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Sprouty1 inhibits angiogenesis in association with up-regulation of p21 and p27

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Abstract

Sprouty1 (Spry1) is a conserved antagonist of FGF signaling. The goal of this study was to further explore the downstream mechanisms governing Spry1 inhibition of endothelial cell proliferation. Up-regulation of Spry1 in HUVECs inhibited tube formation on Matrigel ($n = 6$, $P < 0.001$). This was associated with decreased proliferation as measured by BrdU incorporation ($n = 6$, $P < 0.001$) and increased protein expression of the cyclin-dependent kinase inhibitor 1A (CDKN1A), p21 and cyclin-dependent kinase inhibitor 1B (CDKN1B), p27. A transcriptional analysis using a targeted human angiogenesis array following up-regulation of Spry1 demonstrated a >2-fold increase in an anti-angiogenic factor, serpin peptidase inhibitor, clade F (Serpinf1), and a >2-fold decrease in pro-

angiogenic factors fms-related tyrosine kinase 1 (FLT1), angiopoietin2 (Ang-2), and placental growth factor (PGF) ($n = 2$). To define upstream mechanisms that may regulate endogenous Spry1, we performed a search for responsive elements upstream of the promoter region. This search resulted in the identification of multiple degenerate hypoxia responsive elements. Exposure to hypoxia resulted in a significant increase in Spry1 expression ($n = 8$, $P < 0.01$). These findings shed new light on downstream signaling pathways associated with Spry1 anti-proliferative responses, and provide new evidence that hypoxia stimulates Spry1 expression.

Keywords

HUVEC; Endothelial cell; Hypoxia; HIF; Serpinf1

Introduction

Sprouty (Spry) was first identified in *Drosophila* as a feedback inhibitor of FGF receptor signaling during tracheal development [1]. Loss of function mutations of Spry1 resulted in excessive FGF signaling and ectopic branching in the *Drosophila* trachea [1]. Currently, four mammalian Spry family members, Spry1 – Spry4, have been identified. These Spry isoforms, although differing in the NH3 domains, share a well-conserved cysteine-rich COOH region responsible for translocation of Spry to the plasma membrane [2]. Prior work has shown that both Spry1 and Spry2 had a negative effect on proliferation and differentiation of endothelial cells on Matrigel [3]. Moreover, Spry4 overexpression in endothelial cells was shown to decrease branching and sprouting of small vessels, resulting in abnormal embryonic development of mouse embryos in vivo [4].

The mechanisms by which Spry isoforms inhibit proliferation are only beginning to be elucidated. Lee et al. [4] has demonstrated that Spry 4 up-regulates p21, a well-known cell cycle inhibitor. We extend these findings by providing new evidence that up-regulation of Spry1 is associated with an increase in cell cycle inhibitors p21/p27 and a host of newly identified pro- and anti-angiogenic genes.

To determine how endogenous levels of Spry1 are regulated, we carried out a promoter analysis. This resulted in the identification of multiple degenerate hypoxia responsive elements upstream of the Spry1 promoter region. Exposure to hypoxia resulted in increased expression of Spry1. These findings suggest that exposure to ischemic or hypoxic conditions may up-regulate Spry1 expression and stimulate this anti-proliferative and anti-angiogenic signaling cascade involving p21/p27 (*CDKN1A* and *CDKN1B*).

Methods

Sample preparation

Total RNA was isolated and purified using the RNeasy Mini protocol according to the manufacturer's instructions (Qiagen). Ten micrograms of total RNA was reverse transcribed utilizing a T7-(dT)₂₄ primer, and double-stranded cDNA synthesis was performed according to the manufacturer's instructions (Invitrogen).

Cells and adenoviral infection

HUVECs were obtained from Clonetics (we used pooled aliquots of the same lot number for all studies) as well as Dr. Vercellotti's lab at the University of Minnesota [5, 6]. The cells were maintained in EBM-2MV media (Clonetics) and serum-starved in M199 media (Clonetics). Spry1 and control virus (Ad-Cre, Ad-LacZ) were prepared and titrated by the

Roy J. and Lucille A. Carver College of Medicine Gene Vector Core Lab at the University of Iowa (<http://www.uiowa.edu/~gene/index.htm>) as previously described [7]. Infections were performed using 1,000 moi in serum-free medium for 4 h for all experiments except for the SuperArray real-time PCR experiments (see below). Following infection, the cells were washed thrice with warm PBS and allowed to recover for 48 h in fully supplemented EBM-2 medium.

The Spry and Lac Z viruses used prior to SuperArray real-time PCR were prepared utilizing a Cre-Lox based system [8] (kind gift from P. Robbins, University of Pittsburgh). Approximately 5×10^5 HUVECs were infected with either Ad-LacZ or Ad-Spry1 (1,000 viral particles/cell) in growth medium for 12 h. Cells were then cultured in virus-free growth medium for an additional 24 h before SuperArray analysis was performed. RNAs from transduced cells were extracted using RNeasy Mini Kit (Qiagen) and treated with DNase1 (DNA-free, Ambion) to prevent possible genomic DNA contamination.

Tubule formation assays

Tubule formation assays were performed using growth-factor reduced Matrigel matrix (BD Biosciences). 50 μ l of thawed Matrigel was allowed to solidify in a 96-well plate at 37°C. Both Spry1 expressed HUVECs and controls (13,000 cells) were seeded into each well and continued to be cultured with the support of Matrigel matrix for 6 h at 37°C under either normoxic conditions or 36 h under hypoxic conditions (0.2% oxygen). Digital micro-photographs were taken of the cells using an inverted microscope (Nikon Eclipse TE200). Tubule formation was quantified in a blinded manner using Adobe Photoshop as previously described [9].

Cellular proliferation assays

Cellular proliferation assays were performed using the bromodeoxyuridine (BrdU) colorimetric cell proliferation ELISA (Roche). HUVECs (5,000 cells) were seeded into each well of a 96-well plate and cultured for 48 h prior to BrdU addition. After 24 h, the BrdU labeling solution was added to each well for approximately 10 min, at which time the samples demonstrated a green color clearly distinguishable from the color of pure peroxidase substrate. The amount of BrdU incorporation was determined using an ELISA reader at 450 nm with a reference wavelength of 690 nm according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction

Double-stranded cDNA synthesis was performed as described above. TaqMan assays (Applied Biosystems, Foster City, CA) designed for the ABI 7900HT Fast Real-Time PCR System were utilized for Spry1 (Assay ID Hs00398096_m1), and Hypoxanthine Phosphoribosyl Transferase (HPRT) (# 4333768F). Target amplification and detection were performed on samples and controls in the same thermal cycling reaction in replicated fashion, allowing for minimization of experimental variability and calculation of ΔC_t based on the corresponding control using HPRT (i.e. $\text{Target}^{\Delta C_t \text{ HPRT}}$) as previously described [10].

Superarray real-time PCR analysis

SuperArray real-time PCR analysis (RT² Profiler™ PCR Array Human Angiogenesis, SuperArray Bioscience Corporation) was performed according to the manufacturer's instructions. Three quality control platforms were used to ensure the accuracy of the SuperArray data: control for human genomic DNA contamination, reverse transcriptase control, and positive PCR control. Fold changes were calculated by $2^{[(\text{LacZ Ct-GAPDH Ct})-(\text{Spry1 Ct-GAPDH Ct})]}$.

Western blotting

SDS-PAGE and western blotting were performed as previously described [11, 12]. The following antibodies were used: Spry1 (C-12, sc-18599, Santa Cruz), p21 (c-19, sc-397, Santa Cruz), p27 (F-8, sc-1641, Santa Cruz), Vinculin (V4139, Sigma), Myc (clone 9E10, Santa Cruz, #SC-40), and β -actin (Sigma, cat-#1978). Densitometry was performed on a BioRad ChemiDox XRS with Quality One 4.6.3 software. The bands were analyzed using white transillumination, volume rect tool and volume analysis report supported by 1-D analysis software.

Promoter analysis

FUZZNUC, a program for nucleic acid pattern matching using the EMBOSS interface (Pasteur Institute), was used to search nucleotide sequences in front of the promoter region of Spry1.

Apoptosis

HUVECs (10,000 cells) were seeded in a 6-well plate. The cells were infected with either Ad-Cre or Ad-Spry as described above. After three washes in PBS, the cells were incubated for 5 mins at 37°C with Hoechst33342 (5 μ g/ml; Molecular Probes, Eugene, Oregon) as previously described [11, 12]. Cells were mounted on a coverslip, and 400 cells were counted in each condition. The number of apoptotic nuclei in each condition was then determined.

Statistical analysis

A paired Student's *t*-test was used to statistically analyze differences between two groups. A Kruskal–Wallis one-way ANOVA on ranks was used to statistically analyze differences between more than two groups. Data are expressed as means or as log delta Ct values $\pm$ standard deviation.

Results

At the outset, we demonstrated that up-regulation of Spry1 is sufficient to inhibit tubule formation of HUVECs on Matrigel (Fig. 1, $n = 6$, $P < 0.01$). To understand the nature of the inhibition, we studied the effects of Spry1 up-regulation of the apoptotic index and proliferative index of HUVECs infected with either Ad-Cre (control) or Ad-Spry. There were no statistical differences in the percentage of HUVECs undergoing apoptosis as determined by Hoechst33342 staining in response to Spry1 up-regulation versus control (16 vs. 15%, respectively, $n = 6$, ns). In contrast, Spry1 decreased endothelial proliferation compared with control infected cells as measured by BrdU incorporation (Fig. 2, $n = 6$; $P < 0.01$). The results suggest that Spry1 expression may regulate cell cycle regulatory proteins. Therefore, we assessed the levels of cell cycle inhibitors p21 and p27, the cyclin-dependent kinase inhibitors known to regulate the cell cycle [13, 14]. As shown in Fig. 3a and b, up-regulation of Spry1 resulted in increased protein expression of p21 and p27. Altogether, the data support a working model that Spry1 mediates an anti-proliferative response through the up-regulation of cell cycle inhibitors.

To further define the effects of Spry1 in endothelial cells, gene expression profiling for endothelial cells in response to Spry1 up-regulation was carried out by a targeted array for Human Angiogenesis (RT² Profiler™ PCR Array Human Angiogenesis, SuperArray Bioscience Corporation). Table 1 lists genes with greater than 2-fold change in expression in response to Spry1 up-regulation.

Given the anti-proliferative effects of Spry1 and associated changes in cell cycle and angiogenic related genes, we performed an in silico promoter analysis to assess potential regulatory regions in the Spry1 promoter. We identified multiple hypoxia responsive elements upstream of the promoter region of Spry1 (Fig. 4). To verify whether hypoxia may influence the endogenous expression of Spry1, transcriptional levels of Spry1 in HUVECs cultured in a hypoxic condition (0.2% oxygen) at different time points (4, 8, 12, and 20 h) were examined. Transcriptional levels of Spry1 were generated by real-time PCR quantification. As seen in Fig. 5, Spry1 transcript levels were significantly up-regulated after 12 h of hypoxic exposure.

Discussion

The major findings of this study are that increased Spry1 expression inhibits angiogenesis and endothelial proliferation in association with enhanced expression of cell cycle inhibitors p21 (*CDKN1A*) and p27 (*CDKN1B*). Transcriptional changes in pro and anti-angiogenic factors were also identified. Finally, a promoter analysis and RTQPCR studies demonstrated that Spry1 expression is increased in response to hypoxia. Taken together, these findings suggest that under hypoxic conditions, Spry1 expression is increased and may regulate the extent of angiogenesis through a cell cycle regulatory program.

It has been previously demonstrated that Spry1 over-expression is sufficient to inhibit proliferation [3]. We confirmed and extended these findings to show that these changes were associated with an up-regulation of Serpinf1, a serine protease inhibitor with anti-angiogenic down-stream effects as well as down-regulation of pro-angiogenic factors including PGF and Angiopoietin2 [15, 16]. FLT1, the vascular endothelial growth factor/vascular permeability factor receptor, was also down-regulated. Previous studies have shown a dual function of FLT1 in regulating angiogenesis, with inhibitory effects during embryogenesis and stimulatory effects on pathological angiogenesis [17, 18]. Our findings support the proangiogenic role of FLT1 in endothelial cells subjected to hypoxia. In addition, we found that Spry1 overexpression is sufficient to up-regulate p21 and p27, two well-known inhibitors of cyclin-dependent kinases. Previous work by de Alvaro et al. has demonstrated that Spry2 overexpression induced expression of p21 in myoblast C2C12 cells [19]. Several studies have demonstrated that p27, and not p21, plays an important role in cell growth arrest under hypoxia [7]. We have shown that Spry1 overexpression is sufficient to up-regulate both p21 and p27 consistent with a decrease in proliferation. Although the mechanism(s) by which Spry1 inhibits angiogenesis is not entirely delineated, these findings are an important first step and will require further investigation to ascertain if each of these factors is both necessary and sufficient for Spry1 to exert its inhibitory effects. Our findings suggest that it is the time-dependent balance of both stimulatory and inhibitory regulators of angiogenesis that is perturbed under hypoxic conditions such that at >12 h, anti-angiogenic factors, including Spry1, may play a more predominant role.

Hypoxic stress is generally accepted to be a potent stimulator of pro-angiogenic signals in both physiologic and pathologic processes including tumor growth, embryonic development, wound healing, development of atherosclerotic plaques, and other chronic inflammatory states. Emerging evidence also supports the induction of cell cycle arrest programs in response to hypoxia as well as interwoven links between transcriptional activation, cell death, and cell cycle [20–24]. Our findings suggest that early in hypoxia, proangiogenic factors may predominate. Yet with prolonged exposure to hypoxia (>12 h), anti-angiogenic signals are turned on that may ultimately limit the angiogenic program.

The gene regulation of Spry1 remains poorly understood. Functional promoter analysis has been performed on Spry2, which revealed the presence of several cis-acting elements

including adaptor protein 2 complex (AP2), cAMP response element binding (CREB), stimulating protein 1 (SP1), and E26 Avian leukemia associated oncogene 1 (Ets-1) [25]. Furthermore, the Spry4 promoter has been characterized [26]. Using a one-base degenerate sequence, we identified multiple hypoxia response elements upstream of the Spry1 promoter.

In summary, Spry1 expression is up-regulated by hypoxia. Up-regulation of Spry1 is sufficient to inhibit angiogenesis and endothelial cell proliferation. The anti-angiogenic effects of Spry1 were associated with an increase in cell cycle inhibitors p21, p27, and Serpinf1, as well as a decrease in FLT1, Angiopoietin2 and PGF. These findings shed new light on endogenous anti-angiogenic signaling pathways in the endothelium that are up-regulated in response to hypoxia.

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Fig. 1. Spry1 up-regulation inhibits tubule formation. HUVECs were infected with control adenovirus (a) or Spry1 adenovirus (b) for 4 h. After 48 h, 13,000 cells were plated onto Matrigel and incubated at 37°C. Representative digital microphotographs were taken at 6 h ($n = 6$, $P < 0.01$)

Fig. 2.

a Proliferation was inhibited in HUVECs infected with Spry1 adenovirus, $n = 6$; ** $P < 0.01$. **b** Representative western blot of adenovirus-mediated Spry1 up-regulation, $n = 2$. Quantification of Spry1 protein levels via densitometry were Ad-control, 1.05 ± 0.02 and Ad-Spry1, 2.39 ± 0.13 , arbitrary units)

Fig. 3.

Spry1 increases p21 and p27. **a** Representative western blot showing depicting up-regulation of Spry1 increases p21 expression, $n = 3$. Quantification of p21 protein levels via densitometry were WT, 0.59 ± 0.16 , CRE, 0.0 ± 0.18 and Spry1, 0.82 ± 0.15 , arbitrary units). **b** Representative western blot showing an increase in p27 expression in HUVECs following up-regulation of Spry1, $n = 3$. Quantification of p27 protein levels via densitometry were Ad-control, 1.00 ± 0.02 , and Ad-Spry1, 1.94 ± 0.07 , arbitrary units)

Fig. 4.

The identification of multiple hypoxia responsive elements within the Spry1 gene. Using a one base degenerate sequence, the program FUZZNUC in EMBOSS identified multiple hypoxia responsive elements upstream of the promoter region of Spry1. Gray box and gray rectangle refer to coding sequence. *HIF* Hypoxia inducible factor

Fig. 5. Hypoxia induces Spry1 transcription in a time-dependent fashion. HUVECs were subjected to hypoxia (0.2% oxygen) or normoxia for 4, 8, 12, and 20 h. Real-time PCR quantification was performed using primer probe sets specific for Spry1 ($n = 8$, $P < 0.01$ normoxia vs. hypoxia for the specified time-point)

Table 1

SuperArray real-time PCR analysis of HUVECs infected with Ad-Lac Z or Ad-Spry1

Gene Description	Gene symbol	Fold change	SD
Transforming growth factor, alpha	TGFA	9.22	2.26
Transforming growth factor, beta 2	TGFB2	4.81	2.71
Chemokine (C-X-C motif) ligand 10	CXCL10	3.82	1.08
Tumor necrosis factor (TNF superfamily, member 2)	TNF	2.8	0.25
Fibroblast growth factor 1 (acidic)	FGF1	2.69	0.45
Chemokine (C-X-C motif) ligand 5	CXCL5	2.54	0.27
Chemokine (C-X-C motif) ligand 3	CXCL3	1.86	0.29
Serpin peptidase inhibitor, clade F (prostaglandin G/H synthase and cyclooxygenase)	SERPINF1	1.63	0.6
Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	FLT1	-1.82	0.25
Placental growth factor, vascular endothelial growth factor-related protein)	PGF	-2.69	0.72
Angiopoietin 2	ANGPT2	-5.83	1.22

The fold change presented is in Ad-Spry1 cells compared to control infected cells ($n = 2$)