gap height on the nutrient profiles are then evaluated by numerical solution of Equations 4.1-4.4.

### 4.3 Glucose Consumption Rates

#### 4.3.1 Dependence on Glucose Concentration

The glucose consumption rates were measured as described in Chapter 3. Briefly, the glucose concentration of media was measured in static monolayer cell culture exposed to normoxia (21\% O\textsubscript{2}; \( p_o = 160 \) mmHg) or hypoxia (1\% O\textsubscript{2}; \( p_o = 7.6 \) mmHg) over a 48-hr culture period. Measurements were made for eight conditions (4 in normoxia, 4 in hypoxia): 1) HIF-1\( \alpha^{+} \) ES cells seeded at 100,000 cells/cm\textsuperscript{2} (low seeding density), 2) HIF-1\( \alpha^{+} \) ES cells seeded at 500,000 cells/cm\textsuperscript{2} (high seeding density), 3) HIF-1\( \alpha^{-} \) ES cells at low seeding density, and 4) HIF-1\( \alpha^{-} \) ES cells at high seeding density. The culture conditions (normoxia vs. hypoxia) and glucose
measurements were begun after 24 hours of culture in normoxic conditions. Glucose measurements were made at 0 hr, 6 hr, 16 hr, 24 hr, 31 hr, 41.5 hr, and 48 hr for each condition, for a total of 56 measurements. Since the cells were grown on a mitotically inactive mouse embryonic fibroblast monolayer, the glucose consumed by a fibroblast layer alone was also measured and subtracted from the ES cell glucose measurements to get a consumption rate for the ES cells alone. In a separate experiment, ES cells were seeded under the same eight conditions, and cell counts were made in duplicate by hemocytometer at 6 time points up to 42 hours in culture. The per-cell glucose consumption rates were determined for each time point by dividing the instantaneous slope of the glucose consumption curve by the number of cells at the corresponding time. An example of the method used for the generation of a glucose consumption rate is demonstrated in Figure 4.2 for HIF-1α+/− ES cells seeded at 100,000 cells/cm² in hypoxia.
Figure 4.2 Demonstration of Glucose Consumption Rate Measurement. A) Sample glucose consumption measurements over time. The dashed line represents the instantaneous slope at 24 hrs of culture. B) Sample cell count measurement over time for sandwich culture experiment. Thus, the per-cell glucose consumption rate at 24 hrs is \( \frac{0.025 \text{ mg/cm}^2\text{-hr}}{603,000 \text{ cells/cm}^2} = 4.2 \times 10^{-8} \text{ mg/cell-hr} \).

In Figure 4.3, the glucose consumption rate for the HIF-1α+/+ and HIF-1α-/- cells for low and high seeding densities are plotted together against the glucose concentration at which the glucose consumption rate measurement was made. This provides the glucose concentration dependence
of the glucose consumption rate necessary for the solution of Equations 4.1-4.4. In this figure, the normoxic and hypoxic glucose consumption rates are plotted separately to obtain the oxygen dependence of the glucose consumption rate. It can be seen that glucose consumption rate is roughly linear with glucose concentration. The data for HIF-1α and HIF-1α ES cells, as well as data from experiments with different seeding densities, were combined, as these factors did not affect the relationship between glucose consumption rate and glucose concentration. Also, the glucose consumption rate is increased under hypoxic conditions relative to the normoxic conditions. Since glucose consumption rate should fall to zero at zero glucose concentration, linear approximations of the glucose dependence are determined by performing a least squares fit to a linear equation with a forced zero intercept. This linear approximation fits the data reasonably well at higher glucose concentrations; however, the goodness of fit decreases as the glucose concentration falls below 1 mg/mL. As a first approximation, the linear fit for the normoxic conditions: \( Q_G \text{ (mg/cell-hr)} = 1.95 \times 10^{-8} [C_G \text{ (mg/mL)}] \) will be used for the glucose concentration dependence of the glucose consumption rate in the analytic solution of Equations 4.1-4.4.
Glucose Consumption Rates of HIF-1α⁺/⁺ and HIF-1α⁻/⁻ ES Cells

![Graph showing glucose consumption rates at different oxygen and glucose concentrations](image)

Figure 4.3 Experimental Glucose Consumption Rates for HIF-1α⁺/⁺ and HIF-1α⁻/⁻ cells (data combined) at various glucose concentrations in normoxia (21% O₂; blue diamonds) and hypoxia (1% O₂; pink squares). The solid lines are linear least square fits of the data with the lines forced through the origin (no glucose consumption at zero glucose conditions). There was significant scatter of the data at conditions of combined low glucose and low oxygen concentrations.

4.3.2 Dependence on Oxygen Concentration

It is also necessary to determine an expression for the oxygen concentration dependence of the glucose consumption rate for the solution of Equations 4.1-4.4. Tziampazis and Sambanis(125) measured glucose consumption rate as a function of oxygen concentration in mouse insulinoma βTC3 cells and found that glucose consumption rate was constant at the normoxic value down to an oxygen partial pressure ($p_o$) of approximately 13-15 mmHg. Below this value, the glucose consumption rate increased linearly to a maximum value at anoxic conditions. This well-established rise in glucose consumption under hypoxia, the Pasteur effect, is observed for many cell lines(121). Balin et al. reported no significant difference in glucose consumption rate in WI-38 lung embryonic fibroblasts from 26-134 mmHg, and an increased glucose consumption rate when grown at a $p_o$ of 7.8 mmHg(126). In a model of coupled glucose and oxygen consumption in V79 Chinese hamster lung cells(120), Hlatky et al. also assumed an
approximately linear rise in glucose consumption rate beginning at a $p_o$ of approximately 30 mmHg and rising to a maximum value of twice the normoxic rate in anoxic conditions. The embryonic stem cells used in the sandwich culture experiments had a 33% higher glucose consumption rate at a $p_o$ of 7.6 mmHg than at normoxic conditions (see Figure 4.3: $2.59 \times 10^{-8}$ (mL/cell-hr) / $1.95 \times 10^{-8}$ (mL/cell-hr) = 1.33), consistent with the above results reported in the literature. For the purposes of the solution of Equations 4.1-4.4, it was assumed that the cells have a similar oxygen dependence of glucose consumption rate as reported in the literature. The glucose consumption rate is assumed constant from 15-160 mmHg at the value measured for 160 mmHg ($Q_{G,\text{normoxic}}$). Below 15 mmHg, the glucose consumption rate is assumed to rise linearly by the equation, $Q_G$ (mg/cell-hr) = $(-0.0446 * p_o + 1.67) * Q_{G,\text{normoxic}}$, based on the glucose consumption rate experimentally measured at 1% oxygen.

Combining the glucose and oxygen dependence of the glucose consumption rate gives the following expressions for use in Equations 4.1-4.4.

If $p_o \geq 15$ mmHg:

$$Q_G (\text{mg/cell-hr}) = 1.95 \times 10^{-8} * C_G \quad (4.5)$$

If $p_o < 15$ mmHg:

$$Q_G (\text{mg/cell-hr}) = (-0.0446 * p_o + 1.67) * 1.95 \times 10^{-8} * C_G \quad (4.6)$$

where $p_o$ is in mmHg and $C_G$ is in mg/mL.
4.4 Oxygen Consumption Rate

4.4.1 Measurement of Oxygen Consumption Rate

The oxygen consumption rate of HIF-1α^{+/} and HIF-1α^{-} cells was measured in a stirred chamber closed reactor system as described in Chapter 3. Briefly, cells are counted with a flow cytometer and concentrated to 5-6×10⁶ cells/mL. A volume of cells (210 μL) is loaded into a titanium water-jacketed chamber kept at a constant temperature of 37 °C. The suspension of cells is stirred with a glass-coated stirring bar, and the chamber is sealed and impermeable to oxygen. A fiber-optic oxygen sensor (Instech Laboratories Inc.) within the chamber measures the instantaneous oxygen tension, which is recorded every 11 seconds. The oxygen consumption rate (OCR) is simply calculated from the slope of the oxygen tension vs. time curve. The cell number is assumed to remain constant during the oxygen consumption because the entire experiment is performed in less than 30 minutes. The system is calibrated at room air (p₀ = 160 mmHg) and anoxic (p₀ = 0 mmHg) conditions during each experiment. The OCR was calculated for both HIF-1α^{+/} ES cells and HIF-1α^{-} ES cells in high-glucose media (3.5 mg/mL glucose), with a total of 6 replicate measurements for each cell type.

Figure 4.4 shows a typical experimental measurement for HIF-1α^{+/} cells. The graph begins just before the cells are introduced into the chamber and the chamber is sealed, and the end of the graph is after all of the oxygen has been consumed by the cells. The values have been calibrated such that the value for media exposed to room air (calibration data not shown) is 160 mmHg and the value when all of the oxygen is consumed is set to zero. The linear portion of the curve gives the OCR in terms of overall consumption rate (mmHg/min). The per cell OCR (Q₀) can be
calculated given the starting concentration of cells (5.34×10^6 cells/mL in this case) and the solubility of oxygen in water (k_o = 1.19 nmol/mmHg-mL at 37°C (127)) as follows:

\[ Q_0 (\text{nmol/cell - min}) = \text{Overall OCR (mmHg/min)} \times \frac{k_o (\text{nmol/mmHg - mL})}{\text{Cell concentration (cells/mL)}} \] (4.7)

**Figure 4.4** Oxygen consumption rate measurement of HIF-1α⁺/⁺ ES cells at a concentration of 5.34×10^6 cells/mL in a sealed stirred chamber reactor. The oxygen concentration was measured with a fiberoptic sensor over time, and the resulting slope was used in Equation 4.7 to obtain OCR: OCR = 10.1 mmHg/min * \( \frac{1.19 \text{ nmol/mmHg - mL}}{5.34 \times 10^6 \text{ cells/mL}} \) = 2.24×10^6 nmol/cell-min.

The OCR was measured as in Figure 4.4 for six replicates each of HIF-1α⁺/⁺ and HIF-1α⁻/⁻ ES cells. The results are shown in Figure 4.5, with the units of OCR converted to nmol/10^5 cells-hr for convenience. The oxygen consumption rate of the HIF-1α⁻/⁻ cells was slightly higher (~15% higher; p = 0.02) than that of the HIF-1α⁺/⁺ cells. For the purpose of the calculations in the remainder of this thesis, the average value of the OCR for both cell types, 13.7 nmol/10^5 cells-hr, will be used. This assumption should introduce minimal error because 1) the difference in OCR between the two cell types is small, and 2) the sandwich culture experiments are performed with
a mixture of 50% HIF-1α<sup>+</sup> cells and 50% HIF-1α<sup>-</sup> cells. Thus, the actual consumption rate experienced within the sandwich culture should be close to the average value between the two cell types.

![Oxygen Consumption Rate of ES Cells](image)

**Figure 4.5 Oxygen consumption rate of ES Cells.** Average values of 6 replicates for each cell type. The HIF-1α<sup>-</sup> cell OCR is significantly higher than that of the HIF-1α<sup>+</sup> cells (p = 0.02). The error bars indicate the standard error of the mean for the measurements.

### 4.4.2 Dependence on Glucose Concentration

For comparing relative oxygen consumption rates at various glucose concentrations, an oxygen biosensing microplate was used. The BD Oxygen Biosensor System (OBS; BD Biosciences, Bedford, MA) uses an oxygen-sensitive fluorophore in a silicon rubber matrix in the bottom of a standard 96-well plate to measure oxygen concentrations. The method of preparing the plates has been described previously(128), as has the method of measuring oxygen consumption rates(109). The method is briefly outlined in Chapter 3 of this thesis. While this method has been shown to be very accurate at measuring relative oxygen consumption rates within an experiment, the absolute values of oxygen consumption rate (OCR) in comparative studies to traditional methods
of measurement have been shown to underestimate OCR by a consistent factor of 2-3 [data not published; personal communication with Mark Timmins (BD Biosciences) and Clark Colton (Chemical Engineering, Massachusetts Institute of Technology)]. Therefore, for this thesis, the stirred chamber method described above is used to measure the absolute OCR, and the ability of the OBS system to make multiple measurements simultaneously in a high-throughput manner is used to compare relative OCRs at different glucose concentrations.

The results of the OCR measurements at different glucose concentrations are shown in Figures 4.6 and 4.7. Figure 4.6 demonstrates that using the OBS system, there is no significant difference between OCR of HIF-1α+/+ and HIF-1α−/− cells at any given glucose concentration. It is likely that the OBS is not sensitive enough to pick up the small difference in OCR measured in the stirred chamber method in Section 4.4.1. This is confirmed by the wide error bars showing the standard error of the mean in Figure 4.6. Thus, in Figure 4.7, the data for HIF-1α+/+ and HIF-1α−/− cells are combined to obtain a general relationship between glucose concentration and oxygen consumption rate. The OCR drops slightly with increasingly low glucose concentration, and the linear least-squares fit of the data is shown in Figure 4.7. Comparing the value obtained for OCR obtained for 3.5 mg/mL of glucose in the OBS system with the more accurate value obtained with the stirred chamber system (Section 4.4.1) demonstrated that the OBS system underestimates the value of OCR by a factor of 4.0. Thus, the final dependence of per-cell OCR ($Q_o$) on glucose concentration is:

$$Q_o (\text{nmol}/10^5 \text{cells} \cdot \text{hr}) = 4.0 \times (0.263 \times C_G (\text{mg/mL}) + 2.52)$$
$$= 1.05 \times C_G (\text{mg/mL}) + 10.08$$

(4.8)

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Figure 4.6 Oxygen Consumption Rate of ES cells as Measured by BD Oxygen Biosensor System. N = 2-4 for each data point, and there is no significant difference between OCR for HIF-1α^{++} and HIF-1α^{-} cells at any glucose concentration.

Figure 4.7 Combined data of Oxygen Consumption Rates measured at various glucose concentrations for HIF-1α^{++} and HIF-1α^{-} ES cells. N = 5-7 for each data point.
4.4.3 Dependence on Oxygen Concentration

The oxygen consumption rate of cells generally follows Michaelis-Menten kinetics with respect to oxygen concentration (120, 127, 129, 130):

\[
Q_O = \frac{V_{\text{max}}P_O}{K_M + P_O}
\]  

(4.9)

where \(V_{\text{max}}\) is the maximum consumption rate (at normoxic conditions) and \(K_M\) is the half-maximal oxygen concentration at which the oxygen consumption rate is half of its maximum rate. In general, oxygen consumption rate is constant as the oxygen concentration drops until the cells experience hypoxia at fairly low oxygen concentrations. \(K_M\) values typically fall in the range of 0.5-10 mmHg (120, 127, 129, 130). For the ES cell sandwich culture system, \(V_{\text{max}}\) is equivalent to the expression for the glucose dependence of OCR at normoxic conditions (Equation 4.8). Thus, an estimate of \(K_M\) for this system is sufficient to describe the oxygen dependence of OCR in the ES cells.

The OCR data obtained from the stirred chamber measurements is used to estimate the \(K_M\) value for ES cells. Since the stirred chamber system is making continuous measurements of oxygen tension as the oxygen tension drops, the instantaneous slope of the OCR curve should give the OCR for that particular oxygen concentration. Thus, if the instantaneous slope is plotted against the oxygen tension, a Michaelis-Menten curve should be generated. To calculate the instantaneous slope, at each time point the oxygen tension was recorded along with the change in oxygen tension from the previous time point to the next time point. Figure 4.8 shows the results for one of the HIF-1αc ES cell OCR measurements. Aside from the wide scatter in the leveling off of the curve at \(V_{\text{max}} = 9.4\) mmHg/min, the curve behaves as a standard Michaelis-Menten kinetic curve, with the slope initially rising before leveling off at the “saturation” value, which is
the maximal OCR as measured in Figure 4.4. For each OCR curve, a straight line was fit through the initial rising portion of the curve, as shown in Figure 4.8. The point at which this line crossed the value corresponding to one half of the maximal OCR was taken as the $K_M$ for that curve. These calculations are depicted graphically for the sample curve shown in Figure 4.8.

**Figure 4.8** Method of Calculation of $K_M$ for oxygen dependence of OCR. The data is a representative curve from one of the OCR measurements as depicted in Figure 4.4. The solid line is a best fit line through the initial rising points of the curve, and the large dashed line is the $V_{\text{max}}$ calculated as the slope of the linear portion of the OCR curve. The small dashed lines demonstrate the method used to calculate $K_M$: the $p_o$ value at which the solid line crosses one half of the $V_{\text{max}}$ value.

Curves similar to Figure 4.8 were made for all twelve measurements of OCR in HIF-1α^{+/+} and HIF-1α^{-/} cells. The average value of $K_M$ calculated for HIF-1α^{+/+} cells was not significantly different from that of HIF-1α^{-/} cells ($p = 0.7$), so the average value of all twelve measurements was used as a universal $K_M$ for these cells. The value of $K_M$ calculated in this manner was 5.2 ± 0.9 mmHg (mean ± SEM).
The oxygen- and glucose- dependence of OCR can finally be combined into a general relationship for the per-cell oxygen consumption rate:

\[ Q_0 (\text{nmol/10}^5 \text{ cells - hr}) = (1.05 * C_G + 10.08) \frac{P_o}{P_o + 5.2} \]  

where \( C_G \) is in mg/mL and \( P_o \) is in mmHg. Equation 4.10 takes into account the oxygen dependence and glucose dependence of oxygen consumption rate as determined above, as well as the absolute normoxic, normoglycemic value calculated using the stirred chamber reactor method.

### 4.5 Final Diffusion-Reaction Model

Finally, Equations 4.5, 4.6, and 4.10 can be used in Equations 4.1-4.2 to obtain the final model for the diffusion and consumption of glucose and oxygen within the sandwich culture system. In these final equations, the diffusivities have been converted to units of cm\(^2\)/hr \((D_o = 0.108 \text{ cm}^2/\text{hr}; D_G = 0.0324 \text{ cm}^2/\text{hr})\) and the solubility of oxygen at 37\(^\circ\)C \((k_o = 1.19 \text{ nmol/mmHg-mL})\) has been incorporated to convert the units within the oxygen consumption rate into partial pressure of oxygen (mmHg) from the molar concentration. Thus, the final forms of the differential equations are:

\[ \frac{\partial P_o}{\partial t} = 0.108 \frac{\partial^2 P_o}{\partial x^2} - \left[ \frac{(1.05 * C_G + 10.08) * P_o}{1.19 \times 10^5 * (P_o + 5.2)} \right] * \frac{N}{Z_G} \]  

(4.11)
If \( p_o < 15 \) mmHg,

\[
\frac{\partial C_G}{\partial t} = 0.0324 \frac{\partial^2 C_G}{\partial x^2} - \left[ (-0.045 * p_o + 1.67) \times 1.95 \times 10^{-8} * C_G \right] \frac{N}{Z_G} \tag{4.12}
\]

If \( p_o \geq 15 \) mmHg,

\[
\frac{\partial C_G}{\partial t} = 0.0324 \frac{\partial^2 C_G}{\partial x^2} - \left[ 1.95 \times 10^{-8} * C_G \right] \frac{N}{Z_G} \tag{4.13}
\]

where the units are: \( p_o \) [\( \text{mmHg} \)], \( C_G \) [\( \text{mg/mL} \)], \( t \) [\( \text{hr} \)], \( x \) [\( \text{cm} \)], \( N \) [\( \text{cells/cm}^2 \)], \( Z_G \) [\( \text{cm} \)].

The boundary conditions for these equations are given in Equations 4.3-4.4.

### 4.6 Theoretical Results of Diffusion-Reaction Model

The solutions to the differential equations 4.11-4.13 are determined with a single adjustable parameter, the cell volume density, which is represented by the cell surface density \( N \) divided by the gap height between the two slides of the sandwich culture \( Z_G \). Therefore, the oxygen and glucose concentration profiles will be determined by solving the differential equations for various values of \( N/Z_G \). Then, the solutions will be discussed in terms of optimal selection of the parameters \( N \) and \( Z_G \), which can be adjusted experimentally. In the process, the validity of various assumptions that were made for the development of equations 4.11-4.13 will also be discussed.

#### 4.6.1 Solutions to the Diffusion-Reaction Model

The differential equations were solved by using the partial differential equation solver (pdepe) function in Matlab7.0.4 (The Mathworks, Inc.). This function solves initial-boundary value problems for systems of parabolic partial differential equations in one space variable and time.
Figure 4.9 shows the solution of Equations 4.11-4.13 for a cell density \((N/Z_G)\) of \(3 \times 10^6\) cells/cm\(^3\) in three dimensions. It can be seen from the overall shapes of the curves that there is an initial drop in oxygen and glucose concentration across the entire slide, followed by a steady-state in which the concentration remains constant over time with a higher concentration near the edges and a minimum concentration of oxygen or glucose in the center of the sandwich culture slide. The effect of varying cell density is demonstrated in Figures 4.10-4.12, for values of \(N/Z_G\) of \(3 \times 10^5\), \(3 \times 10^6\), and \(3 \times 10^7\) cells/cm\(^3\), respectively.

Several general observations can be made from examining these three conditions. First, the oxygen profiles reach steady-state faster than the glucose profiles, and their steady-state profiles develop over a much shorter distance from the edge of the sandwich culture. For example, in Figure 4.11, the oxygen profile is constant after approximately one hour, while it takes the glucose profile 24-48 hours to reach steady-state. Also, the oxygen profile reaches its minimum value approximately 3 mm from the edge of the sandwich culture, while the glucose profile does not level off until approximately 1 cm from the edge, near the center of the sandwich culture. Importantly, the glucose is only completely consumed at the highest cell density (Figure 4.12), while oxygen is nearly completely consumed at the center of the sandwich culture even at 300,000 cells/cm\(^3\). These findings are due to the faster consumption rate of oxygen relative to the initial concentration, which is low due to the low solubility of oxygen in media (only 6 µg/mL at 160 mmHg, compared to 3.5 mg/mL of glucose).
Figure 4.9 Glucose and oxygen profiles within the sandwich culture system over a 48 hour time span for a seeding density of $N/Z_G = 3 \times 10^6$ cells/cm$^3$. 
Figure 4.10 Glucose and oxygen profiles from the edge of the sandwich culture for a seeding density of $3 \times 10^5$ cells/cm$^3$. The separate curves are labeled with the corresponding time points, demonstrating the development of the steady-state profile over time.
Figure 4.11 Glucose and oxygen profiles from the edge of the sandwich culture for a seeding density of $3 \times 10^6$ cells/cm$^3$. The separate curves are labeled with the corresponding time points, demonstrating the development of the steady-state profile over time. Note: The scale for the oxygen profile differs from the glucose profile to focus on the first 5 mm from the edge.
Figure 4.12 Glucose and oxygen profiles from the edge of the sandwich culture for a seeding density of $3 \times 10^7$ cells/cm$^3$. The separate curves are labeled with the corresponding time points, demonstrating the development of the steady-state profile over time. Note: The scale for the oxygen profile differs from the glucose profile to focus on the first 5 mm from the edge.
4.6.2 Optimization of $N/Z_c$ for Sandwich Culture System

It is clear from Figures 4.10-4.12 that the volume cell density, which in the sandwich culture system is approximated by the area cell density divided by the gap height between the slides, has a large effect on the oxygen and glucose profiles within the system. In order to determine the optimum cell density, it is necessary to specify the objective of the sandwich culture experiments.

The goal of using the sandwich culture system is to create a system with stable and measurable oxygen gradients over a reasonable distance such that the cells within the gradient can be viewed microscopically. Thus, the gradient should be gradual enough that the desired gradient extends over several millimeters, so that a microscope 10X objective with an 860 μm across field of view (see Chapter 3) can cover the gradient in several fields. On the other hand, if the gradient is too gradual, the cells may not experience hypoxia at all, or may be hypoxic only for a short region in the center of the sandwich culture. Another consideration is the temporal development of the oxygen gradient. Ideally, the oxygen gradient will develop quickly and remain stable for the duration of the sandwich culture experiment (on the order of 24 hours). A slower development would mean that the early time points would not be useful in the analysis. Finally, the glucose levels in the sandwich culture need to be considered. The objective of the thesis is to specifically measure the effects of hypoxia on the HIF-1α+/+ and HIF-1α−/− cells. Concurrent hypoglycemia could confound the results, so the ideal conditions would have a slower development of glucose gradients that do not ultimately result in hypoglycemia for the cells. Normal blood glucose levels are in the range of 1-1.5 mg/mL, and extracellular levels are typically much lower. In measurements of extracellular brain glucose levels, levels in normoglycemic rats have been
measured at 0.25-0.4 mg/mL (131, 132). Therefore, as long as levels are kept well above 0.5 mg/mL in the sandwich culture, the primary effect seen should be due to hypoxia and not hypoglycemia.

Keeping these criteria in mind, Figures 4.10-4.12 can be evaluated to determine the effects of varying \( N/Z_G \) on meeting these criteria. In Figure 4.10, it can be seen that if the cell density is too low, the oxygen gradients are extremely gradual and slow to develop. The steady-state gradient does not develop until 8 hours after the beginning of the experiment, and even at steady-state, the cells do not experience hypoxia until 9-10 mm into the sandwich culture. In Figure 4.12, when the cell concentration is too high, the entire oxygen gradient occurs in less than 1 mm of the sandwich culture, which does not allow for extensive viewing of the cells within the gradient. Also, the cells experience severe hypoglycemia within 3 hours of beginning the experiment. Figure 4.11 demonstrates reasonable conditions for the sandwich culture, with the oxygen gradient developing within 1 hour and extending over the first few mm of the sandwich and glucose levels remaining well above 0.5 mg/mL for the duration of the experiment throughout the sandwich culture.

The diffusion-reaction model in Equations 4.11-4.13 can be used to estimate key parameters that are useful in optimizing the cell density \( N/Z_G \) to be used in the experiments. In particular, six useful parameters are the diffusion time for oxygen and glucose to travel the entire half-width of the sandwich culture (\( T_{D,o} \) and \( T_{D,g} \)), the consumption time necessary for the total amount of oxygen or glucose at any location to be consumed (\( T_{C,o} \) and \( T_{C,g} \)), and the penetration distance for oxygen and glucose (\( X_o \) and \( X_g \)), which is the characteristic diffusion distance for each substance.
during the time period $T_{c,o}$ and $T_{c,g}$, respectively. If $W$ is the entire width of the sandwich culture (2.5 cm) and $p_{o,a}$ and $C_{g,a}$ are the ambient oxygen partial pressure and glucose concentration (160 mmHg and 3.5 mg/mL), then the listed parameters can be described by the following equations ($D_{o} = 3 \times 10^{-5} \text{ cm}^{2}/\text{sec}; D_{g} = 9 \times 10^{-6} \text{ cm}^{2}/\text{sec}; N/Z_{g} = \text{cell density in cells/cm}^{3}$):

$$T_{d,o} = \frac{(W/2)^{2}}{D_{o}} \quad (4.14)$$

$$T_{d,g} = \frac{(W/2)^{2}}{D_{g}} \quad (4.15)$$

$$T_{c,o} = \frac{p_{o,a}}{Q_{o} \ast (N/Z_{o})} \quad (4.16)$$

$$T_{c,g} = \frac{C_{g,a}}{Q_{g} \ast (N/Z_{g})} \quad (4.17)$$

$$X_{o} = (D_{o} \ast T_{c,o})^{0.5} \quad (4.18)$$

$$X_{g} = (D_{g} \ast T_{c,g})^{0.5} \quad (4.19)$$

To gain estimates of these parameters, the consumption rates will be estimated at concentrations midway between ambient conditions and zero concentrations since the actual consumption rates will vary across the width of the sandwich culture ($p_{o} = 80 \text{ mmHg}; C_{g} = 1.75 \text{ mg/mL}$) using Equations 4.5 and 4.10. Therefore,

$$Q_{o} \left( \frac{\text{mmHg}}{\text{hr} \cdot \text{cells/mL}} \right) = \frac{(1.05 \ast 1.75 + 10.08) \ast 80}{1.19 \ast 10^{3} \ast (80 + 5.2)} = 9.4 \ast 10^{-5} \frac{\text{mmHg}}{\text{hr} \cdot \text{cells/mL}}$$

$$Q_{o} \left( \frac{\text{mg/cell} \cdot \text{hr}}{\text{mg/cell} \cdot \text{hr}} \right) = 1.95 \ast 10^{-8} \ast 1.75 = 3.4 \ast 10^{-8} \text{ mg/cell} \cdot \text{hr}$$
Plugging in these values and other known parameters into Equations 4.14-4.19 gives the following parameter values or relationships:

\[
\begin{align*}
T_{D,O} &= 14 \text{ hr} \\
T_{D,G} &= 48 \text{ hr} \\
T_{C,O}(hr) &= \frac{1.7 \times 10^6}{N/Z_G} \\
T_{C,G}(hr) &= \frac{1.03 \times 10^4}{N/Z_G} \\
X_O (mm) &= \frac{4290}{(N/Z_G)^{0.5}} \\
X_G (mm) &= \frac{18200}{(N/Z_G)^{0.5}}
\end{align*}
\]

Figure 4.13 shows the predicted consumption times \((T_c)\) and penetration distances \((X)\) as a function of cell density. As long as the consumption time of oxygen is much less than the time for diffusion through the entire sandwich (14 hr), then a concentration gradient will develop from ambient concentration down to zero oxygen within the sandwich culture. The consumption time also gives an estimate of the time it will take for the system to reach steady-state.
Figure 4.13 A) Characteristic times for consumption of all oxygen or glucose at a given location within the sandwich culture as a function of cell density. The data labels (crosses) mark the values for the cell densities used to generate Figures 4.10-4.12. B) Characteristic penetration distances for oxygen or glucose within the sandwich culture as a function of cell density. The data labels (crosses) mark the values for the cell densities used to generate Figures 4.10-4.12.

It is evident from comparison of Figure 4.13 with the curves generated in Figure 4.10-4.12 that the characteristic consumption time is a good estimate for both oxygen and glucose of the time necessary for the steady-state concentration profile to be reached. The penetration distance for oxygen corresponds to the distance at which the oxygen concentration drops below
approximately 20 mmHg. Further analysis of the curves indicates that the oxygen concentration drops to near zero at a distance approximately 50% greater than the characteristic penetration distance. For glucose, at the two higher cell densities (3×10⁶ and 3×10⁷ cells/cm³), the penetration distance corresponds to a glucose concentration of approximately 0.9 and 0.6 mg/mL, respectively. Importantly, at the lower cell densities, the glucose level does not drop to zero even in the center of the sandwich culture.

To determine an optimum cell density for the sandwich culture, the following stipulations were made:

1) The time to reach steady-state for the oxygen concentration gradient, as estimated by $T_{c.o}$, should be less than 1 hour so that the oxygen gradient develops quickly.

2) The glucose concentration profile should remain above 0.5 mg/mL throughout the entire sandwich culture to avoid the contribution of hypoglycemia to the results.

3) The penetration distance for oxygen should be between 1.3 and 2 mm. This should allow for an oxygen gradient that drops from ambient conditions down to zero oxygen over a distance of 2-3 mm, which corresponds to several fields of view for microscopic analysis.

Given these conditions, Figure 4.13 was used in combination with iterative trials of the MATLAB simulations to give the optimum cell density that meets all three conditions. The resulting optimum cell density is approximately 4.6×10⁶ cells/cm³. Below this cell density, the oxygen penetration distance rises above 2 mm, which may be too gradual a gradient to visualize the behavior of individual cells or groups of cells within the gradient. Above this cell density, the
glucose levels in the center of the cell culture drop below 0.5 mg/mL, and the possibility of cells experiencing hypoglycemia in addition to hypoxia increases.

4.6.3 Effect of $Z_G$ on Assumption of z-Independence of Nutrient Concentrations

The solution of the diffusion-reaction model above required only a specification of the “volume” cell density, $N/Z_G$, to obtain estimates of the concentration profiles. In reality, the area cell density, $N$ (cells/cm$^2$), and the gap height, $Z_G$, can be varied independently. The key limitation for changing the gap height is the desire to maintain constant oxygen and glucose concentrations in the gap above a particular cell within the sandwich culture. The dimensionless Damköhler number can be used to assess the sandwich culture conditions for a given experimental condition. The Damköhler number is a ratio of the characteristic diffusion time for oxygen or glucose to diffuse across the gap to the characteristic reaction time, which is the consumption time $T_c$ for oxygen or glucose (133). Therefore, the Damköhler number ($Da$) can be given for oxygen and glucose by the following expressions:

$$Da_o = \frac{(Z_G^2/D_o)}{P_{o,a}/(Q_o * N/Z_G)} \quad (4.20)$$

$$Da_G = \frac{(Z_G^2/D_G)}{C_{G,a}/(Q_G * N/Z_G)} \quad (4.21)$$

For the assumption of z-independence to hold, the diffusion time should be much less than the reaction time, corresponding to $Da \ll 1$. In this case, the ambient conditions will be used to calculate $Q_o$ and $Q_G$ in order to calculate the maximum Damköhler number within the system (at the edges of the sandwich culture). This gives $Q_o = 1.1 \times 10^4$ mmHg/(hr-cells/cm$^3$) and $Q_G = 6.8 \times 10^8$ mg/cell-hr. Assuming that the area cell density is manipulated along with the gap height
to maintain a constant $N/Z_G$ at the optimum determined cell density above, $4.6 \times 10^6$ cells/cm$^3$, the relationship between Damköhler number and gap height (converted to mm) is:

$$Da_o = \left(\frac{Z_G[\text{mm}]}{1.8}\right)^2 \quad (4.22)$$

$$Da_G = \left(\frac{Z_G[\text{mm}]}{6.0}\right)^2 \quad (4.23)$$

Therefore, as long as $Z_G << 1.8$ mm, the assumption of $z$-independence of oxygen and glucose concentrations will hold. Experimentally, the gap height is created by using glass cover slips that are $175 \mu$m thick, defining the lower limit of the gap height. Figure 4.14 is a graph of the necessary cell area densities for varying gap heights (points on graph are multiples of $175 \mu$m) to give a volume cell density of $4.6 \times 10^6$ cells/cm$^3$.

**Figure 4.14** Experimental conditions that give desired cell density of $4.6 \times 10^6$ cells/cm$^3$. The data points indicate discrete possible experimental values of gap height using $175\mu$m spacers to obtain the desired gap height. The data labels give the value of the area cell density at each marked point on the curve.
For all experiments performed in this thesis, a gap height of 350 \( \mu m \) was arbitrarily chosen, giving a target area cell density of 160,000 cells/cm\(^2\).

4.7 Experimental Validation of Model

The predicted oxygen concentrations by the theoretical model were compared to experimentally observed oxygen concentration profiles that develop within the sandwich culture. In order to obtain approximately 160,000 cells/cm\(^2\) area cell density at the beginning of the sandwich culture experiments, 50% HIF-1\( \alpha^{+}\) ES cells and 50% HIF-1\( \alpha^{-}\) ES cells were seeded onto an inactivated fibroblast feeder layer at a density of 100,000 cells/cm\(^2\). The sandwich culture was started by carefully adding the top slide 24 hours after the initial seeding. The media used contained an oxygen-sensitive palladium-bound porphyrin molecule bound to bovine serum albumin (Oxyphor RO; Harvard Apparatus). Immediately after starting the sandwich culture, the system was placed in a temperature-controlled bath at 37\( ^{\circ}C \) and placed onto an automated stage connected to a microscope that was used to measure the phosphorescence quenching of the porphyrin molecule after excitation at 540 nm (106). The value of the oxygen tension was obtained from the phosphorescence lifetime using a calibration curve made from solutions of known oxygen concentrations (see Chapter 3 for details). The oxygen tension was measured at 1 mm increments across the sandwich culture, and the measurements were repeated at different time points following the beginning of the sandwich culture experiment. The measurements were made for the first three hours of the sandwich culture experiment, long enough to determine that a stable and lasting oxygen gradient had been established.
Figure 4.15 shows the developing oxygen gradient as measured in the sandwich culture experiment. The nature of the experimental measurements prevents accurate $p_o$ values from being obtained above approximately 60 mmHg. Any values above this cutoff fall outside of the calibration curve and are marked on the graph as being above the limits of the measurement technique. It is seen that after 5 minutes, a gradient has already been established that extends 6-7 mm into the sandwich culture. The gradient develops further over the next 15 minutes, and from 20 minutes to 3 hours after beginning the experiment, the gradient has fully developed and is stable over 2-3 mm into the sandwich culture.

![Oxygen Profiles in Sandwich Culture Over Time](image)

**Figure 4.15** Experimental measurements of $p_o$ within sandwich culture started 24 hours after seeding at a density of 100,000 cells/cm$^2$. The control measurement is from a separate slide taken without adding the top slide to the culture.

Figure 4.16 shows the same experimental data from Figure 4.15 with the theoretical curves for 20 minutes and two hours with a cell density of $4.6 \times 10^6$ cells/cm$^3$. While the curves generally predict the behavior of the oxygen profiles within the sandwich culture, the time to steady-state and penetration distance into the sandwich culture are both over predicted. On the one hand, the
model predicts that the steady-state gradient is not reached until 30 minutes after placing the top slide, whereas the experimental curves are clearly equilibrated after only 20 minutes. On the other hand, the final steady-state curve also lies to the right of the experimental data, indicating an overpredicted penetration distance.

Figure 4.16 Theoretical predictions of oxygen concentration curves 20 minutes and 2 hours (steady-state) after starting the sandwich culture experiment. The model overpredicts the time it takes to reach steady-state, and the predicted curves are shifted to the right of the experimental curves. Theoretical curves: $N/Z_0 = 4.6 \times 10^6$ cells/cm$^3$.

The experiments were seeded at 100,000 cells/cm$^2$ to obtain a cell density approximately equal to the theoretical optimum. Given the overprediction of consumption time and penetration distance in the model, it is worthwhile to more accurately measure the cell density after seeding. To measure the actual cell densities 24 hours after seeding with 100,000 cells/cm$^2$, 8 glass slides in 24-well plates were seeded with cells at 100,000 cells/cm$^2$: 4 with HIF-1α$^{+}$ ES cells and 4 with HIF-1α$^{-}$ ES cells. Twenty-four hours after seeding the slides, the cells were trypsinized and counted. The cell densities 24 hours after seeding with 100,000 cells/cm$^2$ were actually (3.2 ±
0.3)×10⁵ cells/cm², which corresponds to an N/Z₀ of 9.2×10⁶ cells/cm³. Solving the diffusion-reaction equations with this value of N/Z₀ gives the theoretical curves in Figure 4.17 for 20 minutes and 2 hours after starting the experiment.

![Oxygen Profiles in Sandwich Culture Over Time](image)

**Figure 4.17** Theoretical predictions of oxygen concentration curves 20 minutes to 3 hours (steady-state) after starting the sandwich culture experiment using a corrected N/Z₀ = 9.6×10⁶ cells/cm³. The theoretical curves remain constant from 20 minutes onward, indicating that steady-state has occurred before 20 minutes. The theoretical curves make an extremely accurate prediction of the experimentally observed behavior.

Although the actual starting cell density in Figure 4.16 was higher than the optimal density determined theoretically, the resulting oxygen gradient is sufficient for the goals of the sandwich culture experiment: the gradient is stable and extends over the first 2 mm of the sandwich culture, allowing six fields of view for observing the cells within the gradient (three on each edge of the sandwich culture). Thus, stipulation 3 in Section 4.6.2 still holds (X₀ = 1.4 mm, causing the entire gradient to extend over 2 mm). The next task is to determine whether the other stipulations outlined in Section 4.6.2 are violated, or whether they can be relaxed. Stipulation 1, that the time to reach steady-state for the oxygen gradient be less than 1 hour, still holds true and
is even lower with the higher seeding density \( T_{c,0} = 11 \) min. As for stipulation 2, although the glucose concentrations do drop below 0.5 mg/mL, the steady-state glucose gradient does not occur until approximately 11 hours into the experiment \( T_{c,G} \), and 7 mm deep into the sandwich culture. Therefore, the primary region of interest (the first 2 mm) remains normoglycemic for the duration of the experiment, as does the majority of the entire slide. For these reasons, it was decided to proceed with the experimental observations in Chapter 5 using initial seeding densities of 100,000 cells/cm\(^2\), giving the oxygen concentration curves depicted in Figure 4.17.

It is also necessary to account for the effects of proliferation during the 24-hour sandwich culture experiments. It is difficult to account for this explicitly because as most of the cells in the system are exposed to hypoxic conditions, the majority of the system will be proliferating very slowly while the edges proliferate rapidly. To get a rough approximation of the average proliferation in 24 hours of the cells in the short distance across which the oxygen concentration falls, the following experiment was performed. As before, 8 glass slides were seeded with 100,000 cells/cm\(^2\) each, 4 with HIF-1α\(^+\) ES cells and 4 with HIF-1α\(^-\) ES cells. The cells were allowed to grow for 24 hours as before. Out of each group of 4 slides, 2 slides were then placed in hypoxia (1% O\(_2\)) for an additional 24 hours, while the other 2 were kept in normoxic conditions. Thus, a total of 4 slides were grown in hypoxia for 24 hours and 4 in normoxia. At the end of the final 24 hours, the total cell density was measured on each of the 8 slides. The normoxic and hypoxic values were averaged together to approximate the average cell density across the oxygen gradient within the sandwich culture. The average cell density obtained in this way was \((7.6 \pm 0.5) \times 10^5\) cells/cm\(^2\), giving an \( N/Z_c \) of 2.2×10\(^7\) cells/cm\(^3\). Figure 4.18 superimposes the theoretical
prediction for the average cell density at 24 hours after beginning the sandwich culture experiment onto the curves from Figure 4.17.

Figure 4.18 Effect of 24 hours of proliferation on oxygen concentration profile within sandwich culture. The gradient becomes steeper and is shifted 0.5 mm to the left.

While the gradient is shifted to the left after accounting for 24 hours of proliferation within the cell culture, the gradient still extends over two fields of view on each edge of the sandwich culture. Therefore, the starting concentration of 100,000 cells/cm² will be sufficient for the experiments performed in Chapter 5.

4.8 Discussion

In this chapter, a diffusion-reaction model was developed to predict the oxygen and glucose concentration profiles within the sandwich culture system, an ideal system for studying the effects of gradients on cells in culture. The glucose consumption rates and oxygen consumption rates were measured for a variety of conditions, and combined with knowledge of cell behavior from the literature, empirical expressions for the glucose and oxygen dependence of the two
consumption rates were developed. These expressions were used in the diffusion-reaction partial
differential equations to obtain explicit oxygen and glucose concentration profiles within the
sandwich culture. The only experimental parameter to be determined in the resulting model is the
volume cell density of cells, which can be experimentally varied by changing either the seeding
cell density within the sandwich culture or the gap height between the top and bottom slides of
the sandwich. The effect of varying this parameter was determined using the model, and an
“optimum” cell density was determined.

The theoretical predictions matched extremely well to experimentally determined oxygen
profiles, after accounting for the proliferation of cells between the initial seeding and the start of
the experiment. These experimental and theoretical profiles allowed for the selection of a
starting cell density of 100,000 cells/cm² for further sandwich culture experiments, which will
allow for the development of stable, measurable oxygen gradients over a wide enough distance
to monitor the cell behavior microscopically within the oxygen gradient. The model as developed
is also a significant contribution in itself, as it allows for the design of further experiments to
measure the effect of oxygen and glucose gradients on various cell types.

One important area of research that could benefit from the use of the sandwich culture system
and the models describing the microenvironmental changes within the system is the area of stem
cell biology. The current studies on the sandwich culture system used mouse embryonic stem
cells as a model to study the effects of oxygen and oxygen gradients on these cells and the
dependence of these effects on HIF-1α expression (see Chapter 5). The possibility of using stem
cells in regenerative medicine requires the removal of the stem cells from their natural
environment and placing them into a foreign environment. It is necessary in doing this to understand how the stem cells interact with their microenvironment (134). The effects of oxygen and oxygen gradients on stem cells are important in many stem cell applications. For example, the potential of stem cells to be used in ischemic myocardium to regenerate the dead or dying heart muscle depends on the response of the stem cells to the ischemic microenvironment, including the effects of hypoxia and HIF expression on stem cell behavior (135). The ability of neural stem cells in the subventricular zone to replace neural cells damaged by brain injuries also depends on their protection against the effects of ischemia and hypoxia (136). Also, the understanding of how hypoxia and HIF expression affects embryonic development and stem cell differentiation is important in understanding how hypoxic responses contribute to serious disease and developmental abnormalities (137). The ability to accurately predict and control key microenvironmental conditions within the sandwich culture system using models such as those described in this thesis make this system ideal for furthering our understanding of stem cell biology and the therapeutic potential of stem cell techniques.

The sandwich culture system also provides a unique microenvironment that mimics the heterogeneous environment experienced by tumor and stromal cells in human cancers in vivo due to the chaotic and poorly perfused tumor vasculature. The mathematical model as developed can be used to predict the characteristics of the nutrient gradients within the sandwich culture and precisely control the gradients by varying the seeding cell density within the sandwich culture system. It is useful to compare the current model elaborated in this chapter with previous modeling descriptions of both tumor spheroids and the sandwich culture system. Casciari et al. (114), in their model of the tumor spheroid, additionally account for the effects of pH, carbon
dioxide, and other metabolic products and ions on the behavior of the cells within the spheroid. These other variables may also change over time within the sandwich culture system; it would be useful in future development of these preliminary models to determine the effect that these changes would have on the predicted oxygen and glucose concentration profiles. Particularly, the effect of pH on the consumption of glucose and oxygen should be evaluated. In the previous study (114), a pH-dependent increase in oxygen consumption rate was seen as glucose concentration decreased. This change was not observed in the current study, but could potentially become important as the pH within the sandwich culture system changes over time. In another study (115), both glucose consumption rate and oxygen consumption rate were found to depend on pH. While the current model did not account for pH effects, it still gave accurate predictions of the initial profile development within the sandwich culture. The ability of the model to accurately predict long-term profiles within the sandwich culture may be enhanced in future modifications by incorporating a model of the hydrogen ion concentrations and pH effects on nutrient consumption rates, similar to Casciari et al (114).

In a previous sandwich culture study modeling the development of oxygen and glucose concentration profiles (120), changes in cell density with time were also incorporated into the model. This is perhaps the biggest limitation of the current model. However, the previous study used measurements of cell density taken directly from the sandwich culture system as inputs to the model describing the profile development. The goal of the current study was to develop a predictive model that could be used to design the sandwich culture experiments in advance, precluding the use of measurements from the sandwich culture as inputs. Also, the primary focus of the current model was on describing the time course of the initial development of the
concentration profiles. Therefore, it was assumed that the cell density would remain reasonably constant for the early time point predictions of the model. Obviously, some of the later time predictions in Figures 4.10-4.12 would be affected by changes in cell density over time, as indicated in Figure 4.18. To incorporate the effects of cell proliferation on the model predictions, it will be necessary in future studies to measure cell proliferation rates as a function of both oxygen and glucose concentrations. Then, the cell density term, \( N \), in the diffusion-consumption equations could be made dependent on oxygen and glucose concentration, while maintaining the predictive nature of the model for designing sandwich culture experiments.

While the above considerations are important for future developments of the models described herein, the current work makes a significant contribution to the field. First, this is the first time that the unsteady-state diffusion-consumption equations have been applied to the sandwich culture system to describe the development of the concentration profiles rather than just the steady-state profiles. Second, the current work provides predictive models for the design of sandwich culture experiments in which all of the parameters of the model were empirically determined rather than fit to the experimental profiles. Third, the current work provides the first validation within the sandwich culture system of the oxygen profile predictions of the diffusion-consumption model by direct, noninvasive measurement of oxygen concentrations by phosphorescence quenching microscopy. The sandwich culture system and the models described in this work provide a valuable system for the future studies of the effects of microenvironment on tumor cells or in the fast-growing field of stem cell biology.
Chapter 5: *In vitro* Investigation of Localization Mechanism for ES Cells with Respect to Hypoxia: Migration, Proliferation, and Apoptosis

5.1 Introduction

As outlined in Chapter 2, one study has shown that HIF-1α+/+ embryonic stem cells are preferentially localized proximal to blood vessels in tumors of mixed HIF-1α++ and HIF-1α−/− cells (80), while another has suggested that HIF-1α+ ES cells are preferentially localized distal to blood vessels (81). The proposed mechanism for this localization has been the hypoxia-induced apoptosis of HIF-1α++ ES cells (1) as cells distant from blood vessels are subjected to hypoxia. However, as outlined in Chapter 2, differences in proliferation or migration of the two cell lines when exposed to hypoxic conditions could also lead to preferential localization of the cell types with respect to blood vessels and/or oxygen. These details are important to study because a decreased vascular dependence of cells upon loss of HIF-1α activity could lead to increased resistance of tumor cells to chemotherapy and radiation, which require access of blood vessels and oxygen to the tumor cells, respectively, to have a maximum therapeutic effect. The understanding of the mechanism for this potential resistance to therapy will be useful in designing targeted therapies to the resistant phenotype.

To study in depth the behavior of the HIF-1α++ and HIF-1α−/− cells with respect to blood vessels, an *in vitro* sandwich culture system is used to mimic the oxygen and nutrient gradients that develop as cells grow in regions distant from the blood supply. In Chapter 4, it was shown theoretically with experimental validation that in the sandwich culture system, stable and measurable oxygen gradients develop across the monolayer cell culture. Thus, cells near the edge
of the sandwich culture system experience normoxic conditions, while cells in the interior of the sandwich culture experience hypoxia. Moreover, in a defined region near the edge of the sandwich culture, the cells experience a gradient of oxygen, which is useful in determining the role of migration in the localization of tumor cells. In this chapter, the experimental sandwich culture system will be used to visualize the behavior of HIF-1α⁺⁺ and HIF-1α⁻ cells in a mixed co-culture system utilizing transfection of fluorescent proteins into the two cell types, fluorescence time-lapse microscopy, and immunofluorescent labeling techniques. In addition, adjunctive measurements of proliferation and apoptosis using more traditional cell culture techniques will provide further insights into the behavior of these cells when exposed to hypoxia and hypoglycemia.

5.2 Transfection of ES Cells with Fluorescent Markers

The HIF-1α⁺⁺ ES cells were transfected with retroviral particles derived from a human embryonic kidney packaging cell line (see Chapter 3 for methods). The retroviral particles contained cyan fluorescent protein (CFP) RNA, which was reverse transcribed and integrated into the ES cell genome. In this way, the HIF-1α⁺⁺ cells expressed CFP constitutively, so that they could be visualized microscopically. Likewise, the HIF-1α⁻ cells were transfected with yellow fluorescent protein (YFP), and expressed YFP stably and constitutively. Figure 5.1 demonstrates the high transfection efficiency of this transfection method, with nearly 100% expression. The cells were cultured on a mouse embryonic fibroblast feeder layer to prevent differentiation, and the non-fluorescent fibroblasts can be seen in Figure 5.1 between the ES cell colonies.
Figure 5.1 Brightfield microscopy image of HIF-1α<sup>+/+</sup> (left) ES cells and HIF-1α<sup>−/−</sup> (right) ES cells merged with fluorescent image of CFP (left; 433 nm Excitation, 475 nm Emission) or YFP (right; 513 nm Excitation, 527 nm Emission). The mouse embryonic fibroblast feeder layer is visible (non-fluorescent) between the ES cell colonies.

5.3 Analysis of HIF-1α Genotype in Transfected ES Cells

The ES cells were obtained as a generous gift from Peter Carmeliet (The Centre for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, University of Leuven, Belgium). The method of generation has been described previously (1). Briefly, the HIF-1α<sup>−/−</sup> cells were generated by homologous recombination with a targeting vector that removes exon 2 from the wildtype allele (see Figure 5.2a and Reference (1)). To test for the presence or absence of the HIF-1α mutation in the ES cells, genomic DNA was isolated from the cells and the HIF-1α genotype was determined by polymerase chain reaction using EcoRI-digested DNA and primers flanking the deletion site (see Chapter 3 for more detailed methods and Reference (80)). The location of the primers used for the wild type and mutated HIF-1α is noted in Figure 2a. Amplication of the wild-type allele with the appropriate primers yielded a product of 270 bp, whereas the HIF-1α<sup>−/−</sup> allele yielded a 340-bp product. Figure 2b confirms the
presence of wild-type HIF-1α in the HIF-1αwt cells and mutated HIF-1α in the HIF-1αmut cells, both before and after transfection with the fluorescent protein marker.

**Figure 5.2** A) HIF-1α allele before and after targeting of exon 2. The top figure shows the wild-type allele with all exons intact. P1 and P2 are the primer sites used for PCR amplification of a 270-bp product incorporating a portion of exon 2. The bottom figure shows the HIF-1αmut allele after gene targeting. Exon 2 has been replaced with the targeting vector containing a neomycin-resistant gene. P3 and P2 are the primer sites for PCR amplification of a 340-bp product. B) PCR analysis of HIF-α status in HIF-1αwt and HIF-1αmut ES cells before and after transfection with fluorescent protein vectors. M - PCR marker for identification of bands; Lane 1 - HIF-1αwt ES cells; Lane 2 - HIF-1αmut ES cells; Lane 3 - HIF-1αwt ES cells with CFP transfection; and Lane 4 - HIF-1αmut ES cells with YFP transfection.
5.4 Localization of Cells with Respect to Hypoxia – Sandwich Culture

In Chapter 4, it was demonstrated that a stable and measurable oxygen gradient developed within a sandwich culture system when the sandwich culture was initiated 24 hours after seeding ES cells at a density of 100,000 cells/cm². All further experiments described herein were carried out in the following sequence:

1) Seed ES cells on bottom slide of sandwich culture at a density of 100,000 cells/cm². The cells were seeded as 50% HIF-1α⁺ CFP ES cells/ 50% HIF-1α⁻ YFP ES cells, unless otherwise noted.

2) Twenty-four hours after seeding, the top slide was placed on the sandwich culture to initiate the development of oxygen gradients.

3) Measurements were made over 24-48 hours of sandwich culture.

4) For immunohistochemical studies within the sandwich culture, the top slide was carefully removed after 24 hours to avoid disturbance of the cell monolayer underneath. The cells were then fixed and stained as necessary.

For visualization of localization of cells with respect to hypoxia, adjacent 860-μm wide fluorescent images were taken from one edge of the sandwich culture to the other (Figure 5.3). For each image, the CFP and YFP fluorescent images were binarized separately, and the total area of cells within the image was quantified (as fraction of total area). A total of 29 images were taken across the sandwich culture at each time point (0, 24, and 48 hours after initiation of the sandwich culture) for both the sandwich culture and a control slide in which no top slide was added. In Figure 5.4, images from a location 3 mm from the edge of the slide are shown from a control slide and a sandwich culture experiment 0 and 24 hours after placing the top slide onto
the sandwich culture. From Figure 4.17, it is seen that at this depth, the cells experience hypoxia for almost the entire 24 hours in the sandwich experiment. The cells on the control slide proliferate to cover the majority of the slide in 24 hours, and the increase in area coverage of blue (CFP-labeled HIF-1α⁺⁺ cells) and green (YFP-labeled HIF-1α⁺⁻ cells) portions of the image is evident. The cells within the sandwich culture decrease in area coverage over the 24 hours due to the hypoxic exposure.

**Figure 5.3** Fluorescent images (CFP and YFP) are captured at adjacent x-locations across the sandwich culture system, and the images are binarized and the fractional areas of CFP-positive cells and YFP-positive cells are quantified.
Figure 5.4 Images taken 3 mm from the edge of culture slide 0 and 24 hours after initiation of experiment. (Left) Control Slide – Cells are exposed to atmospheric conditions and normal tissue culture conditions; (Right) Sandwich Culture – Top slide is placed on sandwich at 0 hours, and cells are rapidly exposed to hypoxic conditions. Blue = CFP-labeled HIF-1α⁺⁺ cells, Green = YFP-labeled HIF-1α⁺ cells.

Images similar to those in Figure 5.4 were quantified for fractional area coverage of HIF-1α⁺⁺ and HIF-1α⁺ cells at 0, 24, and 48 hours after initiation of the sandwich culture environment. By comparing the distribution of HIF-1α⁺⁺ and HIF-1α⁺ cells within the sandwich culture, the difference in localization with respect to hypoxia can be examined. Specifically, if HIF-1α⁺ cells do indeed predominate in areas of hypoxia, the fractional area coverage of HIF-1α⁺ cells should be higher than that of HIF-1α⁺⁺ cells after prolonged hypoxic exposure. Figure 5.5 demonstrates...
the individual measurements of fractional area coverage for the control and sandwich slides at 0, 24, and 48 hours. In the control slide, the cells are exposed to normoxic conditions throughout the 48 hour experiment, and the fractional coverage increases as the cells proliferate. In the sandwich culture, the cells in the interior of the slide experience hypoxia, and the fractional cell area coverage drops within the 48 hours of the experiment, indicating poor survival/proliferation of the cells.

In Figure 5.6, the values of fractional area coverage across the slide are averaged to give the average fractional area coverage of each cell type, and normalized to the initial fractional area coverage. In the control slide graph, it is evident that the cell concentration increases over time as the cells proliferate. The average across the sandwich culture slides gives an indication of the cell concentration with respect to hypoxia, since the majority of the slide is hypoxic over the 48 hours of the experiment. Therefore, for the sandwich culture experiment, the average fractional area coverage across the slide can be used as a comparison of localization of each cell type with respect to hypoxia. Interestingly, although the coverage of both cell types decreases over time, there is no significant difference in the change in cell coverage between the HIF-1α++ and HIF-1α- cells. Thus, at least in the sandwich culture experiment as performed here, there is no difference in cell localization with respect to hypoxia based on HIF-1α status.
Figure 5.5 Individual Measurements of Fractional Cell Area Coverage Across Slide. Slide edges are at 0 and 25 mm. Blue curves indicate HIF-1α+/+ cells and green curves indicate HIF-1α-/- cells. The left graphs indicate the control slides, in which the cells are exposed to oxygen for the entire 48 hours. The right graphs are of the sandwich culture experiment, in which the cells in the interior of the slide experience hypoxia. Note the high cell concentration on either end of the sandwich culture compared to the interior after 48 hours in the sandwich culture.
Figure 5.6 Average fractional cell area coverage across each slide (29 images), normalized to the initial fractional coverage at the beginning of the experiment. In the control slide, both the HIF-1α^+/+ and the HIF-1α^-/- cells proliferate during the 48 hour experiment. In the sandwich culture, the cell area coverage decreases over time, due to poor survival/proliferation of the cells in hypoxia. Interestingly, there is no significant difference in the change in cell coverage between HIF-1α^+/+ and HIF-1α^-/- cells.

5.5 Migration of HIF-1α^+/+ and HIF-1α^-/- ES Cells in Oxygen Gradients

Surprisingly, there was no difference in localization of HIF-1α^+/+ or HIF-1α^-/- cells with respect to hypoxia within the sandwich culture. However, it is possible that the overall cell coverage as measured in Section 5.4 does not change significantly even though differences in behavior are
occurring at the cellular level. For example, if there is a difference in cell migration with respect to oxygen gradients, it may be evident on a smaller length scale than the 860 μm images used for the localization measurements in the previous section. On the other hand, if there are differences in cellular apoptosis between the cell types, there may be cellular changes as apoptotic pathways are activated, but there may not be dramatic changes in cell number during the time course of the experiment. For this reason, the three proposed pathways of cellular localization (migration, apoptosis, and proliferation) were compared in the two cell types during exposure to hypoxic conditions.

To measure cell migration differences within gradients of oxygen, the sandwich culture system was again used. The outermost 3 images on each edge of the sandwich culture contain the areas in which there is a sharp oxygen gradient from atmospheric conditions down to complete anoxia (see Figure 4.17). Therefore, these portions of the sandwich culture were analyzed as follows. Time-lapse images of the regions near the edge of the sandwich culture were analyzed – one image every hour for 24 hours following the introduction of the top slide to the system. The images were separated into CFP fluorescent images of the HIF-1α+/+ cells and YFP fluorescent images of the HIF-1α-/- cells. Clumps of cells were identified and highlighted (see Figure 5.7) at time 0 and at each subsequent time point. At each time point, ImageJ software (NIH; http://rsb.info.nih.gov/ij/) was used to calculate the coordinates of the centroid of the clump of cells. The movement of the clumps over 24 hours was thus followed for HIF-1α+/+ cells and HIF-1α-/- cells individually. The cells were moving on an inactivated mouse embryonic fibroblast feeder layer in order to more closely resemble the environment the cells may experience in vivo.
Figure 5.7 demonstrates the method used for a single clump of cells containing both HIF-1α⁺⁺⁺⁺ and HIF-1α⁻⁻ cells. A total of 61 clumps within 2.5 mm of the edge of the sandwich culture were analyzed. Typically, as in Figure 5.7, the cellular clumps moved as one large unit containing both HIF-1α⁺⁺⁺⁺ and HIF-1α⁻⁻ cells. Clumps would rarely separate into smaller clumps, although frequently smaller clumps would combine and begin to move as one larger clump. The migratory paths of the cellular clumps were analyzed to obtain two parameters. First, the total path length of the cells (A→B in the 24 hour time point image in Figure 5.7) was measured at each time point, and the average clump migration speed was calculated by plotting the average path length (averaged over all 61 clumps) versus time in Figure 5.8. The slope of the curves for HIF-1α⁺⁺⁺⁺ and HIF-1α⁻⁻ cells indicate a clump migration speed of $7.9 \pm 0.3 \, \mu m/hr$ and $8.0 \pm 0.3 \, \mu m/hr$, respectively ($p = $ not significant). Thus, the clumps of cells move an average of approximately 190 $\mu m$ in the 24 hour time frame of the experiment. Second, the x-displacement of each cellular clump (in the direction of the oxygen gradient) from its initial starting point was calculated at each time point during the observation period. This gives an indication of whether the clump is moving in the direction of higher oxygen concentration (arbitrarily chosen as a positive x-displacement) or lower oxygen concentration (negative x-displacement). Figure 5.9 shows that both HIF-1α⁺⁺⁺⁺ and HIF-1α⁻⁻ cells stayed within an average of 10 $\mu m$ from their starting x-position, and there was no significant difference between HIF-1α⁺⁺⁺⁺ and HIF-1α⁻⁻ cells in their net direction of movement at any time point.
Figure 5.7 Method of measuring migration of cellular clumps of HIF-1α^- and HIF-1α^+ ES cells. A merged image of a portion of the sandwich culture 2 mm from the edge is divided into blue (left) and green (right) channels. The blue cells are the CFP-labeled HIF-1α^- cells and the green cells are the YFP-labeled HIF-1α^+ cells. A cellular clump of mixed HIF-1α^- and HIF-1α^+ cells has been highlighted in each image. The CFP and YFP images are analyzed separately, and the location of the centroid of the cell clump is plotted at each time point. In the 8-hr image, the initial starting point is labeled 'A' and the path that the clump has followed is outlined. The 24 hour image delineates the entire path traveled by the clump during the 24-hour measurement, connecting the starting point ('A') to the ending point ('B'). The total path length (A→B) averaged over 61 cellular clumps is plotted versus time in Figure 5.8, and the x-displacement of the clumps from its initial starting point is plotted versus time in Figure 5.9.
Figure 5.8 Average distance traveled by ES Cell Clumps within 2.5 mm from edge of sandwich culture. A total of 61 clumps of each cell type were averaged together to get the displacement vs. time curves. The slope of the curve gives the average clump migration speed. There is no significant difference between the migration speeds of the HIF-1α+/+ and HIF-1α-/- cells.

Figure 5.9 Average displacement in x-direction (direction of oxygen gradient) of cellular clumps from their initial starting position within the sandwich culture system. A total of 61 clumps were averaged together to get the displayed curves. There is no significant difference between HIF-1α+/+ and HIF-1α-/- clump displacements at any time point. Likewise, at no time point is either curve significantly different from its initial starting x-location (p > 0.05). The positive direction has arbitrarily been chosen to be the direction of the source of oxygen (toward the edge of the sandwich culture).
While Figures 5.8 and 5.9 clearly show that HIF-1α<sup>+</sup> and HIF-1α<sup>+</sup> ES cells display no difference in migration behavior within the sandwich culture, it is not as clear in examining Figure 5.9 whether the cells are undergoing directed migration or random cell motility. Indeed, although there is no significant difference in the clump location from its original starting point at any time, there does seem to be a trend toward moving away from the edge of the sandwich culture. More careful analysis is necessary to determine whether this is a true migration or simply random motion. Random cell migration (or in this case, clump migration) in an isotropic environment can be described as a persistent random walk, in which the mean square displacement of the clump from its initial starting point varies linearly with time according to Equation 5.1 (138).

\[ <D^2> = 2n\mu t \]  

(5.1)

\(<D^2>\) is the mean square displacement of the clump from its original location, \(n\) is the dimensionality of the migration, \(\mu\) is the random motility coefficient of the cellular clumps, and \(t\) is the elapsed time since the beginning of the migration measurements.

A characteristic of random motion is that the random motility coefficient is constant such that the mean square displacement varies linearly with time (138). Therefore, one method to determine whether the cellular clumps are undergoing random motility is to analyze the relationship between the mean square displacement and time. Figure 5.10 demonstrates that the mean square displacement of the clumps varies linearly with time, with a random motility coefficient of \(\mu = 30.5 \, \mu m^2/hr\). This suggests that the motion of the cellular clumps is indeed random. Another method of demonstrating that there is no preferential migration in the x-direction (the direction of the oxygen gradient) within the sandwich culture is to compare the mean-square displacement
Figure 5.11 Mean Square Displacement vs. time in x- and y-direction within sandwich culture. The random motility coefficients, calculated from the slope of the curves and dimensionality n=1, were not significantly different for the x- and y-directions ($\mu_x = 32.2 \mu m^2/hr; \mu_y = 29.5 \mu m^2/hr; p = 0.6$).

5.6 Hypoxia-Induced Apoptosis of HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ ES Cells

In Section 5.5, it was demonstrated that there was no significant difference in migration with respect to oxygen gradients between HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ ES cells. The next question addressed is whether there is a difference in hypoxia-induced apoptosis within the sandwich culture system between the two cell types. It has been shown previously that hypoxia and hypoglycemia increase apoptosis and reduce proliferation in HIF-1$\alpha^{+/+}$ ES cells, but not in HIF-1$\alpha^{-/-}$ ES cells (1). Therefore, it was expected prior to the sandwich culture experiments that there would be a difference in apoptosis between the cell types in the hypoxia induced within the sandwich culture system. However, a significant difference in apoptosis and proliferation should lead to a clearly observed difference in cellular localization within the sandwich culture system, with more HIF-1$\alpha^{-/-}$ cells accumulating in the hypoxic regions compared to HIF-1$\alpha^{+/+}$ cells. In Section 5.4 this was shown not to be the case for a mixed cellular system within the sandwich
5.6 Hypoxia-Induced Apoptosis of HIF-1α⁺/⁺ and HIF-1α⁻/⁻ ES Cells

In Section 5.5, it was demonstrated that there was no significant difference in migration with respect to oxygen gradients between HIF-1α⁺/⁺ and HIF-1α⁻/⁻ ES cells. The next question addressed is whether there is a difference in hypoxia-induced apoptosis within the sandwich culture system between the two cell types. It has been shown previously that hypoxia and hypoglycemia increase apoptosis and reduce proliferation in HIF-1α⁺/⁺ ES cells, but not in HIF-1α⁻/⁻ ES cells (1). Therefore, it was expected prior to the sandwich culture experiments that there would be a difference in apoptosis between the cell types in the hypoxia induced within the sandwich culture system. However, a significant difference in apoptosis and proliferation should lead to a clearly observed difference in cellular localization within the sandwich culture system, with more HIF-1α⁻/⁻ cells accumulating in the hypoxic regions compared to HIF-1α⁺/⁺ cells. In Section 5.4 this was shown not to be the case for a mixed cellular system within the sandwich
culture. To gain a better understanding of these discrepancies, the occurrence of hypoxia-induced apoptosis within the sandwich culture system was evaluated.

5.6.1 Apoptosis of ES Cells in Sandwich Culture System

Apoptosis is a regulated physiological process that leads to programmed cell death, characterized by cell shrinkage, membrane blebbing, and DNA fragmentation. Caspases are a family of cysteine proteases that act as central regulators of apoptosis. Caspase activation occurs by either the cell surface death receptor pathway, which involves activation of caspase-8, or a mitochondria-initiated pathway which activates caspase-9. Once activated, a cascade of caspase activation is initiated, which ultimately leads to cleavage of cytoskeletal and nuclear proteins and induction of apoptosis. Caspase-3 is a primary executioner of apoptosis, downstream of both major apoptosis initiation pathways (see Figure 5.12) (139). Activation of caspase-3 involves proteolytic cleavage of the protein into activated fragments.
Figure 5.12 Simplified diagram of apoptotic pathways. The key aspect is that both caspase-8-mediated and caspase-9-mediated pathways converge through the activation of caspase-3. Therefore, activated caspase-3 is an ideal marker of apoptosis.

To detect apoptosis in ES cells within the sandwich culture system, a rabbit monoclonal antibody to cleaved caspase-3 was used (Cell Signaling Technology). The method used is described in detail in Chapter 3. Briefly, HIF-1α+/− and HIF-1α+/− ES cells (non-fluorescent) were seeded at 100,000 cells/cm² in separate sandwich culture systems. After 24 hours of initiating the sandwich culture, the top slide was removed and the cells fixed with 4% paraformaledehdye. The slides were then incubated with rabbit monoclonal antibody to activated caspase-3, followed by incubation with a secondary anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC).
for fluorescent imaging. The slides were mounted with a nuclear dye that fluoresces in the far red region of the spectrum. The slides were viewed with confocal fluorescence microscopy, with FITC images to identify the apoptotic cells, and far red images to identify the cellular nuclei.

Figure 5.13 shows representative images of HIF-1α^{+/+} cells and HIF-1α^{-/-} cells at two different distances from the edge of the slide. The images were analyzed in ImageJ, and the total number of cells (number of nuclei) and number of apoptotic cells were counted in each image. The percentage of apoptotic cells (as a percentage of total cells) is plotted as a function of distance from the edge of the sandwich culture for HIF-1α^{+/+} and HIF-1α^{-/-} cells in Figure 5.14. From the graph, it is evident that apoptosis increases with increasing distance into the hypoxic interior of the sandwich culture. However, as with the previous localization and migration experiments, there is no evident difference between apoptosis of HIF-1α^{+/+} and HIF-1α^{-/-} cells. In order to investigate more deeply, further experiments were done in a controlled environment hypoxic chamber rather than continuing to focus on the sandwich culture (see next section).
Figure 5.13 Activation of Caspase-3 in ES Cells within Sandwich Culture system. The left images are of HIF-1α<sup>+</sup> cells, and the right images are of HIF-1α<sup>-</sup> cells. The top row images were taken at a distance of 1 mm from the edge of the sandwich culture, and the bottom images were taken at 3.5 mm from the edge. The nuclei were stained with a nuclear dye, and are displayed as red in the images. The apoptotic cells were labeled with an antibody to activated caspase-3 and secondary FITC labeling. They are displayed as green in the images.
Figure 5.14 Quantification of apoptosis within the sandwich culture system. Multiple images similar to those in Figure 5.13 were analyzed, and the percentage of cells (nuclear stain) that were apoptotic (Caspase-3 stain) were quantified at different locations in the sandwich culture after 24 hours in culture. The results are displayed for HIF-1α"+" cells (blue) and HIF-1α"−" cells (green). The solid lines are best-fit lines through the data, indicating the remarkable similarity in behavior of HIF-1α"+" and HIF-1α"−" cells.

5.6.2 Apoptosis of ES Cells in Normoxia vs. Hypoxic Chamber

The preliminary apoptosis assay of the ES cells in the sandwich culture system seemed to contradict previously reported results in the literature indicating that HIF-1α"+" cells underwent hypoxia-mediated apoptosis while HIF-1α"−" cells did not (1). This finding was further investigated by revisiting the experiments conducted in the previous report. In the previously cited source, Carmeliet et al. used two \textit{in vitro} assays to assess hypoxia-induced apoptosis. Undifferentiated ES cell monolayers were cultured at 10,000 cells/24-well dish in normal ES cell medium containing 5% fetal calf serum for 24 hours, and stressed for 24 hours in the same medium without LIF (a factor that prevents differentiation) under hypoxic or hypoglycemic conditions. Apoptosis was measured with an ELISA for DNA fragments (oligonucleosomes), and TUNEL-positive cells were visualized with an ApopTag kit. They also used cytokines to measure cytokine-induced apoptosis in both cell lines. Apoptosis in hypoxic tumor zones was
also measured immunohistochemically by measuring the area of TUNEL-immunoreactive cells per hypoxic zone.

In the Carmeliet et al. study, apoptosis was comparable in the HIF-1α+/+ and HIF-1α-/- ES cells when cultured during normoxia and normoglycemia, and increased to a similar extent after stimulation with a cytokine mixture. In contrast, during hypoxia, hypoglycemia, or a combination of the two, apoptosis increased 10-30 fold in the HIF-1α+/+ ES cells but was unaffected in HIF-1α-/- ES cells. The difference in apoptosis between the two cell lines in vivo was less dramatic, with 2.5% apoptotic cells in HIF-1α+/+ hypoxic fields and 1.5% apoptotic cells in HIF-1α-/- hypoxic fields. Thus, even in this earlier report, it is evident that environmental conditions dramatically affect the hypoxia-induced apoptosis of the cells.

Because of the dramatic difference between the previously reported results and the results suggested by the sandwich culture experiment, it is important to closely evaluate the differences between these two studies. First, the cell density used is dramatically different. Carmeliet et al. seeded the ES cells at approximately 5000 cells/cm², while the sandwich culture experiments are performed at 100000 cells/cm². Second, the sandwich culture experiments were performed in normal ES cell media, which contains a high content of fetal calf serum (FCS; 20%v/v) and recombinant LIF to prevent differentiation, while the previous experiments had been performed in 5% FCS and without the use of LIF. Third, the dramatic gradients of oxygen present in the sandwich culture may have a different effect than the static hypoxic culture used in the previous experiments. These dramatic differences in experimental conditions confound the ability to compare the results to the previously reported data; therefore, experiments were conducted to
closely mimic the conditions of the experiments performed in Carmeliet et al (1). While the seeding density, media conditions, and experimental conditions were controlled for in the comparison experiments, the possibility remains that there are clonal differences in phenotype or different degrees of differentiation between the cells used in the current experiments and those used in the experiments reported in Reference (1).

For the comparison experiments, the conditions used in Carmeliet et al. were used to measure apoptosis using a TUNEL-based immunoreactive assay. HIF-1α+ and HIF-1α− ES cells were seeded on gelatinized glass slides within 12-well plates at a low seeding density (~5000 cells/cm²) and grown under various conditions for 24 hours before fixation and immunostaining for TUNEL-positive cells. Two different FCS concentrations were compared, 20% FCS similar to the sandwich culture experiments and 5% FCS similar to Carmeliet et al. In each serum concentration, cells were either grown in high-glucose media (normoglycemia) or in media containing no glucose (hypoglycemia). Likewise, cells were grown either in a normal cell culture incubator containing 5% carbon dioxide (normoxia) or in a hypoxic chamber containing 1% oxygen and 5% carbon dioxide (hypoxia). After 24 hours in the appropriate conditions, the slides were fixed in 4% paraformaldehyde and stained for TUNEL reactivity along with a nuclear dye to stain the cell nuclei. The percentage of TUNEL-positive cells were counted microscopically. Three slides of each cell type were grown in each condition, and a minimum of 300 cells were counted on each slide. The percentage apoptotic cells were averaged for the triplicate experiments, and the results are plotted in Figure 5.15 along with the values reported in Carmeliet et al (1).
Figure 5.15 Results of TUNEL Staining of ES cells after 24 hours of exposure to the stated conditions. FCS = fetal calf serum; Glucose = high glucose ES media (3.5 mg/mL); No Glucose = ES media containing no glucose; Norm = normoxia – Incubator conditions: 5% carbon dioxide in room air; Hyp = hypoxia – 5% carbon dioxide + 1% oxygen. The literature values are the reported values from Carmeliet et al (1). Data from three slides were averaged to obtain the final values, and >300 cells were counted per slide.

Several important observations can be made from the data in Figure 5.15. When cultured in 20% FCS as in the sandwich culture experiments, the ES cells have a 6-7% apoptotic fraction when cultured in normoxia and normoglycemia. When exposed to hypoxia, both HIF-1α+/+ and HIF-1α-/- cells experience an increase in apoptotic fraction to 14-16%. Interestingly, the increase in apoptosis is statistically significant in the HIF-1α+/+ cells, but not statistically significant in the HIF-1α-/- cells. This is likely due to the slightly greater variability in the data for the HIF-1α-/- cells; however, the important point to note is that under hypoxia, there is no significant difference in apoptotic rate between HIF-1α+/+ and HIF-1α-/- cells. Also of interest in the 20% FCS experiments is the complete lack of effect of hypoglycemia on the apoptotic fraction. The conclusion to be made at 20% FCS conditions is that there is no significant difference between the hypoxia-induced apoptosis of HIF-1α+/+ and HIF-1α-/- cells.
Turning to the experiments performed in 5% FCS, it is immediately apparent that the cells behave quite differently from the data reported by Carmeliet et al (1). There is a highly significant increase in apoptosis to greater than 50% apoptotic rate even when cultured in normoxia and normoglycemia. Also, there is no significant increase or decrease in apoptotic fraction under any conditions, except for a statistically significant decrease in apoptosis in HIF-1α+ cells when transitioning from normoglycemia to hypoglycemia under hypoxic conditions, or from normoxia to hypoxia under hypoglycemic conditions. However, while this decrease is significant statistically, the biological significance is questionable since the overall apoptotic rate remains greater than 50%. Again, the key point is that there is no significant difference in apoptosis between HIF-1α+ and HIF-1α− cells under any conditions.

These data contrast dramatically with those from Carmeliet et al(1). In their experiments, the ES cells grown in 5% FCS had a very low apoptotic rate in normoxic, normoglycemic conditions. The HIF-1α+ cells had a minimal increase in apoptosis under any conditions, while the HIF-1α− cells reacted to hypoxia or hypoglycemia with a dramatic increase in apoptosis, with an even greater effect under dual conditions of hypoxia and hypoglycemia together. Thus, the ES cells in the current experiments had a different phenotype of hypoxia-induced apoptosis than those used in the previous experiments in the literature.

5.7 Effects of Hypoxia on Proliferation in HIF-1α+ and HIF-1α− ES Cells

Carmeliet et al. (1) also reported that HIF-1α− ES cells demonstrated suppressed proliferation under hypoxic stress when cultured as either undifferentiated monolayers or as three-dimensional embryoid bodies, as measured by 3H-thymidine or BrdU incorporation. In
HIF-1α⁺⁺ monolayers, proliferation was unaffected, while in embryoid bodies proliferation of HIF-1α⁺ cells significantly increased under hypoxic stress. Given the lack of difference in localization within the sandwich culture or hypoxia-induced apoptosis between HIF-1α⁺⁺ and HIF-1α⁺ ES cells in the current experiments, the next question addressed was whether this difference in proliferation was maintained.

The proliferation of HIF-1α⁺⁺ and HIF-1α⁺ ES cells was compared first by MTT assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (140) relies on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of MTT to form formazan crystals which accumulate in healthy cells due to their membrane impermeability. The cleavage causes a color change from pale yellow to dark blue, and solubilization of the formazan crystals with a detergent allows for colorimetric measurement of the amount of formazan formed. The number of surviving cells is directly proportional to the level of formazan within a certain linear range.

HIF-1α⁺⁺ and HIF-1α⁺ ES cells were seeded in 6 separate 96-well plates at 10000 cells per well, with 4 wells for each cell type per plate. The MTT colorimetric assay was performed at three time points after culturing under normoxic (cell culture incubator with 5% carbon dioxide in room air) or hypoxic (hypoxic chamber with 1% oxygen and 5% carbon dioxide), and the MTT absorbance units are plotted as a function of time in Figure 5.17. Figure 5.16 shows the results from a calibration curve to ensure that the values obtained remained within the linear portion of the MTT assay. Cells were seeded at different seeding densities, and the MTT absorbance was measured after 4 hours of incubation to allow for attachment of the cells. Figure 5.17 shows that...
the cell number increases for both HIF-1α+/+ and HIF-1α−/− cells in either normoxia or hypoxia. Interestingly, at 24 hours, the HIF-1α+/+ cells had proliferated less than the HIF-1α+/+ cells in normoxia, although by 48 hours the number of HIF-1α+/+ cells was again equivalent to the HIF-1α+/+ cells. This difference was unlikely due to HIF-1α activity because HIF-1α is degraded under normoxic conditions, and the reason for this difference is unclear. However, the effect of hypoxia on the HIF-1α+/+ and HIF-1α−/− ES cells was to suppress the proliferation by a similar amount in the two cell types. Therefore, as in the localization and apoptosis experiments, the results of the previous study (1) – increased hypoxia-induced growth arrest in HIF-1α+/+ relative to HIF-1α−/− ES cells – were not confirmed in the current experiments.

![MTT Assay Linear Range](image)

**Figure 5.16** Results of MTT assay performed on multiple cell densities of HIF-1α+/+ and HIF-1α−/− ES cells within 96-well plates demonstrating that the data from Figure 5.17 fall within the linear range of the assay. N = 4 wells per data point. The dashed lines are the least squares linear best fit through the two curves.
Figure 5.17 MTT assay of proliferation of HIF-1α+/+ and HIF-1α−/− cells cultured in normoxia (solid lines) or hypoxia (dashed lines) demonstrating the equivalent effect of hypoxia on the two cell lines.

The effect of hypoxia on proliferation was also measured at higher cell densities by seeding cells in 12-well plates (2 wells per cell type per time point) and performing cell counts using a hemocytometer at various incubation times in normoxia or hypoxia. The cells were initially seeded at 100,000 cells/cm² (low seeding density) to simulate the conditions used in the sandwich culture experiments and 500,000 cells/cm² (high seeding density) to study the effects of greater nutrient deprivation in high density cultures. After seeding, the cells were allowed to grow in normoxia for 24 hours before dividing them into either normoxia or hypoxia treatment for the following 48 hours. The results of the cell counts are demonstrated in Figures 5.18-5.21, and the zero time point in these figures corresponds to the time at which the cells were separated into two groups (24 hours after seeding).
Figure 5.18 Cell counts of HIF-1α⁺⁺ and HIF-1α⁻⁻ ES cells over time cultured under normoxic conditions 24 hours after seeding at 100,000 cells/cm². There is no significant difference between cell density of HIF-1α⁺⁺ and HIF-1α⁻⁻ cells at any time point, nor is there any significant difference between the regression coefficients for a second-order polynomial fit through the data for either curve (solid lines).

Figure 5.19 Cell counts of HIF-1α⁺⁺ and HIF-1α⁻⁻ ES cells over time cultured under hypoxic conditions 24 hours after seeding at 100,000 cells/cm². There is no significant difference between cell density of HIF-1α⁺⁺ and HIF-1α⁻⁻ cells at any time point, nor is there any significant difference between the regression coefficients for a second-order polynomial fit through the data for either curve (solid lines).
Figure 5.20 Cell counts of HIF-1α+ and HIF-1α− ES cells over time cultured under normoxic conditions 24 hours after seeding at 500,000 cells/cm². There is no significant difference between cell density of HIF-1α+ and HIF-1α− cells at any time point, nor is there any significant difference between the regression coefficients for a second-order polynomial fit through the data for either curve (solid lines).

Figure 5.21 Cell counts of HIF-1α+ and HIF-1α− ES cells over time cultured under hypoxic conditions 24 hours after seeding at 500,000 cells/cm². There is a significant difference (p<0.005) between the cell density of HIF-1α+ and HIF-1α− cells at 24 and 43 hours after the beginning of the experiment, but there is no significant difference between the regression coefficients for a second-order polynomial fit through the data for either curve (solid lines).
When cultured under normoxia, both cell types behave similarly. The cells proliferate up to a maximum seeding density of approximately $1.3 \times 10^6$ cells/cm$^2$ (Figures 5.18, 5.20), after which the cell density levels off. The apparent dropoff in cell density at later times is likely an artifact of the polynomial curve fit, although it is possible that cells have begun to die off as they compete for nutrients. When cultured under hypoxia at low seeding densities, the HIF-1α$^{+/+}$ and HIF-1α$^{-/-}$ cells again have similar proliferation rates up to a maximum of approximately 600,000 cells/cm$^2$ (Figure 5.19). When seeded at a higher density under hypoxia, the starting cell density is almost twice the maximum cell density, and the cells quickly begin to die due to overcrowding (Figure 5.21). At first glance, it seems as though the HIF-1α$^{+/+}$ cells die at a more rapid rate than the HIF-1α$^{-/-}$ cells. However, the actual cell density is only significantly different between the two cell types at 24 and 43 hours after the introduction to hypoxia; and due to the wide variability in cell counts, the difference in the regression curves in Figure 5.21 is not statistically significant. Therefore, it appears that in the proliferation experiments as well, there is no difference in behavior between the HIF-1α$^{+/+}$ and HIF-1α$^{-/-}$ cells when exposed to hypoxia.

5.8 Discussion

The objective of this study was to determine the mechanism of localization of ES cells within tumors with respect to hypoxia based on HIF-1α status. Previous reports (80, 81) had suggested both a preferential localization of HIF-1α$^{+/+}$ ES cells close to blood vessels as well as a preferential localization of HIF-1α$^{-/-}$ ES cells distant from blood vessels. The mechanism of localization had previously been presumed to be decreased hypoxia-induced apoptosis and increased stress-induced proliferation in HIF-1α$^{+/+}$ ES cells (1), although no evidence for this had been presented. Another possibility for differential localization could be enhanced migration of
the HIF-1α" cells (60, 61), allowing them a greater opportunity to migrate away from hypoxic areas.

The sandwich culture system (103) was assumed to be an ideal system to investigate the localization phenomenon because of the ability to develop stable gradients of oxygen and view the behavior of the two cell types microscopically. Fluorescent labeling of the HIF-1α" and HIF-1α' cells with CFP and YFP, respectively, also allowed for simultaneous viewing of the two cell types within a single system, ensuring that the cells would experience identical microenvironmental stimuli. The first significant finding of the current study was that there was no difference in localization of the HIF-1α" and HIF-1α' cells with respect to oxygen levels within the sandwich culture system. Three possible general reasons for this discrepancy of the behavior of these cells from previous reports are:

1) The ES cells or conditions used in these experiments are inherently different from those used in previous experiments.

2) The sandwich culture system is not an adequate system for viewing differences with respect to hypoxia; the differences may become apparent with the use of a more controlled system.

3) The cells may behave differently in vitro than in vivo: the reason for the localization may involve stimuli other than hypoxia or in addition to hypoxia that are a result of the complex tumor microenvironment experienced by the cells.

Following up on these possibilities, it was also demonstrated that there was no difference in migration or apoptosis within the sandwich culture system, and there was no difference in
apoptosis or proliferation upon exposure to hypoxia in a well-controlled hypoxic chamber environment. The fact that the lack of difference between the cell types is also seen in hypoxic chamber experiments indicate that the results do not stem from a problem with the sandwich culture system. The fact that repeating the conditions as closely as possible to the conditions used to measure apoptosis by Carmeliet et al. (1) gave drastically different results (Figure 5.14) indicate a strong likelihood that there is a fundamental difference between the cells used in the two studies or another factor that was not controlled between the two experiments.

One possible source of the discrepancy found in the two experiments is the fetal calf serum used for the experiments. It was seen in the current experiments that the concentration of serum used in the cell culture media dramatically affected the inherent survivability of the cells, even when grown under normoxic, normoglycemic conditions, whereas in the previous experiments the cells grew well in lower serum concentrations. The unfortunate reality of the use of serum in cell culture is that the composition and other characteristics of the serum varies a great deal from source to source. The fact that different batches of serum were used in the two experiments could have had a large effect on the behavior of the cells in response to the serum. In future experiments, it would be interesting to follow up on this avenue by testing different batches of serum on the cells to see if they have differing effects on cell viability when grown in low serum conditions.

Aside from any differences in media, it is also possible that there was an inherent difference in the cell phenotypes used in the two experimental studies. The fact that the source of the cells in these experiments is an embryonic stem cell line increases the likelihood that the cells differed in
some way. First, the cells used in all experiments were clonal in nature. They had been clonally selected from a variety of colonies that were successfully recombined to alter the HIF-1α status of the cells. Because different clones were used in the two studies, it is impossible to tell whether the differences in results could be simply due to clonal variation in cellular phenotype. This possibility could be further investigated by repeating the experiments with several new clones to see if there is variability in the results. Also, the cells are maintained in as undifferentiated state as possible by limiting the number of cell passages and using LIF (leukemia inhibitory factor) in the ES cell media as well as growing the cells on a fibroblast feeder layer. However, it is not known whether the cell handling and culturing, especially during the transfection process with fluorescent proteins, resulted in a change in the differentiation status of the cells. If the two cell types had not only undergone some differentiation, but possibly differentiated along different paths, the results of the experiment could have been affected. It would be interesting in future experiments to investigate the effect of differentiation on the differential apoptosis, proliferation, and migration in response to hypoxia.

Finally, it is not known whether the change from the tumor microenvironment in vivo to the artificial environment of the sandwich culture in vitro abolishes the localization phenomena. In the tumor environment, the cells are not only subjected to deprivation of oxygen and nutrients, but there is a complex interrelationship with tumor stromal cells such as fibroblasts, endothelial cells, and smooth muscle perivascular cells. The role that HIF-1α plays in these interactions has not been carefully studied, and there may be an unforeseen difference in how the HIF-1α+ and HIF-1α− cells interact with their environment, leading to the observed localization. Although the in vitro differences seen between the current apoptosis studies and previously reported results
suggest a more fundamental difference, there still may be a role for other microenvironmental factors in the observed differences. The localization of ES cells in vivo with respect to hypoxia will be further investigated in Chapter 6.

The implications of the current studies when examined critically are profound. Regardless of the reasons for the discrepancies between the current results and previous results, the fact that the cells behave differently under different conditions seriously call into question the appropriateness of the ES cell model in making more general observations about tumor behavior. The ES cell teratoma results were already ambiguous because of the discrepancy in tumor growth results between different studies using different ES cell lines (1, 83). The current results indicate that even with the same cell source, cell phenotype depends on undefined experimental factors that may not be easily controlled. The lack of consistency in this cell type calls into question its usefulness as a tumor model.

Finally, the current results raise another important question. The enhanced hypoxia-induced apoptosis and decreased proliferation of HIF-1α ES cells was postulated as the mechanism for HIF-1α’s role as a tumor suppressor in this model. For whatever reason, the cell line used in the current experiments does not exhibit a difference in either apoptosis or proliferation when exposed to hypoxia. If in fact this is the mechanism for suppressed tumor growth of ES cell teratomas, it would be expected that the tumor growth would be similar in these cells regardless of HIF-1α status. These and other interesting questions led to further studies of tumor growth and localization in vivo, which will be addressed in the next chapter.
Chapter 6: *In vivo* Studies of HIF-1α Effect on Tumor Growth and Localization of Tumor Cells with Respect to Hypoxia

6.1 Introduction

In Chapter 5, it was demonstrated that HIF-1α*+/+* and HIF-1α*+/−* ES cells showed no differences in migration, apoptosis or proliferation under hypoxic exposure *in vitro*. Also, the localization of HIF-1α*+/+* cells in well-oxygenated regions observed *in vivo* was not recapitulated in the *in vitro* sandwich culture system. The *in vitro* hypoxia-induced apoptosis studies demonstrated that the cells used in these experiments are responding differently than has been previously shown in the literature (e.g. (1)). Several questions arise from these studies regarding the extension to *in vivo* tumor behavior. Specifically:

1) Subcutaneous HIF-1α*+/+* ES cell tumors grew slower than HIF-1α*+/−* tumors, despite increased angiogenesis (1). The proposed mechanism for this delayed tumor growth was increased hypoxia-induced apoptosis and growth inhibition in HIF-1α*+/+* ES cells; however, the possibility of other mechanisms, such as the normalization of tumor blood vessels within HIF-1α*+/−* tumors, has not been ruled out.

2) A preliminary study reported by Brown et al. (81) using multiphoton fluorescent microscopy to view HIF-1α*+/−* ES cells within mixed ES cell tumors grown in a dorsal skinfold chamber suggested that HIF-1α*+/−* ES cells may be preferentially localized distant from blood vessels.

3) HIF-1α*+/+* ES cells in mixed ES cell tumors (50% HIF-1α*+/+* and 50% HIF-1α*+/−*) grown subcutaneously were preferentially located proximal to blood vessels (80). The
authors referred to this phenomenon as ‘selective vascular dependence’ of the HIF-1α\textsuperscript{+/−} cells and also attributed this observation to increased hypoxia-induced apoptosis and growth inhibition.

The goal of this chapter is to explore these previous observations in a quantitative manner to determine the complex dependence of these findings on the in vitro phenotype of the cells. On the one hand, if hypoxia-induced apoptosis and growth inhibition is truly the mechanism for these phenomena, the cells in question would not be expected to exhibit the same behaviors, since they do not differ in their apoptotic or proliferative response to hypoxia in vitro (Chapter 5). On the other hand, it is quite possible that the behaviors seen in vivo are due to interactions between more complex sets of mechanisms such as changes in blood flow, pH, glucose availability, metabolic waste products, growth factor concentrations, and cell-cell interactions with tumor stromal cells. Therefore, the conditions necessary for the tumor growth and localization differences may be present in vivo, but not evident in the controlled and artificial in vitro environment.

In addition to repeating the above experiments, the work in this thesis will also address some limitations of the previous studies. First, the growth of mixed tumors of varying concentrations of HIF-1α\textsuperscript{+/−} vs. HIF-1α\textsuperscript{+/−} ES cells will be measured. In studies in which HIF-1α acts as a tumor promoter, a small fraction of HIF-1α\textsuperscript{+/−} cells mixed into a HIF-1α\textsuperscript{−/−} tumor recapitulates the growth of a HIF-1α\textsuperscript{−/−} tumor (107), likely due to a soluble factor produced by the cells that promotes tumor growth. If HIF-1α\textsuperscript{+/−} cells in the current study have such a nonlinear effect on
tumor growth, that would indicate a possible alternative mechanism to hypoxia-induced apoptosis and growth inhibition for delayed tumor growth.

There are also limitations of the methods used for the dorsal skinfold chamber observation of HIF-1α- cell localization distant from vasculature (81). The previous study cited this preferential localization of HIF-1α- ES cells as a qualitative observation made in tumors in which only the HIF-1α- ES cells could be visualized. In the current study, the localization of HIF-1α- and HIF-1α- ES cells with respect to vasculature within the dorsal skinfold chamber will be measured quantitatively using CFP- and YFP-labeled cells and a quantitative image analysis software.

Finally, the study that reported the behavior of ES cells within subcutaneous tumors (80) had several limitations. Much of the analysis relied on cell sorting of a disaggregated tumor, and only the 5% “most proximal” and 5% “most distal” cells with respect to vasculature were analyzed. Therefore, the extension of the results to the entire tumor is unclear. The authors also attempted to directly visualize the localization by using lac-Z positive HIF-1α- cells mixed with non-labeled HIF-1α- cells. Qualitatively, the lacZ-positive cells seemed to cluster around the tumor vasculature (CD31-positive cells). However, as in the dorsal chamber study, this observation also suffered from a lack of quantification of the distance of the two cell types from vasculature as well as the ability to visualize only one of the cell types, in this case the HIF-1α- cells. It is possible that more viable cells in general would be found around blood vessels, such that lacZ staining of HIF-1α- ES cells would also have shown such clustering. These limitations will be addressed in the current study by quantifying the distance from vessels of CFP-labeled HIF-1α-
and YFP-labeled HIF-1α− ES cells within a mixed tumor. Also, the distance of cells from hypoxic regions will also be compared between the two cell types using a pimonidazole-derived hypoxic marker.

6.2 Effect of HIF-1α on Growth of ES Cell Tumors

Subcutaneous tumor growth of ES cell tumors was measured as described in Chapter 3. Briefly, either HIF-1α+/+ cells, HIF-1α− cells, or a mixture of the two cell types were injected into the subcutaneous space on the right flank of severe combined immunodeficient (SCID) mice at a total cell concentration of 2×10⁶ cells in 0.1 mL of Hank’s Balanced Salt Solution (Sigma). The tumors were measured in two dimensions at regular intervals after injection with calipers, and the volume of tumors at each time point was calculated as $V = (\text{Length})*(\text{Width})^2*(\pi/6)$. The mice were injected in six groups (the parenthetical values of n give the number of mice in each group): 100% HIF-1α+/+ non-fluorescent ES cells (n = 10), 100% HIF-1α− non-fluorescent ES cells (n = 9), 100% CFP-labeled HIF-1α+/+ ES cells (n = 5), 100% YFP-labeled HIF-1α− ES cells (n = 5), 50% CFP-labeled HIF-1α+/+ ES cells + 50% YFP-labeled HIF-1α− ES cells (n = 4), and 10% CFP-labeled HIF-1α+/+ ES cells + 90% YFP-labeled HIF-1α− ES cells (n = 5).

In the following studies comparing tumor growth, the tumor growth curves are generated as follows. Each individual tumor growth curve was analyzed, and it was found that the tumors all exhibit exponential growth between the sizes of 100 and 1000 mm³; that is, the individual growth curves form a straight line when plotted on a log-linear plot (data not shown). These volume points were also chosen because all of the tumors were allowed to reach at least 1000 mm³ before the mice were sacrificed. For each individual tumor, the growth curve was plotted on a
log-linear scale, and the time in days to reach the chosen tumor volumes in the following figures (100-1000 mm³) was calculated. For each given volume, the times for all tumors within the group of interest to reach that volume were averaged and plotted as volume vs. time. The error bars in the following figures indicate one standard error of the mean time to reach each volume.

The first test was to see if the transfection of fluorescently labeled protein into the cells had any effect on tumor growth. Figure 6.1A compares the non-fluorescent HIF-1α⁺⁺ tumors to the CFP-labeled HIF-1α⁺⁺ tumors, and Figure 6.1B compares the non-fluorescent HIF-1α⁺⁺ tumors to the YFP-labeled HIF-1α⁺⁺ tumors. These figures make clear that the fluorescent protein does not affect the tumor growth behavior, and for the remaining figures the non-fluorescent and fluorescent tumors are combined in presenting the data.

The tumor growth curves for the subcutaneous tumors are shown in Figure 6.2. Figure 6.2A shows the curves for all of the individual tumors. The purpose of this figure is simply to show the wide variability in tumor growth curves, with the overall curves shifting to the right (slower growing) as the percentage of HIF-1α⁺⁺ cells are increased. This is made clearer in Figure 6.2B, which depicts the average tumor growth curves for each group of tumors as described above. The p-values indicated are for the time in days to reach 1000 mm³, although the indicated curves remain significantly different for all volumes from 400-1000 mm³.
Figure 6.1 A) Comparison of tumor growth curves for HIF-1α+/+ non-fluorescent ES cell tumors (n=10) and HIF-1α+/+ CFP-labeled ES cell tumors (n=5) grown subcutaneously. B) Comparison of tumor growth curves for HIF-1α−/− non-fluorescent ES cell tumors (n=9) and HIF-1α−/− YFP-labeled ES cell tumors (n=5) grown subcutaneously. Error bars in both figures are ± 1 SEM.
Figure 6.2 A) Individual tumor growth curves for all tumors in all groups. The nonfluorescent and fluorescent labeled tumors have been included together for the statistical analysis. B) Average tumor growth curves for each group of tumors. P-values indicate significance for the time to reach 1000 mm$^3$, although the difference between the indicated curves is significant (p<0.05) for all volumes from 400-1000 mm$^3$. Error bars in both figures indicate + 1 SEM.

Several interesting observations can be made regarding the curves in Figure 6.2. First of all, the action of HIF-1α as a tumor suppressor in the ES cells is confirmed, despite the lack of difference in hypoxia-induced apoptosis and growth arrest in the in vitro studies. Therefore, the
mechanism for increased tumor growth may be more complex than originally proposed by Carmeliet et al. (1). Second, a preliminary inspection of Figure 6.2B would seem to indicate that HIF-1α has a nonlinear effect on tumor growth: the 90% HIF-1α tumor growth curve is shifted to the right more than would be expected for only 10% HIF-1α cells and there is no significant difference between tumor volumes for the HIF-1α curves and 50% HIF-1α curves. However, Figure 6.3 indicates the doubling times calculated from the log-linear slopes of the tumor growth curves (doubling time = ln(2)/[slope of ln(volume) vs. time]), and they decrease in value in proportion to the fraction of HIF-1α cells in each tumor group. Therefore, Figure 6.2 and 6.3 together indicate that while HIF-1α may confer a growth delay that is disproportionate to the amount of HIF-1α present, the growth rate of the tumors once they are established is proportionate to the amount of HIF-1α present.

**Tumor Doubling Times**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Doubling Time (days)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α/+/+</td>
<td>5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>50% HIF-1α+/−</td>
<td>6</td>
<td>0.0002</td>
</tr>
<tr>
<td>90% HIF-1α+/−</td>
<td>7</td>
<td>0.04</td>
</tr>
<tr>
<td>HIF-1α+/−</td>
<td>8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Figure 6.3* Doubling times of tumors based on HIF-1α status. The doubling time for 50% HIF-1α tumors is significantly different from both HIF-1α and HIF-1α tumors and lies almost exactly halfway between the two values for the pure tumor types. The 90% HIF-1α doubling time is not significantly different from either the HIF-1α tumors or the 50% HIF-1α tumors.
Thus, it was determined that the growth delay and decreased tumor growth rate reported previously for HIF-1α<sup>+</sup> ES cell tumors relative to HIF-1α cells were confirmed in the present studies. Moreover, it was determined that the growth rate of tumors is inversely proportional to the amount of HIF-1α present in the tumors, although HIF-1α does seem to delay the initiation of tumor growth in a nonlinear manner.

### 6.3 Quantification of Localization within ES Cell Tumors Grown in Dorsal Skinfold Chambers

In Chapter 2, Figure 2.1 demonstrates the subjective observation published by Brown et al. (81) indicating that HIF-1α<sup>-</sup> cells (GFP-labeled) seem to be localized distant from the blood vasculature (visualized in red after tetramethylrhodamine-dextran injection). In order to objectively measure the localization of both HIF-1α<sup>+</sup> and HIF-1α<sup>-</sup> cells within the dorsal skinfold chamber, dual-labeled cells were used (CFP-labeled HIF-1α<sup>+</sup> cells and YFP-labeled HIF-1α<sup>-</sup> cells). A mixture of 50% HIF-1α<sup>+</sup> cells and 50% HIF-1α<sup>-</sup> cells were injected into the dorsal skinfold chamber as described in Chapter 3. Three weeks after tumor implantation, the tumors were examined microscopically after injection of 2×10<sup>6</sup> MW tetramethylrhodamine-labeled dextran to label the blood vessels. Using a multiphoton laser scanning microscope, confocal images were taken up to a depth of 150 μm (spaced 5 μm apart) via intravital microscopy (see Chapter 3). In these images, HIF-1α<sup>+</sup> ES cells are seen in blue (CFP), HIF-1α<sup>-</sup> ES cells are seen in green (YFP), and blood vessels are seen in red (rhodamine). Figure 6.4 shows representative composite images of maximum intensity projections of the entire 150-mm thickness of adjacent sections within three such mixed tumors.
Examination of Figure 6.4 makes it clear that it is extremely difficult to make subjective evaluations of the tendency for one or the other cell type to be localized closer to the blood vessels. In some areas of the image, HIF-1α⁺⁺⁺ cells seem to dominate around the blood vessels (top right of Figure 6.4A) while in others HIF-1α⁺⁺ cells cluster around the vessel (bottom middle of Figure 6.4C). Therefore, the images from mixed tumors grown in dorsal skinfold chambers were analyzed quantitatively to measure directly the distribution of HIF-1α⁺⁺⁺ and HIF-1α⁺⁺ cells around the blood vessels. Figure 6.5 illustrates the image analysis technique that was used. An automated program was written in NIH Image (http://rsb.info.nih.gov/) to binarize the red, green, and blue channels of the multifluorescent image. The blood vessels were skeletonized to obtain
an outline of the area covered by the blood vessels. Ten concentric outlines spaced approximately 9 μm apart were drawn starting at the vessel wall and in most cases covering the majority of the image. The area of green overlapping with each outlined area (HIF-1α−/− cells) and the area of blue overlapping with each outlined area (HIF-1α+/− cells) was measured and normalized to the total green area or total blue area, respectively, within the entire image. This number gives an approximation of the fraction of HIF-1α+/− or HIF-1α−/− cells that are within each given distance away from the tumor vessels within the image. This technique was repeated on five separate tumors, with 5-9 random locations analyzed per tumor for a total of 30 images. For this analysis, 50 μm deep maximum intensity projections were used to give a deep enough image to have all three colors present in significant amounts without getting a significant overlap in the perpendicular direction due to vessels that are out of plane with respect to the cells.

The final graph in Figure 6.5D gives information about the localization of tumor cells with respect to vasculature. The slope of the graph at any point is proportional to the amount of cells that are in that specific location. For example, in Figure 6.5, it is clear that most of the HIF-1α+/− cells (blue) are clustered around the vessels running vertically down the center of the image. Therefore, the slope of the graph for HIF-1α+/− cells increases sharply within the first 20 μm from the vessels. From 20 μm to 60 μm, the blue curve levels off as there are very few HIF-1α+/− cells in this region. The curve then rises slightly again at 60-80 μm, as there are a few more cells near the edge of the image. Conversely, the HIF-1α−/− ES cells are spread rather evenly across all distances from the vessel, so the slope of the green curve is fairly constant. Therefore, in this image, the HIF-1α−/− ES cells would be described as localized proximal to the blood vessels.
Looking solely at the image in Figure 6.5 would give the impression that HIF-1α^+/+ cells are localized proximal to the blood vessels while HIF-1α^-/- cells are more evenly dispersed within the tumor. However, Figure 6.4 clearly demonstrates a marked heterogeneity in cell distribution. Indeed, another selected image in Figure 6.6 demonstrates the opposite trend, with the HIF-1α^-/-
cells located closer to the blood vessels as seen by the initially steeper slope of the HIF-1α\textsuperscript{+} curve in Figure 6.6B.

Because of this heterogeneity within the tumors, it was desirable to gain an overall picture of the cellular localization with respect to blood vessels. Therefore, 30 total images were analyzed, and for each given distance from the blood vessels, the average fractional cell overlap for HIF-1α\textsuperscript{+/+} and HIF-1α\textsuperscript{+/-} cells for all images was calculated and plotted vs. distance in Figure 6.7. If there was a consistent picture of one cell type or another being localized proximal to the vessels, the average graph would show a sharper initial slope for that cell type compared to the other. In actuality, when many images are analyzed and the results averaged, there is no significant difference in localization of HIF-1α\textsuperscript{+/+} or HIF-1α\textsuperscript{+/-} ES cells with respect to blood vessels. Therefore, contrary to the initial subjective observation made by Brown et al. (81), there is no evidence of localization of ES cells with respect to blood vessels in tumors grown in dorsal chambers on the basis of HIF-1α status.
Figure 6.6 Another representative image of mixed HIF-1α⁺⁄⁺ and HIF-1α⁻⁄⁻ tumors. In this case, the HIF-1α⁻⁄⁻ cells are closer to the vessels overall than the HIF-1α⁺⁄⁺ cells, which are more evenly spread at all distances from the vessels.
Figure 6.7 Average cell distribution for HIF-1α^{+/+} cells (blue) and HIF-1α^{−/−} cells (green) with respect to vasculature in mixed tumors. There is no statistically significant difference between the localization of HIF-1α^{+/+} vs. HIF-1α^{+} ES cells within the tumors (p > 0.35 for all distances). The error bars indicate the mean ± standard error of the mean.

6.4 Quantification of Localization within ES Cell Tumors Grown Subcutaneously

Yu et al. (80) reported the heterogeneous vascular dependence of ES cells within tumors, with HIF-1α^{+/+} ES cells localized proximal to blood vessels in subcutaneous tumors of mixed HIF-1α^{+/+} and HIF-1α^{−} ES cells. The limitations of this study have been stated above, and the question also arises as to whether the localization phenomenon still occurs in the cell lines studied in this thesis even though there is no difference in hypoxia-induced apoptosis between the cell types. In order to study localization in subcutaneous tumors, ES cells were injected subcutaneously in SCID mice at a total cell concentration of 2×10^6 cells in 0.1 mL (50% CFP-labeled HIF-1α^{+/+} and 50% YFP-labeled HIF-1α^{−} ES cells).
The methods used to study the localization are detailed in Chapter 3. Briefly, after the tumors reached a volume of 1000 mm³, the mice were injected intravenously with Hypoxyprobe-1 (Chemicon, Inc.), a marker of hypoxia that can be visualized by immunohistochemistry, one hour before sacrifice. One hour later, the mice were perfused by direct infusion into the left ventricle with 4% paraformaldehyde, and the tumors were excised and fixed in 4% paraformaldehyde, followed by immersion in 30% sucrose solution overnight and freezing of the tumors in OCT freezing solution at -80°C.

Tumor sections were cut on a cryostat and divided into two groups. Group 1 was incubated with a primary antibody against CD31 to label all blood vessels within the tumor, and Group 2 was incubated with a primary antibody against the hypoxic piminidazole marker to label areas of hypoxia. Groups 1 and 2 were then incubated with a secondary antibody conjugated to a far-red fluorescent label (Excitation = 650 nm, Emission = 710 nm). Four tumors of mixed HIF-1α status (50% HIF-1α⁺⁺ and 50% HIF-1α⁻⁻) were sectioned, with 3 sections from each tumor for each staining group (spaced 300 μm apart). For each section, 4 random areas were analyzed for a total of 12 locations per tumor per group, or 48 locations per group.

In each selected location, fluorescent images were taken of the blue, green, and far red fluorescence to visualize the HIF-1α⁺⁺ cells, HIF-1α⁻⁻ cells, and label of interest (CD31 or hypoxic marker). The images were analyzed similar to the method used for analyzing multiphoton microscopic images of the dorsal skinfold chamber above. The red, green, and blue channels of the image were binarized similar to the multiphoton image in Figure 6.5. The red channel, corresponding to the vessels or hypoxic area, was skeletonized and ten concentric
regions around the outlined vessels or hypoxic areas were drawn, spaced approximately 13 μm apart. The automated NIH Image program used in Section 6.3 was again used to measure the overlapping blue and green areas in each consecutive distance from vessels/hypoxia. Figures 6.8-6.9 show representative graphs for two representative images each of CD31-positive vessels and hypoxic areas. The graphs demonstrate the fraction of HIF-1α+ cells (blue) or HIF-1α- cells (green) within each given distance of the labeled areas.

**Figure 6.8** Representative images (A and B) of mixed HIF-1α+ (blue) and HIF-1α- (green) tumor sections stained for CD31-positive vessels (red). The graphs on the right indicate the fraction of cells of each type within a certain distance of the vessels for the corresponding left image. The graph was calculated by the same method depicted in Figure 6.5, except with concentric outlines drawn approximately 13 μm apart.
Figure 6.9 Representative images (A and B) of mixed HIF-1α<sup>+</sup> (blue) and HIF-1α<sup>-</sup> (green) tumor sections stained for hypoxic marker (Hydroxyprobe-1; red). The graphs on the right indicate the fraction of cells of each type within a certain distance of the hypoxic areas in the corresponding left image. The graph was calculated by the same method depicted in Figure 6.5, except with concentric outlines drawn around the hypoxic regions approximately 13 μm apart.

Again, as in the case for the dorsal skinfold chambers, the cell distribution around blood vessels or hypoxia is extremely dependent on the location that is analyzed. This is especially evident in Figure 6.9, where in 6.9A it seems as though the HIF-1α<sup>-</sup> cells are localized in the hypoxic area whereas in 6.9B it is the HIF-1α<sup>+</sup> cells that are in the hypoxic region. To analyze more quantitatively the tendency of the two cell types to localize within the tumor, 48 images in each group were analyzed and the results averaged together to obtain average curves for the distance of HIF-1α<sup>-</sup> and HIF-1α<sup>+</sup> cells from blood vessels and hypoxia. These curves are depicted in Figure 6.10, and it is again evident that in the subcutaneous tumors as well, there seems to be no
tendency for either HIF-1α+/+ or HIF-1α−/− cells to localize with respect to blood vessels or hypoxia. While the HIF-1α+/+ curve seems to be displaced to a larger fraction of cells at any given distance from hypoxia, the difference between the curves is statistically insignificant, and the slopes of the curves are similar throughout the graph.

![Graph A](image)

**Figure 6.10** Average cell distribution for HIF-1α+/+ cells (blue) and HIF-1α−/− cells (green) with respect to CD31-positive vessels (A) and hypoxia (B). The HIF-1α+/+ curves are not statistically different than the HIF-1α−/− curves (p > 0.55 for all distances for CD31; p > 0.12 for all distances for hypoxia). The error bars indicate the mean ± standard error of the mean.

### 6.5 Discussion

Several important findings were elucidated from the studies presented in this chapter. First, the HIF-1α−/− ES cells displayed a much faster tumor growth than the HIF-1α+/+ ES cells, in agreement with the previously reported model using ES cells xenografted into SCID mice (1). This agreement is actually surprising since the cells did not display a difference in hypoxia-mediated apoptosis *in vitro* that was seen in previous studies. It had been postulated that hypoxia-induced apoptosis and growth arrest in the HIF-1α+/+ tumors led to the decreased tumor growth rate relative to the HIF-1α−/− ES cells. The current studies indicate that the mechanism
may indeed be more complex than previously thought, although the possibility of a difference in hypoxia-induced apoptosis \textit{in vivo} remains.

While further studies are warranted to further tease out the mechanism of increased tumor growth, some possibilities can be speculated in order to direct future research in this area. It may be that HIF-1\(\alpha\) does impart a survival disadvantage to cells \textit{in vivo}, which may be due to interactions between hypoxia, glucose availability, interactions with various growth factors present in the tumor microenvironment, and interactions between tumor cells and stromal cells. Further \textit{in vivo} studies would be necessary to determine whether the effects on apoptosis and proliferation \textit{in vivo} differ from those seen \textit{in vitro}. Interestingly, in the previous study, the \textit{in vivo} differences in apoptosis and proliferation were actually less dramatic than those seen \textit{in vitro}. It is also not known to what extent stromal cell expression of HIF-1\(\alpha\) affects the tumor phenotype. The contribution of stromal cell HIF expression to the overall HIF activity in ES cell tumors can be measured by comparing HIF-1\(\alpha\) protein levels in HIF-1\(\alpha^{+}\) ES cell tumors to that in HIF-1\(\alpha^{-}\) ES cell tumors. Any HIF-1\(\alpha\) expression seen in the HIF-1\(\alpha^{-}\) tumors would presumably be due to expression by the endogenous stroma in which the tumor cells are growing.

Another mechanism by which HIF-1\(\alpha\) may affect the tumor growth may be in the functionality of the blood vessels which are recruited. HIF-1\(\alpha\) is known to upregulate VEGF levels and thus angiogenesis within tumors, and indeed in previous studies, VEGF levels were seen to be decreased in HIF-1\(\alpha^{-}\) tumors by approximately 40\% compared to HIF-1\(\alpha^{+}\) tumors (1, 79). This paradoxical increase in tumor growth despite decreased VEGF levels and angiogenesis suggests
that the remaining vessels within the HIF-1α⁺ tumors may be more efficient in delivering oxygen and nutrients to the tumor. Indeed, there is growing evidence for this concept of “normalization” of blood vessels following the downregulation of VEGF or other antiangiogenic therapies (2). Blockade of VEGF signaling in transplanted tumors in mice prunes the immature and leaky blood vessels and remodels the remaining vasculature so that the resulting vascular network is less leaky, less dilated, and less tortuous with greater pericyte coverage. In addition, functional changes are evident in the remaining vessels, such as decreased interstitial fluid pressure, increased tumor oxygenation, and improved penetration of drugs in these tumors (141, 142). Evidence of normalization has also been seen clinically in patients treated with a combination of an antibody to VEGF, chemotherapy, and radiation (143).

Previously, the increased tumor growth of HIF-1α⁻⁻ tumors was attributed to hypoxia-induced apoptosis and growth arrest in the HIF-1α⁺⁺ tumors (1); however, the contribution of vessel normalization was not investigated. It was noted in one previous study that the HIF-1α⁺⁺ tumors lacked medium-sized and large vessels but not small vessels and capillaries (1). Thus, the vessels that remain in the HIF-1α⁺⁺ tumors may be more efficient and more similar to normal tissue blood vessels than the large and chaotic vessels typically seen in tumors. In future studies, both the structure and the function of the blood vessels in HIF-1α⁺⁺ vs. HIF-1α⁺⁻ ES tumors should be carefully analyzed. In particular, vessel permeability, pericyte coverage of blood vessels, basement membrane integrity, vascular density, vessel diameter, interstitial fluid pressure, degree of hypoxia, and drug penetration should be investigated in these tumors (2).
The role of HIF-2α in these ES cell tumors should also be investigated. It has been shown that HIF-1α and HIF-2α can have distinct roles in tumors, and that changes in the expression of one isoform can affect the expression levels of the other (144). Therefore, the status of HIF-2α in the ES cells used within this thesis should be investigated, as well as its corresponding role in apoptosis, proliferation, and tumor growth. Likewise, the role of HIF-1α as a tumor suppressor should also be addressed in other tumor cell lines, particularly those with more malignant phenotype. Recent findings suggesting that HIF-1α may play a tumor suppressor role in certain glioma tumors may be further understood by performing similar experiments to those described in this thesis with the glioma cells (88).

A second important finding from these studies is that in tumors grown from mixed HIF-1α+/− and HIF-1α−/− ES cells, the tumor growth rate was inversely proportional to the amount of HIF-1α+/− cells initially present, while there was a disproportionate initial tumor growth delay with increasing fractions of HIF-1α−/− cells. In other words, it took longer for the mixed tumors to start growing than would be predicted, but once they were established, the rate of growth was proportionally between the growth rate of HIF-1α−/− and HIF-1α+/− tumors. Thus, HIF-1α seems to play a tumor suppressor role in these tumors at early stages, even suppressing the growth of the fraction of cells that are HIF-1α−/−. However, as the tumors grow past a certain point, they seem to ‘escape’ this tumor suppressor phenotype and the resulting growth rate is determined by the ratio of HIF-1α−/− cells to HIF-1α+/− cells. It would be expected if this were the case that as the HIF-1α−/− cells outgrew the HIF-1α+/− cells, the tumor exponential growth rate would increase over time. In the current studies, this increased growth rate over time was not observed, but
could be investigated in further studies by monitoring changes in the ratio of CFP-labeled cells to YFP-labeled cells.

Finally, a third important finding of these studies was that a quantitative analysis of mixed HIF-1α+ and HIF-1α− tumors in both the dorsal skinfold chamber and the subcutaneous space indicated that there was no difference in localization of tumor cells in vivo with respect to blood vessels or hypoxia based on HIF-1α status. This is in contradiction to previous observations made in two separate studies (80, 81). However, in both of these studies, only a small fraction of tumor cells was analyzed within the tumor or only one of the tumor cell types was visualized. The current study is also the first study to look directly at localization with respect to hypoxia, rather than just blood vessels. Although the results in this case were the same for both vasculature and hypoxia, it is important to investigate hypoxia directly because it has been shown that oxygen levels in tumors do not necessarily correlate with the location of blood vessels due to the chaotic nature and heterogeneous perfusion of tumor vessels (106).

Two possible interpretations could be advanced for the lack of difference in localization between HIF-1α+ and HIF-1α− cells in the current study. First, it is possible that the apparent localization seen in previous studies was an artifact of the limitations of the previous studies outlined above. The ability to observe both cell types directly throughout the tumor and the quantitative nature of the current studies may allow for more accurate representation of the tumor phenotype in vivo. On the other hand, the difference in phenotype of the cells in the current study with respect to hypoxia-induced apoptosis and growth arrest in vitro may extend into differing
tumor phenotype \textit{in vivo} as well. These issues highlight the complexities of HIF-1\(\alpha\) in tumor biology and raise intriguing and provocative directions for future investigations.
Chapter 7: Conclusion

Hypoxia is an important aspect of the tumor microenvironment and occurs due to the chaotic nature of the blood vessels formed within tumors and the overall poor perfusion compared to the vasculature of normal tissue. The response of tumors to this hypoxic microenvironment depends in large part on the activity of HIF transcription factors, and primarily on the upregulation of HIF-1α in hypoxia. Because HIF-1α has been reported to promote increased tumor growth and tumor aggressiveness, it has been viewed as a potential target for tumor therapy. However, studies demonstrating the role of HIF-1α as a tumor suppressor in certain tumor types have caused a more cautionary approach to targeting HIF-1α, and it is extremely important for the contradicting role of HIF-1α in different situations to be understood at a deeper level. This thesis provides an important contribution to this ongoing investigation into the multifaceted role of HIF-1α.

It was first demonstrated in Chapter 4 that a sandwich culture system could be used to study the development of oxygen gradients and the response of cells to such gradients in a well-controlled environment. A diffusion-reaction mathematical model was developed that accurately predicts the oxygen concentration profiles within the sandwich culture. Even beyond the studies reported in this thesis, this model provides an invaluable tool for the future studies of tumor cells or stem cells in hypoxic microenvironments. The advantage of the sandwich culture system is that it can reproduce a single aspect of the microenvironment of tumors – the gradient of oxygen and nutrients that develops – while controlling for other variables. The other in vitro system that provides these features, the spheroid system, has the disadvantages of requiring fixation and sectioning to see the entire gradient, as well as the extremely small distance over which the
gradient is formed. The sandwich culture system provides a ‘tunable’ gradient, in which the distance over which the gradients develop can be predictably adjusted by changing the cell density or the sandwich gap used in the system. Thus, these initial studies and modeling provide fertile ground for further experimentation and model development for future work.

This thesis also provides essential insights into the behavior of ES cell tumors through the *in vitro* and *in vivo* studies in Chapters 5 and 6. Previously, three related findings had been reported regarding the role of HIF-1α in ES cell tumor phenotype. First, the removal of HIF-1α from the cells led to an increased tumor growth relative to HIF-1α⁺⁺ cells, suggesting a unique tumor suppressor role for HIF-1α (1). Second, the HIF-1α⁺⁺ cells were demonstrated to localize preferentially within tumors proximal to blood vessels (80) while HIF-1α⁻⁻ cells were observed in some instances to localize distal to the blood vessels (81), suggesting an increased vascular dependence of HIF-1α⁺⁺ cells compared to the HIF-1α⁻⁻ cells. Third, HIF-1α seemed to confer upon these ES cells an increased apoptosis and growth arrest when exposed to hypoxia compared to cells lacking HIF-1α (1). These findings were thought to be related in that the increased apoptosis and growth arrest by HIF-1α⁺⁺ cells in hypoxic regions was thought to be the mechanism for the decreased tumor growth and localization within oxygenated areas.

However, this thesis demonstrated the surprising finding that the ES cells used in this study exhibited no difference in hypoxia-induced apoptosis or proliferation *in vitro*, or in localization with respect to blood vessels or hypoxia *in vivo*. In addition, there was no difference in migration of the cells when exposed to oxygen gradients *in vitro*. On the other hand, the cells used in these
studies did demonstrate an increased tumor growth of HIF-1α−/− ES cell tumors, with the exponential growth rate inversely proportional to the amount of HIF-1α+/+ cells within the tumor.

Two important results of these new findings arise. First, the difference in behavior of the ES cells in various studies seems to depend on factors unrelated to the HIF-1α status of the cells. The difference in apoptosis, proliferation, and localization may have been due to clonal differences in the cells unrelated to the presence or absence of HIF-1α. It is also unknown what effect differentiation of the cells has on the observed behavior. While the ES cells are initially pluripotent, the inevitable handling and culturing of the cells when performing such studies may cause differentiation of the cells into a new, undetermined phenotype which may further confound the results. As far as the previous localization studies are concerned, it is unknown whether the limitations of the previous studies outlined in this thesis could have led to the artificial appearance of a difference between the two cell types that was not borne out upon more careful quantification of the localization.

Another potential contributor to the conflicting results is the unresolved role of HIF-2α in mediating the effects of HIF-1α on apoptosis, proliferation, migration, and tumor growth. It has clearly been shown in some renal cell carcinomas that HIF-1α and HIF-2α have distinct, non-overlapping, and sometimes opposing roles in tumor development (94, 95, 144). Raval et al. (144) demonstrated that HIF-1α has a pro-apoptotic function and HIF-2α a pro-tumorigenic function in VHL-defective renal cell carcinoma cells, and that HIF-1α retards tumor growth while HIF-2α enhances tumor growth. However, in a recent study of HIF-2α in rat glioma cells, human malignant glioblastoma cells, and embryonic stem cells, it was found that decreased
expression of HIF-2α led to decreased apoptosis, decreased angiogenesis, and increased tumor growth relative to wild type tumors (145). Therefore, the role of HIF-2α in tumor phenotype also seems to be tumor-dependent. Future studies should be performed on the ES cells used in this thesis to determine the HIF-2α expression of these cells and whether HIF-2α compensates for the loss of HIF-1α in these cells, or whether HIF-2α and HIF-1α have opposing roles. This question is especially important as it has been shown in certain renal cell carcinoma cells that suppression of one HIF isoform can cause increased expression of other isoforms (144). Experiments should be performed using siRNA to block HIF-2α in the ES cell lines used in this thesis to answer these questions.

Although the current studies demonstrated no difference in apoptosis or proliferation based on HIF-1α status in vitro, there remained a marked difference in tumor growth in vivo. Therefore, the true mechanism for the increased tumor growth remains an open question for future studies. A fertile area for future investigation is the question of whether the vessels in HIF-1α−− tumors are more “normalized” than those in HIF-1α+− tumors due to differences in VEGF levels and perhaps other angiogenic factors. The structure and function of the blood vessels in these tumors should be carefully analyzed as outlined in Chapter 6. Another potential avenue of investigation raised by the normalization hypothesis is whether chemotherapy and radiation would be more effective in the HIF-1α−− ES tumors than in the HIF-1α+− tumors. It is known that combining antiangiogenesis therapy with chemotherapy or radiation can produce synergistic effects, and it has been proposed that this is due to a “normalization window” created by the remodeling of blood vessels (2). If HIF-1α−− tumors are truly normalized, they may be more susceptible to treatment by traditional therapies. The clinical implications of this possibility are evident. A
prime concern of clinically treating tumors by targeting the HIF-1α pathway is that the removal
of HIF-1α may cause a tumor-promoting effect in some tumors. But if this enhanced tumor
growth is due to vessel normalization, then the combination of HIF-1α targeting therapies with
chemotherapy or radiation could turn this potential pitfall into a treatment advantage. Thus,
initial concerns of pursuing HIF-1α as a clinical target may be alleviated by future studies that
elucidate further the vessel normalization effect of removing HIF-1α in these ES cell tumors.

While various observations made in both previous studies and the current thesis are interesting
and worthy of further investigation, particularly the continued counterintuitive increased tumor
growth of HIF-1α- tumors compared to HIF-1α+ tumors, the variability of findings in the ES
cell tumor model make it extremely difficult in the context of this model to perform careful
studies to analyze these behaviors. Fortunately, there are reports of other animal models in which
HIF-1α plays a tumor suppressor role, particularly the finding that HIF-1α+ astrocytomas have a
more malignant phenotype when grown in an orthotopic environment (88). These models may
provide a more stable and reliable source for investigating further the multifaceted role of
HIF-1α in tumor growth and phenotype.

Like any research study, the current thesis raises many more questions to be addressed in the
future. The precise reasons for the discrepancy between the results presented here and previous
results are unclear, although several careful studies were performed to eliminate possible
reasons. The discrepancy initially seen in the sandwich culture system was investigated more
closely with further in vitro studies to ensure that it was not an artifact of the nature of the
sandwich culture that was different from previous studies. Likewise, the studies were carried into
animal models to demonstrate that the difference was also seen in vivo as well as in vitro, indicating that the previously reported localization behavior did not simply depend on the in vivo microenvironment to be observed. As HIF-1α continues to be investigated as a potential target for tumor therapy, these and other studies will continue to be necessary in order to ensure that the research findings can be translated into a clinical setting in a careful and well-informed manner. Thus, these studies provide an important piece in the unfolding understanding of HIF-1α’s role in tumor growth and treatment, particularly by raising provocative challenges to previously held notions about the role of HIF-1α as a tumor suppressor in certain types of cancer.


