

## Identification and regulation of Sprouty1, a negative inhibitor of the ERK cascade, in the human heart

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**Huebert, Robert C., Qinglu Li, Neeta Adhikari, Nathan J. Charles, Xinqiang Han, Mohammed-Karim Ezzat, Suzanne Grindle, Soon Park, Sofia Ormaza, David Fermin, Leslie W. Miller, and Jennifer L. Hall.** Identification and regulation of Sprouty1, a negative inhibitor of the ERK cascade, in the human heart. *Physiol Genomics* 18: 284–289, 2004; 10.1152/physiolgenomics.00098.2004.—We screened a compendium of gene profiles from 19 paired human heart samples harvested at the time of implant and explant of a left ventricular assist device (LVAD) for novel genes regulating the Ras/MEK/ERK cascade. From this analysis we identified Sprouty1, an evolutionally conserved gene that acts as an intrinsic inhibitor of the Ras/MEK/ERK pathway. Sprouty1 mRNA and protein were significantly upregulated in the heart in response to mechanical unloading with a LVAD. The upregulation of Sprouty1 in the heart following mechanical unloading was accompanied by a significant decrease in phosphorylated ERK1/2. Gain of function experiments demonstrated that upregulation of Sprouty1 in isolated cardiac myocytes led to a significant decrease and altered kinetics of ERK1/2 phosphorylation. Immunohistochemistry of human hearts revealed that Sprouty1 was also expressed in the microvasculature. Upregulation of Sprouty1 in endothelial cells led to a significant decrease in VEGF-induced endothelial cell proliferation. To our knowledge, these findings are the first to define Sprouty expression in the heart and suggest that Sprouty1 may serve as an intrinsic mediator governing ventricular remodeling through a coordinated coupling of both myocyte and vascular alterations in response to mechanical load.

heart failure; remodeling; genes; molecular biology; signal transduction

CONGESTIVE HEART FAILURE is newly diagnosed in over 500,000 Americans each year and contributes to more than 250,000 deaths annually (18). The progression to heart failure is normally marked by remodeling of the ventricle characterized by an increase in chamber size, wall thickness, apoptosis, interstitial fibrosis, and a reduction in contractility and compliance (20). Recent evidence suggests that a mechanical ventricular assist device significantly reduces myocardial wall stress and decreases mortality in patients with refractory heart failure (21, 29). The near complete unloading of the failing ventricle and significant reduction in myocyte size in response to a ventricular assist device allows an opportunity to extend our current understanding of the critical genes governing myocardial re-

modeling in response to alterations in mechanical stretch and strain.

Early work by Sadoshima and Izumo (22, 23) demonstrated that cell stretch rapidly induced p21Ras. These findings were followed by the generation of a transgenic mouse model in which constitutive activation of human Ha-Ras led to myocyte hypertrophy and diastolic dysfunction (15). Activation of Ras stimulates the Ras/MEK/ERK pathway in cardiac myocytes and leads to cardiac hypertrophy (2, 3, 5, 13, 15, 22–24, 26, 28, 31). The majority of studies have demonstrated that ERK1/2 is activated by agonists and stretching (2, 22, 23). Glennon et al. (8) demonstrated that depletion of ERK using an antisense oligonucleotide approach led to a downregulation of phenylephrine-induced hypertrophy. Furthermore, a transgenic model harboring heart-specific upregulation of an activated MEK1 transgene led to concentric hypertrophy and augmented cardiac function (3).

The objective of this paper was to screen a small compendium of gene profiles from paired human heart samples harvested at the time of left ventricular assist device (LVAD) implant and explant for genes regulating Ras/ERK signaling. Utilizing this approach, we identified Sprouty1, a novel inhibitor of Ras/ERK signaling in the heart. Sprouty1 was significantly upregulated following mechanical unloading with a LVAD in the failing human heart in parallel with a coordinate inhibition of ERK1/2 phosphorylation. Gain of function experiments demonstrated that Sprouty1 inhibited ERK phosphorylation in cardiac myocytes and reduced endothelial cell number. To our knowledge, this is the first evidence of Sprouty1 expression in the heart. Our data suggest that Sprouty1 may serve as an intrinsic mediator governing ventricular remodeling through a coordinated coupling of both myocyte and vascular alterations in response to mechanical load.

### MATERIALS AND METHODS

**Tissue collection.** Nineteen paired human left ventricular apex samples were collected at the time of LVAD implant (pre-LVAD) and at the time of LVAD explant and subsequent transplantation (post-LVAD). All tissue was immediately frozen in liquid nitrogen. IRB-approved informed consent was obtained from all participating patients.

**Sample preparation.** Total RNA was isolated using the TRIzol-chloroform-isopropanol technique. The RNA was further purified using the RNeasy Mini Protocol according to the manufacturer's directions (Qiagen) as we have described (4, 11, 14). Ten micrograms of total RNA was reverse transcribed utilizing a T7-(dT)<sub>24</sub> primer, and double-stranded cDNA synthesis was performed according to the manufacturer's directions (Invitrogen). Biotin-labeled RNA targets were obtained by using the Enzo BioArray High Yield RNA Transcript Labeling Kit according to the

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Table 1. Patient data

Unique ID	Age	Sex	Days of LVAD Support	LVEF, %	Medications
I-4	59	M	123	10	dopamine, epinephrine, Milrinone, heparin, vasopressin
I-7	60	M	134	30	dobutamine, antiarrhythmic, digoxin, beta-blocker, diuretic, Natricor
I-8	52	M	221	15	Milrinone, antiarrhythmic, ACE-I, digoxin, nitrates, diuretic
I-12	55	M	198	25	anticoagulant, ACE-I, diuretic, beta-blocker
I-13	68	M	106	15	diuretic, ACE-I, beta-blocker
IM-1	51	M	151	35	antiarrhythmic, dobutamine, dopamine, heparin,
IM-3	63	M	28	15	none
IM-4	46	M	301	30	dopamine, epinephrine
IM-5	50	M	681	20	heparin, diuretic, beta-blocker
IM-6	50	M	21	10	none
IM-7	59	M	57	23	dobutamine, antiarrhythmic, ACE-I, digoxin, beta-blocker, heparin, diuretic, vasodilator
N-2	38	F	40	NA	none
N-3	46	M	65	15	heparin, vasodilator
N-4	42	F	86	15	dopamine, Milrinone, antiarrhythmic, beta-blocker, diuretic, nitrate, aspirin
N-6	52	M	186	20	heparin, Milrinone, antiarrhythmic, ACE-I, digoxin, beta-blocker, diuretic, vasodilator, Prozac
N-7	41	M	6	15	Milrinone, antiarrhythmic, vasodilator
N-8	33	F	29	15	none
N-9	46	M	521	20	dobutamine, Milrinone, digoxin, beta-blocker, diuretic, angiotensin receptor blocker
N-22	54	F	39	15	none

M, male; F, female; NA, not available; LVAD, left ventricular assist device; I, ischemic; IM, acute myocardial infarction within 10 days of LVAD implant; N, nonischemic; LVEF, left ventricular ejection fraction obtained prior to LVAD implant.

manufacturer's directions (Enzo). Fragmented and biotin-labeled cRNA was then hybridized to the HG-U133A GeneChip (Affymetrix) as previously described (4, 11, 14).

**Microarray analysis.** All GeneChips were processed through Gene Expressionist (GeneData, Basel, Switzerland) utilizing a hierarchical clustering algorithm to assess similarity (4, 11, 14). Data are expressed as means  $\pm$  SE. Statistical analysis of Sproutyl expression pre- and post-LVAD was completed with significance analysis of microarrays (SAM) and a paired Student's *t*-test. All 38 expression data files have been uploaded for access on the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database with the following accession numbers: GSM14936 to GSM14973.

**Real-Time quantitative polymerase chain reaction.** Real-time quantitative PCR was used to further confirm and quantify the detected gene expression changes as we have previously described (27). Primers were specific for human Sproutyl (5'-TGGCAGTGGCAGTTCGTTAGTTGT-3' and 5'-TCCGGATTGCCCTTTCAGACC-3').

**Cell isolation and culture.** Cos cells (ATCC), bovine pulmonary aortic endothelial cells (CPAE; ATCC), and isolated neonatal murine cardiac myocytes were used in this study. In brief, myocytes were isolated by digesting myocardial tissue from 1- to 3-day old mice with multiple rounds of collagenase/pancreatin/ADS buffer treatment. Cells were spun at 1,000 rpm followed by resuspension in ADS buffer (116 mM NaCl, 20 mM HEPES, 0.87 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.5 mM glucose, 5.4 mM KCl, 0.83 mM MgSO<sub>4</sub>) prior to counting and viability assessment with trypan blue staining. Cells were plated in a plastic dish for 1 h to allow for fibroblast adherence, and the supernatant containing the myocytes was removed and plated onto laminin-coated culture plates. Approximately 500,000 myocytes are collected per neonatal mouse heart.

**Western blotting.** SDS-PAGE and Western blotting were performed as previously described (27) utilizing an antibody specific for Sproutyl (gift from Gerhard Christofori) (16) and pERK1/2 (Cell Signaling Technology).

**Immunohistochemistry.** Paraffin sections of human heart samples pre- and post-LVAD were deparaffinized with xylene and rehydrated with a graded ethanol series. After washing and quenching of endogenous peroxidase, the sections were blocked and incubated with rabbit primary antibody against Sproutyl or nonimmune rabbit IgG overnight at 4°C. The remaining steps were carried out using an immunoperoxidase detection procedure (Vectastain Elite ABC Kit; Vector Laboratories). The peroxidase was visualized by reaction with diami-

nobenzidine (DAB) and hydrogen peroxide (DAB substrate kit; Vector Laboratories). Slides were counterstained with hematoxylin, mounted, and visualized.

**Sproutyl upregulation.** Cos cells were transiently transfected with a Sproutyl expression vector (kind gift G. Guy) or an enhanced green fluorescent protein (eGFP) control vector with Effectene (Qiagen) as previously described (27). Isolated neonatal cardiac myocytes and CPAE cells were infected with Sproutyl or GFP adenovirus for 4 h in serum-free media after which the media was replaced with fresh growth medium. Twenty-four hours postinfection, myocytes were serum-starved for 4 h then treated with bFGF (20 ng/ml) or vehicle

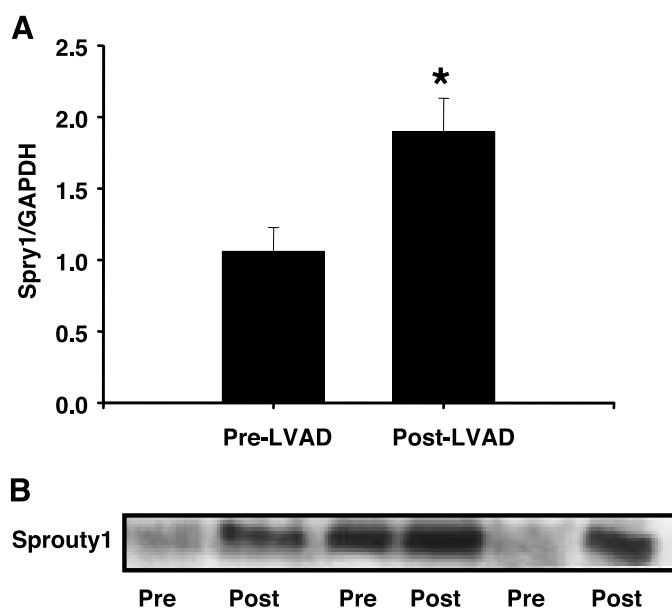


Fig. 1. A: Sproutyl (Spry1) mRNA is increased in the human heart following ventricular unloading with LVAD as measured by real-time quantitative PCR. Sproutyl expression was normalized to GAPDH (which was not significantly changed in response to left ventricular unloading) ( $n = 9$ ,  $P < 0.008$ ). B: representative Western blot demonstrating upregulation in Sproutyl protein expression following ventricular unloading with an LVAD in paired human heart samples.

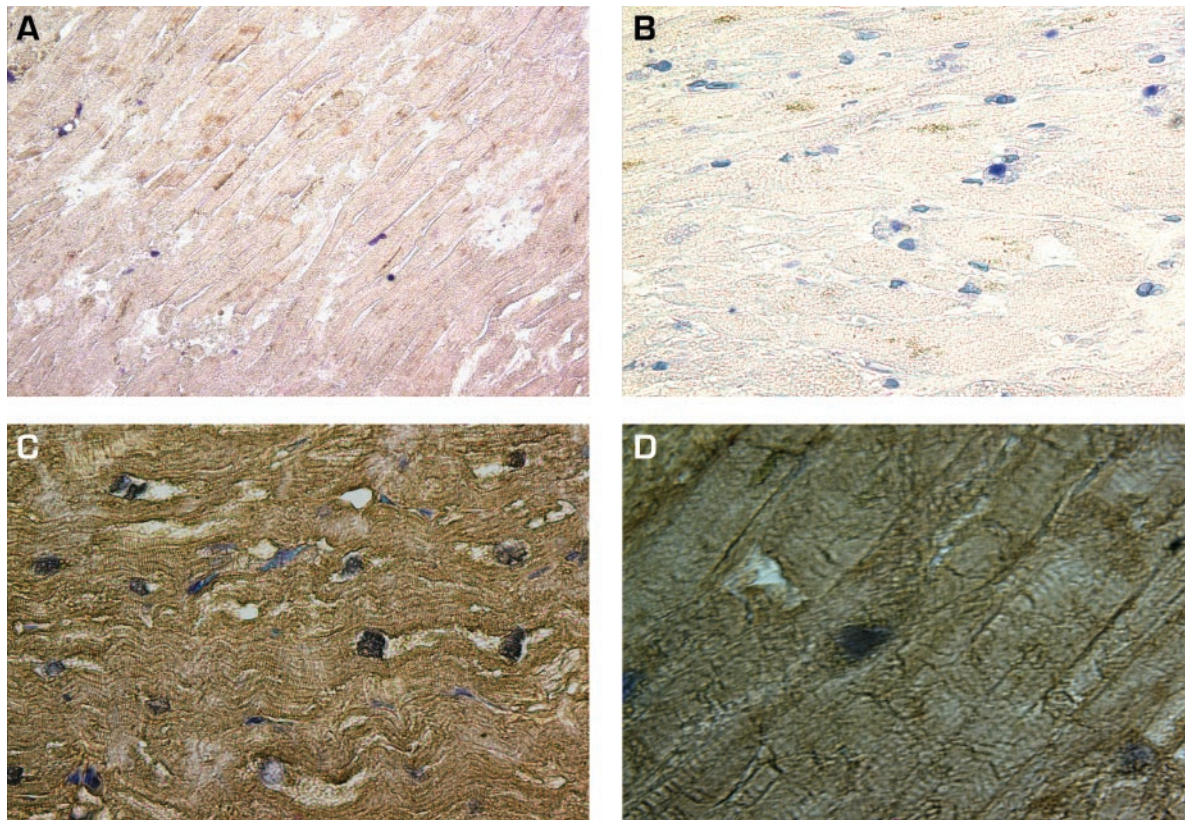


Fig. 2. Immunohistochemistry demonstrating expression of Sprouty1 in the human heart. Sections were cut from a left ventricular apex specimen (pre and post-LVAD) included in this study. *A*: Sprouty1 staining on pre-LVAD specimen ( $\times 40$ ). *B*: nonimmune IgG control ( $\times 40$ ). *C*: Sprouty1 staining on post-LVAD specimen ( $\times 40$ ). *D*: Sprouty1 staining on post-LVAD specimen ( $\times 60$ ).

and harvested at 0, 3, 10, and 30 min to assess pERK expression. For endothelial cell experiments, equal numbers of cells were plated into 24-well plates and infected with GFP or Sprouty1 adenovirus. Cell number was quantitated with a Coulter counter at *time 0* (24 h postinfection), at which time cells were placed in serum-free or serum-free + VEGF (25 ng/ml) containing media. Cell number was again determined at 18 and 24 h posttreatment. Viruses 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.html>) as previously described (1). The Sprouty1 adenovirus was created by ligating Sprouty1 cDNA into an Ad5 adenoviral shuttle vector containing a CMV promoter. A GFP adenovirus purchased from the Gene Vector Core Lab containing a CMV promoter was used as a control.

**Statistical analysis.** A paired Student's *t*-test was used to statistically analyze differences between two groups. Data are expressed as means  $\pm$  SE.

## RESULTS

Gene expression profiles from 19 paired human left ventricular apex samples taken at the time of LVAD implant and explantation were compiled from the HG-U133A Affymetrix GeneChip and screened for genes involved in the Ras/MEK/ERK signaling pathway. Patient characteristics are summarized in Table 1. Average age of the 19 patients was  $51 \pm 2$  yr, mean time on support was  $159 \pm 40$  days, and average ejection fraction was  $19 \pm 2\%$  (average  $\pm$  SE). Five of the 19 patients had clinical evidence of coronary artery disease and were classified as ischemic, 8 patients were classified as nonischemic (no evidence of coronary artery disease), and 6 patients

presented with an acute myocardial infarction within 10 days of LVAD implant. At the time of LVAD implant the majority of patients were on inotropic agents, intravenous vasodilators, digoxin, angiotensin converting enzyme inhibitor (ACE-I) or angiotensin receptor blocker, beta-blockers, and diuretics (Table 1).

In our search for novel genes regulating the Ras/MEK/ERK cascade, Sprouty1 was identified as statistically significant with both SAM (false-discovery rate less than 1%) and a paired Student's *t*-test ( $1.4 \pm 0.1$ -fold,  $P < 0.01$ ). This modest but significant and consistent upregulation of Sprouty1 was confirmed by real-time quantitative PCR (Fig. 1A). The upregulation of Sprouty1 in response to mechanical unloading was consistent in the nonischemic, ischemic, and ischemic-acute myocardial infarction cohorts, suggesting that regulation of Sprouty1 following ventricular unloading may act as a common node in the response of the failing myocyte to changes in stretch and strain.

Western blotting demonstrated that Sprouty1 protein expression was also increased following mechanical unloading of the heart (Fig. 1B). By immunohistochemistry, Sprouty1 was localized throughout the myofibril with more intense staining

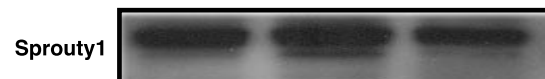


Fig. 3. Representative Western blot demonstrating Sprouty1 protein expression in 3 nonfailing unmatched human donor hearts.

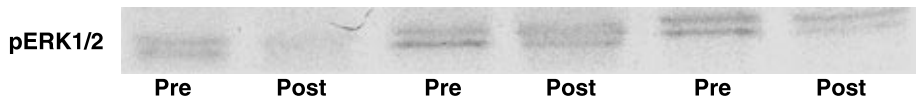


Fig. 4. Representative Western blots demonstrating a decrease in pERK1/2 protein expression in paired human heart samples following ventricular unloading.

surrounding the nucleus as well as in the microvasculature within the heart (Fig. 2). Sprouty1 was also expressed in nonfailing unmatched donor hearts (Fig. 3). Thus Sprouty1 is expressed in the myocyte and microvasculature and appears in both failing and nonfailing human hearts.

Based on previous work, we hypothesized that the upregulation in Sprouty1 following mechanical unloading would lead to a significant decrease in ERK activity. In line with our hypothesis, phosphorylated ERK1/2 was decreased in the human heart following mechanical unloading with the LVAD (Fig. 4). Thus, Sprouty1, a previously described ERK inhibitor, was significantly increased while phosphorylated ERK was decreased in post-LVAD samples compared with the paired pre-LVAD samples.

To test whether Sprouty1 upregulation directly inhibited ERK signaling, we transiently upregulated Sprouty1 in Cos cells and assessed pERK expression in response to bFGF. We chose to induce pERK with bFGF, since Sprouty1 was originally identified as an inhibitor of bFGF signaling in *Drosophila* (10). In line with previous work in other cell systems, upregulation of Sprouty1 led to a significant decrease and delay in pERK following bFGF treatment (Fig. 5). Sprouty1 also decreased baseline pERK expression. Taken together, these findings demonstrate that direct upregulation of Sprouty1 inhibits ERK activity. To extend these findings to a more physiologically relevant model, an adenoviral approach was used to upregulate Sprouty1 expression in isolated neonatal cardiac myocytes. As in the Cos experiments, the increase in Sprouty1 expression resulted in a loss of pERK at baseline and 3 min as well as at 30 min following stimulation with bFGF (Fig. 6).

Given that we observed Sprouty1 expression in the microvasculature of the human heart, we assessed the role of Sprouty1 in endothelial cells. Adenoviral upregulation of Sprouty1 in CPAE cells led to a significant (13–15%) decrease in endothelial cell number at 18 and 24 h in response to VEGF (Fig. 7). Sprouty1 statistically increased the percentage of apoptotic nuclei compared with GFP-infected cells under identical VEGF conditions (GFP,  $2 \pm 1\%$  apoptotic nuclei vs. Sprouty1,  $4 \pm 1\%$ ;  $n = 6$ ,  $P < 0.02$ ). However, the physiological significance of 2% vs. 4% apoptosis is likely to be minor. A fivefold increase in the titer resulted in a greater Sprouty1 inhibition of VEGF-induced endothelial cell number at 24 h ( $14 \pm 4\%$  inhibition at 18 h and  $22 \pm 2\%$  at 24 h;  $n = 6-8$ ,  $P < 0.02$ ).

## DISCUSSION

The purpose of this study was to identify novel Ras/MEK/ERK signaling molecules that may play a critical role in ventricular remodeling. Based on a genomic screen of 19 paired human heart samples, we identified Sprouty1, a highly conserved, negative inhibitor of Ras signaling. To our knowledge, this is the first evidence of Sprouty expression in the heart. The fact that Sprouty1 is significantly upregulated following mechanical unloading suggests that it may play a critical role in governing the regulation of the ERK signaling cascade in response to mechanical stress and strain in the heart.

Four mammalian genes (Sprouty1–4) have been identified that encode protein homologues of *Drosophila* Sprouty (6, 19) and have been further characterized as general inhibitors of the Ras/MEK/ERK signaling induced by EGF, FGF, and IGF pathways in human vascular smooth muscle, lung epithelium, fibroblasts, kidney epithelial cells, and PC12 cells (9, 12, 16, 25, 30). In line with these findings, we identified a significant increase in Sprouty1 in the heart following mechanical unloading with the LVAD that correlated with a significant loss of ERK phosphorylation. Our findings of decreased phosphorylated ERK1/2 in human hearts following ventricular unloading confirms earlier work by Flesch and colleagues (7).

We further demonstrated that direct upregulation of Sprouty1 in Cos or isolated neonatal cardiac myocytes results in a significant decline in ERK phosphorylation. Specifically, Sprouty1 decreased ERK phosphorylation at baseline and significantly dampened ERK activity in response to bFGF, inhibiting the early increase in phosphorylation as well as hastening the loss of the signal over time. A link between ERK activity and the regulation of myocyte size has been demonstrated by Bueno et al. (3) by showing that overexpression of MEK1 in a transgenic model is sufficient to induce concentric hypertrophy. Support for ERK in the regulation of myocyte size in the human may be best exemplified by work from Flesch et al. (7) demonstrating that loss of ERK activity correlates with a significant decrease in myocyte size in response to mechanical unloading with a VAD. Our data suggest that a mechanism governing the loss of ERK activity in the heart following mechanical unloading may be the upregulation of Sprouty1, an intrinsic evolutionally conserved inhibitor of ERK.

Sprouty was first identified as an inhibitor of *Drosophila* FGF receptor signaling during tracheal development (10). The

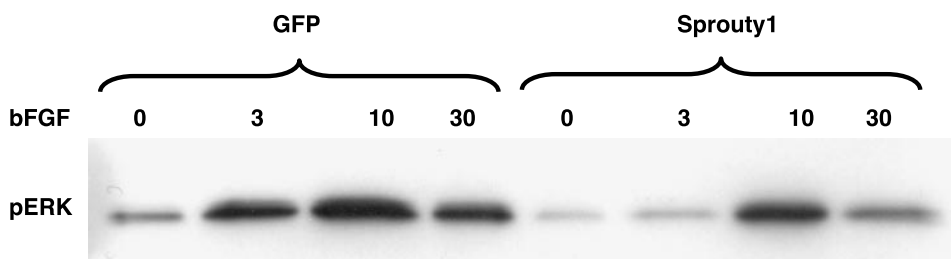
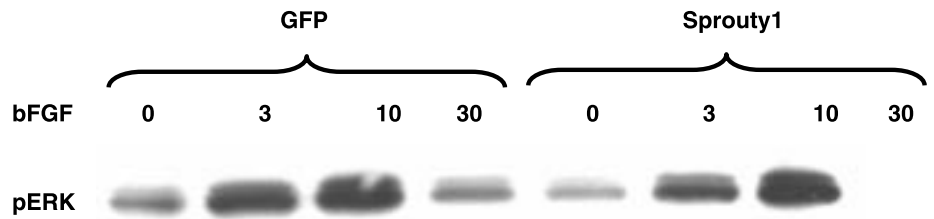


Fig. 5. Upregulation of Sprouty1 led to a decrease and delay in pERK1/2. Cos cells were transiently transfected with Sprouty1 or a control vector (GFP), treated with bFGF (20 ng/ml), and harvested at 0, 3, 10, and 30 min.

Fig. 6. Sprouty1 upregulation in isolated neonatal cardiac myocytes led to a decrease in Sprouty1 at baseline and in response to bFGF. Myocytes were infected with Sprouty1 or GFP adenovirus (100 pfu/cell) 24 h prior to treatment with bFGF (20 ng/ml) and harvested at 0, 3, 10, and 30 min.



molecular regulation of branching of the trachea in *Drosophila* (which possesses an open circulatory system) shares many similarities with genes that govern angiogenesis in higher organisms. Lee et al. (17) demonstrated that upregulation of Sprouty4 in vivo inhibited branching and sprouting of small vessels and also significantly decreased VEGF-induced endothelial cell proliferation. Our findings demonstrate that upregulation of Sprouty1 also inhibits VEGF-induced endothelial cell proliferation. These findings would suggest that Sprouty1 may govern ventricular remodeling through the coordinated regulation of both myocyte size and vascular networks.

In conclusion, these findings represent the first evidence of Sprouty1 in the heart. Furthermore, this is the first evidence that Sprouty is regulated by mechanical stretch and strain. Considered in the context of previous findings, our data suggest that Sprouty1 may play an integral role as a critical intrinsic mediator governing the regulation of the ERK cascade in response to alterations in workload in the heart.

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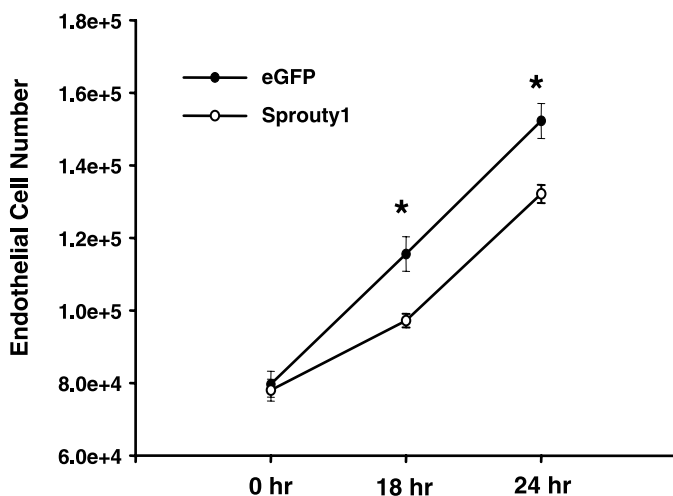


Fig. 7. Sprouty1 upregulation in endothelial cells results in a significant decrease in cell number at 18 and 24 h. Equal numbers of cells were plated into 24 well plates and infected with GFP or Sprouty1 adenovirus (1,000 pfu/cell). Cell number was assessed with a Coulter counter ( $n = 6-8$ ,  $*P < 0.002$ ).

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