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**Upregulation of Hypoxia-Inducible Genes in Endothelial Cells to Create
Artificial Vasculature.**

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Robert B. Schonberger

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UPREGULATION OF HYPOXIA-INDUCIBLE GENES IN ENDOTHELIAL
CELLS TO CREATE ARTIFICIAL VASCULATURE

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Abstract:

This study explored the possibility that upregulation of Hypoxia Inducible

Factor-1 (Hif-1)-responsive genes in Human Umbilical Vein Endothelial Cells

(HUVEC) would promote and stabilize HUVEC formation into inchoate vascular

beds within artificial collagen gels. This experiment was designed to explore the

above possibility by sub-cloning Hif-1α, the related chimeric construct Hif-1α/VP16,

and the marker gene dsRed into retroviral expression vectors, producing retroviral

vectors containing these genes, and stably transducing HUVEC using these

retroviruses. Transduced HUVEC were to be observed in cell culture as well as after

implantation into artificial collagen gels that have previously supported vascular bed

formation by HUVEC. Our results show, preliminarily, that HUVEC transduced with

Hif-1α/VP16 go into cell-cycle arrest. Attempts to transduce HUVEC with Hif-1α

failed to achieve high enough transduction efficiency to determine the cells'

angiogenic potential. This study concluded that more experiments need to be

conducted to better characterize the effects of hypoxia-responsive gene upregulation

in controlling HUVEC angiogenesis and cell-cycle signaling and that straightforward

transduction of HUVEC by Hif-1α/VP16 is probably not sufficient, in itself, to

induce *in vitro* vascular bed formation.

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Background:

Hypoxia-inducible factor 1 (Hif-1) is a transcription factor primarily responsible for regulating the cellular response to hypoxia.(1) Hif-1 affects the expression of numerous genes involved in nutrient delivery, extracellular matrix formation, the glycolytic pathway,(2) and cell survival. In the context of its ability to govern the above processes, Hif-1 represents a potent tool for possible gene therapeutic manipulation in the treatment of several disease processes that are either constituted by or related to pathologic changes of the vasculature. Upregulation of angiogenesis offers a potential therapy for several conditions including chronic cardiac ischemia and peripheral vascular disease. Downregulation of angiogenesis offers a potentially important avenue for the treatment of a variety of tumors and other diseases associated with vascular proliferation. More specific to the present study, Hif-1's position as a transcription factor involved in regulating angiogenesis and the cell-cycle indicates that Hif-1 manipulation may also have important applications to tissue engineering, specifically in promoting the formation of viable vascular beds by endothelial cells.(3)

The potential relevance of manipulation of angiogenesis for the treatment of human disease:

The prospect of a harnessing control of angiogenesis to treat disease has broad appeal in a variety of clinical situations. As mentioned above, upregulation of angiogenesis would have particular relevance for a person suffering the effects of chronic coronary ischemia or peripheral vascular disease. The possibility that tissues

could be revascularized via upregulation of angiogenesis rather than via surgically delivered vascular grafts represents a potentially revolutionary advance in the treatment of these disorders.

Regarding upregulation of angiogenesis in humans, currently, results of phase II or III clinical trials of therapeutic angiogenesis in humans have shown some promising results. Adenovirus-mediated Vascular Endothelial Growth Factor $(VEGF)_{121}$ and $VEGF_{165}$ as well as Fibroblast Growth Factor-4 (FGF-4) have been used with some signs of success in the treatment of both coronary artery disease and peripheral vascular disease.(4) Specifically, while adenovirus-mediatedVEGF transfection during Percutaneous Transluminal Coronay Angiography (PTCA) and stenting did not reduce clinical restenosis rates or extent of restenosis (defined as minimal lumen diameter), it did result in improved myocardial perfusion (as measured by perfusion at rest minus perfusion during adenosine administration).(5, 6) The improved perfusion, however, was not associated with any improvement in functional status. A randomized, double-blind, placebo-controlled trial of adenovirus mediated FGF-4 for chronic coronary ischemia showed more clinically significant results. In particular, at eight week follow-up of this cohort of 52 patients, freedom from angina was observed in 30% of the treatment group versus 13% of the placebo group, and 43% of the treatment group was not taking nitrates versus 17% of the placebo group.(7)

In a randomized double-blind placebo-controlled trial of VEGF administration for limb ischemia in 36 patients, digital subtraction angiography showed statistically significant increases in the vascularity of limbs treated with VEGF in comparison to

placebo. However, similar to the coronary VEGF trial mentioned above,(6) no clinically significant difference in limb perfusion was detected. Specifically, Ankle-Brachial Index and mean Rutherford class were similarly improved in both placebo and treatment groups.(8)

The discordance between statistically significant improvements in laboratory endpoints of vascular status and lack of clinical improvement in some of the above trials may be due to the fact that induction of angiogenesis, to the extent that it occurs in these trials, is most likely happening at the level of the microcirculation. According to Poiseuille's Law, resistance decreases (and hence flow increases) proportionally to the fourth power of the radius of a vessel. Increasing the numbers of tiny capillaries in an ischemic region may therefore do little to increase actual blood flow significantly. It has been estimated that 42,000 capillaries of 7-μm diameter would be needed to provide the equivalent blood flow found in one small artery of 100-μm in diameter.(4) Hence, although initial trials have shown promise,(9) the possibility that gene therapeutic upregulation of angiogenesis will reach the level of common clinical practice as the sole or primary treatment for ischemic disorders may await the development of methods to engineer larger caliber vasculature.

While the above discussion has illustrated the limited success of efforts to "turn on" the angiogenic switch, efforts to turn the angiogenic switch off have made further inroads into the realm of standard clinical practice. The ability to inhibit angiogenesis has the potential of great clinical utility. In the field of oncology, downregulation of angiogenesis has been recognized as a potent therapeutic target at

least since Judah Folkman and others first isolated soluble angiogenic factors from tumors and postulated the necessity of angiogenesis for tumor growth.(10, 11) Beyond the laboratory, minimally invasive treatment modalities leading to regression of tumors by starving them of their blood supply have already entered clinical practice. One notable example of this technology is the VEGF monoclonal antibody bevasizumab (Avastin®), which has shown convincing benefits in the treatment of colorectal cancer and has shown promise in the treatment of renal cell cancer, non-small cell lung cancer, pancreatic cancer and metastatic breast cancer.(12) The broad variety of cancers susceptible to this single treatment modality points to the near universal utility that control of angiogenesis would afford the oncologist.

The effort to block vascular proliferation has applications beyond oncology. For example, the ability to induce regression of proliferative diabetic retinopathy, macular degeneration, or a benign hemangioma via administration of soluble angiogenesis inhibitors or by altering the genome of the pathological vasculature has obvious appeal.

A brief history of gene therapy in the treatment of human disease and the need for improved delivery systems:

One of the earliest trials that provided a foundation for using gene therapy for the treatment of human disease occurred in 1987 when Hershfield, *et al.* had limited success in treating children with Severe Combined Immuno-Deficiency (SCID) secondary to Adenosine Deaminase (ADA) deficiency with enzyme replacement via administration of bovine ADA to peripheral lymphocytes.(13) Later work by Kohn,

et al. improved on this method by using retroviral-mediated gene therapy to stably transduce lymphocytic precursors, rather than using transient non-genomic alteration of mature immune cells. Kohn, *et al.* successfully harvested CD34+ lymphocyte progenitor cells from the umbilical blood of 3 SCID babies and then transduced those cells with full copies of the human Adenosine Deaminase gene before autologous reimplantation into the peripheral blood of the three patients.(14) His group demonstrated the proliferation of the transduced cells and a corresponding long lasting therapeutic benefit to these patients.(15)

The success of these trials was promising and led to a proliferation of clinical studies of gene therapy. However, the news in 1999 that an 18-year-old subject enrolled in a gene therapy trial at the University of Pennsylvania died four days after adenovirus-mediated gene therapy for ornithine transcarbamylase deficiency highlighted the potential danger and experimental nature of the technique.(16) In 2002, there was further cause for caution in gene therapy trials after two patients in a trial to treat X-linked SCID (independent from the Kohn, *et al.* trials mentioned above) were found to have contracted a lymphoproliferative disorder similar to leukemia.(17) Unlike the adenovirus-mediated trial at the University of Pennsylvania, the SCID trial used a retroviral vehicle for delivery and stable expression of the desired gene. While it was known that retroviral insertion into the genome could potentially be oncogenic, it was thought that such events would be exceedingly rare, requiring that gene insertion occur at one of several particular points in the genome. The fact that 2 out of 10 patients developed a leukemia-like disorder demonstrated that the scientific understanding of the implications of

manipulating the genome remained stubbornly rudimentary. This second story of the

dangers of gene therapy was, along with the U Penn tragedy, picked up by the

popular press(18) and highlighted the need for further work on safe and effective

delivery methods for gene therapeutic agents. The X-linked SCID patients developed

problems years after treatment, demonstrating that studies of safety would require

follow-up both in the immediate post-treatment period as well as years into the future.

Despite the above well-publicized setbacks for gene therapy, the broader

picture shows that scientific interest in gene therapy remains robust, and it continues

to hold much promise as a potential treatment modality. As evidence for this claim, a

recent search of the clinicaltrials.gov database using "gene therapy" as a search term

in the "treatment" field resulted in a list of 68 registered trials that were recently in

the process of actively recruiting study subjects.(19)

In addition, it is worth noting that the desire for safe and effective delivery

systems points to one potential benefit of the artificial vascular bed project described

herein. If gene therapeutic manipulations can succeed in creating viable vascular

beds, these artificial tissues could potentially serve as a removable nexus for delivery

of soluble factors via genetically modified cells within the artificial vascular beds. By

their susceptibility to minimally invasive monitoring and their ability to be removed if

necessary, such vascular beds could potentially increase the safety of a significant

subset of gene therapies.

Having briefly reviewed the clinical potential and current usage of angiogenic

alteration as well as some of the recent history of gene therapy, I will now return to

the biological basis for the present study's effort to alter Hif-1α specifically, and

hypoxia-inducible gene expression in general, for the creation of artificial vasculature.

The structure of Hif-1 and its regulation in cells:

In its transcriptionally active form, Hif-1 is a heterodimer(20) composed of a hypoxia-inducible Hif-1α subunit and a β subunit comprised of the constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT).(20, 21) While the alpha subunit is unique to Hif-1, the beta subunit is active in a number of pathways through its ability dimerize with a number of the bHLH-PAS family of proteins.(20, 21) After dimerizing, Hif-1 controls gene transciption by binding to hypoxia response elements (HREs) in the promoters of target genes containing the core sequence 5'-CGTG-3'.(21) Genes containing HREs are numerous and include vascular endothelial growth factor (VEGF), erythropoietin, glucose transporter-1 (GLUT-1), and several glycolytic enzymes.(22) Teleologically, a cell encountering hypoxia might be expected to upregulate these very genes as it attempted to increase its blood supply, increase available glucose, and switch from oxygen-dependent oxidative phosphorylation to anaerobic glycolysis for energy.

According to the current model, regulation of the action of Hif-1 occurs primarily though the control of post-translational ubiquitinylation and subsequent degradation of Hif-1α.(23) In normoxic conditions, Hif-1α is constitutively expressed and then quickly ubiquitinylated and broken down. This breakdown is mediated by the action of elongin prolyl hydroxylases which hydroxylate two proline residues of Hif-1α.only under normoxic conditions.(23) With Hif-1α in its

hydroxylated state, Von Hippel Lindau (VHL) protein organizes a complex leading to

activation of the E3 ubiquitin ligase which ubiquitinylates Hif-1α, targeting it for

degradation by a proteosome.(22),(24) In hypoxic conditions, Hif-1α remains

unhydroxylated and hence avoids ubiquitinylation by VHL, remaining free to

dimerize with ARNT into its active DNA-binding form. The degradation process has

been shown to be VHL-dependent, so that in the absence of VHL, even after Hif-1α

is hydroxylated, it remains stable and available for dimerization.(25) The loss of Hif-

1α ubiquitinylation by VHL is believed to be responsible for the highly vascular

nature of tumors in which VHL is inactivated.(23) This phenomenon of VHL-loss-

associated angiogenesis is well-known in the context of the Von-Hippel Lindau

syndrome in which genetic errors in VHL are associated with retinal and central

nervous system hemangiomas.(26)

Hif-1 and its role in Apoptosis and Cell-Cycle Regulation:

While Hif-1 is clearly a promoter of angiogenesis, its effects on apoptosis and

the cell cycle are less well-defined. Hif-1 has been associated both with pro and anti-

apoptotic processes.(22) For example, hypoxia increases the Hif-1-mediated

expression of Nip3 which is a pro-apoptotic gene.(22) Hypoxia also induces

stabilization of p53 which results in increased expression of several pro-apoptotic

genes, an effect not seen in Hif-1α deficient cells.(22) On the other side of the coin,

upregulated expression of Hif-1α has been shown to induce resistance to apoptosis in

pancreatic cancer cells.(27) In another experiment, hypoxia was shown to confer

protection from radiation-induced apoptosis perhaps by elevating levels of bcl-2 as

well as altering intracellular trafficking of the pro-apoptotic bax protein.(28) Further evidence of Hif-1's role as an anti-apoptotic factor comes from the observation that iron chelators confer protection on cortical neuron cultures from oxidative stress-induced apoptosis and that this effect is associated with increased DNA binding of Hif-1.(29) In sum, Hif-1 appears to alter several apoptosis-associated genes, and whether its ultimate effect is toward or away from apoptosis probably depends on the specific cell type and cell environment in which it is upregulated.

Regarding cell-cycle regulation, conflicting data indicate that Hif-1's role in inhibiting cell proliferation needs further delineation. In general, hypoxia is known to induce cell-cycle arrest,(30) with different levels of hypoxia required to arrest cells in at least 3 different points in the cell cycle.(31) Extreme hypoxia will arrest cells in S-phase and is associated with a pRetinoblastoma (pRB)-independent decrease in cyclin A protein levels.(31) In G1, at least two different points of hypoxia-induced arrest have been characterized .(31)   Transfection of HUVEC with Hif-1α has been associated with increased expression of p21, decreases in Bcl-2 expression, and subsequent cell-cycle arrest in G1.(32) In murine embryonic fibroblasts and splenic B-lymphocytes, exposure to hypoxia leads to arrest in G1 through a Hif-1α-dependent pathway.(30) In contrast, Gardner, *et al*. found that Hif-1α-null embryonic stem cells arrested in G1 in a similar fashion to Hif-1α-competent stem cells.(33) Elucidating the precise contribution of Hif-1α in regulating the cell-cycle will likely await the better characterization of several arrest-points within G1 in which Hif-1α will play differing roles. Moreover, Hif-1s role at each of these arrest points may also vary among different cell lineages.

Hif-1-responsive genes as a potential target of manipulation in the creation of artificial vascular beds by endothelial cells:

Given Hif-1's well-established role in angiogenesis, its potential role as an anti-apoptotic agent, and its ambiguous effects on cell proliferation, the present study attempted to assess the possibility that upregulation of Hif-1-responsive genes would promote and stabilize artificial vascular bed formation by endothelial cells. Schechner, *et al*.(3) have succeeded in getting human umbilical vein endothelial cells (HUVEC) to form tubes in a three-dimensional collagen-fibronectin gel, which upon implantation into immuno-compromised mice, form complex microvessels lined by human endothelium. They have observed in their experiments that the HUVEC in these gels, if not implanted into mice, apoptose after 24 hours. Overcoming this tendency of HUVEC to apoptose has been a major focus of attempts at creating artificial vascular beds.(34, 35) Schechner, *et al*. were able to delay HUVEC apoptotis *in vitro* for over 7 days by retroviral transduction of HUVEC with a gene expressing caspase-resistant Bcl-2.(3) Such transduced cells have not only lived longer *in vitro*, but when implanted subcutaneously into immuno-compromised mice, have been shown to organize by 60 days into HUVEC-lined multi-layered microvessels invested with smooth muscle which anastomose into the native mouse circulation.

Other investigators have tried anti-apoptotic agents other than Bcl-2 to aid in HUVEC angiogenesis. One successful strategy at inducing HUVEC to undergo inchoate angiogenesis *in vitro* has employed the use of oncogenic phorbol esters,

specifically phorbol myrestate acetate (PMA) which both prevents HUVEC apoptosis and induces HUVEC invasion into artificial collagen gels.(34, 35)

Though specific alteration of the expression of Hif-1-responsive genes in HUVEC has not been attempted previously in the effort to create artificial vascular beds, there are reasons to believe that such experiments may prove fruitful. Hif-1, as mentioned previously, is a known promoter of VEGF expression, and VEGF has been used in HUVEC angiogenesis experiments with some positive results. Although VEGF alone has not induced angiogenesis by endothelial cells in artificial collagen gels, it has shown a role in stabilizing inchoate endothelial tubule formation. When HUVEC are exposed to VEGF without concomitant exposure to PMA, they fail to penetrate and proliferate in artificial collagen beds, however VEGF has been shown to prevent regression of endothelial tubules after they have been formed in the presence of PMA.(35)

Beyond its upregulation of VEGF, Hif-1 may also prove important in promoting HUVEC tubule formation through a mechanism similar to that of PMA. The mechanism by which PMA induces tubule formation by HUVEC is not fully known, however one possibility is that it acts in part through upregulation of Ap-1, a transcription factor which is activated both by phorbol esters and hypoxia.(36) Multiple possible Ap-1 binding sites have been identified in the promoter sequence of Hif-1α,(36) and it is possible that phorbol esters may ultimately promote HUVEC tubule formation through downstream upregulation of Hif-1α via Ap-1.

Strategies for artificially upregulating Hif-1 activity and the expression of Hif-1-responsive genes:

Several strategies have been successful in the effort to artificially induce transcription of Hif-1-responsive genes. Perhaps the most straightforward method for upregulating Hif-1-responsive gene expression is to expose cells artificially to hypoxia which results in almost immediate increases in Hif-1 DNA binding.(37) Likewise, restoration of normoxic conditions results in rapid dissociation of Hif-1 from its binding sites, with half-lives for both association and dissociation of under one minute.(37) Although hypoxia is quite effective in inducing Hif-1-responsive genes, it is clearly an impractical strategy for long-term induction of such genes in cells outside of tightly regulated cell culture conditions. Desferrioxamine and Cobalt Chloride have also been shown to upregulate Hif-1 DNA binding with kinetics similar to those of hypoxia,(38) however neither is compatible with long-term *in vivo* upregulation of Hif-1-responsive genes.

Hif-1-responsive genes have also been successfully upregulated by gene therapeutic manipulation of cellular levels of Hif-1α.(39) Given that Hif-1 activity is primarily regulated by post-translational destruction of Hif-1α, the fact that increasing cellular Hif-1α levels results in alteration of Hif-1-responsive gene expression was not obvious. It might have been expected that increased Hif-1α expression would have been cancelled-out by increased destruction of Hif-1α, leading to no change in Hif-1-responsive transcriptional activity. With acute increases in Hif-1α protein concentration, however, the Hif-1-responsive genes VEGF and GLUT-1 have been shown to be upregulated, indicating that the Hif-1α degradation pathway

cannot immediately compensate for elevated Hif-1α levels. Nevertheless, there is evidence to suggest that a feedback upregulation of Hif-1α degradation may happen in response to elevated Hif-1α levels. Cells that were induced to produce an over 100-fold increase in Hif-1α expression through a Tetracycline-controlled induction method were found after 1 week to have decreasing levels of Hif-1α expression despite continued exposure to doxycycline-free media.(39) Such cellular behavior indicates that the upregulation of Hif-1-responsive genes obtained by artificially raising Hif-1α levels may ultimately be a transient phenomenon.

An alternative strategy, which may lead to a more sustained upregulation of Hif-1-responsive genes, has been to employ the use of artificial transcription factors. One such transcription factor with demonstrated success is the chimeric Hif-1α/VP16 (Genzyme). Hif-1α/VP16 encodes a hybrid protein composed of the DNA-binding and dimerization domains of Hif-1α protein and the trans-activation domain of the Herpes Simplex Virus (HSV).(40) Direct intramuscular gene transfer of Hif-1α/VP16 into ischemic rabbit hind-limbs has resulted in therapeutic levels of angiogenic response.(40) In a porcine model of cardiac ischemia, adenovirus-mediated transduction of Hif-1α/VP16 led to increased myocardial perfusion and increased left ventricular function, an effect not seen with Hif-1α plasmid delivery.(41) The specific pattern of gene upregulation after transfection with Hif-1α/VP16 is related to, but not identical with, the pattern induced by hypoxia. Gene expression patterns following Hif-1α/VP16 transfection have been studied in cardiac myocytes,(42) and several known Hif-1-responsive genes including VEGF, Glut-1,

insulin-like growth factor binding protein 3, and several glycolytic enzymes have been upregulated. However, adenovirus-mediated transfection of myocytes with Hif-1α/VP16 also resulted in upregulation of several genes not previously known to be regulated by hypoxia, HIF-1, or adenovirus infection including plasminogen activator inhibitor 1 (PAI1) and endothelial differentiation factor 1.

The gene-expression pattern of Hif-1α/VP16 makes it potentially even more promising than wild Hif-1α for inducing HUVEC angiogenesis. Insulin-like growth factor binding proteins control the activity of insulin-like growth factor which when upregulated has been associated with VEGF-dependent neovascularization in diabetic retinopathy.(43) In addition, the fact that Hif-1α/VP16 upregulates PAI1 may be especially promising since PAI1 has been implicated in the control of endothelial cell migration and tube formation *in vitro* as well as of *in vivo* tumor invasion and angiogenesis.(42, 44, 45) Beyond its potentially superior gene-induction profile for angiogenesis, Hif-1α/VP16 may also have more benign effects on apoptosis than wild Hif-1α. As above, Hif-1 has been implicated in the induction of apoptosis via affects on p53 and bcl-2.(42, 46) In cardiac myocyte transfection, however, Hif-1α/VP16 was found to upregulate the pro-survival genes Nip-3 and TNF-α, with no observed increase in apoptotic activity.(42) While Hif-1-responsive genes have been implicated in both pro and anti-apoptotic processes, it is not known to what extent Hif-1α or Hif-/VP16 affect apoptosis and cell-cycling in HUVEC.

The practical appeal of upregulating Hif-1α or employing Hif-1α/VP16 to induce HUVEC angiogenesis:

The possibility that Hif-1-responsive gene manipulation might both delay apoptosis in HUVEC as it has done in other cell lines, as well as stabilize and promote inchoate vascular bed formation through upregulation of VEGF and other angiogenic genes makes it a promising strategy for inducing *in vitro* HUVEC angiogenesis. If such an approach were to work, not only would it facilitate the development of artificial vasculature, but it might make such artificial tissue more readily acceptable for use in human subjects by avoiding the use of known oncogenic alterations to HUVEC. If the implantation of artificial vascular beds into human subjects has any hope of one day playing a therapeutic role in human disease, avoiding the direct alteration of Bcl-2, the use of phorbol esters, or other oncogenic alterations would certainly be preferable.(47) Efforts in this regard have recently found success through co-implantation of endothelial cells with mesenchymal precursor cells.(47) In any case, observations of the effects that upregulation of Hif-1-responsive genes have on the behavior of HUVEC may have important implications both for tissue engineering as well as the understanding of Hif-1's role in endothelial cell biology in general.

Methods:

In order to test the hypothesis that upregulation of Hif-1-responsive genes would induce HUVEC angiogenesis, we sub-cloned three constructs for retroviral-mediated transduction into HUVEC. The first construct consisted of human Hif-1α cDNA which we sub-cloned into pLNCX2 (Clontech), a retroviral packaging plasmid capable of producing high-titre, replication-incompetent retroviruses. The second

construct consisted of Hif-1α/VP16 cDNA (Genzyme) which we sub-cloned into pLNCX2, and the third construct was dsRed (Clontech), a gene coding for a protein exhibiting red phosphorescence under ultraviolet light, which we sub-cloned into pLNCX2. dsRed was to be used as an easily identifiable marker for transduction success in parallel with other transductions.

Sub-Cloning human Hif-1α cDNA into the viral packaging plasmid pLNCX2:

Plasmid containing human wild Hif-1α cDNA within pBSK(Bluescript/Stratagene) was obtained from Reed Hickey (Giordano Lab, Yale University School of Medicine). The Hif-1α gene was removed from pBSK using a 5' EcoRV/NotI 3' restriction digest, and an appropriately-sized 3.6 Kbp fragment was gel isolated. Preparation of pLNCX2 was accomplished first by cutting with HindIII restriction digest at the unique HindIII site in the multiple-cloning site (MCS) of pLNCX2. This cut plasmid was then blunt-ended using Klenow Fragment exonuclease (New England Biolabs) in EcoPol Buffer. This blunt-ending was meant to correspond to the blunt-cut EcoRV site on the Hif-1α fragment. Blunted pLNCX2-HindIII digest was then cut at a single MCS point with a NotI restriction digest, corresponding to the 3' Not I cut on the Hif-1α fragment. The 6.1 Kbp fragment from this Not I digest was gel isolated. Ligation of Hif-1α into pLNCX2 was then attempted after treatment of pLNCX2 with Shrimp Alkaline Phosphatase (Roche).

After the attempted ligation, we attempted to transform a JM-109 strain of *e. coli* with the sub-cloned Hif-1α/pLNCX2 via heat-shock. JM-109 were then plated

on ampicillin-containing media to select for pLNCX2-containing *e.coli* (pLNCX2

contains an ampicillin-resistance gene). 10 ampilcillin-resistant colonies were picked

and mini-prepped.

To check if the correct plasmid containing the full Hif-1α/pLNCX2 construct

was present, DNA from each of these 10 mini-prepped colonies was cut with

BglII/SalI digest and run on a gel. The expected fragment sizes would be 127bp,

652bp, 2.9Kbp, and 6Kbp which corresponded with the gel for 7 of the colonies, one

of which was then maxi-prepped (see figure 1 in appendix).

Sub-Cloning Hif-1αVP16 cDNA into the viral packaging plasmid pLNCX2:

Plasmid containing the chimeric Hif-1α/VP16 cDNA within pBSK was

obtained from Reed Hickey (Giordano Lab, Yale Universtiy School of Medicine).

The plasmid was cut with ClaI restriction digest and then blunt-ended using Klenow

Fragment exonuclease followed by another cut with a Not I restriction digest, leading

to a 1.5 Kbp Hif-1α/VP16 fragment with 5' blunt end and 3'Not I-cut restriction site.

This 1.5Kbp Hif-1α/VP16 fragment was gel isolated. As in the above section,

pLNCX2 with 5' blunt end and 3'Not I cut restriction site was made. As above,

ligation of Hif-1α/VP16 into pLNCX2 was attempted after treatment of pLNCX2

with Shrimp Alkaline Phosphatase (Roche).

 After the attempted ligation, a JM-109 strain of *e. coli* was transformed with

the sub-cloned Hif-1α/VP16/pLNCX2 via heat-shock. JM-109 were then plated on

ampicillin-containing media to select for plasmid-containing *e.coli*. 6 ampilcillin-

resistant colonies were picked and mini-prepped.

To check if the correct plasmid containing the full Hif-1αVP16/pLNCX2 construct was present, DNA from these 6 mini-prepped colonies was cut with AvaI restriction digest and run on a gel. The expected visible fragment sizes would be 600bp, 1.2Kbp, 2.5Kbp, and 2.9Kbp which corresponded with the gel for 4 of the 6 colonies, one of which was then maxi-prepped (see figure 2 in appendix).

Sub-Cloning of dsRed cDNA into the viral packaging plasmid pLNCX2:

Plasmid containing the dsRed cDNA was obtained from Reed Hickey (Giordano Lab, Yale University School of Medicine). The plasmid was cut with 5' HindIII/NotI 3' restriction digest and the corresponding 700bp dsRed fragment was gel isolated. Similarly, pLNCX2 was cut with 5' HindIII/NotI 3' restriction digest and the corresponding 6.1 Kbp fragment was gel isolated. As above, ligation of dsRed into pLNCX2 was attempted after treatment of pLNCX2 with Shrimp Alkaline Phosphatase (Roche).

As above, after the attempted ligation, a JM-109 strain of *e. coli* was transformed with the sub-cloned dsRed/pLNCX2 via heat-shock. JM-109 were then plated on ampicillin-containing media to select for pLNCX2-containing *e.coli*. 10 ampilcillin-resistant colonies were picked and mini-prepped.

To check if the correct plasmid containing the full dsRed/pLNCX2 construct was present, DNA from each of these 10 mini-prepped colonies was cut with HindIII/NotI restriction digest and run on a gel. The expected visible fragment sizes would be 6.1Kbp and 700bp which corresponded with the gel for all 10 colonies, one of which was then maxi-prepped (see figure 3 in appendix).

Transfection of the packaging cell line and determination of retroviral titre:

Retropack PT67 packaging cells were obtained from BD Biosciences (www.bdbiosciences.com) and grown in cell culture using DMEM (Gibco) supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin solution. 24 hourse before transfection, packaging cells were plated at approximately 60% confluency on 60mm plates. Transfection with plasmid DNA containing dsRed was accomplished by mixing 16mcg of dsRed in 3mL of DMEM along with 40mcL Lipofectamine 2000 (Invitrogen), and after 20 minutes of incubation, pipetting the mixture onto PT67 cells.

Eighteen hours post-tranfection, culture medium was removed, and packaging cells were washed with phosphate buffered saline twice. Cells were then plated with selection medium containing 100mcg/mL of geneticin (pLNCX2 contains a neomycin resistance gene). After 7 days, surviving cells were examined under ultraviolet light and glowed red as would be expected for dsRed-containing cells. Retrovirus-containing media was harvested every 24 hours. To assess retroviral titre, NIH-3T3 cells were plated in six well plates to a density of approximately 10^5 cells per well with medium as above. Six 10-fold serial dilutions of retrovirus-containing medium were made, and added to 3T3 plates. 48 hours after attempted transduction, 3T3s were examined under ultraviolet light. No cells beyond the 10^{-4} dilution glowed red. Because a density of at least 10^6 virions/mL was desired, the above transduction was attempted with the addition of 4mg/mL of polybrene, a polycation capable of diminishing electro-static repulsion between cells and virions. However, no cells

were successfully transduced after numerous attempts, indicting either that virions

had lost their infectivity or that polybrene was interfering in the process. Multiple

repeat attempts at re-transfection of packaging cells and retrovirus production proved

unsuccessful. Because 10^4 infective virions/mL was considered too low for

transduction of HUVEC, and because the viability of these virions was questionable

after subsequent attempts failed to observe infectivity, David Enis of the Schechner

Lab, Yale University School of Medicine, re-attemtpted transfection of a different

retroviral packaging cell line with our three constructs.

For the further attempts at retroviral production, the GP2-293 packaging cell

line (Clontech) was used. This packaging cell line requires co-transfection with

pVSV-G, a plasmid expressing VSV-G from the cytomegalovirus promoter.   VSV-G

is toxic to the packaging cells, so retrovirus particles must be transfected with pVSV-

G simultaneously with the desired genes, and retroviral harvesting must be done

shortly after transfection. Accordingly, GP2-293 cells were grown in cell culture

using DMEM (Gibco) supplemented with 10% fetal bovine serum, 1% glutamine,

and 1% penicillin/streptomycin solution. 24 hourse before transfection, packaging

cells were plated at approximately 60% confluency on 60mm plates. Dave Enis

accomplished transfection with the above pLNCX2 constructs containing dsRed, Hif-

1α, and Hif-1α/VP16 and pVSV-G by mixing equal amounts of each plasmid with

pVSV-G and pipetting the three mixtures over plated GP-2 cells. Ninety hours post-

transfection, retrovirus-containing supernatant was harvested and added to cultured

HUVEC to which Geneticin was added to select for transduced cells.

HUVEC Cell Culture and Creation of Artificial Collagen Gels:

David Enis of the Schechner laboratory maintained and transduced HUVEC in cell culture. Isolation and culture of HUVEC has been described elsewhere(48), however briefly, HUVEC can be harvested from flushed umbilical veins via use of collagenase solution and cutured in Medium 199 containing 15mM HEPES organic buffer.(48) Had the transduction produced a stable line of replicating HUVEC, collagen gels would have been formed as described elsewhere.(3) Briefly, as described by Schechner *et al*., HUVEC would be suspended in Hepes and NaHCO3 buffered Medium 199 with rat-tail type 1 collagen (1.5 mg/ml) and human plasma fibronectin (90mcg/ml) and then brought to pH 7.5 with 0.1 molar HCl at 4 degrees Celsius. The suspended HUVEC would then be inserted into rat-tail type 1 collagen-coated C-6 transwells and warmed to 37 degrees for collagen polymerization and then covered with Medium 199 supplemented with 20% FBS, 50 mcg/ml endothelial cell growth factor, 200 mcg/ml streptomycin and penicillin, 2mM L-Glutamine and 100 mcg/ml heparin.(3) The above collagen-gel formation was not performed, however, due to the results of the transduction trial.

Results:

The majority of HUVEC in the plate transduced with dsRed glowed red when exposed to ultraviolet light, appearing to validate our transduction methods. After exposure to Geneticin, only glowing, dsRed-containing HUVEC survived, further indicating the success of the transduction as well as the selection method. Because the dsRed transduction was conducted in parallel with Hif-1α and Hif-1α/VP16

transductions, it was taken as reassurance that the latter two transduction procedures were also valid. In the Hif-1α-transduced plate, however, very few of the cells survived exposure to Geneticin, and it was felt that transduction efficiency was insufficient to warrant implantation of the few remaining cells into collagen gels. HUVEC transduced with Hif-1α/VP16 appeared to achieve good transduction efficiency similar to the success of dsRed transduction, as evidenced by the majority of Hif-1α/VP16-transduced HUVEC surviving exposure to Geneticin. The surviving Hif-1α/VP16-transduced HUVEC were observed to grow larger and stop replicating in cell culture shortly after selection and prior to attaining the requisite cell count for collagen-gel insertion trials. Following these observations, an effort was made to confirm the success of HUVEC transduction with Hif1-α/VP16 by conducting Western Blot analysis of the transduced HUVEC using antibody to the HSV VP16 protein. David Enis conducted this Western Blot and, by report, confirmed the presence of VP16 protein in transduced HUVEC.

While further analysis of the Hif-1α/VP16-transduced HUVEC was felt to be warranted to document the gene expression pattern associated with their arrested replication, laboratory priorities were more focused on engineering successful angiogenesis, and it was not felt that the further study of these arrested cells would be immediately fruitful in this effort.

Discussion:

While additional trials of HUVEC transduction with Hif-1α and Hif-1α/VP16 would be necessary for any firm conclusion about their effects on HUVEC behavior, our initial trial does not give cause for optimism that such transductions will, in themselves, successfully promote HUVEC *in vitro* tubule formation or *in vivo* angiogenesis. If further trials continued to show arrested proliferation of Hif-1α/VP16-transduced HUVEC, it would be helpful to document their gene-expression pattern as well as the stage at which they arrested. Specifically, gene microarray analysis of expression patterns could be used for the former, and for the latter, flow cytometry has been used in previous experiments to document the stage of cell cycle arrest as well as signs of early apoptosis.(32) Nevertheless, while further trials and experiments are needed, we have several speculative theories about why HUVEC appeared to stop replicating after Hif-1α/VP16 transduction.

As was mentioned above, hypoxia's effects on endothelial cell cycling have revealed conflicting data, but cell-cycle arrest has previously been associated with Hif-1α and is therefore not particularly surprising to see with Hif-1α/VP16.(26, 32) Furthermore, there are reasons beyond our own observation in this experiment to suspect that Hif-1α/VP16 may be more prone to cause cell-cycle arrest than Hif-1α. To see this, it is useful to return to the gene expression patterns seen in previous Hif-1α/VP16 transfection experiments. Of note, it has been observed that at least some Hif-1-responsive genes show intensely increased expression by Hif-1α/VP16 at rates far in excess of those produced by hypoxia. Specifically, TaqMan quantitative PCR

analysis of Hif-1α/VP16-transfected cardiac myocytes revealed a 102-fold increase in VEGF expression over control myocytes as compared to a 4-fold increase from hypoxia alone. Regarding VEGF induction, Hif-1α/VP16 was over 25-times more potent than hypoxia alone in cardiac myocytes. It is possible that while physiologic, mild hypoxic signaling could induce endothelial cell proliferation, Hif-1α/VP16 may have mimicked HUVEC exposure to extreme hypoxia, which has been shown to induce cell-cycle arrest (49) as mentioned above.

Another observation of Hif-1α/VP16-transfected cells is that they have previously shown "dramatically" elevated (numeric data was not presented) levels of brain natriuretic peptide (BNP), a gene only "moderately" induced by hypoxia.(42) It is possible that the Hif-1α/VP16-transduced HUVEC in our experiment were not truly quiescent. Among other things, BNP elevation is associated with cell hypertrophy and has even been used as a marker for cell hypertrophy in myocyte studies.(50) It is possible that the Hif-1α/VP16-transduced HUVEC in our experiment were not entirely arrested but instead had begun a hypertrophic process. To assess this possibility, transduced HUVEC would need more extended observation than we conducted, including measurements of BNP expression. The observation that they were growing larger was consistent with continued protein synthesis in the absence of proliferation, which could be indicative either of physiologic hypertrophy or with failure to replicate due to cell-cycle arrest.

Another possibility of why our Hif-1α/VP16-transduced HUVEC stopped replicating rests on the fact that retroviral transduction with any construct is bound to cause multiple alterations in the biology of a cell, independent of any specific process

related to hypoxia-inducible gene expression. Specifically the use of the HSV VP16 activation domain, might be particularly prone to interfere with cell-cycling. During HSV infection, VP16 protein is required for immediate early transcription of viral proteins. It binds with several proteins in a host cell to form a complex to induce transcription, and one of these host proteins is known as Host Cell Factor (HCF).(51) HCF is primarily a protein necessary for cell cycling, specifically it is one of a number of proteins necessary for the progression of cells from G1 to S phase.(52) Although cell cycle arrest has not previously been reported in gene therapy trials using chimeric constructs containing a VP16 domain, it is theoretically possible that in our trial, expression of VP16 protein in HUVEC interfered with the action of the native HCF protein and caused arrest in G1.

If Hif-1α/VP16 transduction is shown in repeated attempts to induce arrest in HUVEC, there still may be a role for this artificial transcription factor in promoting artificial angiogenesis. To overcome its affects on cell cycling while still benefiting from upregulation of hypoxia-responsive genes, a fruitful strategy for angiogenesis might be to transduce endothelial or mesenchymal cells with Hif-1α/VP16 and co-implant them with wild-type endothelial cells into artificial collagen gels. The basic strategy of co-implantation of transduced cells and wild-type cells has proved fruitful previously in supporting angiogenesis.(47) Whatever proteins from Hif-1α/VP16 have been responsible for previous successful efforts to promote angiogenesis could then act on wild-type HUVEC through paracrine affects without interfering with HUVEC replication directly. Such paracrine affects could be responsible for the *in vivo* success of previous Hif-1α/VP16 angiogenesis experiments.(40)

Assessment of the paracrine effects of HIF-1α expression in parenchymal cells on glucose metabolism and on the expression of adhesion complexes:

Germane to the above discussion, the Giordano laboratory has explored the possibility of paracrine effects of hypoxia-inducible gene upregulation and has confirmed their presence on both glucose metabolism and adhesion-molecule expression. In one series of experiments, the laboratory tested the effects of conditioned media from Hif-1α-transduced endothelial cells on glucose uptake in myoblasts. As shown below (figure 4), the conditioned media taken from plates of Hif-1α-transduced endothelial cells, when added to myoblasts in cell-culture, significantly increased glucose uptake in the myoblasts in a paracrine manner, establishing that there is indeed cross-talk from the endothelium to parenchymal cells that can effect metabolism.(53)

Figure 4:

Glucose Utilization by Cultured Myoblasts After Exposure to Conditioned Media
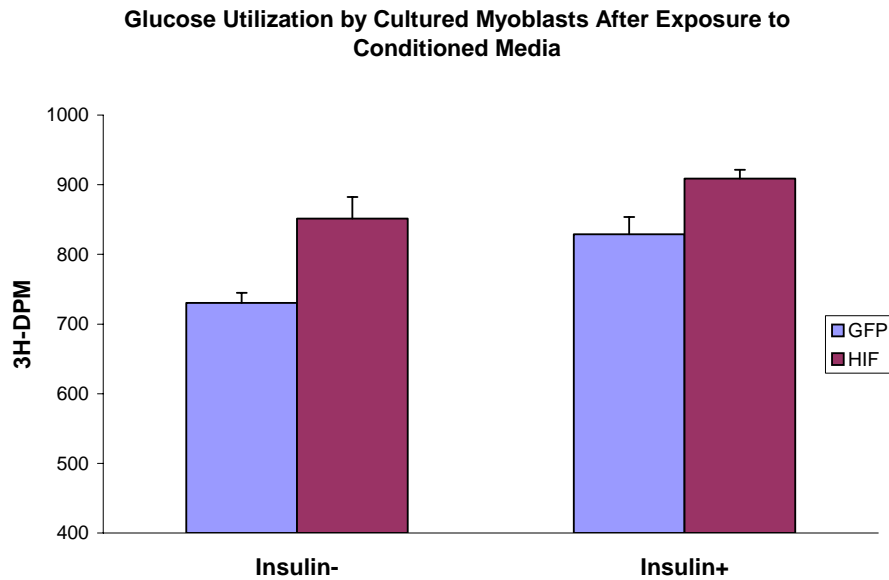
p<0.05 for GFP vs Hif difference

In other experiments, conditioned media from HIF-1α-transduced myoblasts was incubated with HUVEC monolayers. By Fluorescence Activated Cell Sorting analysis (not shown) there was a significant increase in the expression of ICAM-1, VCAM, and MadCAM in response to the conditioned media. By focused microarray analysis there were several endothelial adhesion molecule genes upregulated in response to the conditioned media. The table below (figure 5) is an abridged list of these genes. The designation on the left is the gene identity, and the number on the right is the fold altered relative to controls. Positive numbers indicate upregulation, whereas negative numbers indicate downregulation. For example, ICAM-1 is upregulated 1.65 x control; integrin alpha 1 (ITGA1) is upregulated 2.96 x over control.(53)

Figure 5:

HUVEC Gene Expression After Exposure to Conditioned Media

Gene	Fold Altered
ICAM1	0.649332
ITGA1	1.963328
ITGA10	-0.24591
ITGA11	0.316086
ITGA2	0.299741
ITGA2B	0.565757
ITGA3	0.444684
ITGA4	0.451907
ITGA5	0.745917
ITGA6	0.649002
ITGA7	1.223483
ITGA8	0.258984

In addition to its implications for gene therapy, the above have significant

implications for the understanding of endothelial cell biology in general. The

endothelium, far from being simply a passive layer of boundary cells, can have

critical effects on whole-organ and whole-organism metabolism via paracrine effects

as well as via altered molecular transport.

The critical effects of Hif-1α alteration on whole-organ and whole-organism

biology:

To further elaborate on the role of endothelial Hif-1α alteration on whole

organ and organism biology, a mouse model was developed with cre-lox technology

to obtain an endothelium-specific Hif-1α knockout. Tie-2 Cre mice were crossed

with mice in which Exon 3 of the Hif-1α gene was flanked by loxP sites. This cross

resulted in viable litters of mice in which Hif-1α was deleted specifically in the

endothelium (Complete Hif-1α-knockout mice are not viable). The efficiency of the

endothelial-specific knockout deletion, as determined by quantitative PCR, was >90%

in each isolated batch of endothelial cells evaluated by the Giordano lab. Hif-1α was

not deleted discernibly in other cell types tested, including cardiac muscle.(53)

Basal glucose levels in these mice were measured with a strip-based

commercial glucose monitoring system used by diabetics (figure 6).

Figure 6:

Basal Glucose in Hif EC KO Mice vs. Controls

p<0.05
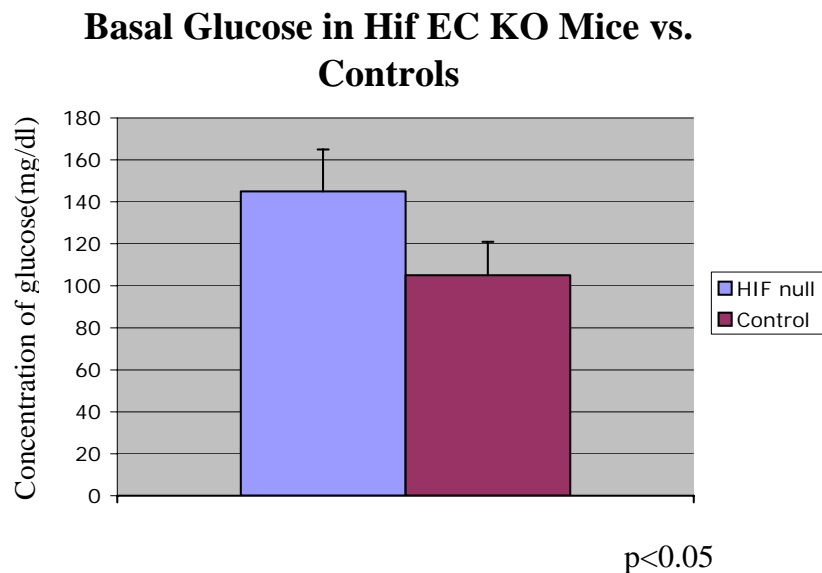
To determine glucose uptake in vivo, tritiated 2-D glucose was injected by

jugular vein into wild-type as well as endothelium-specific Hif-1α-knockout mice.

Following injection, the heart (figure 7) and brain (figure 8) were harvested and

assessed for uptake of the labeled glucose.(53)

Figure 7:

Heart Glucose Uptake

$p < 0.05$

Figure 8:

Brain Glucose Uptake
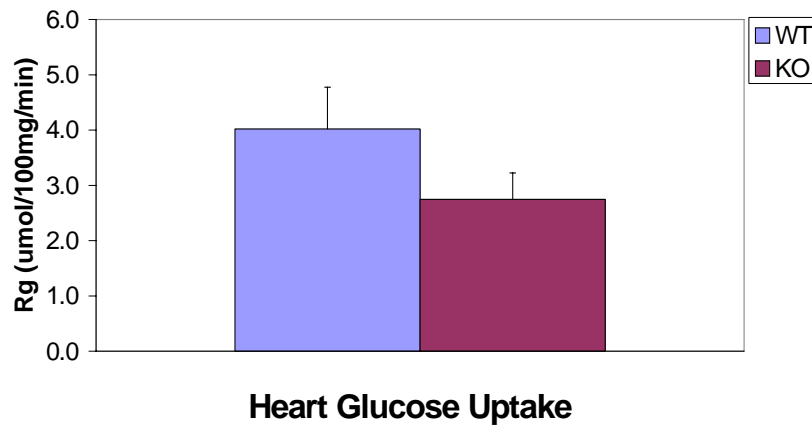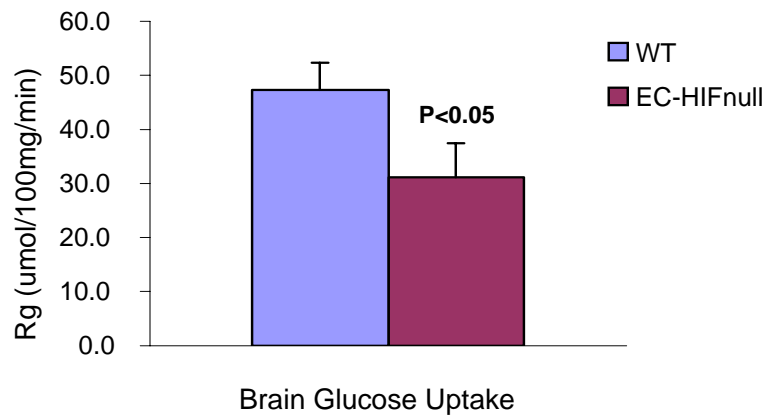
$p < 0.01$

The above data on the local intra-cellular, regional inter-cellular, and organismal effects of Hif-1α manipulation within endothelial cells point to the potential relevance of gene therapeutic manipulation of Hif-1α-responsive genes. Such manipulation may prove therapeutic not only when specifically diseased cells

are targeted for treatment. On the contrary, because of the wide-ranging effects that Hif-1α manipulation has, broader regions or systems might prove to cause significant alterations to the biology of a distant locus of pathology in response to such manipulation.

Such a strategy is not entirely novel, as downregulation of angiogenesis for the control of tumor growth is but another manifestation of this idea. That is, rather than targeting the specific cancerous cells for gene therapeutic manipulation, one can target the milieu in which those cells live – the surrounding blood vessels in the case of a tumor – and effect a cure at the adjacent pathological locus.

Conclusion:

Returning to the effort to engineer artificial vasculature, which was the original goal of this project, while the initial transduction trials described herein were not successful at inducing angiogenesis, this work suggests that there are possible uses of Hif-1α/VP16 and Hif-1α for alteration of endothelial cell behavior, as well as that of surrounding tissues. By altering the cell-cycle and apoptosis-associated genes, changing expression of angiogenic factors, modifying energy metabolism, and affecting adhesion-molecule expression, both intracellularly and in a paracrine manner, hypoxia-inducible gene alteration may have a significant and potentially useful role in the effort to create artificial vasculature.

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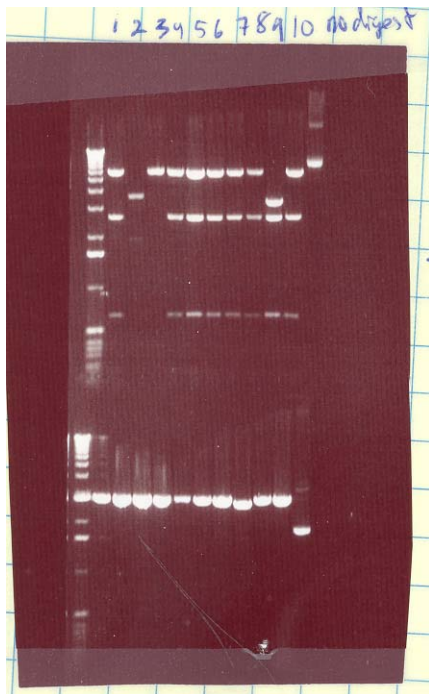
Appendix:

Fig.1: BglII/SalI restriction digest
of Hif-1α/pLNCX2 ligation. Lanes 1, 4-8 and 10 contain the expected bands.
Lane 0 is a standard1Kbp ladder.

Fig.2: AvaI restriction digest of
Hif-1α/VP16/pLNCX2 ligation. Lanes 1, 3, 4, and 6 contain the expected bands.
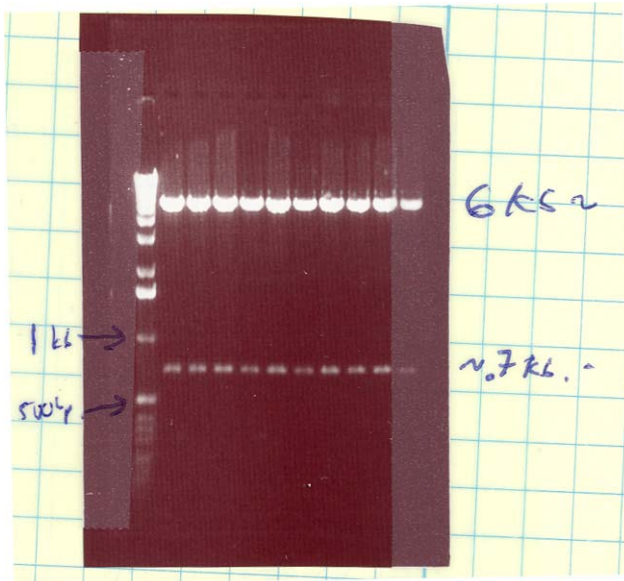
Lane 0 is a standard 1Kbp ladder.

Fig. 3: HindIII/NotI restriction digest of dsRed/pLNCX2 ligation.
All ten lanes show the expected 6.1Kbp and 700bp fragment sizes.