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Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells

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Abstract

The Notch ligand, Dll4, is essential for angiogenesis during embryonic vascular development and is involved in tumour angiogenesis. Several recent publications demonstrated that blockade of Dll4 signalling inhibits tumour growth, suggesting that it may constitute a good candidate for anti-cancer therapy. In order to understand the role of Dll4 at the cellular level, we performed an analysis of Dll4-regulated genes in HUVECs. The genes identified included several angiogenic signalling pathways, such as VEGF, FGF and HGF. In particular we identified downregulation (VEGFR2, placenta growth factor PlGF) of VEGF pathway components resulting in the overall effect of limiting the response of HUVEC to VEGF. However extensive upregulation of VEGFR1 was observed allowing continued response to its ligand PlGF but the soluble form of the VEGFR1, sVEGFR1 was also upregulated. PlGF enhanced tubulogenesis of HUVEC suggesting that downregulation of PlGF and upregulation of VEGFR1 including sVEGFR1 are important mechanisms by which Dll4 attenuates PlGF and VEGF signalling. Dll4-stimulated HUVECs had impaired ERK activation in response to VEGF and HGF indicating that Dll4 signalling negatively regulates these pathways. Dll4 expression reduced vessel sprout length in a 3D tubulogenesis assay confirming that Dll4 signalling inhibits angiogenesis. Altogether, our data suggest that Dll4 expression acts as a switch from the proliferative phase of angiogenesis to the maturation and stabilisation phase by blocking endothelial cell proliferation and allowing induction of a more mature, differentiated phenotype. The regulation of sVEGFR1 provides a novel mechanism for Dll4 signalling to regulate cells at distance, not just in adjacent cells.

Keywords: Dll4; Notch; cDNA microarray; HUVEC; Sprout formation; Angiogenesis

Introduction

Notch signalling has recently been implicated in vascular development and homeostasis since genes encoding components of Notch signalling are mutated in two human diseases (Alagille Syndrome and CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy)) which exhibit vascular defects (Karsan, 2005; Shawber and Kitajewski, 2004). Furthermore, many Notch receptors and ligands are expressed in cells of the vasculature (Iso et al., 2003a; Villa et al., 2001). Finally studies of transgenic mice have revealed an essential early role for Notch signalling in angiogenesis (Iso et al., 2003a).

Notch signalling is an evolutionarily conserved intercellular signalling pathway mediated by membrane-tethered receptor–ligand interactions between adjacent cells (Artavanis-Tsakonas et al., 1999; Lai, 2004). Receptor–ligand binding induces sequential cleavages of the Notch receptor, the last of which is performed by the γ-secretase complex, releasing the Notch intracellular domain (NICD) which translocates to the nucleus (Artavanis-Tsakonas et al., 1999; Lai, 2004). In the nucleus NICD typically interacts with RBP-Jκ (recombination signal binding protein Jκ) leading to the transcription of Notch target genes such as members of the Hes and Hey families of

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transcriptional repressors (Iso et al., 2003b). In mammals there are four Notch receptors (Notch1–4) and 5 Notch ligands: two serrate-like ligands named Jagged1 and Jagged2 and three delta-like ligands (Dll) named Dll1, 3, 4.

We and others have shown that Dll4 is expressed specifically at sites of vascular development and angiogenesis and is confined to arterial ECs (Benedito and Duarte, 2005; Claxton and Fruttiger, 2004; Mailhos et al., 2001; Shutter et al., 2000). Dll4 expression is particularly critical for angiogenesis as haploinsufficiency of Dll4 leads to embryonic lethality in mice due to vascular defects (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004) demonstrating the essential role of Dll4 in angiogenesis during development.

Thus Dll4 expression is essential for normal angiogenesis during development but Dll4 expression in adults is confined to areas of physiological angiogenesis such as in the ovary around developing follicles and areas of pathological angiogenesis such as tumour vasculature (Mailhos et al., 2001). Recent evidence suggests that Dll4 expression is upregulated in tumour endothelium and that this correlates with tumour vessel maturation and remodeling (Hainaud et al., 2006; Patel et al., 2006). In addition Dll4 expression is induced by hypoxia, a common feature of tumour development known to induce angiogenesis (Mailhos et al., 2001) making Dll4 an attractive anti-tumour target. Indeed recent papers have demonstrated that blockade of Dll4 signalling promotes non-productive angiogenesis thus inhibiting tumour growth (Noguera-Troise et al., 2006; Ridgway et al., 2006) though the mechanistic basis for this remains unclear. In the present study we performed a cDNA microarray screen to identify Dll4 regulated genes in order to understand the function of Dll4 signalling in physiological and pathological angiogenesis.

Materials and methods

Cell culture, cells and reagents

HUVECs were isolated from fresh human umbilical cords by infusion with 0.2% collagenase. Single donor HUVECs were used between passages 3 and 7 and were cultured in M199 media supplemented with 20% fetal calf serum (FCS: Sigma-Aldrich, St. Louis, MO), 12 mM L-glutamine, 50 mg/L ECGS (endothelial cell growth supplement; BD Biosciences Bedford MA, USA), 10 units/mL heparin (Sigma) and an antimycotic/antibiotic (Gibco). The Phoenix amphotropic viral packaging cell line (gift from Garry Nolan) was cultured in DMEM supplemented with 10% FCS, 12 mM L-glutamine and penicillin/streptomycin. Recombinant human Dll4 extracellular domain (rDll4) was purchased from R&D Systems (Minneapolis, USA). The γ-secretase inhibitor DAPT (Calbiochem) was dissolved in DMSO (Sigma) and used at a final concentration of 2 μM.

Retroviral packaging and infection

The retroviral vector and full length human Dll4 construct have been previously described (Williams et al., 2006). Low passage, 50% confluent HUVECs were infected using 0.4-μm filtered virus containing supernatant supplemented with 4 ng/mL polybrene. After 5 h at 37 °C an equal volume of normal HUVEC culture media was added. Infection efficiencies were from 60% to 90%.

Microarray analysis

Microarray analysis was performed in triplicate and each replicate used HUVECs from a different donor. Total RNA was extracted from HUVECs using TRI-reagent (Sigma) followed by DNase I treatment (DNase-free; Ambion) according to the manufacturer's instructions. First and second strand cDNA synthesis was performed using Superscript dscDNA Synthesis Kit (Invitrogen) and 10 μg of total RNA. Clean-up of double stranded cDNA was carried out using Phase Lock Gels, 2 mL light (Eppendorf), followed by synthesis of labeled cRNA with the BioArray High Yield RNA Transcript Labeling Kit (ENZO, Affymetrix). Purification of cRNA and quantification was done with RNeasy Mini Kit (Qiagen), followed by cRNA Fragmentation using 30 μg cRNA and Fragmentation Buffer (200 mM Tris-acetate pH 8.1, 500 mM MKOAc, 150 mM MgOAc). Hybridisation Cocktail for human HG-U133A Affymetrix GeneChip® utilised the Gene Chip Eurkaryotic Hybridisation Control Kit (ENZO, Affymetrix) and hybridisation, washing, staining, and scanning of the chip was performed according to the manufacturer's instructions. Data was labeled as MIAME compliant. Raw signal files were background corrected and normalised using the gcrma modification of the rma normalisation procedure (Irizarry et al., 2003) available from the Bioconductor project (www.bioconductor.org) for the R statistical language. Log2 ratios of Dll4 signal to control signal were generated for each probeset. A list was generated that contained those probesets for which an average absolute fold change of at least 1.5 was observed between Dll4 and control samples. Where a gene was represented by more than one probeset the average signal value was used. This list was used to generate a list of statistically significant ($P \leq 0.01$) probesets with the eBayes approach (Smyth, 2004) as implemented in the limma package (Smyth and Speed, 2003) of BioConductor. Visualisation of this gene list was performed using the hierarchical clustering algorithm with euclidean distance and single linkage. Enrichment analysis was conducted using the DAVID algorithm and significance of enrichment is measured using the Fisher's Exact Test for each GO term relative to the background of the Affymetrix HG-U133A chip.

Quantitative real time PCR (Q-PCR)

Reverse transcription was performed using 1 μg total DNase 1-treated RNA and the High Capacity cDNA Archive Kit (Applied Biosystems). The resulting cDNA was used for Q-PCR using the Exiqon system (Roche, Basel, Switzerland). Briefly Q-PCR reactions were set up in triplicate using the Corbett Research Roto Gene RG-3000 robot (Corbett, Sydney, Australia). Each 25 μl reaction contained the equivalent of 25 ng reverse transcribed cDNA, 0.4 μM of each oligonucleotide, 12.5 μl of 2× Absolute QPCR master mix (AbGene, Epsom, UK) and 0.25 μl of the appropriate Exiqon probe. GAPDH or Flo2 were used as reference genes to normalise results. The cycling conditions used were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The relative quantitation was performed as previously described (Patel et al., 2005). The differences between Q-PCR experimental groups were analyzed using one sample Student's t test and $p < 0.05$ was considered significant.

Oligonucleotides

Gene	Forward 5′–3′	Reverse 5′–3′
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
Flo2	CTCAGCTTCACCATCAAGGAC	TCAGCATCTCTCTGCACCAC
Sulfatase1	CCAATGCTTCCCAACACATA	GCATTGGTCCTGTGTACTGC
Elastin	CACTGGGGTATCCCATCAAG	GTGGTGTAGGGCAGTCCATAG
PCDH12	GGAGGAGCTCACTGTGGATT	CTGATGCAGCAAGGACAGC
INHBA	CTCGGAGATCATCACGTTTG	CCTTGGAAATCTCGAAGTGC