Novel Cross-Talk between Three Cardiovascular Regulators: Thrombin Cleavage Fragment of Jagged1 Induces Fibroblast Growth Factor 1 Expression and Release

Maria Duarte,^{*†} Vihren Kolev,^{*‡} Doreen Kacer,^{*} Carla Mouta-Bellum,^{*} Raffaella Soldi,^{*§} Irene Graziani,^{*||} Aleksandr Kirov,^{*} Robert Friesel,^{*} Lucy Liaw,^{*} Deena Small,^{||} Joseph Verdi,^{*} Thomas Maciag,^{*} and Igor Prudovsky^{*}

^{*}Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, ME 04074; and

^{||}Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH 03824

Submitted December 12, 2007; Revised July 23, 2008; Accepted August 29, 2008

Monitoring Editor: J. Silvio Gutkind

Angiogenesis is controlled by several regulatory mechanisms, including the Notch and fibroblast growth factor (FGF) signaling pathways. FGF1, a prototype member of FGF family, lacks a signal peptide and is released through an endoplasmic reticulum–Golgi-independent mechanism. A soluble extracellular domain of the Notch ligand Jagged1 (sj1) inhibits Notch signaling and induces FGF1 release. Thrombin, a key protease of the blood coagulation cascade and a potent inducer of angiogenesis, stimulates rapid FGF1 release through a mechanism dependent on the major thrombin receptor protease-activated receptor (PAR) 1. This study demonstrates that thrombin cleaves Jagged1 in its extracellular domain. The sj1 form produced as a result of thrombin cleavage inhibits Notch-mediated CBF1/Suppressor of Hairless [(Su(H))/Lag-1–dependent transcription and induces FGF1 expression and release. The overexpression of Jagged1 in PAR1 null cells results in a rapid thrombin-induced export of FGF1. These data demonstrate the existence of novel cross-talk between thrombin, FGF, and Notch signaling pathways, which play important roles in vascular formation and remodeling.

INTRODUCTION

Fibroblast growth factor (FGF) family members exhibit a variety of biological activities. During embryogenesis, they regulate mesoderm induction, neurulation, and formation of the circulatory and skeletal systems (Fallon *et al.*, 1994; Friesel and Maciag, 1999). During postnatal development, they play a crucial role in angiogenesis, tissue regeneration, inflammation, and pathogenesis of some tumors (Christofori

and Luef, 1997; Friesel and Maciag, 1999; Woolley *et al.*, 2000; Javerzat *et al.*, 2002; Okunieff *et al.*, 2003). The biological effects of FGFs are mediated through the activation of four transmembrane phosphotyrosine kinase receptors (FGFR1–4) (McKeehan *et al.*, 1998), with the participation of cell surface proteoglycans (Ornitz and Itoh, 2001), and consequently require FGF release. FGF1 is a potent proangiogenic factor, which supports the survival of endothelial cells in vitro, stimulates the growth of vessels, and enhances the repair of infarctic lesions in vivo (Sellke *et al.*, 1996; Schumacher *et al.*, 1998; Friesel and Maciag, 1999; Buehler *et al.*, 2002). FGF1 lacks a signal peptide in its primary structure (Friesel and Maciag, 1999), and, similar to other signal peptide-less extracellular regulatory proteins, it undergoes nonclassical export (for review, see Prudovsky *et al.*, 2003; Nickel, 2005; Prudovsky *et al.*, 2008).

Thrombin, the key protease of the blood coagulation cascade, induces the expression of several growth factors (Basus *et al.*, 2001; Cucina *et al.*, 2002; Cao *et al.*, 2006) and exhibits proangiogenic activity (Steinhoff *et al.*, 2005). We recently demonstrated that thrombin, through activation of the protease-activated receptor (PAR) 1, rapidly induces FGF1 expression and its release under nonstress conditions, revealing an interplay between thrombin signaling and nonclassical FGF1 release (Duarte *et al.*, 2006).

PAR1 is activated through the proteolytic mechanism in which thrombin binds and cleaves the amino-terminal domain of the receptor (Vu *et al.*, 1991). Activated PAR1 molecules are rapidly internalized, which results in the desensitization of cells to continued thrombin stimulation (Paing *et al.*, 2002). Surprisingly, we observed that FGF1 release into

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-12-1237>) on September 10, 2008.

Present addresses: [†] Alentejo Biotechnology Center (CEBAL), Edifício NERBE, 7800-904 Beja, Portugal; [‡] Cutaneous Biology Research Center, Harvard Medical School and Massachusetts General Hospital, Charlestown, MA 02129; [§] Department of Pharmacology and Toxicology, University of Utah, Salt Lake City, UT 84112; ^{||} Department of Clinical Care Medicine and Surgery, University of Florence, Florence 50139, Italy.

Address correspondence to: Igor Prudovsky (prudoi@mmc.org).

Abbreviations used: caN1, constitutively active Notch1; CL, cell lysate(s); CSL, CBF1/Suppressor of Hairless [(Su(H))/Lag-1; EGF, epidermal growth factor; DSL, Delta/Serrate/Lag-2; FGF, fibroblast growth factor; FLJ1, full-length Jagged1; dnFGFR1, dominant-negative form of fibroblast growth factor receptor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEF, mouse embryo fibroblast; NCSC, neural crest stem cell; PAGE, polyacrylamide gel electrophoresis; PAR, protease-activated receptor; PCR, polymerase chain reaction; RT, reverse transcription; sj1, soluble Jagged1; TRAP, thrombin receptor-activated peptide.

the conditioned medium upon thrombin treatment did not decrease over time, suggesting that when PAR1 receptors get desensitized, thrombin may continue to induce FGF1 export through another mechanism(s) that is independent of PAR1 activation (Duarte *et al.*, 2006).

We reported previously that the expression of a soluble nontransmembrane form of the Notch ligand Jagged1, an 117 kDa soluble Jagged1 (sJ1 117 kDa), induced nonclassical release of FGF1 under nonstress conditions mediated by the inhibition of Notch signaling (Small *et al.*, 2003). We hypothesized that thrombin could cleave Jagged1 and produce sJ1, thus stimulating the export of FGF1 into the extracellular compartment after the desensitization of PAR1 population. This hypothesis is based on the following data: 1) premature truncations, leading to the production of nontransmembrane forms of human Jagged1, result in Alagille syndrome, a disease characterized by spontaneous bleeding, congenital heart defects, and pulmonary stenosis (Joutel and Tournier-Lasserre, 1998); 2) Jagged1 null mice hemorrhage at days 11–12 of embryonic development (Hrabe de Angelis *et al.*, 1997; Xue *et al.*, 1999); 3) Jagged1 is an FGF response gene in human endothelial cells undergoing differentiation on fibrin clots (Zimrin *et al.*, 1995, 1996); and 4) the enzymatic activity of thrombin was implicated in tissue repair (Fenton *et al.*, 1998) and angiogenesis (Herbert *et al.*, 1994). We questioned whether thrombin cleaves Jagged1, and, if so, what are the effects of the resultant cleavage fragment(s) on FGF1 expression and export. We found that thrombin mediates the production of a short extracellular form of Jagged1 (sJ1 39 kDa) that inhibited CBF1/Suppressor of Hairless [(Su(H))/Lag-1 (CSL)-mediated transcription of Notch signaling and induced FGF1 expression and release. Moreover, the overexpression of Jagged1 resulted in rapid thrombin-induced FGF1 release from PAR1 null cells. These results suggest the existence of novel cross-talk between the FGF, Notch, and thrombin signaling pathways; all three important in angiogenesis and tissue repair.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Transfection

NIH 3T3 cells (American Type Culture Collection, Manassas, VA), FGF1R136K NIH 3T3, and full-length Jagged1 (FLJ1) NIH 3T3 transfectants were maintained as described previously (Small *et al.*, 2001; Duarte *et al.*, 2006). PAR1 null mouse embryonic fibroblasts (MEFs) and PAR1 MEFs transfected with PAR1 (gifts from S. Coughlin, University of California, San Francisco, CA), human embryonic kidney (HEK) 293 cells (American Type Culture Collection), and bEnd.3 cells (American Type Culture Collection) were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). All cells used in FGF1 release experiments were grown on human fibronectin-coated (10 $\mu\text{g}/\text{cm}^2$) dishes, as described previously (Jackson *et al.*, 1992).

The N-terminal V5-tagged full-length Jagged1 (FLJ1NV5) construct was obtained by cloning the complete human Jagged1 open reading frame (ORF) into BamHI and XhoI restriction sites of pcDNA3.1/Hygro(+) (Invitrogen). The V5-His tag was excised from the pcDNA4/V5-His vector and inserted into the FLJ1-pcDNA3.1/Hygro(+) between the signal peptide and the Delta, Serrate, Lag-2 (DSL) domain of FLJ1, originating FLJ1NV5. For this purpose, the two new restriction sites, NotI and EcoRI, were introduced in FLJ1-pcDNA3.1/Hygro(+) construct by polymerase chain reaction (PCR) mutagenesis. sJ1 39 kDa was obtained from the N-terminally V5-His-tagged FLJ1-pcDNA3.1/Hygro(+) construct by insertion of a stop codon at position 349, followed by a PmeI restriction site that was used to clone the fragment back into the pcDNA3.1/Hygro(+) vector. A point mutation resulting in the change of arginine in position 348 to lysine was introduced into FLJ1NV5 resulting in thrombin uncleavable FLJ1NV5 R348K mutant. A deletion mutant of J1 corresponding to its first 229 amino acids (sJ1 DSL), containing the signal peptide, DSL domain and devoid of all epidermal growth factor (EGF)-like repeats, was produced by introducing a stop codon after the first 687 base pairs of human Jagged1 in the N-terminally Myc-tagged sJ1 117-kDa construct (Wong *et al.*, 2000) and subsequently cloned in the pCS2 vector. All mutagenesis reactions were performed using the QuikChange site-direct mu-

tagenesis kit (Stratagene, La Jolla, CA), and the sequences were confirmed by DNA sequencing.

NIH 3T3 cells transfectants expressing sJ1 39 kDa and insert-less control vector pcDNA3.1/Hygro(+) were generated by using the FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN), and selected by using 200 $\mu\text{g}/\text{ml}$ hygromycin (Roche Diagnostics). Transfectants were screened for gene expression by using an anti-V5 antibody (Invitrogen). The genomic incorporation of insert-less control vector pcDNA3.1/Hygro(+) was screened by PCR. All experiments involving transient cell transfections were performed by using the FuGENE 6 reagent.

Construction, Production, and Transduction of FLJ1NV5 and sJ1 39-kDa Adenoviruses

A cDNA insert encoding FLJ1NV5 3740 base pairs and sJ1 39-kDa 1069 base pairs was excised from pcDNA3.1/Hygro(+) and cloned in the BamHI and SmaI sites of pAdlox shuttle vector. Recombinant adenoviruses were produced, purified, and titrated as described previously (Hardy *et al.*, 1997). Briefly, CRE8 cells were transfected with SfiI-digested FLJ1NV5 and sJ1 39-kDa pAdlox DNA and infected with the ϕ 5 virus. Lysates were prepared 2 d after infection. Viruses were passed twice through CRE8 cells and purified from the second passage by using a cesium density gradient. The viruses were quantified by optical density at 260-nm readings, and the bioactivity was determined by the plaque-forming unit assay.

The adenoviral transduction was performed in serum-free DMEM with $\sim 10^3$ viral particles/cell in the presence of poly-D-lysine hydrobromide (Sigma-Aldrich, St. Louis, MO) (5×10^3 molecules/viral particle) for 2 h at 37°C. Then, the adenovirus-containing media were removed and replaced with serum-containing medium. The cells were plated for experiments 24–48 h after transduction. FGF1R136K, β -galactosidase, and the constitutively active form of Notch1 (caN1) pAdlox shuttle vector constructs have been described previously (Small *et al.*, 2003; Duarte *et al.*, 2006). The efficiency of transduction for FLJ1NV5 and caN1 was assessed by immunofluorescence using an anti-V5 monoclonal antibody (Invitrogen). In addition, a dominant-negative (dn) form of *Xenopus laevis* FGFR1 (Neilson and Friesel, 1996) was cloned in the pAdlox shuttle vector for adenovirus-mediated cell transduction. Its expression was assessed by immunofluorescence using rabbit anti-FGFR1 antibodies (Neilson and Friesel, 1996).

The Cell-free Translation of Jagged1, Thrombin Cleavage, and Automated Edman Microsequencing

A plasmid containing FLJ1 (Zimrin *et al.*, 1996) was transcribed and translated in vitro in the presence of a [^{35}S]Met/Cys protein-labeling mixture (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), by using the T7-coupled reticulocyte lysate system according to the manufacturer's instructions (Promega, Madison, WI) in a total volume of 50 μl . After 60 min of incubation at 30°C, the reaction was stopped by the addition of 0.05% dithiothreitol. Half of the reaction mixture was incubated with 1 U of thrombin (Sigma-Aldrich) for 15 min at 37°C, and the reaction was stopped by boiling in the presence of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The samples were resolved by 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by autoradiography. The bands corresponding to the thrombin cleavage products were excised and subjected to automated Edman microsequencing (Applied Biosciences; Protein, Nucleic Acid, and Cell Imaging Core, Maine Medical Center Research Institute, Scarborough, ME). The products of each cycle were collected before resolution by high-pressure liquid chromatography, added to liquid scintillation fluid (Beckman Coulter, Fullerton, CA), and the ^{35}S samples were quantified by liquid scintillation spectroscopy (Beckman Coulter).

Cleavage of Jagged1 Expressed on the Cell Surface, and Immunoprecipitation of Soluble Jagged1 from Conditioned Medium

HEK 293 or PAR1 null cells were transduced with the FLJ1NV5 adenovirus. Control cells were transfected with the adenovirus expressing β -galactosidase. Forty-eight hours after transduction, the cells were washed with DMEM and incubated with 1 U/ml thrombin for 1 h in serum-free medium at 37°C. In control dishes, the cells were incubated with or without 1 U/ml thrombin in the presence of hirudin (Sigma-Aldrich) at a final concentration of 5 U/ml or in the presence of protease inhibitor cocktail (Sigma-Aldrich). Conditioned media were collected and concentrated by Centricon devices (Millipore, Billerica, MA). Then, 1 ml of conditioned medium was immunoprecipitated with 1 μg of rabbit anti-V5 antibody overnight at 4°C, followed by incubation for 2 h at 4°C with 50 μl of 50% (wt/wt) protein A-Sepharose beads (GE Healthcare). After repeated washes with cell lysis buffer, the immunoprecipitated proteins were separated on 12% SDS-PAGE and immunoblotted using the mouse anti-V5 antibody.

Dual-Luciferase Reporter Assay of CSL-dependent Transcription

Nontransfected NIH 3T3 cells, insert-less vector control, sJ1 117-kDa (Small *et al.*, 2003) and sJ1 39-kDa NIH 3T3 cell transfectants were plated on fibronectin-

tin-coated ($10 \mu\text{g}/\text{cm}^2$) cell culture dishes at $\sim 50\%$ confluence and transiently cotransfected using FuGENE 6 reagent with 500 ng of a luciferase construct

driven by four tandem copies of the CBF1 response element. Cotransfection with 100 ng of the TK *Renilla* (Promega) construct was used as internal

Figure 1. Thrombin cleaves Jagged1. (A) In vitro translated human Jagged1 is cleaved by thrombin. The FLJ1 transcript was in vitro translated in the presence of a [^{35}S]Met/Cys mixture, and the translation product was incubated with or without thrombin (1 U/reaction) for 15 min at 37°C. The reaction products were visualized by autoradiography. (B) Schematic diagram of Jagged1, FLJ1NV5, sJ1 39 kDa, and sJ1 DSL. First panel, schematic diagram of full-length Jagged1 and the position of the thrombin cleavage site. The Jagged1 amino acid sequence between residues 345 and 364 in context with the thrombin cleavage site (arrow) between the third and fourth EGF repeats is presented. Asterisks identify Cys residues used in the identification of the NH₂-terminal thrombin cleavage product by automated Edman sequencing of the [^{35}S]Cys/Met-labeled Jagged1 translation product. Second panel, The FLJ1NV5 construct sequence showing the V5 tag insert between the signal peptide and the DSL domain of the Jagged1. Third panel, FLJ1NV5 construct with the R348K mutation. Fourth panel, sJ1 39-kDa deletion mutant corresponding to the thrombin cleavage product of the human Jagged1. Fifth panel, sJ1 DSL mutant. Domains are as follows: ■, signal peptide; ▨, DSL domain; □, EGF repeat domain; ▩, Cys-Rich (CR) domain; and ▤, transmembrane.

(C) Automated Edman protein sequencing combined with liquid scintillation spectroscopy. Amount of ^{35}S measured by liquid scintillation spectroscopy in high-performance liquid chromatography fractions of the N-terminal 95-kDa excised fragment. Each cycle analyzed is indicated by numbers. (D) Thrombin cleaves Jagged1 expressed in HEK 293 cells. HEK 293 cells were transfected with the FLJ1NV5 adenovirus. Forty-eight hours after transfection, the cells were treated either with thrombin, thrombin

plus hirudin, or thrombin plus protease inhibitor cocktail for 1 h at 37°C. Control FLJ1NV5-transduced cells were incubated in serum-free media without thrombin. Cleaved Jagged1 was immunoprecipitated from the conditioned medium by using anti-V5 antibodies. Immunoprecipitated 39-kDa Jagged1 fragment was visualized, using anti-V5 immunoblotting. The corresponding cell lysates (CL) from the FLJ1NV5 transfectant HEK 293 cells are shown (top).

control for transfection efficiency. In additional experiments, stable FLJ1 NIH 3T3 cell transfectants were treated with or without 1, 2, or 4 U/ml thrombin (Sigma-Aldrich) or 10, 20, or 40 nM thrombin receptor-activated peptide (TRAP; Sigma-Aldrich) for 12 h before and 48 h after transfection. Forty-eight hours after transfection, the cells were harvested, and the luciferase/*Renilla* activity measured by utilizing the Dual Luciferase Reporter Assay System (Promega). Each experiment was performed in triplicate.

Reverse Transcription (RT)-PCR and Real-Time RT-PCR Analysis

RT-PCR was performed with total RNA isolated, using the RNeasy kit (QIAGEN, Valencia, CA) from PAR1 null MEFs, insert-less vector control, sj1 117 kDa (Small *et al.*, 2001), and sj1 39 kDa NIH 3T3 cell transfectants, as well as from NIH 3T3 cells adenovirally transduced with β -galactosidase or caN1. Total RNA (1 μ g) was used as a template for the PCR reaction performed with the Platinum Tap One Step RT-PCR kit (Invitrogen). The following PCR primers were used: *Jagged1*: forward 5'-GGCGGCTGGGAAGGAA-CAAC-3' and reverse 5'-TCACCGGCTGGAGACTGGAAG-3'; *fgf1*: forward 5'-ATGGCTGAAGGGGAGATCACAACC-3' and reverse 5'-CGCGCTTA-CAGCTCCCGTTC-3', originating 620-base pair and 578-base pair products, respectively. RT-PCR was performed with 1 μ g of RNA, using the Platinum Taq One Step RT-PCR kit (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) or β -actin expression served as a control for RNA loading. The amplification products were visualized by 1.5% agarose gel electrophoresis. Real-time PCR was performed using the Icyler IQ real-time PCR (Bio-Rad, Hercules, CA), according to the manufacturer's recommendations. Amplification of the *gapdh* or β -actin cDNA was used as the endogenous normalization standard. Each sample was amplified in triplicate.

Heat Shock and Thrombin/TRAP Stimulation Assays, and Immunoblot Analysis of FGF1 Release

The heat shock-induced FGF1 release assay was performed by incubation of cells at 42°C for 110 min in serum-free DMEM containing 5 U/ml heparin (Sigma-Aldrich), as described previously (Jackson *et al.*, 1992).

Control cultures were incubated at 37°C for the same time. Thrombin or TRAP stimulation experiments were performed by incubation of cells at 37°C, for different times, in the presence of 1 U/ml (equivalent to 10 nM) thrombin (Sigma-Aldrich) or 5.7 μ M TRAP (Sigma-Aldrich). Control cells were incubated in the absence of thrombin or TRAP for the same times. Furthermore, conditioned media were collected, filtered, and FGF1 was isolated for immunoblot analysis by using heparin-Sepharose chromatography. In both heat shock or thrombin/TRAP stimulation experiments, cell viability was assessed by measuring lactate dehydrogenase activity in conditioned medium after filtration (Bergmeyer, 1965; LaVallee *et al.*, 1998). Densitometric analysis of the Western blots was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Testing the Thrombin Effect on NCSC Multipotentiality

Flow cytometry was used to purify and enrich murine neural crest stem cells (NCSCs) from the sciatic nerve of embryonic day 12–13 mice by staining cell suspension with anti-p75 and anti- α 3 integrin antibodies (Morrison *et al.*, 1999). Double-positive cells were placed in nontissue culture plastic plates with Morrison media (Morrison *et al.*, 1999), with or without 1 U/ml thrombin or 7.5 nM recombinant sj1 (gift of M. Bhatia, McMaster University). The expanding spheres were visible 48 h later and allowed to expand for seven full days, and the media were refreshed every other day. On the seventh day, the cells were adenovirally transduced with dnFGFR or β -galactosidase. Then, individual colonies were isolated with a sterile Pasteur pipette and trypsinized. NCSCs were plated on poly-D-lysine laminin matrix on tissue culture plastic Nunc plates in Morrison media. The differentiation was allowed to continue for seven additional days. At the end of the incubation period, the arising secondary clones were fixed in the mixture of acetic acid and ethanol (1:1) and stained for markers of neural crest differentiation (nestin to identify stem cells; glial fibrillary acidic protein to identify glial cells, peripherin to identify neurons; and smooth muscle actin to identify myofibroblasts), as described previously (Nikopoulos *et al.*, 2007). The clones were scored for tripotential differentiation.

Figure 2. The Jagged1 thrombin cleavage product (39-kDa fragment) inhibits CSL-mediated transcription. (A) sj1 39 kDa decreases the CSL-mediated transcription in NIH 3T3 transfectants. Vector control, FLJ1NV5 and sj1 117-kDa, and sj1 39-kDa NIH 3T3 stable transfectants were transiently cotransfected with luciferase construct driven by four copies of CBF1 response element and *Renilla* construct. The assessment of CSL-mediated transcription was performed, as described in *Materials and Methods*. *Renilla* activity served as internal control for transfection efficiency. The data represent the normalized ratio of luciferase to *Renilla* activity $\pm$ SEM. (B) Coexpression of FLJ1 attenuates the negative effect of sj1 39 kDa on CSL-mediated transcription. sj1 39-kDa NIH 3T3 stable transfectants were transiently cotransfected with luciferase construct driven by four copies of CBF1 response element, *Renilla* construct, and transduced with the FLJ1 or β -galactosidase (control) adenoviruses (0.5, 5.0, or 10.0 μ l of 10^{12} plaque-forming units/ml virus suspension per 10-cm dish). The assessment of CSL-mediated transcription was performed, as described in *Materials and Methods*.

RESULTS

Thrombin Cleaves Jagged1

We demonstrated that the nontransmembrane form of Jagged1 (sJ1 117 kDa) repressed Notch-mediated CSL-dependent transcription and induced transcription and stress-independent release of FGF1 (Small *et al.*, 2003). In an attempt to further define this pathway, we sought to determine whether the Jagged1 translation product were susceptible to proteolytic cleavage by thrombin. Examination of human Jagged1 amino acid sequence revealed two evolutionary conserved putative thrombin cleavage sites within the extracellular domain of Jagged1 (R113 and R348). To assess Jagged1 as a thrombin substrate, FLJ1 was *in vitro* transcribed and translated in the presence of [³⁵S]Cys/Met mixture and the 134-kDa Jagged1 translation product incubated either with or without thrombin. Radiographic analysis of the reaction product revealed cleavage of FLJ1 protein into 95- and 39-kDa fragments (Figure 1A). Because the size of these fragments was consistent with Jagged1 cleavage between residues R348 and G349 (Figure 1B, top), which eliminated position R113/G114 from further consideration as a thrombin cleavage site, we sought to confirm the identity of the putative site. The Jagged1 transcript was translated *in vitro* in the presence of [³⁵S]Cys and resolved by SDS-PAGE. The 95-kDa band was excised and subjected to automated Edman chemistry. The product of each cycle was monitored by liquid scintillation spectroscopy. We observed [³⁵S]Cys radioactivity in cycles 2, 11, and 13 (Figure 1C), which agrees with the position of Cys at residues 351, 360, and 362, but not in the cycles corresponding to either residues 352 through 359 or in the cycles corresponding to residues 361 and 363 (Figure 1B). These analyses suggest that thrombin is able to cleave Jagged1 between arginine 348 (R348) and glycine 349 (G349) that are located between EGF repeats 3 and 4 (Shimizu *et al.*, 1999) in the extracellular portion of Jagged1. This cleavage yields an amino-terminal fragment with a molecular mass of approximately 39 kDa.

To verify that thrombin cleaves Jagged1 expressed in living cells, HEK 293 were transduced with a FLJ1NV5 adenoviral construct (Figure 1B, second panel) for 48 h, and transduced cells were used for thrombin treatment. After 1 h of treatment at 37°C with 1 U/ml thrombin, the serum-free

medium was collected, concentrated, and immunoprecipitated with the anti-V5 antibody, resolved by SDS-PAGE, and immunoblotted with the anti-V5 antibody. As shown in Figure 1D (bottom, lane 2), thrombin induced the cleavage and release into the medium of an N-terminal fragment of Jagged1 with the molecular mass of approximately 39 kDa. Thrombin-induced Jagged1 cleavage was completely blocked in the presence of hirudin, a highly specific thrombin inhibitor (Figure 1D, bottom, lane 3). Moreover, the appearance of sJ1 39 kDa in the conditioned medium, after thrombin incubation, was fully inhibited in the presence of a protease inhibitor cocktail (Figure 1D, bottom, lane 4).

sJ1 39-kDa Expression Results in the Inhibition of the CSL-dependent Transcription

The extracellular domain of Jagged1 is involved in receptor binding, and contains the N-terminal DSL domain and 16 tandem EGF-like repeats (Rebay *et al.*, 1991). Because thrombin cleaves Jagged1 between the third and fourth EGF repeat, we sought to evaluate the biological activity of the resulting soluble Notch ligand, particularly its ability to regulate Notch signaling. We prepared a construct coding for the product of thrombin-mediated cleavage of Jagged1, sJ1 39 kDa (Figure 1B, bottom). We reported previously that the ectopic expression of sJ1 117 kDa significantly diminished the CSL-mediated transcription (Small *et al.*, 2001). To determine whether sJ1 39 kDa carries the same capacity to decrease Notch signaling as does sJ1 117 kDa, which represents the whole extracellular domain of Jagged1 (Small *et al.*, 2001), we assayed vector control, FLJ1, sJ1 117 kDa, and sJ1 39 kDa NIH 3T3 stable transfectants for CSL-dependent transcription by using a luciferase reporter assay (Jarriault *et al.*, 1995; Hsieh *et al.*, 1996). Although FLJ1 transfectants exhibited an increase in CSL-mediated transcription, NIH 3T3 sJ1 39-kDa transfectants displayed a decrease of the CSL-dependent transcription (Figure 2A), which was similar to sJ1 117-kDa transfectants (Small *et al.*, 2001).

Does sJ1 39 kDa inhibit Notch signaling by acting in a dominant-negative manner? Thus, does it compete with FLJ1 for Notch binding? To answer this question, we assessed the effect of FLJ1 coexpression on CSL-dependent transcription in cells transfected with sJ1 39 kDa. We found a FLJ1 dose-dependent rescue of the CSL-dependent tran-

Figure 3. sJ1 39-kDa induces FGF1 expression and its release. (A) sJ1 39 kDa induces FGF1 expression. RT-PCR (top) and quantitative-RT-PCR (bottom) on total RNA extracted from untransfected NIH 3T3 cells, vector control, sJ1 117- and sJ1 39-kDa transfectants was performed to assay *fgf1* expression by using primers and conditions described in *Materials and Methods*. GAPDH served as mRNA loading control. (B) sJ1 39 kDa induces FGF1 release under normal growth conditions. Immunoblot analysis of FGF1 export into the extracellular compartment by vector control and sJ1 39-kDa stable NIH 3T3 transfectants transduced with FGF1 adenovirus for 48 h, and then subjected to heat shock (42°C) or maintained under normal growth conditions (37°C). CL from these cells is shown (left) (1/10 of total CL was loaded). Bar graphs represent the percentage of FGF1 released. Densitometric gel analysis was used to quantify FGF1 release. The densitometric values for different conditions were normalized for total FGF1 expression levels. Each bar represents the mean of the normalized FGF1 release $\pm$ SEM from three independent experiments.

scription in sJ1 39-kDa transfectants (Figure 2B). These results suggest that sJg1 39 kDa competes with FLJ1 for Notch binding and that its effect can be attenuated by the overexpression of FLJ1.

sJ1 39 kDa Induces FGF1 Expression and Release

Before this study, we reported (Small *et al.*, 2003) that suppression of endogenous Notch signaling mediated by ectopic expression of either sJ1 117 kDa or dominant-negative mutants of Notch1 or Notch2 resulted in prolonged FGFR stimulation caused by an increase in the expression of several FGF family members and the nonclassical export of FGF1 into the extracellular compartment (Small *et al.*, 2003). To further explore the biological activity of sJ1 39 kDa, we assessed by RT-PCR untransfected NIH 3T3 cells, vector-transfected control, and sJ1 117- and sJ1 39-kDa transfectant NIH 3T3 cells for the expression of *fgf1*. sJ1 117-kDa and sJ1 39-kDa transfectants expressed *fgf1*, whereas both untransfected and vector control-transfected cells did not (Figure 3A, top). Further quantitative RT-PCR analysis demonstrated that sJ1 39-kDa induced significantly higher *fgf1* mRNA levels than sJ1 117 kDa (Figure 3A, bottom). Given that FGF1 is expressed in the sJ1 39-kDa transfectants, we next examined these cells for FGF1 release.

Because expression of the FGF1 steady-state translation product is undetectable in the NIH 3T3 (Jackson *et al.*, 1992) cells and not sufficiently high in sJ1 39-kDa transfectants, we examined vector control and sJ1 39 kDa NIH 3T3 transfectants for the release of adenovirally transduced FGF1 under normal (37°C) and heat shock (42°C) conditions (Figure 3B). Thrombin can cleave FGF1; however, proteolytic cleavage of extracellular FGF1 can be prevented by its binding to heparan sulfate proteoglycans of the cell surface (Rosengart *et al.*, 1988). Thus, for better detection of FGF1 released into the medium, we are using in our experiments the thrombin uncleavable FGF1R136K mutant (Erzurum *et al.*, 2003; Duarte *et al.*, 2006). Both vector control and sJ1 39-kDa transfectants exported FGF1 in response to temperature stress (42°C), whereas FGF1 release under nonstress conditions (37°C) was only observed in the sJ1 39-kDa NIH 3T3 transfectants (Figure 3B). Thus, sJ1 39 kDa resulting from thrombin cleavage was not inferior to sJ1 117 kDa in its ability to repress Notch signaling and subsequently induce FGF1 expression and release.

Expression of the DSL Domain of Jagged1 Results in the Inhibition of CSL-dependent Transcription and the Induction of FGF1 Transcription and Export

sJ1 39-kDa contains the N-terminal DSL domain followed by three EGF repeats. During the interaction between Jagged1 and Notch, DSL presents the minimal binding unit, which is indispensable for Notch binding (Shimizu *et al.*, 1999). To assess the role of DSL in the biological effects of the thrombin cleavage product of Jagged1, we cloned a deletion mutant of Jagged1, which encompasses only the N-terminal DSL domain (sJ1 DSL) and does not contain EGF repeats. The luciferase assay (Figure 4A) demonstrated that the expression of sJ1 DSL in NIH 3T3 cells inhibits CSL-dependent transcription as efficiently as sJ1 39 kDa. Furthermore, expression of sJ1 DSL results in *fgf1* transcription (Figure 4B) and in stress-independent export of transfected FGF1 (Figure 4C). Thus, the biological activities of sJ1 DSL are similar to those of sJ1 117 kDa and sJ1 39 kDa.

Figure 4. sJ1 DSL attenuates CSL-dependent transcription and induces FGF1 transcription and export in NIH 3T3 cells. (A) sJ1 DSL decreases the CSL-mediated transcription in NIH 3T3 transfectants. Vector control, sJ1 39 kDa, or sJ1 DSL were transiently cotransfected to NIH 3T3 cells with luciferase construct driven by four copies of CBF1 response element and *Renilla* construct. The assessment of CSL-mediated transcription was performed, as described in *Materials and Methods*. *Renilla* activity served as internal control for transfection efficiency. The data represent the normalized ratio of luciferase to *Renilla* activity $\pm$ SEM. (B) sJ1 DSL induces FGF1 expression. RT-PCR on total RNA extracted from NIH 3T3 cells transfected with sJ1 DSL or vector control was performed to assay *fgf1* expression by using primers and conditions described in *Materials and Methods*. β -actin served as mRNA loading control. (C) sJ1 DSL induces FGF1 release under normal growth conditions. Immunoblot analysis of FGF1 export into the extracellular compartment by NIH 3T3 transiently cotransfected with FGF1 and sJ1 DSL or vector control and then subjected to heat shock (42°C) or maintained under normal growth conditions (37°C). CL from these cells is shown (left) (1/10 of total CL was loaded).

Thrombin but Not TRAP Attenuates CSL-dependent Transcription

Because sJ1 39-kDa cell transfectants display down-regulation of CSL-dependent transcription, we questioned whether thrombin and TRAP, an agonist peptide of PAR1 devoid of proteolytic activity, are able to attenuate CSL-dependent transcription. The

Figure 5. Thrombin treatment attenuates CSL-dependent transcription in Jagged1 NIH 3T3 cell transfectants. FLJ1 NIH 3T3 transfectants were treated with thrombin or TRAP for 12 h before and 48 h after luciferase and *Renilla* cotransfection. CSL1-mediated transcription was assayed, as described in *Materials and Methods*. The bar graphs represent the normalized ratio of luciferase to *Renilla* activity, $\pm$ SEM as a function of the concentration of thrombin or TRAP. The results from unstimulated NIH 3T3 cells served as a control.

treatment of the FLJ1 NIH 3T3 cell transfectants with thrombin but not TRAP reduced the level of CSL-dependent transcription, in a dose-dependent manner (Figure 5). Apparently, the thrombin-induced proteolytic cleavage of Jagged1 inhibits CSL-mediated Notch signaling.

Constitutively Active Notch1 Inhibits the Thrombin-Induced Release and Expression of FGF1
Because FGF1 release is up-regulated in Notch-repressed cells (Small *et al.*, 2003), we wanted to determine whether canonical Notch1 signaling is involved in thrombin-induced

Figure 6. Constitutive activation of Notch signaling blocks thrombin-induced FGF1 release and expression. (A) Thrombin-induced release of FGF1 is repressed by the expression of caN1. FGF1R136K NIH 3T3 cell transfectants were adenovirally transduced with caN1, and the levels of FGF1 in media conditioned by addition of thrombin were assessed using FGF1 immunoblot analysis. β -Galactosidase-transduced cells were used as a control. The representative CL from FGF1R136K-transduced cells is shown (left). Note that stress-induced release of FGF1 is not repressed by the expression of caN1. Bar graphs represent the percentage of FGF1 release as described in Figure 3B. (B) Thrombin-induced expression of FGF1 is repressed by caN1. NIH 3T3 cells transduced with caN1 adenovirus and vector control were treated for 30 min with 1 U/ml thrombin. RT-PCR experiments were performed as described in *Materials and Methods*. *gapdh* served as mRNA loading control.

FGF1 release. For these experiments, we adenovirally transduced FGF1R136K NIH 3T3 cell transfectants with caN1, and then we stimulated them with thrombin. CaN1 corresponds to the intracellular domain of Notch1, which is produced as a result of proteolytic cleavage induced by Notch1 binding to its ligand (Gridley, 2007). Approximately 90% of the cells expressed caN1 48 h after transfection (data not shown). As shown in Figure 6A, thrombin stimulated the release of FGF1R136K from β -galactosidase-transduced control cells; however, it was unable to initiate the release from cells expressing caN1 (Figure 6A). Conversely, the expression of caN1 did not affect the heat shock-induced FGF1 release pathway (Figure 6A). Analysis by RT-PCR revealed that adenoviral transduction of caN1 also inhibited the thrombin-induced expression of endogenous *fgf1* (Figure 6B). These data demonstrate that the induction of FGF1 expression and release by thrombin can be negatively regulated by Notch signaling.

Overexpression of Jagged1 Accelerates FGF1 Release from PAR1 Null Cells in Response to Thrombin, and This Effect Depends on Jagged1 Cleavage

We demonstrated that thrombin stimulates rapid FGF1 release through a mechanism mediated by PAR1 activation (Duarte *et al.*, 2006). However, the ability of sJ1 39 kDa to stimulate FGF1 export prompted us to hypothesize that even in the absence of PAR1, thrombin cleavage of the endogenous Jagged1 could result in FGF1 release, providing that thrombin treatment is long enough to allow the accumulation of sJ1. To test this hypothesis, PAR1 null cells were first treated with thrombin or TRAP in complete cell culture medium for 2 or 48 h. In parallel, other PAR1 null cells were transduced with FGF1R136K adenovirus. Forty-eight hours after FGF1 transduction, the cells were washed in serum-free medium containing heparin (5 U/ml) and incubated for an additional 2 h in the medium conditioned by untransduced PAR1 null cell cultures treated with thrombin or TRAP.

At the beginning of these experiments, we assessed Jagged1 expression in PAR1 null cells and found that Jagged1 transcript level in these cells is similar to that observed in NIH 3T3 cells (Figure 7A). As shown in Figure 7B, the medium conditioned for 2 h by thrombin-treated cells failed to induce FGF1 release. However, the medium conditioned for 48 h by cells treated with thrombin, but not with TRAP, efficiently stimulated FGF1 release from PAR1 null cells (Figure 7B).

We suggest that the extended incubation with thrombin resulted in the accumulation of extracellular sJ1 39 kDa in the amounts sufficient to induce FGF1 release in the absence of PAR1. The modest expression levels of Jagged1 in the cells we studied and insufficient sensitivity of the available commercial anti-Jagged1 antibodies did not allow us to reliably detect the cleaved endogenous Jagged1. However, we found that thrombin induces the cleavage of transfected FLJ1 not only in HEK 293 cells but also in PAR1 null MEFs (Figure 8A).

To further assess the hypothesis that thrombin-dependent cleavage of Jagged1 results in FGF1 export, we overexpressed FLJ1NV5 by adenoviral transduction in PAR1 null cells. Cells transduced with FLJ1NV5 were stimulated with thrombin for 2 h. The ratio of FLJ1NV5-transduced cells was >40% (data not shown). Conditioned media were collected and added for another 2 h to PAR1 null cells transduced with FGF1R136K adenovirus. As shown in Figure 8B, conditioned medium from thrombin-treated PAR1 null cells overexpressing Jagged1 induced FGF1 release, whereas conditioned medium from thrombin-treated control β -galactosidase-transduced cells did not exhibit such an effect.

Next, we produced a form of Jagged1 with a point mutation in the thrombin cleavage site (R348K). HEK 293 cells were

Figure 7. Long-term thrombin incubation induces FGF1 release from PAR1 null mouse embryonic fibroblasts. (A) Jagged1 expression in PAR1 null mouse embryonic fibroblasts. The expression of *Jagged1* in PAR1 null, PAR1 WT control mouse embryonic fibroblast and NIH 3T3 cells was determined by RT-PCR, using primers and conditions described in *Materials and Methods*. *gapdh* served as controls for mRNA loading. (B) Long-term thrombin incubation induces FGF1 release from PAR1 null cells. PAR1 null cells were stimulated with either thrombin or TRAP for 2 or 48 h. Conditioned media were collected and added for 2 h to PAR1 null cells transduced with FGF1R136K adenovirus. Detection of FGF1 in the conditioned media was performed as described in *Materials and Methods*. The CL from these cells (1/10 of total) is shown (left). Bar graphs represent the percentage of FGF1 release as described in Figure 3B.

transfected with wild-type (WT) or R348K FLJ1NV5. Thrombin treatment of cells transfected with FLJ1NV5R348K did not result in the appearance of sJ1 39 kDa in the medium (Figure 9A). We assessed the ability of the thrombin uncleavable mutant of Jagged1 (R348K) to support thrombin stimulated FGF1 export in PAR1 null cells. As expected, the medium conditioned for 2 h by thrombin-treated FLJ1NV5R348K HEK 293 transfectants did not induce FGF1 export (Figure 9B). Collectively, these data demonstrate that thrombin-induced cleavage of Jagged1 results in PAR1-independent FGF1 release.

Thrombin Induces FGF1 Export from Endothelial Cells

Our experiments on thrombin-induced FGF1 export were performed on immortalized mouse fibroblastoid cells. To verify the thrombin effect on a cell type more relevant to the cardiovascular system, we assessed thrombin-induced FGF1 export in the mouse cerebroendothelial cell line bEnd.3, which expresses Jagged1 (Figure 10A). As expected, short-term thrombin treatment induced FGF1 export from bEnd.3 cells (Figure

Figure 8. Short-term thrombin stimulation induces FGF1 release from PAR1 null cells overexpressing Jagged1. (A) Thrombin cleaves Jagged1 expressed in PAR1 null cells. PAR1 null cells were transfected with the FLJ1NV5 adenovirus. Forty-eight hours after transfection, the cells were treated with thrombin for 1 h at 37°C. Control FLJ1NV5-transduced cells were incubated in serum-free media without thrombin. Cleaved Jagged1 was immunoprecipitated from the conditioned medium by utilizing anti-V5 antibodies. Immunoprecipitated 39-kDa Jagged1 fragment was visualized, using anti-V5 immunoblotting. The corresponding CL from the FLJ1NV5 transfectant PAR1 null cells are shown (top). (B) Short-term thrombin stimulation induces FGF1 release from PAR1 null cells overexpressing Jagged1. PAR1 null cells transfected with FLJ1NV5 or β -galactosidase adenoviruses for 48 h were stimulated with thrombin for 2 h. Conditioned media were collected and added for 2 h to PAR1 null cells transduced with FGF1R136K. Detection of FGF1 in the conditioned medium was performed as described in *Materials and Methods*. The representative CL from these cells (1/10 of total) is shown (left). Bar graphs represent the percentage of FGF1 release as described in Figure 3B.

10B). This release was inhibited by pertussis toxin, indicating its dependence on PAR1 signaling (Chalmers *et al.*, 2003). However, medium conditioned by bEnd.3 cells exposed to thrombin for 48 h, induced FGF1 export from bEnd.3 cells even in presence of pertussis toxin (Figure 10B), which apparently reflects the accumulation of sJ1 39 kDa in this medium.

Similar to sJ1, Thrombin Enhances the Multipotentiality of Neural Crest Stem Cells, and This Effect Is Dependent on FGFR Signaling

We recently reported that sJ1 expression or treatment with recombinant sJ1 enhanced the multipotentiality of NCSCs (Nikopoulos *et al.*, 2007). We found that thrombin exhibited a similar effect (Figure 11). Interestingly, the enhancement of NCSC multipotentiality (assessed as the percentage of secondary NCSC clones with tripotential differentiation) both by sJ1 and by thrombin was eliminated by their adenoviral transduction with a dominant-negative form of FGFR1 (dn-

FGFR1) (Figure 11). Because NCSC express significant levels of Jagged1 (Nelson *et al.*, 2007), these results suggest that proteolytic production of sJ1 and the release of FGF1 underlie the effect of thrombin on NCSC multipotentiality.

DISCUSSION

Thrombin regulates a broad range of biological processes as a result of its proteolytic activity (Fenton *et al.*, 1998). Proteolysis of cell surface molecules and extracellular matrix components has long been known to play an important role in both *in vitro* and *in vivo* angiogenesis (Maciag *et al.*, 1982). Here, we report that thrombin can stimulate expression and release of FGF1, a potent angiogenic factor, through its ability to cleave Jagged1 and to produce sJ1 39 kDa, which inhibits CSL-mediated Notch signaling. Our FGF1 release experiments on cells stably transfected or virally transduced with FGF1 demonstrate that the stimulation of FGF1 expres-

Figure 9. Accelerating effect of Jagged1 expression on thrombin-induced FGF1 export depends on Jagged1 cleavage. (A) R348K mutation abolishes thrombin-induced cleavage of Jagged1. HEK 293 cells were transfected with the FLJ1NV5 WT or FLJ1NV5 R348K. Forty-eight hours after transfection, the cells were treated with thrombin for 1 h at 37°C. Control WT- or R348K-transduced cells were incubated in serum-free media without thrombin. Cleaved Jagged1 was immunoprecipitated from the conditioned medium by utilizing anti-V5 antibodies. Immunoprecipitated 39-kDa Jagged1 fragment was visualized, using anti-V5 immunoblotting. The corresponding CL (1/10 of total) from the FLJ1NV5 WT or R348K transfectant HEK293 cells are shown (top). (B) Accelerating effect of Jagged1 expression on thrombin-induced FGF1 export depends on Jagged1 cleavage. HEK 293 cells transfected with FLJ1NV5 WT or FLJ1NV5 R348K for 48 h were stimulated with thrombin for 2 h. Conditioned media were collected and added for 2 h to PAR1 null MEFs transduced with FGF1R136K. Detection of FGF1 in the conditioned medium was performed as described in *Materials and Methods*. The representative CL (1/10 of total) from these cells is shown (left).

sion and export by sJ1 are two independent phenomena. Indeed, sJ1 efficiently induces the release of transfected FGF1, whose expression is not dependent on PAR1 or Notch signaling. Numerous studies demonstrated that the activity of Notch signaling is highly dependent on cell type and environmental context, and this is particularly true for the activity of soluble ligands and for Notch regulation of cell growth (Aho, 2004). As we demonstrated here, in fibroblastoid cells Jagged1 thrombin cleavage product sJ1 39 kDa acts as a dominant-negative regulator of Notch signaling, and induces FGF1 expression and release. Interestingly, sJ1 39 kDa is roughly three times smaller than sJ1 117 kDa, but it has similar effect on CSL-dependent transcription and FGF1 export. Thus, we narrowed down the extracellular Jagged1 region involved in the down-regulation of Notch signaling, to the DSL and the first three EGF repeats, because those were the common domains between the two forms of sJ1. Further deletion mutagenesis demonstrated that Jagged1 DSL is sufficient to inhibit CSL-dependent transcription and induce FGF1 transcription and export. The biological roles of soluble Notch ligands are insufficiently understood, although we know that naturally occurring soluble forms of Notch ligands arise due to proteolytic cleavage (Qi *et al.*, 1999), including the cleavage stimulated by their interaction

Figure 10. Thrombin induces FGF1 export from endothelial cells. (A) Jagged1 expression in bEnd.3 cells. The expression of Jagged1 in PAR1 null MEF, NIH 3T3 cells, and bEnd.3 cells was determined by RT-PCR, using primers and conditions described in *Materials and Methods*. β -actin served as control for mRNA loading. (B) Thrombin stimulates FGF1 export from bEnd.3 cells. bEnd.3 cells were stimulated with thrombin for 2 or 48 h. Conditioned media were collected and added for 2 h to bEnd.3 cells transduced with FGF1R136K adenovirus in presence or absence of pertussis toxin (inhibitor of PAR1 signaling; Sigma-Aldrich). Detection of FGF1 in the conditioned media was performed as described in *Materials and Methods*. The CL from these cells (1/10 of total) is shown (left).

with Notch receptors (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003) or differential mRNA processing (Zimrin *et al.*, 1996; Li *et al.*, 1997; Aho, 2004). Several reports indicate that soluble forms of Jagged1 may play crucial roles in hematopoietic and nerve stem cell self-renewal, cell proliferation, angiogenesis, and vascular repair (Masuya *et al.*, 2002; Vas *et al.*, 2004; Nikopoulos *et al.*, 2007; Scehnet *et al.*, 2007). Our *in vitro* studies demonstrated that fibroblastoid cells expressing sJ1 117-kDa form chord-like structures, similar to those formed by endothelial cells during angiogenesis (Wang *et al.*, 2000; Small *et al.*, 2001; Trifonova *et al.*, 2004). In addition, intradermal injection of these cells into the flank of nude mice resulted in formation of tissue masses with prominent vascularization, underlying a crucial role of sJ1 in the presence of angiogenesis (Wang *et al.*, 2000).

We hypothesize that the cross-talk between thrombin/PAR1, Jagged1/Notch, and FGF1/FGFR can play a role in the organization of the angiogenic response to ischemic tissue damage. This response can proceed through several stages. Initially, tissue damage activates the coagulation cascade, generating thrombin, which in turn stimulates PAR1 activation and PAR1-dependent FGF1 expression and release. Released FGF1 subsequently promotes angiogenesis and stimulates Jagged1 expression in the damaged tissue. Indeed, FGF stimulation induces Jagged1 transcription (Zimrin *et al.*, 1996). Although PAR1 receptors get desensitized over time, the release of FGF1 at the later stages of tissue response to ischemia becomes dependent upon sJ1 39 kDa produced due to thrombin-dependent cleavage of Jagged1. Accumulation of sJ1 39 kDa results in the inhibition of Notch signaling and further stimulation of FGF1 transcription and export. As cell-cell interactions disturbed by initial

Figure 11. Similar to sj1, thrombin enhances the multipotentiality of NCSC, and this effect is dependent on FGFR signaling. Through the duration of the experiment, mouse NCSCs were incubated with or without 7.5 nM recombinant sj1 or 1 U/ml thrombin. Freshly isolated NCSCs formed colonies for 7 d on nonadhesive Petri dishes. On the seventh day, the cells were adenovirally transduced with dnFGFR or β -galactosidase. Then, individual colonies were isolated and trypsinized. NCSCs were plated on poly-D-lysine laminin matrix in tissue culture plastic plates. The differentiation was allowed to continue for seven additional days. At the end of the incubation period, the arising secondary clones were fixed and stained for markers of neural crest differentiation as described in *Materials and Methods*. Percentage of secondary clones with tripotential (glia, neuron, and myofibroblast) differentiation is presented.

tissue damage reestablish, and the content of thrombin in the damaged site declines, the interaction between Notch receptors and their transmembrane ligands on neighboring cells results in the inhibition of FGF1 expression and export and the stabilization of newly formed vascular structures.

It is noteworthy that the expression of caN1 completely blocks the induction of FGF1 expression and release by 5- to 30-min thrombin treatment, when the sufficient accumulation of sj1 is hardly plausible and the induction is mediated solely through PAR1 receptors (Duarte *et al.*, 2006). Apparently, the pathway(s) downstream of PAR1, which are responsible for stimulation of both FGF1 expression and release, are negatively regulated by Notch signaling. Indeed, p38 mitogen-activated protein kinase (MAPK), one of major PAR1 effectors, is inhibited by MAPK phosphatase 1, the expression of which is under the positive control of Notch (Kondoh *et al.*, 2007). It remains to be elucidated, which genes induced by Notch signaling are responsible for the repression of FGF1 expression and release and whether these two processes are regulated by the same or different Notch-dependent genes. In addition, although the stress-induced FGF1 export is sufficiently well studied (Prudovsky *et al.*, 2008), the mechanisms of thrombin-induced FGF1 release and their similarity to stress-dependent secretion are subjects of future studies.

Biological effects of thrombin, including the stimulation of PAR1, are based on its serine protease activity. Besides stimulating FGF1 export through the activation of PAR1 (Duarte *et al.*, 2006) thrombin also exerts the same effect through the proteolytic generation of sj1, which attenuates Notch signaling. It was conceivable that other serine proteases that are involved in the regulation of blood coagulation, such as Factor Xa, Arg-C protease, tissue plasminogen activator (tPA), and plasmin, could stimulate FGF1 export through Jagged1 proteolysis. However, using the PeptideCutter program, we found that Factor Xa and Arg-C protease cleavage

sites are absent in Jagged1. Our analysis also demonstrated that consensus tPA cleavage sites (Ding *et al.*, 1995) are not present in Jagged1. Prediction of plasmin cleavage sites based on primary protein structure is unreliable; however, using PAR1 null cells, we found that unlike thrombin, long-term plasmin treatment fails to induce FGF1 release (Supplemental Figure 1). We believe that the search for additional extracellular proteases able to stimulate the nonclassical protein export is an exciting subject for future studies.

Given that thrombin, FGF, and Notch pathways play important roles in the cardiovascular system, the further understanding of the cross-talk between them can open new opportunities for the development of therapeutics and diagnostic strategies for vascular-related diseases and other pathological conditions.

ACKNOWLEDGMENTS

This article is dedicated to the memory of Tom Maciag. Recombinant sj1 was a kind gift of M. Bhatia and PAR1 null MEFs of S. Coughlin. This work was supported in part by National Institutes of Health grants HL-32348, HL-35627, and RR-15555 (Project 4) (to I. P.). M. D. contributed to this study to partially fulfill the requirements for the Ph.D. degree from the University of Minho, Portugal.

REFERENCES

- Aho, S. (2004). Soluble form of Jagged 1, unique product of epithelial keratinocytes and a regulator of keratinocyte differentiation. *J. Cell Biochem.* 92, 1271–1281.
- Bassus, S., Herkert, O., Kronemann, N., Gorch, A., Bremerich, D., Kirchmaier, C. M., Busse, R., and Schini-Kerth, V. B. (2001). Thrombin causes vascular endothelial growth factor expression in vascular smooth muscle cells: role of reactive oxygen species. *Arterioscler. Thromb. Vasc. Biol.* 21, 1550–1555.
- Bergmeyer, H. U. (1965). *Methods of enzymatic analysis*, New York: Academic Press.
- Buehler, A. *et al.* (2002). Angiogenesis-independent cardioprotection in FGF-1 transgenic mice. *Cardiovasc. Res.* 55, 768–777.
- Cao, H., Dronadula, N., and Rao, G. N. (2006). Thrombin induces expression of FGF-2 via activation of PI3K-Akt-Fra-1 signaling axis leading to DNA synthesis and motility in vascular smooth muscle cells. *Am. J. Physiol. Cell Physiol.* 290, C172–C182.
- Chalmers, C. J., Balmanno, K., Hadfield, K., Ley, R., and Cook, S. J. (2003). Thrombin inhibits Bim (Bcl-2-interacting mediator of cell death) expression and prevents serum-withdrawal-induced apoptosis via protease-activated receptor 1. *Biochem. J.* 375, 99–109.
- Christofori, G., and Luef, S. (1997). Novel forms of acidic fibroblast growth factor-1 are constitutively exported by beta tumor cell lines independent from conventional secretion and apoptosis. *Angiogenesis* 1, 55–70.
- Cucina, A., Borrelli, V., Lucarelli, M., Sterpetti, A. V., Cavallaro, A., Strom, R., Santoro-D'Angelo, L., and Scarpa, S. (2002). Autocrine production of basic fibroblast growth factor translated from novel synthesized mRNA mediates thrombin-induced mitogenesis in smooth muscle cells. *Cell Biochem. Funct.* 20, 39–46.
- Ding, L., Coombs, G. S., Strandberg, L., Navre, M., Corey, D. R., and Madison, E. L. (1995). Origins of the specificity of tissue-type plasminogen activator. *Proc. Natl. Acad. Sci. USA* 92, 7627–7631.
- Duarte, M., Kolev, V., Soldi, R., Kirov, A., Graziani, I., Oliveira, S. M., Kacer, D., Friesel, R., Maciag, T., and Prudovsky, I. (2006). Thrombin induces rapid PAR1-mediated non-classical FGF1 release. *Biochem. Biophys. Res. Commun.* 350, 604–609.
- Erzurum, V. Z., Bian, J. F., Husak, V. A., Ellinger, J., Xue, L., Burgess, W. H., and Greisler, H. P. (2003). R136K fibroblast growth factor-1 mutant induces heparin-independent migration of endothelial cells through fibrin glue. *J. Vasc. Surg.* 37, 1075–1081.
- Fallon, J. F., Lopez, A., Ros, M. A., Savage, M. P., Olwin, B. B., and Simandl, B. K. (1994). FGF-2, apical ectodermal ridge growth signal for chick limb development. *Science* 264, 104–107.
- Fenton, J. W., 2nd, Ofosu, F. A., Breznjak, D. V., and Hassouna, H. I. (1998). Thrombin and antithrombotics. *Semin. Thromb. Hemost.* 24, 87–91.
- Friesel, R., and Maciag, T. (1999). Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling. *Thromb. Haemost.* 82, 748–754.

- Gridley, T. (2007). Notch signaling in vascular development and physiology. *Development* 134, 2709–2718.
- Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., and Phipps, M. L. (1997). Construction of adenovirus vectors through Cre-lox recombination. *J. Virol.* 71, 1842–1849.
- Herbert, J.M., Dupuy, E., Laplace, M.C., Zini, J.M., Bar Shavit, R., and Tobelem, G. (1994). Thrombin induces endothelial cell growth via both a proteolytic and a non-proteolytic pathway. *Biochem. J.* 303, 227–231.
- Hrabe de Angelis, M., McIntyre, J., 2nd, and Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue DIII. *Nature* 386, 717–721.
- Hsieh, J. J., Henkel, T., Salmon, P., Robey, E., Peterson, M. G., and Hayward, S. D. (1996). Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Mol. Cell Biol.* 16, 952–959.
- Ikeuchi, T., and Sisodia, S. S. (2003). The Notch ligands, Delta1 and Jagged2, are substrates for presenilin-dependent “gamma-secretase” cleavage. *J. Biol. Chem.* 278, 7751–7754.
- Jackson, A., Friedman, S., Zhan, X., Engleka, K. A., Forough, R., and Maciag, T. (1992). Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* 89, 10691–10695.
- Jarriault, S., Brou, C., Loegeat, F., Schroeter, E. H., Kopan, R., and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* 377, 355–358.
- Javerzat, S., Auguste, P., and Bikfalvi, A. (2002). The role of fibroblast growth factors in vascular development. *Trends Mol. Med.* 8, 483–489.
- Joutel, A., and Tournier-Lasserre, E. (1998). Notch signalling pathway and human diseases. *Semin. Cell Dev. Biol.* 9, 619–625.
- Kondoh, K., Sunadome, K., and Nishida, E. (2007). Notch signaling suppresses p38 MAPK activity via induction of MKP-1 in myogenesis. *J. Biol. Chem.* 282, 3058–3065.
- LaVallee, T. M., Tarantini, F., Gamble, S., Carreira, C. M., Jackson, A., and Maciag, T. (1998). Synaptotagmin-1 is required for fibroblast growth factor-1 release. *J. Biol. Chem.* 273, 22217–22223.
- LaVoie, M. J., and Selkoe, D. J. (2003). The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. *J. Biol. Chem.* 278, 34427–34437.
- Li, L. *et al.* (1997). Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat. Genet.* 16, 243–251.
- Maciag, T., Hoover, G. A., and Weinstein, R. (1982). High and low molecular weight forms of endothelial cell growth factor. *J. Biol. Chem.* 257, 5333–5336.
- Masuya, M. *et al.* (2002). The soluble Notch ligand, Jagged-1, inhibits proliferation of CD34+ macrophage progenitors. *Int. J. Hematol.* 75, 269–276.
- McKeehan, W. L., Wang, F., and Kan, M. (1998). The heparan sulfate-fibroblast growth factor family: diversity of structure and function. *Prog. Nucleic Acid Res. Mol. Biol.* 59, 135–176.
- Morrison, S. J., White, P. M., Zock, C., and Anderson, D. J. (1999). Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell* 96, 737–749.
- Neilson, K. M., and Friesel, R. (1996). Ligand-independent activation of fibroblast growth factor receptors by point mutations in the extracellular, transmembrane, and kinase domains. *J. Biol. Chem.* 271, 25049–25057.
- Nelson, B. R., Hartman, B. H., Georgi, S. A., Lan, M. S., and Reh, T. A. (2007). Transient inactivation of Notch signaling synchronizes differentiation of neural progenitor cells. *Dev. Biol.* 304, 479–498.
- Nickel, W. (2005). Unconventional secretory routes: direct protein export across the plasma membrane of mammalian cells. *Traffic* 6, 607–614.
- Nikopoulos, G. N., Duarte, M., Kubu, C. J., Bellum, S., Friesel, R., Maciag, T., Prudovsky, I., and Verdi, J. M. (2007). Soluble Jagged1 attenuates lateral inhibition, allowing for the clonal expansion of neural crest stem cells. *Stem Cells* 25, 3133–3142.
- Okunieff, P., Fenton, B. M., Zhang, L., Kern, F. G., Wu, T., Greg, J. R., and Ding, I. (2003). Fibroblast growth factors (FGFs) increase breast tumor growth rate, metastases, blood flow, and oxygenation without significant change in vascular density. *Adv. Exp. Med. Biol.* 530, 593–601.
- Ornitz, D. M., and Itoh, N. (2001). Fibroblast growth factors. *Genome Biol.* 2.
- Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J., and Trejo, J. (2002). beta-Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. *J. Biol. Chem.* 277, 1292–1300.
- Prudovsky, I. *et al.* (2003). The non-classical export routes: FGF1 and IL-1alpha point the way. *J. Cell Sci.* 116, 4871–4881.
- Prudovsky, I., Tarantini, F., Landriscina, M., Neivandt, D., Soldi, R., Kirov, A., Small, D., Kathir, K. M., Rajalingam, D., and Kumar, T. K. (2008). Secretion without Golgi. *J. Cell Biochem.* 103, 1327–1343.
- Qi, H., Rand, M. D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T., and Artavanis-Tsakonas, S. (1999). Processing of the notch ligand delta by the metalloprotease Kuzbanian. *Science* 283, 91–94.
- Rebay, I., Fleming, R. J., Fehon, R. G., Chervas, L., Chervas, P., and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* 67, 687–699.
- Rosengart, T. K., Johnson, W. V., Friesel, R., Clark, R., and Maciag, T. (1988). Heparin protects heparin-binding growth factor-I from proteolytic inactivation in vitro. *Biochem. Biophys. Res. Commun.* 152, 432–440.
- Scehnet, J. S., Jiang, W., Kumar, S. R., Krasnoperov, V., Trindade, A., Benedito, R., Djokovic, D., Borges, C., Ley, E. J., Duarte, A., and Gill, P. S. (2007). Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood* 109, 4753–4760.
- Schumacher, B., Pecher, P., von Specht, B. U., and Stegmann, T. (1998). Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation* 97, 645–650.
- Sellke, F. W., Li, J., Stamler, A., Lopez, J. J., Thomas, K. A., and Simons, M. (1996). Angiogenesis induced by acidic fibroblast growth factor as an alternative method of revascularization for chronic myocardial ischemia. *Surgery* 120, 182–188.
- Shimizu, K., Chiba, S., Kumano, K., Hosoya, N., Takahashi, T., Kanda, Y., Hamada, Y., Yazaki, Y., and Hirai, H. (1999). Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods. *J. Biol. Chem.* 274, 32961–32969.
- Small, D., Kovalenko, D., Kacer, D., Liaw, L., Landriscina, M., Di Serio, C., Prudovsky, I., and Maciag, T. (2001). Soluble Jagged 1 represses the function of its transmembrane form to induce the formation of the Src-dependent chord-like phenotype. *J. Biol. Chem.* 276, 32022–32030.
- Small, D. *et al.* (2003). Notch activation suppresses fibroblast growth factor-dependent cellular transformation. *J. Biol. Chem.* 278, 16405–16413.
- Steinhoff, M., Buddenkotte, J., Shpacovitch, V., Rattenholl, A., Moormann, C., Vergnolle, N., Luger, T. A., and Hollenberg, M. D. (2005). Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. *Endocr. Rev.* 26, 1–43.
- Trifonova, R., Small, D., Kacer, D., Kovalenko, D., Kolev, V., Mandinova, A., Soldi, R., Liaw, L., Prudovsky, I., and Maciag, T. (2004). The non-transmembrane form of Delta1, but not of Jagged1, induces normal migratory behavior accompanied by fibroblast growth factor receptor 1-dependent transformation. *J. Biol. Chem.* 279, 13285–13288.
- Vas, V., Szilágyi, L., Pálóczi, K., and Uher, F. (2004). Soluble Jagged-1 is able to inhibit the function of its multivalent form to induce hematopoietic stem cell self-renewal in a surrogate in vitro assay. *J. Leukoc. Biol.* 75, 714–720.
- Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64, 1057–1068.
- Wang, X., Romanic, A. M., Yue, T. L., Feuerstein, G. Z., and Ohlstein, E. H. (2000). Expression of interleukin-1beta, interleukin-1 receptor, and interleukin-1 receptor antagonist mRNA in rat carotid artery after balloon angioplasty. *Biochem. Biophys. Res. Commun.* 271, 138–143.
- Wong, M. K., Prudovsky, I., Vary, C., Booth, C., Liaw, L., Mousa, S., Small, D., and Maciag, T. (2000). A non-transmembrane form of Jagged-1 regulates the formation of matrix-dependent chord-like structures. *Biochem. Biophys. Res. Commun.* 268, 853–859.
- Woolley, P. V., Gollin, S. M., Riskalla, W., Finkelstein, S., Stefanik, D. F., Riskalla, L., Swaney, W. P., Weisenthal, L., and McKenna, R. J., Jr. (2000). Cytogenetics, immunostaining for fibroblast growth factors, p53 sequencing, and clinical features of two cases of cystosarcoma phylloides. *Mol. Diagn.* 5, 179–190.
- Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G., and Gridley, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum. Mol. Genet.* 8, 723–730.
- Zimrin, A. B., Pepper, M. S., McMahon, G. A., Nguyen, F., Montesano, R., and Maciag, T. (1996). An antisense oligonucleotide to the notch ligand jagged enhances fibroblast growth factor-induced angiogenesis in vitro. *J. Biol. Chem.* 271, 32499–32502.
- Zimrin, A. B., Villeponteau, B., and Maciag, T. (1995). Models of in vitro angiogenesis: endothelial cell differentiation on fibrin but not matrigel is transcriptionally dependent. *Biochem. Biophys. Res. Commun.* 213, 630–638.