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Differential but complementary HIF1α and HIF2α transcriptional regulation

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Abstract

Effective vascular regeneration could provide therapeutic benefit for multiple pathologies, especially in chronic peripheral artery disease (PAD) and myocardial ischaemia. The hypoxia inducible factors (HIFs) mediate the cellular transcriptional response to hypoxia and regulate multiple processes that are required for angiogenesis to ultimately restore perfusion and oxygen supply. In endothelial cells, both HIF1α and HIF2α are known to contribute to this role, however the extent and individual roles of each of these HIFα remains unclear. To characterise the individual roles of HIFα, we sequenced the transcriptional outputs of stabilised forms of HIF1α and HIF2α, where they regulated 701 and 1454 genes, respectively. HIF1α transcription primarily regulated metabolic reprogramming, whereas HIF2α exerted a larger role in regulating angiogenic extracellular signalling, guidance cues and extracellular matrix remodelling factors. Furthermore, HIF2α almost exclusively regulated a large and diverse subset of transcription factors and coregulators that contribute to its diverse roles in hypoxia. Further understanding of how HIFs regulate cellular processes in hypoxia and angiogenesis could offer new avenues to modulate physiological angiogenesis to enhance revascularisation in ischaemic conditions and other pathologies.
Introduction

Atherosclerotic stenosis and other arteriopathies can result in chronic vascular insufficiencies, which manifest as coronary, cerebrovascular or peripheral vascular disease. In addition to being strongly associated with elevated risk for fatal cardiovascular events, peripheral vascular disease can cause chronic and debilitating complications such as critical limb ischaemia, resulting in claudication, ulcers, coagulative necrosis and distal limb loss. Reduced perfusion in these tissues impedes regeneration through oxygen and nutrient depletion. Tissue hypoxia results in the activation of the hypoxia inducible factors (HIFs) that mediate adaptive cellular processes and the secretion of angiogenic growth factors and cytokines to stimulate revascularisation and restoration of functionality. Loss-of-function studies have shown that HIF1α is central to the recovery of tissue perfusion, maintenance of tissue viability and expression of angiogenic cytokines in models of hind limb ischaemia and wound healing.

The HIFs are heterodimeric transcription factors that comprise of an oxygen labile and regulated α-subunits (HIFα), encoded by three genes HIF1A, EPAS1 (HIF2α) and HIF3A, and a stably expressed β-subunit, ARNT (HIF1β). Both HIF subunits, and their respective isoforms retain a conserved domain structure and are members of the PER-ARNT-SIM (PAS) subfamily of basic helix-loop-helix (bHLH) proteins. To enable a rapid response to hypoxic insults without the need for de novo protein synthesis, HIFα are continuously transcribed and translated but rapidly degraded through oxygen dependent post-translational hydroxylation, ubiquitination and subsequent degradation. In conditions of low oxygen tension, this process is inhibited and HIFα are stabilised enabling them to heterodimerise and translocate to the nucleus in order to undertake their transcriptional program. Beyond oxygen-dependent hydroxylation, HIFα are extensively post-translationally regulated by various modification at different residues altering its stability and activity. HIF1α and HIF2α are considered to be the major HIFα isoforms that mediate the positive HIF transcriptional program. Both isoforms exhibit a highly conserved domain structure and functional similarities, made evident by their binding to the same hypoxia responsive element (HRE) characterised by a conserved RCGTG DNA motif.

Despite these generalised regulatory, structural and functional similarities, it is becoming increasingly evident that HIF1α and HIF2α exhibit significant differences in their
transcriptional regulation in response to hypoxia and disease states. This has most strikingly been observed in several forms of cancer, such as renal cell carcinoma, where the regulation of HIF1α and HIF2α is perturbed resulting in HIFα specific activities which are frequently inconsistent, and occasionally contradictory between different cancer types. Despite utilising the same HRE and sharing canonical hypoxia inducible target genes, HIF1α and HIF2α exhibit diverse biological activities and occupy roles outside of hypoxia and metabolic reprogramming. The mechanism accounting for target gene divergency remains incompletely understood but is known to be largely independent of DNA binding, rather differences in the C-terminus of HIFs enables interaction with alternative transcription factors and coregulators. Furthermore, HIFs are themselves regulated in a non-equivalent manner where HIF2α is typically stabilised at higher relative O₂ concentrations compared with HIF1α. These phenotypic differences and variation in HIF1α and HIF2α activity suggest that they exert non-equivalent regulation on divergent target genes in a context dependent manner.

In an attempt to delineate the HIFs transcriptional outputs, we expressed stabilised versions of HIF1α and HIF2α in primary human endothelial cells (ECs), a cell type central to angiogenesis and revascularisation. Quantitating the resulting transcriptomes using RNA-seq, we sought to identify the biological processes preferentially associated with either of the HIFα isoforms. HIF1α was seen to specifically regulate energy metabolism compared with HIF2α’s relativity larger role in extracellular signalling, transcription and extracellular remodelling. These results suggest that in EC HIF1α is associated with acute cellular metabolic adaptations to rapid changes in O₂ concentrations, whereas HIF2α mediates a wider role in tissue revascularisation and regenerative processes.
Results

Transcriptional profiles of HIF1α and HIF2α

In order to further dissect the collective and individual roles of HIF1α and HIF2α on gene expression, primary human ECs were transduced with adenovirus vectors expressing either a stabilised form of HIF1α, HIF2α or an empty CMV early-intermediate promoter construct (eCMV) as a control. Mutations of the proline residues within the HIF oxygen-dependent degradation domain enabled HIF1α and HIF2α to be expressed in normoxic conditions as confirmed by western blotting (fig. S1) and qPCR for HIF targets (fig. S2). Cells from 3 biological replicates were harvested 48h post-infection and their RNA was isolated, depleted of ribosomal RNA and used to construct directional cDNA libraries that were sequenced on a NextSeq 550 using 75bp single-end reads. Each replicate yielded an average of 24 million reads that were aligned using STAR\(^\text{11}\) for differential gene expression analysis in edgeR.\(^\text{12}\) After filtering out transcripts with low counts across all libraries, up to 27,648 transcripts from a total of 48,118 were identified as being expressed. Multi-dimentional scaling of the normalised log\(_2\) counts per million (CPM) for eCMV, HIF1α and HIF2α showed strong association between replicates and the largest variation between HIF2α and eCMV in dimension 1, followed by HIF1α and eCMV in dimension 2, with both accounting for 84% of the overall variance (fig. S3).

HIF1α and HIF2α overexpression resulted in 1485 and 3067 differentially expressed transcripts representing 701 and 1454 genes respectively, using a fold change (FC) of 2 and FDR < 0.01 (Table S1). Of the differentially expressed genes (DEGs), for both HIF1α and HIF2α the majority, 85.5% and 73.6%, respectively were up-regulated as seen in figure 1 and 2, in agreement with previous reported findings.\(^\text{13}\) Hierarchical clustering of the variance of log\(_2\) FC revealed 4 primary gene clusters (HIF1α upregulated, HIF2α upregulated, upregulated by both and downregulated by both) which exhibited distinct forms of regulation under HIF1α and HIF2α (fig. 2; clusters). HIF1α and HIF2α exhibited either parallel or non-overlapping regulation of their target genes; only 9 genes were inversely regulated between the HIF isoforms suggesting that their forms of regulation are not antagonistic.

When comparing the genes regulated by HIF1α and HIF2α, less than 20% were regulated by both HIF isoforms, however these overlapping genes constitute 48% of the
HIF1α regulated genes, as shown in figure 3. Comparing the regulated RNA classes, HIF1α and HIF2α exerted a significantly different proportion of non-coding to coding RNA species (7.67% and 12.7% respectively, p-value < 0.01, Chi-Square). The proportion of up and downregulated genes between HIFs remained approximately equivalent in both RNA classes (data not shown).

Ontology and major functional categories of HIF regulated genes

In order to comprehend the complex biological functions, processes and phenotypes that occur downstream of HIF transcriptional regulation, we sought to cluster the DEGs based on semantic similarities, expression patterns and interaction networks which could reveal system level differences in how HIF1α and HIF2α regulate their target genes. In order to identify predominant biological themes associated with the regulated genes of HIF1α and HIF2α, overrepresented Gene Ontology (GO) terms for each set of HIF DEGs was analysed using the clusterProfiler package. Redundant GO terms were removed and the most informative GO terms were selected using minimum FDR and Rel similarity. The top enriched biological processes unique and shared between HIF1α and HIF2α are shown in figure 4. Overlap between top enriched biological processes (FDR < 0.05) was limited to response to hypoxia. HIF1α specific enriched processes centred on glycolysis, NADH regeneration and other metabolic processes. In contrast, HIF2α enriched processes included angiogenesis, endothelial cell migration and extracellular matrix reorganisation.

To further identify biological processes positively or negatively regulated by HIF1α and HIF2α, we performed GO analysis on the genes categorised by the unsupervised gene clustering determined previously in figure 2. Consistent with the terms previously identified, the HIF1α upregulated cluster 1 was associated with glycolysis and NADH regeneration whereas the HIF2α upregulated cluster 3 and 4 was associated with endothelial cell migration, pattern specification process and activation of GTPase signalling. Processes negatively regulated by both HIF1α and HIF2α in cluster 2 included responses to reactive oxygen species, and negative regulation of immune system processes. Furthermore, CAMERA was used to identify inter-gene correlation enrichment against the MSigDB (Broad Institute) hallmark collection to represent well defined and non-redundant biological processes. As anticipated, hypoxia was the highest enriched hallmark for both HIF1α and HIF2α but interestingly, both showed decreased activation for c-Myc regulated genes (fig. S4a). In addition to the general negative trend, HIF2α overexpression in particular resulted in
the significant downregulation of 15 c-Myc regulated genes (fig. S4b). Whilst HIF2α overexpression only resulted in a modest downregulation of c-Myc expression (FC -0.7, FDR < 7e-04), both HIF1α and HIF2α induced robust expression of the c-Myc suppressor MXII.

Since several known HIF target genes mediate their effects through a variety of signalling pathways, DEGs were analysed using Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City) to identify activation or repression of cellular signalling and metabolic processes downstream of HIF1α and HIF2α. 13 and 17 canonical pathways with an activation Z-score were seen to be enriched (p-value < 0.05) for HIF1α and HIF2α, respectively as shown in figure 5. The majority of the enriched pathways were seen to be activated with only 4 pathways between HIF1α and HIF2α had an activation z-score < 0. Consistently with the GO analysis, there was minimal overlap between the significantly enriched pathways where only 4 were shared by HIF1α and HIF2α.

Differentially regulated HIF target genes and processes

Energy metabolism

HIF1α DEGs were most significantly related to anaerobic metabolic reprogramming (fig. 6a) where HIF1α regulated almost 80% of the glycolytic related genes expressed in EC (GO:0061621), including the regulatory rate limiting enzyme PFK. Both HIFs upregulated several glucose transporters also listed in figure 6a. HIF1α further regulated the switch to glycolysis by upregulating pyruvate dehydrogenase kinase (PDK) which redirects pyruvate metabolism away from the TCA cycle to instead be anaerobically reduced to lactate for NAD+ regeneration, a process catalysed by the HIF1α-induced lactate dehydrogenase A (LDHA). Furthermore, coordinate HIF1α regulation of the lactate and proton symporter SLC16A3 and carbonic anhydrase 9 (CA9) prevents acidification from intracellular accumulation of lactate. Hydroxyacylglutathione hydrolase (HAGH), encoding an enzyme used to remove the cytotoxic glycolysis by-product methylglyoxal, was also seen to be induced under HIF1α overexpression.

HIF1α also regulates the autophagy related genes BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 like (BNIP3L) which have also been shown to increase mitochondrial autophagy in order to reduce mitochondrial mass and as an adaptive survival mechanism to maintain ATP levels. Conversely, HIF2α induced the expression of PPARα and PPARγ and
other genes related to fatty acid and lipoprotein uptake including the very low density lipoprotein receptor (VLDLR), LDL receptor related protein 2 (LRP2), lipase A (LIPA), phospholipid transfer protein (PLTP), perilipin 2 (PLIN2) and the fatty acid transporter SLC27A3.

Extracellular signalling and guidance cues

The HIFs primary means of regulating angiogenesis is through the induction of multiple extracellular signalling ligands and receptors with diverse functional activities on multiple vascular, mural and immune cell types. HIF1α and HIF2α regulated multiple growth factors of the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), Wnt and the transforming growth factor (TGF) families (fig. 6b). VEGF-A, the principle pro-angiogenic growth factor, was ranked as one of the highest induced HIF2α target genes (fig. 1b) but was also regulated by HIF1α to a lesser degree. The other VEGF members were exclusively regulated such that VEGFC was a HIF2α specific target whilst VEGFB was regulated by HIF1α. Angiopoietin-like 4 (ANGPTL4) ranked as the highest upregulated growth factor genes for both HIF1α and HIF2α. HIF2α regulated several other signalling cytokines related to angiogenesis including interleukin 33 (IL33), C-X-C motif chemokine ligand 8 (CXCL8), C-C motif chemokine ligand 28 (CCL28), and bone morphogenetic protein 4 (BMP4). Furthermore, HIF2α was also seen to regulate several corresponding receptors including receptor tyrosine kinases VEGF receptor 1 (FLT1), VEGF receptor 2 (KDR), FGF receptor 3 (FGFR3), insulin receptor (INSR), and the cytokine receptors C-X-C chemokine receptor type 4 (CXCR4), leptin receptor (LEPR) and tumour necrosis factor receptor superfamily member 10B (TNFRSF10B). Multiple genes encoding vasodilator peptide hormones and receptors including adrenomedullin (ADM), natriuretic peptide receptor (NPR1/3), natriuretic peptide B (NPPB), vasoactive intestinal peptide (VIP) and prostaglandin synthases (PTGS1, PTGIS) were regulated by HIF1α and HIF2α. HIF2α, unlike HIF1α, regulated juxtacrine signalling pathways including the Notch related genes Delta-like 4 (Dll4), hairy and enhancer of split-1 (HES1), Deltex E3 Ubiquitin Ligase 4 (DTX4) and Lunatic Fringe Homolog (LFNG) as shown in figure 6c. Other guidance pathways regulated by HIF2α included the Sonic Hedgehog pathway related genes Patched 1 (PTCH) and Smoothened, Frizzled Class Receptor (SMO), in addition to axon guidance related signalling molecules and receptors (class 3 and 4 semaphorins, ephrins and plexins) also shown in
figure 6c. Overall, both HIF1α and HIF2α regulated a variety of extracellular signalling processes but HIF2α appeared to regulate a larger and more diverse subset of target genes.

Transcription

Analysis of the molecular function GO terms associated with HIF1α and HIF2α showed the largest difference was for multiple transcription factor activity associated terms, where HIF2α regulated 66 genes classified under the term GO:0000982 relative to 20 regulated by HIF1α, several of which are shown in figure 6d. Both HIF1α and HIF2α regulated multiple immediate-early response genes such as AP-1 subunits (FOS, JUNB, ATF3), ETS and MAPK7, which can act as downstream terminal effector nodes for extracellular signalling pathways. Interestingly, HIF2α virtually exclusively upregulated a significant fraction (n=24) of the class I HOX genes from the HOXA, HOXB and HOXD clusters. Furthermore, HIF2α regulated another 36 homeobox related genes from various subclasses including PRD, NKL, LIM, POU, SINE, TALE, CUT and ZF. Forty one of these genes were associated with the pattern specification GO term (GO:0007389) which represents the highest enriched HIF2α biological process (fig. 4). Other regulated transcription factor families include GATA, KLF, NFATC and FOX. In addition to transcription factors, HIF1α and HIF2α both modestly regulated various histone modifiers involved in chromatin remodelling and epigenetic regulation (fig. 6e). HIF1α and HIF2α regulated a number of repressive histone deacetylases (HDACs) known to act on H3K27ac of promoters and enhancers. Alternatively, they both regulated several Jumonji domain containing lysine-specific demethylases (KDMs) responsible for demethylating multiple histones including H3K9, H3K27 and H3K36 which require O2 as a cofactor. HIF1α upregulated the histone acetyltransferase KAT6B, whereas HIF2α upregulated the classical HIF histone acetyltransferase cofactors EP300 and CREB binding protein (CREBBP) which mediates activating H3K27 acetylation.

Extracellular remodelling

HIF2α, and to a lesser degree HIF1α, appear to regulate extracellular matrix (ECM) remodelling through induction of the basement membrane components laminin and collagen IV, in addition to enzymes required for collagen post-translational modification and fibril assembly (fig. 6f). HIF1α and HIF2α both induce gene expression of enzymes responsible for mediating the hydroxylation of the procollagen proline residues by the
collagen prolyl 4-hydroxylases $P4HA1$ and $P4HA2$, along with procollagen lysine residues by the lysyl hydroxylases, procollagen-lysine,2-oxoglutarate 5-dioxygenase ($PLOD$) 1 and 2. Furthermore, the lysyl oxidases $LOX$ and $LOXL2$ genes that mediate the lysine derived inter-polypeptide chain crosslinks required for the stabilisation of collagen fibrils, are also HIF regulated. Likewise, both HIFs regulated a number of matricellular proteins associated with wound healing and tissue repair including fibulin 2 and 7 (FBLN), peristin (POSTN), versican (VCAN) and tenascin X (TNXB) that contribute to vessel formation and vessel wall integrity. Additionally, Claudin 3 (CLDN3), component of endothelial tight junctions as part of the paracellular barrier between endothelial cells, was highly induced by both HIF1α and HIF2α. Conversely, the anti-angiogenic matricellular protein thrombospondin 1 (THBS1) was downregulated by HIF2α.

Interaction networks of HIF1α and HIF2α target genes

To identify functional interactions between HIF DEGs, we used Cytoscape with the Reactome-FI plug-in to create modules of known functional complexes. In figure 7a, HIF1α DEGs formed 3 modules where HIF1α was seen to act as a central hub, forming 21 interactions with its regulated genes with the majority associated with glycolysis ($N = 9$, $FDR \leq 4e-15$). In figure 7b, HIF2α DEGs formed 4 modules, the largest consisting of 69 connected nodes with transcription activity as the most overrepresented function ($FDR \leq 1.7e-4$). The transcription factors GATA2 and FOS formed the central hubs with 17 and 13 first neighbour nodes, collectively. Pathway analysis of the GATA2 and FOS hubs were associated with the AP-1 transcription factor network (GATA2 $FDR < 0.01$, FOS $FDR < 3e-5$) and NFAT transcription (GATA2 $FDR < 0.01$, FOS $FDR < 6e-5$). A HIF2α DEG module with PPARα as a hub was associated to the already identified fatty acid uptake related proteins SLC27A1, PLTP, and LIPA.

HRE motif occurrence in promoters of target genes

To further explore the role of HIF transcriptional regulation, the enrichment of the consensus HRE motif and other motifs was analysed in regions proximal to HIF1α and HIF2α target gene transcriptional start sites (TSS). Transcription factor motifs enriched in HIF1α and HIF2α target gene TSSs is shown in figure 8a and 8b, respectively. As anticipated, the HRE motif was identified as the most significantly enriched motif present in 34.42% of HIF1α target genes ($p$-value $< 1e-27$), unexpectedly however, the HRE motif
wasn’t significantly enriched in HIF2α DEG TSSs over the GC% matched background. Using public ChIP-seq data for H3K4me2 H3K27ac with co-occupancy as a mark of regulatory regions, we performed motif enrichment analysis for those intergenic regions occurring within 100kb of HIF DEGs. Motif analysis revealed a similar trend in that the HRE was only significantly identified in HIF1α DEG regulatory regions, with the top motifs shown in figure S5. These findings suggest that HIF2α may either regulate a significant subset of its DEGs either directly through mechanisms independent of its DNA-binding or through indirect regulation of other transcription factors.
Discussion

Critical limb ischaemia resulting from advanced PAD requires extensive intervention to maximise limb salvage, vessel patency and wound healing. The capacity for physiological angiogenesis is frequently impaired in patients with PAD due to the presence of multiple comorbidities, such as aging and diabetes mellitus which contribute to endothelial dysfunction\textsuperscript{24,25} and reduced HIF1\(\alpha\) activity.\textsuperscript{1,2} Current revascularisation strategies to preserve limb viability include surgical vascular bypass and endovascular angioplasty, with or without stenting.\textsuperscript{26} Nevertheless, surgical revascularisation carries periprocedural complications and remains of limited use to patients exhibiting diffuse and distal forms of the disease that is frequently associated with diabetes mellitus.\textsuperscript{27}

Therapeutic vascular regeneration through the use of minimally invasive gene or cell based therapies is anticipated to circumvent the disease constraints placed on endogenous angiogenesis and to stimulate robust collateral growth as to alleviate ischaemia and restore limb functionality. Angiogenesis however, represents a complex process that consists of multiple spatially and temporally regulated processes such as migration, sprout/tube formation, proliferation, vascular and stromal remodelling. As the master systemic and cellular transcriptional regulators of the hypoxia response, the HIFs remain an attractive, if complex and incompletely understood candidate for vascular regeneration. Whilst several studies have attributed a critical role to HIF1\(\alpha\) in angiogenesis\textsuperscript{28}, the full extent of its transcriptional activity in comparison with HIF2\(\alpha\) has yet to be defined.

Our present findings indicate that in ECs, HIF2\(\alpha\) transcriptionally regulates a larger and more functionally diverse set of target genes compared with those of HIF1\(\alpha\). These findings contrast results from studies in other cell types where HIF1\(\alpha\) was the predominantly active isoform and HIF2\(\alpha\) was seen to be largely redundant.\textsuperscript{29,30} We hypothesise that these differences could result from the more limited specific expression profile of HIF2\(\alpha\)\textsuperscript{31} or a prerequisite for cell-specific transcriptional cofactors required for productive HIF2\(\alpha\) transcriptional elongation.\textsuperscript{8} Not only did HIF1\(\alpha\) and HIF2\(\alpha\) exhibit substantially different degrees of gene regulation, but there was also limited overlap in their target genes which was surprising given their close structural, functional and regulatory similarities.

HIF1\(\alpha\) was seen to primarily regulate genes affecting metabolic reprogramming, suggesting it mediates a shift in energy production away from O\(_2\) dependent
oxidative phosphorylation to anoxic glycolysis. As part of a comprehensive metabolic transcriptional program, HIF1α concurrently regulated genes associated with NADH redox balance, lactate efflux, methylglyoxal detoxification and cellular autophagy, likely to enable ECs to adapt and survive in an acute, low O₂ environment. Conversely, the predominantly HIF2α regulated fatty acid and lipoprotein uptake may appear counterintuitive, but may represent a mechanism to acquire energy reserves for proliferation, membrane biosynthesis and lipid signalling.

HIF2α, and to a lesser degree HIF1α, transcriptionally regulate biological processes involved in angiogenesis through the regulation of numerous extracellular signalling ligands and receptors, comprised of growth factors, cytokines, vasoactive compounds and guidance cues. The growth factors VEGFs, FGFs and TGFβ have well characterised roles in vascular biology where they can stimulate EC proliferation, sprouting, migration and survival. Within angiogenesis, vascular patterning is critical for optimal vascular network density requiring precisely regulated spatiotemporal signalling between EC and their environment. This process is primarily controlled through chemoattractant gradients, EC tip/stalk phenotype selection and other attraction and repulsion cues. Migratory sprout “tip” ECs closest to the chemoattractant gradient express high levels of the Notch ligand Dll4 which is regulated by MEF2 and HIF2α. Subsequent juxtacrine signalling between tip cell Dll4 and the trailing stalk cell Notch receptors laterally suppresses the migratory tip cell phenotype through reducing Dll4 and KDR expression to prevent excessive branching. In addition to Notch signalling, HIF2α also appeared to specifically induce a number of axonal guidance associated molecules including semaphorins, ephrins, plexins and their receptors, which are known to act as attraction or repulsion cues in vascular morphology and branching but also arterial/venous vascular patterning.

This additional layer of regulation of angiogenesis through Notch signalling and vascular guidance cues may enhance the formation of organised, well perfused and functional vasculature. A similar observation has been reported in conditional EC knockout mice where EPAS1 deletion resulted in poor perfusion caused by disordered vasculature in a Dll4 dependent manner. In addition to effects on EC, HIF2α also contributes to vessel normalisation by inducing the expression of multiple collagens, collagen modifying enzymes and other secreted ECM proteins. Collagen IV, peristin, fibulin, and laminins are used to synthesise the basement membrane of the immature vascular structure with parallel
recruitment of pericytes. Since oedema and poor perfusion caused by leaky, immature and irregular collateral vessels is a major clinical limitation when using gene transfer with single growth factor like VEGFA\textsuperscript{39,40}, HIF2α may alternatively support normalised collateral growth due to its capacity to regulate multiple balanced pro-angiogenic pathways.

Strikingly, the largest difference between HIF1α and HIF2α transcriptional profiles was the degree of regulation of other transcription factors. HIF2α overexpression resulted in over 100 genes characterised as transcription factors or transcriptional regulators being induced. Motif analysis of HIF2α DEGs promoters or distal regulatory regions failed to identify enrichment for the canonical HRE, suggesting that HIF2α regulation is either indirectly mediated through other induced transcription factors or through independent DNA-binding mechanisms. HIF2α has been show to preferentially bind to intergenic regions rather than proximal promoters relative to HIF1α.\textsuperscript{6} Several HIF2α regulated transcription factors have well defined roles in EC angiogenic processes such as GATA2 and FOS, both of which are known to interact with a network of other HIF2α DEG including NFATC1/2 and TFAP2A. The role of other transcription factors, such as the large magnitude of Hox and non-Hox homeobox genes, remains less well characterised in the context of HIF activation. Various individual Hox genes are known to be differentially expressed in conditions of vascular quiescence, sprouting angiogenesis and vessel normalisation in addition to wound healing.\textsuperscript{41} However, how HIF2α induced Hox cluster expression and subsequent spatiotemporal gene regulation would influence revascularisation is currently poorly defined. It would be interesting to delineate the role of HIF induced Hox activity in the spatiotemporal regulation of gene expression in development and disease. Induction of various transcriptional cofactors and histone modifiers likely represents an additional layer of intricate transcriptional regulation.

It is becoming increasingly evident that HIF1α and HIF2α fulfil non-overlapping niche roles in regulating the cellular and systemic hypoxia response and their related biological processes. Further understanding of their activities and downstream effects will hopefully enable appropriate clinical use of either coordinate or individual HIFs in vascular regeneration.
Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from cords obtained from the Kuopio University Hospital with approval from the Kuopio University Hospital Ethics Committee. HUVECs were maintained in endothelial growth medium (EGM; basal medium with CC-4133 SingleQuots: 2% FBS, 0.1% hEGF, 0.1% Hydrocortisone, 0.4% bovine brain extract, 0.1% Gentamicin-Amphotericin B; Lonza) on T-75 cell culture flasks coated with 10g/ml fibronectin and 0.05% gelatin (Sigma, St. Louis, MO) and maintained in humidified 5% CO₂.

Adenovirus transductions

Sub-confluent HUVECs (70%) at passage 5 were infected 24h post-seeding with E1/E3 deleted replication incompetent adenovirus serotype 5 overexpressing a stabilised HIF1α (P402→A, P563→A), HIF-2α (P405→A, P530→A) or an empty construct containing the CMV minimal promoter (eCMV) as a control. Prior to transduction, the media was exchanged for endothelial basal media without supplements, diluted adenovirus at a MOI of 20 was added and incubated for 4 hours. Subsequently, media was exchanged for complete EGM with supplements. Cells were collected for RNA and protein isolation 48 hours post infection.

Protein extraction and western blotting

Cells were lysed using RIPA buffer (50mM Tris-Cl, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Roche)) and the total protein content was determined with the BCA protein assay (Pierce, Thermo Fisher Scientific). 9µg of protein was separated on Mini-PROTEAN TGX Stain-Free gels (BioRad) and transferred to a 0.2µm nitrocellulose membrane (BioRad). The membrane was blocked with TBST containing 5% nonfat dry milk overnight at 4 °C and incubated with primary antibodies diluted 1:1000 in TBST containing 5% BSA for 1 hour at room temperature. Antibodies used were: anti-HIF1α (Thermo Fisher Scientific, MA1-16504), anti-HIF2α (Thermo Fisher Scientific, MA1-16519), anti-PECAM1 (DAKO, M0823). HRP-conjugated goat anti-IgG (Thermo Fisher Scientific, 32430) and was diluted 1:5000 with TBST.
containing 5% nonfat dry milk and the membranes incubated for 1 hour at room temperature before ECL detection (ChemiDox XRS).

Library preparation, sequencing and qPCR

Total RNA from cells was isolated using RNeasy Kit (Qiagen) followed by DNase treatment using the Turbo DNase kit (Ambion). 250ng RNA was reverse transcribed using SuperScript IV First-Strand Synthesis System (ThermoFisher Scientific). qPCR was performed using a StepOne Plus (Applied Biosystems) with TaqMan universal master mix (Applied Biosystems) to confirm induction of HIF target genes using TaqMan assays (Applied Biosystems) for VEGFA (Hs00903129) and GAPDH (Hs99999905_m1) with RPLP0 (Hs00420895_gH) as an endogenous control. Gene expression was estimated using the $2^{-\Delta\Delta CT}$ method. RNA-seq libraries were prepared from 250ng of total RNA using the TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold (Illumina). Sequencing was performed with the Nextseq 550 using 75 cycles in single end high output mode.

Data analysis

RNA-Seq was mapped using STAR\textsuperscript{11} allowing up to two mismatches and reporting only one alignment for each read. Poor quality reads were filtered out (minimum 97% of bp over quality cutoff 10) and tag per base value was set to 3. Transcripts with low counts of CPM < 1 not present in at least 2 libraries above this threshold were considered not expressed and removed from the analysis. Differential gene expression was estimated using edgeR\textsuperscript{12}, using TMM library size normalisation and quasi-likelihood F-testing. Transcripts with a log\textsubscript{2} FC of $\geq 1$ or $\leq -1$ and $FDR < 0.01$ were considered differentially expressed. Plots were produced and visualised in R using ggplot2. MDS was performed using cmdscale. Heatmaps were produced using Heatmap.2 and Pheatmap. Hierarchical clustering was performed using Ward’s least absolute error with Manhattan distance\textsuperscript{43}. Gene ontology was performed using clusterProfile\textsuperscript{14} with $FDR < 0.05$ and the most informative GO terms were selected with ‘Simplify’ using minimum $FDR$ with the Rel method. Interaction analysis of DEGs was performed using the Cytoscape software with the Reactome functional interaction (FI) plugin\textsuperscript{44}. Pathway analysis of the DEGs was performed using Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City). Promoter and proximal regulatory element motif analysis of differentially regulated genes was performed using the ‘findmotifs.pl’ command in HOMER using +/- 750bp region of the TSS with a motif length of 8, 10 and 12 bases. A random set of
genomic positions with matched GC% composition was used as background for enrichment. Co-bound HUVEC H3K4me2 and H3K27ac intergenic regions were determined using annotation from the ‘annotatePeaks.pl’ command. Motif analysis was performed on those regions within 100kb of HIF target genes using ‘findMotifsGenome.pl’ command with default settings with the total co-bound H3K4me2 and H3K27ac peaks used as background.
Acknowledgements

We thank the EMBL GeneCore Sequencing Service (http://www.genecore.embl.de) for RNA-Seq library preparation and sequencing. Viral vectors were obtained from the National Virus Vector Laboratory (Kuopio) which is supported by Biocentre Finland and EU EATRIS infrastructure networks. We would also like to thank Annakaisa Tirronen for assistance with the western blots and Henri Niskanen for assistance with the RNA-seq data.

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Author Contributions

N.L.D conducted the experiments, analysed the data and wrote the manuscript. S.Y.H, M.U.K and N.L-K conceived and supervised the project.

Data Access

The experiments performed in this study are available in GEO under the accession number GSE98060. The public ChIP-seq data for H3K4me2 and H3K27ac are available in GEO under accession numbers GSE29611 and GSE38555.
References


Figure Legends

**Figure 1. HIF1α and HIF2α expression profiles.** Volcano plot of log₂ fold change and -log₁₀ (p-value) of gene expression under HIF1α (a) and HIF2α (b) overexpression. Differentially regulated genes (fold change > 2, FDR < 0.01) are highlighted with the top 20 genes annotated.

**Figure 2. Heatmap of HIF1α and HIF2α differentially regulated genes.** Expression values are shown as log₂ fold change ratios. Rows are hierarchically clustered (k=4) using Ward’s least absolute error with Manhattan distance. The highest enriched GO terms associated with genes each cluster is shown on the right.

**Figure 3. Venn diagram of the proportion of unique and shared differentially regulated genes of HIF1α and HIF2α.**

**Figure 4. Gene ontology enrichment for biological processes regulated by HIF1α and HIF2α.** Enriched biological processes are shown in a descending order according to their -log₂ FDR values. The size of the dots are representative of the percentage of genes regulated for the given GO term.

**Figure 5. Ingenuity canonical pathway analysis of HIF1α and HIF2α differentially expressed genes.** Enriched pathways are shown descending according to their -log₂ p-values. The size of the dots is proportional to the -log₂ p-values. The activation z-score on the x-axis is used to predict the regulation direction based on the observed differentially expressed genes.

**Figure 6. Heatmaps of selected HIF1α and HIF2α regulated genes central to HIF regulated biological processes.** (a) Metabolic reprogramming constitutes genes involved in glycolysis, NADH regeneration, glucose transport, fatty acid transport and autophagy. (b) Genes involved in extracellular signalling including ligands and receptors of growth factors, cytokines and vasodilatory molecules. (c) Diverse transcription factors from ETS, GATA, AP-1/2, FOX, NFATC, HOX and other families. (d) Guidance cues and receptors related to axon guidance including semaphorins, ephrins and plexins. (e) Genes encoding matricellular basement membrane proteins, collagen fibril assembly, adhesion and junction molecules. (f) Histone modifiers including lysine demethylases, methyltransferases, deacetylases and acetyltransferases. (g) Genes that are inversely regulated by HIF1α and HIF2α.

**Figure 7. Functional interaction networks of HIF regulated genes.** Cytoscape with the Reactome-FI plug-in was used to identify genes regulated by HIF1α (a) or HIF2α (b) that
formed functional interaction networks. Gene nodes are coloured according to their log₂ fold change. Genes that act as central hubs are highlighted with borders whereas edge genes are shown without borders.

**Figure 8. Motif analysis of the proximal regulatory regions of HIF target genes.** Enriched motifs identified in the proximal regions flanking (+/- 750bp) the transcription start site of HIF1α (a) and HIF2α (b) target genes calculated relative to a GC% matched background sequence.

**Figure S1. Western blots of stable HIF1α and HF2α expression.** (a) HIF1α expression in HUVECs transduced with AdCMV or AdHIF1α. (b) HIF2α expression in HUVECs transduced with AdCMV or AdHIF2α. (c) Expression of endothelial marker PECAM1 (CD31) in AdCMV treated controls.

**Figure S2. RT-qPCR confirmation of HIF target gene expression** (a) Fold change of GAPDH expression under HIF1α and HIF2α overexpression compared with eCMV control. (b) Fold change of VEGFA expression under HIF1α and HIF2α overexpression compared with eCMV control. Comparative fold change of (c) GAPDH and (d) VEGFA obtained by RNA-seq.

**Figure S3. Multi-dimensional scaling (MDS) plot of RNA-seq expression profiles.**

**Figure S4. HIF regulation of c-Myc target genes** (a) Enrichment of Hallmark biological processes in HIF1α and HIF2α DEGs (b) antagonistic HIF log₂ fold change expression values of c-Myc target genes.

**Figure S5. Top enriched motifs present in HIF target gene distal regulatory regions.** Overrepresented motifs identified within H3K4me2 and H3K27ac intergenic regulatory regions within 100kb to (a) HIF1α and (b) HIF2α target genes.

**Figure S6. Expression of cell marker genes.** logRPKM expression values of cell marker genes used to identify endothelial, fibroblast and vascular smooth muscle cells.

**Table S1. Differential expression of HIF1α and HIF2α target genes.**
Cluster 1

Cluster 2

Cluster 3

Cluster 4

HIF1α

HIF2α

GO term  
-\log FDR
Canonical glycolysis  
30.65
NADH regeneration  
30.65
Response to hypoxia  
12.84

GO term  
-\log FDR
Pattern specification process  
29.00
Angiogenesis  
20.09
Endothelial cell migration  
11.03

GO term  
-\log FDR
Pattern specification process  
43.04
Cell fate commitment  
28.41
Axon guidance  
21.92

GO term  
-\log FDR
Negative regulation of immune system  
8.32
Response to reactive oxygen species  
7.68
Primary alcohol metabolic process  
7.33
HIF1α  HIF2α

- 398 (21.5%)
- 303 (16.4%)
- 1151 (62.1%)
Negative Regulation Of Cell Adhesion

Epithelial Cell Proliferation

Neuron Fate Commitment

Endothelial Cell Migration

Ribonucleoside Diphosphate Metabolic Process

Canonical Glycolysis

Pyruvate Metabolic Process

Extracellular Matrix Organization

Tube Morphogenesis

Angiogenesis

Response To Hypoxia

Pattern Specification Process

HIF1α HIF2α

Gene Ratio %

-\log \text{FDR}

5.0

7.5

10.0

12.5

Gene Ratio %

20

30

40

50

60
### a)

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Hypoxia provokes a diverse cellular transcriptional response that is primarily mediated by the hypoxia inducible factors. Ylä-Herttuala et al. delineate the downstream genes and their associated processes regulated by the major HIF isoforms HIF1α and HIF2α, as to further understand how their non-overlapping activity could affect vascular disease and regeneration.