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Research Article

Hypoxia-mediated activation of Dll4-Notch-Hey2 signaling in endothelial progenitor cells and adoption of arterial cell fate

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ABSTRACT

Adequate response to low oxygen levels (hypoxia) by hypoxia inducible factor (HIF) is essential for normal development and physiology, but this pathway may also contribute to pathological processes like tumor angiogenesis. Here we show that hypoxia is an inducer of Notch signaling. Hypoxic conditions lead to induction of the Notch ligand *Dll4* and the Notch target genes *Hey1* and *Hey2* in various cell lines. Promoter analysis revealed that *Hey1*, *Hey2* and *Dll4* are induced by HIF-1α and Notch activation. Hypoxia-induced Notch signaling may also determine endothelial identity. Endothelial progenitor cells (EPCs) contain high amounts of COUP-TFII, a regulator of vein identity, while levels of the arterial regulators Dll4 and Hey2 are low. Hypoxia-mediated upregulation of Dll4 and Hey2 leads to repression of *COUP-TFII* in eEPCs. Finally, we show that Hey factors are capable of repressing HIF-1α-induced gene expression, suggesting a negative feedback loop to prevent excessive hypoxic gene induction. Thus, reduced oxygen levels lead to activation of the Dll4-Notch-Hey2 signaling cascade and subsequent repression of *COUP-TFII* in endothelial progenitor cells. We propose that this is an important step in the developmental regulation of arterial cell fate decision.

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Introduction

Adequate oxygen (O_2) levels are a principal requirement for life of all vertebrates. They can sense low oxygen levels (hypoxia) and react by various means to reduce energy consumption. Although there is a general reduction of gene expression and protein synthesis during hypoxia, a series of genes like *vascular endothelial growth factor* (*Vegf*), *glucose transporter type 1* (*Glut1*) or *erythropoietin* (*Epo*) is significantly upregulated to reduce deleterious effects of oxygen deprivation [1,2]. This is accomplished by modifying cellular and systemic processes like glucose transport, glycolysis, erythropoiesis, angiogenesis, vasodilation or respiratory rate [3,4].

Transcriptional activation of most hypoxia-regulated genes is mediated by hypoxia-inducible-factor (HIF). HIF-1 is a heterodimer consisting of HIF-1α and ARNT (also known as HIF-1β). In recent years, two additional HIF-α subunits, HIF-2α (EPAS1/HRF/HLF/MOP2) and HIF-3α, have been isolated (for review see [4]). It is well established that HIF-1 activity is regulated by protein stability of its α subunit. Although

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constitutively expressed, HIF-1α protein is rapidly degraded under normoxia due to oxygen-dependent prolyl hydroxylation and ubiquitination by the von Hippel-Lindau E3 ubiquitin ligase complex (VHL). Under hypoxia, hydroxylation and subsequent degradation are reduced and the HIF-α subunit translocates to the nucleus where it engages ARNT. The protein complex activates hypoxia-responsive genes by binding to hypoxia response elements (HRE) (reviewed in [5,6]).

Notch signaling is an evolutionary conserved signaling mechanism for intercellular communication and cell fate decision during metazoan development. Notch proteins (Notch1–4 in vertebrates) are single-pass transmembrane proteins that are activated by Delta (Dll1, 3, 4) and Jagged/Serrate (Jag1, 2) ligands. This interaction leads to proteolytic cleavage and release of the intracellular domain of Notch (NICD), which translocates into the nucleus and interacts with the DNA-binding protein RBP-Jκ (also known as CBF1 or suppressor of Hairless). This transcriptional activator complex induces transcription of target genes, most notably the *Hey* and *Hes* genes (for review see [7]).

The Hey bHLH transcription factor family (Hey1, Hey2 and HeyL) is essential for various steps of embryonic development, where their role in cardiovascular development is best understood (for review see [8]). *Hey2* knockout mice suffer from cardiac hypertrophy and lethal congenital heart defects [9–11]. While *Hey1* null mice do not show obvious defects, *Hey1/2* double deficient embryos die during midgestation due to severe angiogenesis defects in yolk sac, placenta and embryo proper and a failure to generate arterial endothelial cells [12]. *Notch1* and *Dll4* mutant mice show similar defects suggesting a linear cascade that is essential for arterial endothelial development [13–16]. Very recently, it was shown that venous fate, which was believed to be the default pathway in the absence of Notch signaling, is regulated by a member of the orphan nuclear receptor superfamily, COUP-TFII [17].

In *Hey1/2* mutants we had observed a significant upregulation of *Vegf*, indicating that the embryos suffer from hypoxia. In addition, it has been shown that the Hey2/CHF1 protein can interact with ARNT and repress ARNT/HIF-2α-dependent transcription [18]. This prompted us to examine the role of *Hey* genes during hypoxia. Here we show that *Hey1* and *Hey2* are upregulated by hypoxia in an HIF and Notch-dependent manner and that elevated Hey levels subsequently limit HIF-driven gene expression. Hypoxia activates the artery-specific Dll4-Notch-Hey2 signaling axis in endothelial progenitor cells, leading to a repression of the venous regulator COUP-TFII and adoption of arterial cell fate.

Materials and methods

Plasmids

Expression vectors encoding Hey1, Hey2, HA-Notch1-ICD, HA-Notch4-ICD, HIF-1α, HIF-2α, constitutive active HIF-1αDPA and HIF-2αDPA have been described [19–21]. The full-length human *Dll4* coding region was released from IMAGE clone IRAKp961M09174Q (RZPD, Berlin, Germany) with *Sac*II and cloned into pIRES2-EGFP (Clontech).

For luciferase assays, promoter fragments upstream of the translational start codon of human *Dll4* (6 kb) and murine *Hey2* (12 kb) and *COUP-TFII* (10 kb) promoters were inserted into pGL3basic (Promega) by BAC recombineering [22] as described in Supplementary data 1. Shortened versions were generated by restriction enzyme digestion. *Hey1* promoter and HRE-luciferase reporters have been described [19–21]. RBP-Jκ and HRE sites in the *Hey1*−95/+87 luciferase reporter were mutated by PCR using mutagenized oligonucleotides.

Luciferase assay

HEK293T, HeLa and eEPCs were transiently transfected in 24 well plates using polyethylenimine, GeneJuice (Novagen) or Lipofectamine2000 (Invitrogen). 36 to 48 h post-transfection luciferase activity was measured (MicroLumat Plus LB96V, Berthold, Germany). Each experiment was repeated three times with each reaction measured in triplicate. Results are shown as mean values with standard deviations.

Quantitative real-time RT-PCR

mRNA was purified with Trizol Reagent (Invitrogen) and cDNA prepared using the RevertAid kit (Fermentas). Quantitative real-time PCR was performed in a BioRad iCyler as described [12]. Primer sequences are listed in Supplementary data 2. Biostatistical analysis was performed using the SPSS 11.0 program and the Wilcoxon test.

Cell culture and hypoxia

HEK293T, HEK293, HeLa and bEnd5 were cultured in DMEM with 10% FCS and 20 mM HEPES in 5% CO_2 and 95% air. Embryonic endothelial progenitor cells (eEPCs; line T17b) were cultured as described [23]. CHO cells were maintained in DMEM/F12 with 10% FCS and 20 mM HEPES. Stable CHO lines expressing Dll4 were generated by transfection with linearized pDll4-IRES2-EGFP. Clones with strong eGFP fluorescence and the ability to stimulate *Hes1* and *Hey1* expression in HeLa cells were expanded. CHO cells expressing Dll1 were a kind gift of Dr. Achim Gossler (Hannover, Germany). For costimulation experiments, CHO and eEPCs were mixed in a 3:1 ratio. All PCR primers were designed to specifically amplify cDNA derived from eEPCs but not from CHO cells.

Inhibition of Notch signaling was done using the gamma-secretase inhibitor DAPT (*N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-Butyl Ester (Calbiochem) at 2 μM.

Results

Hey1, Hey2 and Dll4 are upregulated under hypoxia

To test if *Hey* genes are affected by low oxygen tension, we initially cultured HeLa cells under normoxia (95% air, 5% CO_2) or anoxia (100% N_2) and measured mRNA expression by quantitative real-time RT-PCR (qRT-PCR). We found a 3.5-fold upregulation of *Hey1* mRNA expression after 8 h under anoxic conditions (data not shown). When repeated under hypoxic conditions (1% O_2), Hey1 was upregulated 2.7-fold

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