

Jedi—A Novel Transmembrane Protein Expressed in Early Hematopoietic Cells

Andrei V. Krivtsov,^{1,3,5} Fedor N. Rozov,^{2,4} Marina V. Zinovyeva,² P. Jan Hendriks,¹ Yajuan Jiang,¹ Jan W.M. Visser,^{1,3} and Alexander V. Belyavsky^{1,2,3*}

¹Lindsley F. Kimball Research Institute, New York Blood Center, 310 East 67 Street, New York, NY 10021

²Engelhardt Institute of Molecular Biology RAS, Vavilov Str. 32, Moscow 119991, Russia

³ViaCell Inc., Cambridge Research Center, 26 Landsdowne Street, Cambridge, MA 02139

⁴University of Oslo, Centre for medical studies Russia, Vavilov Str. 34/5, Moscow 119334

⁵Department of Hematology/Oncology, Children's Hospital Boston, Karp Building 08005H, 1 Blackfan Circle, Boston MA 02115, 617 919 2501

Abstract Self-renewal and differentiation of hematopoietic stem and progenitor cells are defined by the ensembles of genes expressed by these cells. Here we report identification of a novel gene named Jedi, which is expressed predominantly in short- and long-term repopulating stem cells when compared to more mature bone marrow progenitors. Jedi mRNA encodes a transmembrane protein that contains multiple EGF-like repeats. Jedi and two earlier reported proteins, MEGF10 and MEGF11, share a substantial homology and are likely to represent a novel protein family. Studies of the potential role of Jedi in hematopoietic regulation demonstrated that the retrovirally mediated expression of Jedi in bone marrow cells decreased the number of myeloid progenitors in *in vitro* clonogenic assays. In addition, expression of Jedi in NIH 3T3 fibroblasts resulted in a decreased number of late and early myeloid progenitors in the non-adherent co-cultured bone marrow cells. Jedi shares a number of structural features with the Jagged/Serrate/Delta family of Notch ligands, and our experiments indicate that the extracellular domain of Jedi, similar to the corresponding domain of Jagged1, inhibits Notch signaling. On the basis of obtained results, we suggest that Jedi is involved in the fine regulation of the early stages of hematopoietic differentiation, presumably through the Notch signaling pathway. *J. Cell. Biochem.* 101: 767–784, 2007.

© 2007 Wiley-Liss, Inc.

Key words: Hematopoiesis; transmembrane protein; Notch ligand; progenitor cells; colony formation; tyrosine phosphorylation

Hematopoiesis proceeds from stem to mature cells through a number of tightly regulated proliferation and differentiation steps [Visser et al., 1991; Morrison et al., 1995]. The properties of stem and progenitor cells important for these processes are the subject of intensive

investigation [Lemischka, 2001]. Although during the last few years a number of attempts to elucidate a global gene expression profile of hematopoietic stem cells (HSCs) revealed several hundred genes expressed preferentially in early HSCs [Phillips et al., 2000; Ramalho-Santos et al., 2002; Terskikh et al., 2003], relatively few of the novel genes identified in these screens, were characterized in substantial detail. Among the factors likely to play an essential role in the regulation of the early hematopoietic compartment are undoubtedly cytokines, membrane proteins, and the components of the extracellular matrix, which provide or relay environmental cues influencing cell fate. Among several signaling pathways known to contribute to commitment and proliferation of cells, the Notch pathway is believed to be one of the crucial mechanisms controlling cell fate [Milner and Bigas, 1999; Kojika and Griffin,

Grant sponsor: The New York Blood Center; Grant number: 32211616; Grant sponsor: Russian Foundation for Basic Research; Grant numbers: 99-04-49252, 03-04-48786; Grant sponsor: U.S. Civilian Research and Development Foundation; Grant numbers: RB1-2453-MO-02, RUXO-002715-MO-06.

*Correspondence to: Alexander V. Belyavsky, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov Street 32, 119991 Moscow, Russia.
E-mail: abelyavs@yahoo.com

Received 1 October 2006; Accepted 16 November 2006

DOI 10.1002/jcb.21232

© 2007 Wiley-Liss, Inc.

2001; Staal and Clevers, 2001]. Binding of the Notch receptor to its ligands tethered on adjacent cells [Schweisguth, 2004] triggers proteolysis of the receptor, eventually leading to transcriptional activation of gene ensembles which inhibit certain differentiation programs or induce self-renewal [Kojika and Griffin, 2001]. Both Notch receptors and Notch ligands are single-pass transmembrane proteins, characterized by multiple EGF-like repeats in the extracellular domain. Two families of Notch ligands that have been identified in mammals, namely Delta and Serrate/Jagged [Bettenhausen et al., 1995; Lindsell et al., 1995; Dunwoodie et al., 1997; Li et al., 1998], bear also a conserved DSL domain (after *Delta/Serrate/Lag-2*) [Tax et al., 1994; Nye and Kopan, 1995], which is required for binding to Notch. Besides Notch ligands and receptors, a multitude of other extracellular proteins contain EGF-like repeats, and some of these proteins may influence the fate of early HSCs [Moore et al., 1997b]. In this article we describe a novel cDNA, Jedi, that encodes a transmembrane protein containing multiple EGF-like repeats and a DSL-like domain. On the basis of sequence similarity, Jedi and the earlier reported MEGF10 and MEGF11 proteins are likely to represent a novel protein family. Expression of Jedi mRNA and protein was detected in the early compartment of bone marrow hematopoietic cells, stromal cells supporting hematopoiesis and in several adult murine tissues. Jedi overexpression in hematopoietic progenitors resulted in decrease in the number of committed myeloid progenitor cells. Our data indicate that Jedi may be involved in the regulation of hematopoietic differentiation.

MATERIALS AND METHODS

Isolation and Analysis of Jedi cDNA

A cDNA tag corresponding to Jedi was isolated as a result of the Gene Expression Fingerprinting screening procedure [Ivanova and Belyavsky, 1995; Shmelkov et al., 2001] aimed at identification of genes downregulated during hematopoietic differentiation. A longer cDNA copy was isolated using the GeneTrapper (Invitrogen, Carlsbad, CA) kit. Full-length Jedi cDNA was isolated using a PCR screen from the mouse testis Rapid-Screen arrayed cDNA library panel (Origene, Rockville, MD). Five independent clones were isolated, two of which

(designated as F4 and E7) were analyzed in more detail. Sequence analysis was performed using GeneRunner 3.0 software (Hastings Software Inc., Hastings, NY), the BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST>) and the ExPASy Proteomics Tool Web server (<http://www.expasy.ch/tools>).

Animals

Mice C57B/6 (Jackson Laboratories, Bar Harbor, ME) 8 to 12 weeks old were used as bone marrow (BM) donors. Rabbits, 1 kg, female (Covance, Princeton, NJ) were used to generate antiserum. Mice and rabbits were housed at the New York Blood Center animal facility.

Cell Lines

The following cell lines were used in this work: retrovirus packaging lines 293GPG, E86 and Phoenix Ampho, embryonic fibroblast line NIH/3T3, lymphoma ABE-8.1/2, leukemia BC3A, lymphoma EL4, lymphocytic leukemia L1210, acute myelocytic leukemia LT12, acute promyelocytic leukemia LT12NL15, adenocarcinoma (epithelial) MCF-7, mastocytoma P815, myelomonocytic leukemia WEHI 3B, lymphoma WEHI 7, lymphoma LBRM-33, stromal cell lines FBMD-1 and AFT024, hematopoietic cell line FDCP-1.

Northern and Southern Blots, RT-PCR

The adult mouse poly(A)⁺ RNA blot membrane (Origene) was hybridized with a ³²P-labeled Jedi probe (nt 178–2452) in a buffer provided by the manufacturer. The analysis of Jedi RNA expression in the hematopoietic stem/progenitor cell subpopulations of bone marrow was performed by Southern hybridization with PCR amplified cDNA samples from sorted cell fractions as described by Zinovyeva et al. [2000] RT-PCR with total RNA from AFT024 cells was performed using the RT-PCR kit (Invitrogen) and the Advantage 2 polymerase (BD Biosciences, Palo Alto, CA). First cDNA strand was used as a template for amplification with primers Jedi-9 (5'GTCTGACTGGGACACTCAACTC 3'), and Jedi-25 (5'CCCTCTGCTCTCGTAGTA 3').

Retroviral Constructs and Viral Supernatants

Jedi cDNA fragments corresponding to full length (FL, aa 1–1034), membrane tethered extracellular (MT, aa 1–756), or intracellular (IC, aa 776–1034) forms of the protein were

cloned into the IRES-GFP cassette of the pSFG retroviral vector [Rivière et al., 1995; Maher et al., 2002] using Xho I and Nco I restriction sites, resulting in three expression vectors—SFG-FL, SFG-MT, and SFG-IC. The 293GPG packaging cell line was transfected with these or control SFG vectors using the calcium phosphate method [Rovira et al., 2000]. Supernatants were collected 2–4 days thereafter and used to infect E86 cells. Four days later E86 cells expressing GFP were selected using FACS. The resulting cell populations, termed E86-FL, E86-MT, E86-IC, and E86-SFG, were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS and penicillin/streptomycin. Cell supernatants were collected after 12 h cultivation. The virus titer in these supernatants was assayed using the NIH 3T3 cell line.

For Notch signaling assays, a pcDNA3.1/Hygro(+) vector with inserted Jedi ORF was used as a starting construct. To prepare a construct expressing a V5 epitope-tagged version of Jedi, its ORF was subcloned into the pcDNA3.1/V5-HisA vector (Invitrogen) using EcoR I and Xho I restriction enzymes. To produce a C-terminal fusion with V5 epitope, the Jagged1 ORF in the plasmid hJagged1/pcDNA3.1/Hygro(+) [Small et al., 2001] was amplified by eight cycles of PCR with Pfu polymerase (Stratagene), and primers T7 and hJagV5A (ACTCTCGAGTACGATGTACTIONCA-TTC). The PCR product was cloned into the pcDNA3.1/V5-HisA vector using BamH I and Xho I sites. The inserts (fused and non-fused versions of Jedi and Jagged1) were then subcloned into the pBabe Puro retroviral vector using appropriate combinations of restriction sites.

The Phoenix Ampho packaging cell line was transfected with Jagged/pBabe puro, Jedi/pBabe Puro, JaggedV5-His6/pBabe Puro, JediV5-His6/pBabe Puro, and control vector pBabe Puro using Lipofectamine 2000 (Invitrogen). Viral supernatants were collected 1–3 days thereafter and used to infect NIH 3T3 cells.

Retroviral Transduction of NIH3T3 Cells

Supernatants of E86-FL, E86-MT, E86-IC, and E86-SFG cells were applied to 30% confluent NIH 3T3 cells (grown for 24 h in DMEM supplemented with 10% FBS) for 16 h with addition of polybrene 5 $\mu\text{g/ml}$ (Sigma-Aldrich, St. Louis, MO). Three days later NIH 3T3 cells

were sorted to select GFP expressing cells resulting in four cell populations termed NIH 3T3-SFG, NIH 3T3-FL, NIH 3T3-MT, or NIH 3T3-IC.

For the Notch signaling coculture assay, viral supernatants of Phoenix Ampho packaging cells were applied to NIH 3T3 cells for 24 h with addition of polybrene 5 $\mu\text{g/ml}$. Four days later infected cells were selected using puromycin 3 $\mu\text{g/ml}$. The resulting cell populations, termed 3T3-pBabe, 3T3-Jagged, 3T3-Jedi, 3T3-Jagged-V5, 3T3-Jedi-V5, and 3T3-Jedagged-V5, were used for Notch assays.

Retroviral Transduction of Bone Marrow Mononuclear Cells

Bone marrow mononuclear cells (BM MNC) were isolated by centrifugation in a NycoPrep (Axis-Shield PoC AS, Oslo, Norway) for 20 min, 1,200g at room temperature. For retroviral gene transfer, BM MNC were plated at 2×10^7 in 10 ml IMDM supplemented with 20% fetal calf serum (FCS), stem cell factor (SCF) at 50 ng/ml (Amgen, Thousand Oaks, CA), interleukin-3 (IL-3) at 10 ng/ml (BD, CA) and penicillin/streptomycin, as previously described [Yan et al., 1999]. After 40 h of culture non-adherent cells were combined with retrovirus-containing supernatants from E86-FL, E86-MT, E86-IC, or E86-SFG cell populations, and transferred to Retronectin-coated Petri dishes. SCF and IL-3 were added to the final concentration 50 and 10 ng/ml, respectively. After culturing for 3 days, non-adherent GFP-positive cells were collected using a MoFlo flow cytometer (Cytomation, Fort Collins, CO) equipped with a 488 nm argon laser. For analysis of clonogenic efficiency (Day 10 CFUs, namely CFU-G, CFU-M, and CFU-GM), 3×10^2 cells were plated in triplicates in 2 cm dishes containing 1 ml IMDM of methylcellulose media 3534 (StemCell Technologies, Vancouver, Canada) supplemented with GM-CSF 10 ng/ml, penicillin 100 U/ml and streptomycin 100 $\mu\text{g/ml}$, and incubated at 37°C, 5% CO₂. GFP-positive colonies were scored on Day 10 using an inverted fluorescent microscope. Numbers of colonies (CFU-c) were averaged for three dishes.

BM MNC Co-Culture With Retrovirally Transduced NIH 3T3 Cells

Retrovirally transduced NIH 3T3 cells were plated in 12-well plates at 10^5 per well in the IMDM-GlutaMax medium supplemented with

10% FCS and penicillin/streptomycin, and grown at 37°C, 5% CO₂ for 2 days to reach full confluence. For the CFU-c Day 10 analysis, 1 × 10⁵ BM MNC were layered in triplicate on NIH 3T3 stroma. Co-cultures were maintained in IMDM supplemented with 10% FCS and penicillin/streptomycin (2 ml/well). At each time point (2, 5, 7, 9, and 12 days), 1 ml of media from each well was aspirated after gentle shaking of the plate, and 300 µl aliquots (in triplicate) used for the methylcellulose colony-forming assay described above.

For the high proliferative potential (HPP) cells and quiescent HPP (HPP-q) analyses, 10⁵ BM MNCs were layered on the retrovirally transduced NIH 3T3 stroma and cultured in 2 ml of IMDM with 10% FCS and penicillin/streptomycin for 7 days. Non-adherent cells were centrifuged, and half of the sample was transferred to 1 ml of MethoCult GF 3434 (Stem Cell Technologies Inc., Vancouver, BC, Canada) supplemented with 10 ng/ml GM-CSF, and incubated for 21 days at 37°C. The HPP-q assay [Fortunel et al., 2000] was performed with the remaining half of the sample as described above, with anti-TGF-beta antibody (clone 1D11, R&D Systems) added at a concentration of 20 µg/ml. HPP and HPP-q colonies equal to or exceeding 0.3 mm in diameter were scored using light microscopy.

Protein Expression and Purification

sJagged1 plasmid (encoding the myc-6xHis-tagged extracellular domain of Jagged1) was a gift of Dr. D. Small. To prepare the plasmid expressing the myc-6xHis-tagged extracellular domain of Jedi, the extracellular part of Jedi was amplified with primers S1ORF-5_Eco 5' GATCGAATTCCTCTGCAATGCCACTTTGTC and Jedi-EC-A 5' GATCTCGAGTTATGGGT-CACGGGAGAGGT using Pfu polymerase (Fermentas), and cloned into EcoR1 and Xho1 sites of pcDNA6MycHisB vector (Invitrogen).

COS1 cells were transfected with sJagged1 or sJedi plasmids. Twenty-four hours post-transfection the concentration of the FCS in culture medium was decreased to 0.5%, and after cultivation for additional 2 days cell culture supernatants were collected. The supernatants were dialyzed against 20 mM Tris-Cl pH 8.0, 300 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol at +4°C, proteins purified using Ni-NTA resin (Qiagen, Valencia, CA) according to the manufacturer's instructions. Protein

yields were estimated by Western blotting using anti-myc antibodies.

Cell-Based Assays for Notch Signaling

Analysis of the effects of soluble Jedi and Jagged1 proteins was performed as described [Small et al., 2003]. HEK293 cells in a 12-well plate were co-transfected with 700 ng of full-length Notch1 plasmid, 700 ng of CBF1-luc reporter construct [Hsieh et al., 1996] and 10 ng of pRL-CMV (Promega, Madison, WI) using Lipofectamine 2000. The transfected cells were incubated with either COS1-conditioned medium or partially purified sJagged1 or sJedi proteins. The amounts of sJagged1 or sJedi proteins applied onto HEK293 cells were the same as in initial COS1-conditioned medium (as measured by semi-quantitative Western blotting). Twenty-four to forty-eight hours post-transfection cell lysates were prepared, and firefly and *Renilla* luciferase activities were measured using Dual Luciferase Reporter System (Promega).

The CBF1 co-culture assay was performed as described [Moloney et al., 2000]. NIH 3T3 cells were co-transfected with Notch1, CBF-luc, and pRL-CMV plasmids. After incubation at 37°C for 16 h, transfected cells were overlaid with 2.5 × 10⁵ retrovirally transfected NIH 3T3 cells. After another 32 h, cell lysates were prepared, and firefly and *Renilla* luciferase activities were measured. All experiments were performed in triplicate at least twice.

Production of Polyclonal Antibodies to Jedi

The cDNA fragment corresponding to the N-terminal part of Jedi (aa 20–67) was generated by PCR using primers 5' ATGGATCCTCACTG-CAGGCGTGGGCGGGA and 5' ATACATAT-GAACTCCAATGATCCCAATGTC, and cloned into the pET-14b vector (Novagen, Madison, WI). Expression and purification of the protein was conducted as described [Beresten et al., 1999]. For the generation of polyclonal antibodies, two rabbits were injected with bacterially expressed N-terminal Jedi fragment. After four consecutive immunizations, serum was collected and tested by Western blotting. Affinity purification of antibodies [Harlow and Lane, 1988] was performed using the same N-terminal Jedi fragment immobilized on CNBr-activated Sepharose (Amersham-Pharmacia Biotech, Piscataway, NJ).

Cell Staining and FACS Analysis

Primary anti-Jedi rabbit antibody or control non-immune rabbit IgG and secondary goat anti-rabbit-PE (Vector Labs, Burlingame, CA) antibody were used for Jedi detection. The lineage specific antibody (Lin) cocktail consisted of anti-B220-FITC, anti-CD3-FITC, anti-CD4-FITC, anti-CD8-FITC, anti-Mac1-FITC, anti-Gr1-FITC, and anti-Ter119-FITC (BD Biosciences). Labeling was performed using 3 μ l of each antibody per 10^7 BM MNC in PBS supplemented with 1% FBS on ice.

For analysis of Jedi expression, BM MNC labeled with anti-Jedi and Lin cocktail antibodies were analyzed on a FASCalibur (BD Biosciences). Lineage negative cells, gated as 5% of low fluorescent events in FITC channel, were analyzed for Jedi-PE fluorescence. The thymus was dissected from 8-week-old mice; cells were strained through a 40 μ m cell strainer (BD Biosciences) and washed twice before incubation with anti-Jedi antibody.

Analysis of Tyrosine Phosphorylation

The pEGFP-Jedi-IC plasmid was constructed by amplification of the fragment of Jedi cDNA corresponding to aa 778–1034 with primers mJ1–7 (5' ATAAGATCTTACCGCCAGTGGC-AAAAGGG) and mJ1–3 (5' ATGAATTCTCTT-CAGCGGTCCTGGCG), followed by insertion of the fragment into pEGFP-N2 vector using EcoRI and BamHI restriction sites. The pEGFP-Jedi-ICA plasmid was constructed using primers mJ1–7 and mJ1–5 (5' ATGAATTCTCAGT-CAGCGGGCAGTGTGAC) to amplify a Jedi fragment corresponding to aa 778–868.

NIH 3T3 cells were transfected using Lipofectin (Invitrogen) with control pEGFP-N2, pEGFP-Jedi-IC or pEGFP-Jedi-ICA fusion plasmids. The transfected cells were incubated with 5 mM peroxyvanadate (prepared by mixing 500 μ l of 100 mM ortho-vanadate and 17 μ l hydrogen peroxide) at 37°C for 20 min. Cells were lysed in HNTG buffer (20 mM Hepes pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100, 100 μ g/ml PMSF, 5 mM peroxyvanadate). Cell lysates were subjected to immunoprecipitation with 1 μ g of anti-GFP antibody (BD Biosciences) and 20 μ l of Protein A Sepharose (Amersham-Pharmacia Biotech) at +4°C overnight. Immunoprecipitates were washed three times with the HNTG buffer, mixed with equal volume of 2 \times Laemmli loading

buffer, and incubated at 100°C for 5 min. Immunoprecipitates were resolved on 10% SDS-PAGE, transferred onto Hybond ECL (Amersham-Pharmacia Biotech) and probed with anti-phosphotyrosine antibody (UBI, Lake Placid, NY) or anti-GFP antibody (Clontech, Palo Alto, CA). Binding of primary antibody was detected using the ECL system (Amersham-Pharmacia Biotech).

RESULTS

Jedi is a Novel Protein Containing EGF-Like Repeats and a DSL-Like Domain

Screening of the OriGene cDNA library to identify longer sequences corresponding to the initial cDNA tag yielded five positive clones, two of which (E7 and F4), with the longest 5' regions, were taken for further analysis. Clone E7 cDNA had an open reading frame of 3,105 bp corresponding to a predicted 1,034 amino acid-long protein with a putative initiation methionine followed by a hydrophobic leader peptide (Fig. 1A). The E7-encoded protein appears to be a transmembrane protein with a large extracellular domain of 753 amino acids and an IC domain of 258 amino acids. The protein shares a structural similarity with Notch ligands such as Jagged and Delta. These similarities include, first of all, 14 EGF-like repeats [Nye and Kopan, 1995] in the extracellular domain. Additionally, a region of the protein immediately preceding EGF repeats shares a considerable similarity with the DSL domain. This includes a position in the protein molecule, and a significant, but not complete, conservation of the consensus amino residues of the DSL domain, as illustrated by the alignment of the DSL domains of several DSL family proteins and a corresponding region of Jedi (Fig. 1B). On the basis of similarity with Jagged and Delta, the cloned cDNA was named *Jedi*.

Clone F4, in comparison to E7, had a 152 bp long insertion after nucleotide 2277 (nucleotide 2344 of the E7 clone) leading to a stop codon shortly after the insertion point (Fig. 1C). The predicted protein lacks a transmembrane domain and most likely represents a secreted form of Jedi. Searches for sequence homologies in the GeneBank revealed a human cDNA sequence (Acc. No. XM_371320) which encodes a predicted protein FLJ00193 with a substantial homology to the entire protein sequence of Jedi (overall degree of aa identity 77%). This

A

1	<u>MPLCPLLLLLLALGLRLTGTLSNDPNVCTFWESFTTTTTKESHLRP</u>	leader peptide
45	<u>FSLLPAESCHRWPEDPHTCAQPTVVYRTVYRQVVKMDSRPLRQ</u>	
88	<u>CCRGYYESRGACVPLCAQECVHGRCVAPNQCCAPGWRGGDCS</u>	DSL-like
131	<u>SECAPGMWGPQCDKFCHCGNNSCDPKSGACFCPSGLQPPNCL</u>	EGF-like 1
174	<u>QPCPAGHYGPACQFDCQCYG-ASCDPQDGACFCPPGRAGPSCN</u>	EGF-like 2
216	<u>VPCSQGTDFGFFCPRTYPCQNGGVPQGSQGS CSCPPGWMGVICS</u>	EGF-like 3
259	<u>LPCPEGFHGPNCCTQECRCHNGGLCDRFTGQCHCAPGYIGDRCQ</u>	EGF-like 4
302	<u>EECPVGRFQGDAETCDAPGARCFPANGACLCEHGLTGDRCTE</u>	EGF-like 5
346	<u>RLCPDGRYGLSCQEPCTCDPEHLSLCHPMHGECSQCPGWAGLHCN</u>	EGF-like 6
391	<u>ESCPQDTHGPGQEHCLCLHGGLCLADSGLCRCAPGYTGPHCA</u>	EGF-like 7
434	<u>NLCPPDTYGINCSSRCSCENAIACSPIDGTICKEGWQRGNCS</u>	EGF-like 8
477	<u>VPCPLGTWGFNCNASQCVAHDGVCSPTGACTCTPGWHGAHCQ</u>	EGF-like 9
520	<u>LPCPKQFGEGCASVCDCHSDGCDPVHGQCRCQAGWMGTRCH</u>	EGF-like 10
563	<u>LPCPEGFWGANCSNTCTCKNGGTCVSENGNCVCAPGFRGPSCQ</u>	EGF-like 11
606	<u>RPCPPGRYGRKRCVQCKCANNHSSCHPSDGTCSCLAGWTGPDCS</u>	EGF-like 12
649	<u>EACPPGHWGLKCSQLCQCHHGGTCHPQDGSICITPGWTGPNCCL</u>	EGF-like 13
692	<u>EGCPPRMFGVNCSQLCQCDLGEMCHPQTGACVCPGHSADCK</u>	EGF-like 14
735	<u>MGSQESFTIMPTSPVTHNSLGA</u> VIGIAVLGTLVVALIALFIGYRQ	TM
780	<u>WQKGEKEHEHLAVAYSTGRLDGSYVMPDVSPSYSHYYSNPSYHTL</u>	
825	<u>SQCSNPNNPPNKVPGSQLFVSSQAPERPSRAHGRENHVTLPADW</u>	
869	<u>KHRREPHERGASHLDRSYSCSYSHRNGPGPFCHKGPISEEGLGAS</u>	
914	<u>VMSLSENENPYATIRDLPSLPGEPRESGYVEMKGPSPVSPRQSLH</u>	
959	<u>LRDRQORQLQPQRDSGTYEQPSPLSHNEESLGSTPPLPPGLPPGO</u>	PEST
1004	<u>YDSPKNSHIPGHYDLPPVRHPPSPPSRRQDR</u>	

B

Delta	<u>CDLNYYGSGCAKFCRPRDSSFHSTCSETGEILCLTGWQGDYIC</u>
LAG2	<u>CARNYFNRCENFCDAHLAKAARKRCDAMGRLRCDIGWMGPHC</u>
APX1	<u>CSSNYHGKRCNRYCIA-NAKL-HWECSTHGVRRCASGWSGEDC</u>
hJagged1	<u>CDDYYYGFGCNKFCRPRDDFFGHYACDQNGNKTCEMGWMGPEC</u>
mJagged2	<u>CDENYYSATCNKFCRPRNDFFGHYTCDQYGNKACMDGWMGKEC</u>
mD111	<u>CDEHYYGEGCSVFCRPRDDAFGHFTCGDRGEKMCDPGWKGQYC</u>
xD112	<u>CDEHYYGDCSDYCRPRDDNFGHYTCDQGNRLCMSGWKGEYC</u>
mD113	<u>CEPPAVGAACARLCRSRSAP---SRCG-PGLRPCTPF--PDEC</u>
hD113	<u>CAP-LEDECEAPLVCRCAGCSPEHGFCEQPGECRCLEGWTGPLC</u>
mD114	<u>CSDNYYGDNCSRLCKKRNDHFGHYVCQPDGNLSCLPGWTGEYC</u>
Jedi	<u>CCRGYYESRGACVPLCAQE-CVHGRCVAPNQCCAPGWRGGDC</u>
MEGF10	<u>CCPGFYESGEMCVPHCADK-CVHGRCIAPNTCQCEPGWGGTNC</u>
MEGF11	<u>CCPGYYENGDFCIPLCTEE-CVHGRCVSPDTCHEPGWGGPDC</u>

consensus C Y C C H C G C G W G C

C

E7 Jedi	723	<u>PPGHSGADCKMGSQESFTIMPTSPVTHNS</u> LGAVIGIAVLGTLVVALIALF
F4 Jedi	723	<u>PPGHSGADCKMGESFAPLTLVFL*</u>

Fig. 1. Sequence and protein motifs of Jedi. **A:** Predicted amino acid (aa) sequence of Jedi (accession No. [AF444274](#)). Leader peptide (aa 1–19) and DSL-like domain (aa 89–130) are underlined. EGF-like repeat motifs 1–14 are aligned and indicated on the right side of the sequence. Highly conserved amino acids of EGF-like repeats are marked with gray. The transmembrane domain (aa 757–775) is boxed. The PEST motif (aa 984–1007) is double underlined. **B:** Alignment of DSL domains of different Notch ligands with the corresponding regions of the Jedi/MEGF proteins (accession Nos. are provided in parentheses): *D. melanogaster* Delta ([X06289](#)), *C. elegans* Lag-2 and APX-1 ([X77496](#) and [U50143](#), respectively), human Jagged

1 ([U61276](#)), mouse Jagged 2 ([MN 010588](#)), mouse Dll1 ([AB050457](#)), *X. laevis* Dll2 ([S74210](#)), mouse and human Dll3 ([NM 007866](#) and [NM 016941](#), respectively), mouse Dll4 ([XM 123881](#)), human MEGF10 ([AB058676](#)), and human MEGF11 ([BC126313](#)). Highly conserved amino acids are marked with gray. **C:** Alignment of fragments of amino acid sequences of Jedi cDNA clones E7 and F4 (GenBank accession No. [AF461685](#)). Identical sequences are underlined. Insertion of the 152 nucleotides in the cDNA sequence after nucleotide 2,277 of the F4 clone (amino acid 734) results in a stop codon (asterisk). The transmembrane domain is boxed.

protein is likely to represent a human homolog of Jedi. Two earlier reported human transmembrane proteins with EGF-like repeats, MEGF10 and MEGF11 (Acc. Nos. AB058676 and AB058677, respectively) [Nagase et al., 2001] also demonstrate lower but still substantial homology with Jedi, as seen in the amino acid alignment shown in Figure 2. The highest degree of similarity of these proteins to Jedi was found in their extracellular regions, which contain 17 EGF-like repeats and have roughly 50% of amino acids identical to those of Jedi. In the IC region, the similarity was largely limited to the three blocks of amino acid sequences surrounding conserved tyrosine residues. Little is known about the function of these MEGF proteins. The initial insight may come from the protein interaction studies, which indicate that MEGF11 interacts with Atrophin 1 [Lim et al., 2006]. For MEGF10, several diverse interacting partners were identified using a yeast two-hybrid system, including growth factor receptor-bound protein 10, ATP dependent RNA helicase 3, translokin (Cep57), ALMS1 and others [Nakayama et al., 2002].

The Jedi amino acid sequence similarity with other proteins, including DSL family proteins, was substantially lower. On the basis of these data, we conclude that Jedi, MEGF10 and MEGF11 represent a new family of transmembrane proteins with multiple EGF-like domains.

Expression Pattern

Studies of the tissue distribution of Jedi mRNA in mouse tissues using Northern blotting (Fig. 3A) revealed broad distribution of the message, with maximal expression detected in kidney and heart, followed by lung, spleen, liver, brain, testis, skin, and stomach. Three distinct hybridizing bands 3.6, 4.3, and 5.8 kb in length were detected on the blots, with the 4.3 kb band most likely corresponding to the 4,290 bp long clone E7. Two other mRNA species may represent different splice variants of Jedi mRNA. We also analyzed Jedi expression in hematopoietic stem/progenitor cells by Southern hybridization with amplified 3' terminal total cDNA fragment populations from sorted cells as described previously [Zinovyeva et al., 2000]. Jedi mRNA, as judged by the abundance of the cDNA fragment of the expected size 149 bp, was expressed at the highest level in $\text{Rho}^-/\text{Rho(Ver)}^+$ fraction corresponding to short

term repopulating stem cells, and at a lower level in $\text{Rho}^+/\text{Rho(Ver)}^+$ fraction (long term repopulating stem cells) (Fig. 3B). Comparably lower expression was found in Rho^{dull} cells (oligopotential progenitors), whereas little if any expression was detected in $\text{Rho}^{\text{bright}}$ cells (committed progenitor cells).

Additionally, we tested several murine hematopoietic and stromal cell lines (listed in Material and Methods section) for expression of Jedi protein by FACS using an anti-Jedi antibody. Jedi expression was detected only in the AFT024 fetal liver stromal cell line (Fig. 3C). This result was also confirmed by RT-PCR (Fig. 3C, inset). Expression of Jedi by AFT024 cells known to support transplantable long-term repopulating murine stem cells [Moore et al., 1997a], prompted us to study expression of the gene in hematopoietic stroma and related cells. RT-PCR analysis using identical amounts of RNA isolated from total bone marrow, from stromal cells derived from long-term bone marrow hematopoietic culture [Dexter et al., 1984], from cultured mesenchymal stem cells (MSCs) and from MSCs differentiated along osteogenic lineage [Peister et al., 2004] demonstrate clearly that Jedi RNA is expressed at several-fold higher levels in cells supporting hematopoiesis, namely stromal cells and MSCs differentiated along osteogenic lineage.

Hematopoietic Progenitors and Thymocytes Express Jedi

From hybridization experiments with cDNA libraries we expected a very small number of cells in the bone marrow to be Jedi-positive; therefore, we studied Jedi expression in lineage negative cells. The mononuclear fraction of bone marrow was stained with FITC-labeled anti-lineage antibody cocktail along with anti-Jedi antibody. Lin^- cells were gated and analyzed for Jedi expression. We found that around 15% of Lin^- cells were Jedi-positive (Fig. 4A).

The thymus is one of the important hematopoietic tissues that also demonstrated expression of Jedi message. Using FACS analysis, we investigated Jedi protein expression in the thymus. A single cell thymocyte suspension was stained with anti-Jedi antibody or control rabbit IgG. On the forward scatter/side scatter graph, all thymocytes grouped into three distinct cell populations (Fig. 4B). Each group was gated and analyzed for Jedi staining. Jedi positive cells (4.1%) were detected in the

hMEGF10	(1)	MVISLNSCSLFTICLLCHWIGTASPANLEDPNVCSHWESYSYTVQSSYPPEFDQIYYTSCITDILNWFKCTRHRVRYRARRHEKTYRRESQCCPFYSEEMCVHCAKCKVHGRCI
hMEGF11	(1)	-----MVLSTLGIARFLOALNTPDPNVCSHWESYAVTVQSSYAPFDQIYYRCTDILNWFKTRHRYSYKTYARRGRTMYRRSQCCPYENGFCTICTEFCVHGRCV
mJedi	(1)	-----PLCLPLLLALGLTANLSTANLDPNVCTFWESFVTKESHLRSEFSLPAECHRWFEDHTCAQPTVVRVYRQVWMDSRPALCCRGVYERGACVLCACVHGRCV
Consensus	(1)	-----LS-----L--T--LN-EDPNVCSHWESY-VTVQSSY-HPFQDIYYTSCITDILNWFKTRHRYSYRARRGRTMYRRSQCCPFYSEEMCVHCAKCKVHGRCV
hMEGF10	(120)	APVCCQEPGWTNCSACDGDHGHPHTRCCKGALCNPIITGACHAAGFRGWRCEDEQEQRYNDHQRCQQQNGAICDHPVTEERRCPQCYTGAFCEDCPFKSHGQRCRCP
hMEGF11	(114)	SPDICHCEPQWGPDPSSGDHGHGPHSNRCCQNGALCNPIITGACHAAGFRGWRCEELCAPCHKGLPCCRHGASCDPRAGECLCAPCYTGVYCEELCPFKSHGHAHELRCRCP
mJedi	(114)	APQCCACGWRGDCSECAPMGWQDKFKHCGNNSDDPKSACAFPSGLPPNGLQPPAHPGEPACDFDCCQVY-ASCDPQDCACTFPQRAQPSCNVPSQSGHDFPFRPYF
Consensus	(121)	APNTCCQEPGWG-DCSS-CD-DHWGPHC--RCQC-NGALCNPIITGAC-CAAGFRWRCE--C--GTYG--CQ--CQC--GASCDP--GEC-CPPGYTG--CE-ICPFG-HG--CE-RCP
hMEGF10	(240)	CONGVCHHVTCECSFSGMGTVCQPCPEGRFKKCSQEQCHNGTCDAAITGQCHCSPSYTGERCCDFCPYTVGLCAETCCQVNGGKCYHVSQAICCEAGFAFEREARLCEPEGL
hMEGF11	(234)	CONGGTCHHTCEACPCPMTCAVAPCPPEPTQNCSDPCHGGQDDHVTCQCHCTAGMVDRCOCEPFFSFGQCSORCDHNGGQSPSTGACCEPKYKPCORCLRCPEGL
mJedi	(233)	CONGGVPOGSSCSCHPFGMGLVLSLPCPEGFHPNCTLRCHNGGLDHRFGTQCHCAFSITGDRCCOECPCYRFQDCAETCDAPAKFPANAGALCEHGFTRCERLRCDDGR
Consensus	(241)	CONGGVCHH-TGRCSCPPGMMG-VC-QPCPEG-FG-NCQEC-CHNGG-CD--TGQCHC-PGY-GDRQCEPCVY-FG--CAETCDC-NGG-C-P--GALICE-GF-G-RC-ERLRCPEGL
hMEGF10	(360)	YGLKDKRCPCHLENTSCHPFSQACKRPFMSGLYCNEMTSPGFEAQQLISQNGADDSVITCKTCAQPKGIDSTPPLFTYGINCCSSCKKIDAVESPDVGSCTCKAGWGG
hMEGF11	(354)	HGGTLIPCPDADNTHSCHPVTGACTQCPGWSHHCNESPQVGYGDGQLPITQNGADHSITGCTCAPGMEVCAVSAAGTYGPNCCSSTICSONGCTGSPVDGSCTKCKAGWGG
mJedi	(353)	YGLSQEPTCDPPEHSLSCHPWHBSCQPGMAIDHCNESPQDTEPEQEHLLHGLLADSLRCAPGTPPHANLPPDIYGINCCSSRCSCEAIAACSPIDGTITCKEAGWQR
Consensus	(361)	YG--C--PCPCD-ENT-SCHPM-GEC-CQPGWSLHCNESP-G-YG-GCQ--C--CQNGADC-S-TG-CTCAPGF-G--CA--CP-GTYGINCCSSRCS-C--CSPVDGSCTKCKEAGWGG
hMEGF10	(480)	VDCIRCFSGTWFVGCNLTCCQNGGANTLDCITCAFGRREKELPCQDQYGLNCAERDCSADGGHPTTGCHRLCPGWSVHRCDSYCAEERWGNCSYPCYKNGASCSPDDGI
hMEGF11	(474)	LDCTLPCSGTWGLNCHESCTANGAASPLDSCSTPGMLGDTLPCPDGELNCHSEHCDCHADGDPTVGHCCLAGTLEFCDSCTPPWRWGNCSYCSLENGSCSPEDGS
mJedi	(473)	GNCSVPCLTGTFNCKNASQCAHDVCSFQTACTTPGHRGAHQQLPCPKQEGEASVCDCCDSQDQVFGCQQAQGMGTFCHLPEPEPFMGANCSNTCTKNGEFCVSNENGN
Consensus	(481)	-DCS-PCPSTGTFNCHN-SCQANGACSP-DG-CTCTPGW-G--CELPCPDGTFGLNCAE-CDCSHADGDPVTVGHCRCLAGW-G-RCDS-CPEGRWGNCS--C-CKNGGSCSPEDG-
hMEGF10	(600)	CECAPFRGTCQRIKCSFGYCHR-SOTPCQVHSSGPHHTIGLDCLPFGTALCHNEVCPSEFRKNCAGICTNMGTCNIDRSCQYVPGWISGDCSQPCPPAHWGNPIHTNCH
hMEGF11	(594)	CECAPFRGTCQRIKCPGYVCHR-SOTPELVCHSSRCHHLSGIECLGFSGALCNVCAQYFGQDCAQLCSANNGTCSIDRSCQYVPGWISGDCSQPCPPAHWGNPIHTNCH
mJedi	(593)	CVCAFPFRGTCQRIKCPGYVCHR-SOTPELVCHSSRCHHLSGIECLGFSGALCNVCAQYFGQDCAQLCSANNGTCSIDRSCQYVPGWISGDCSQPCPPAHWGNPIHTNCH
Consensus	(601)	CECAPFRGTCQRIKCPGYVCHR-SOTPELVCHSSRCHHLSGIECLGFSGALCN-VC--G-FG--CA--C-C-NNGTG-C-PGWIG-DCSQPCPPAHWGNPIHTNCH
hMEGF10	(720)	NGAFCSAYDGEKCTPGWTLGCTQRCPLCYKDKDALTCCQONGADCDHISGQTCRTGFMGRHCKOKPSGTGYGGRQIQCILNNTCDBHITCTCYCSPGKARGCDQAGVIVGNL
hMEGF11	(714)	NGAFCSAEDGACHCTPGWTLGCTQRCPLCYKDKDALTCCQONGADCDHISGQTCRTGFMGRHCKOKPSGTGYGGRQIQCILNNTCDBHITCTCYCSPGKARGCDQAGVIVGNL
mJedi	(668)	HGGTCHPQDGLICTPGWTLGCTQRCPLCYKDKDALTCCQONGADCDHISGQTCRTGFMGRHCKOKPSGTGYGGRQIQCILNNTCDBHITCTCYCSPGKARGCDQAGVIVGNL
Consensus	(721)	NGA-CSA-DG-C-CTPGWTLGCTQRCPLCYKDKDALTCCQONGA-CDHISG-CTQRTGF-G-HCEQ-C-C-GT-GYGC-Q-C-C-NNSTCDH-TCYCYCSPG-KG-RCDQA-----L
hMEGF10	(840)	NSLSRSTALPADSYQIGATAGLILIVLVLFLLALFIVRHKQKH-ESSMPAITYTPAMRVVNAQYTIISGTLPHSNGGNANSHYFNPSYHTLQCAIYSPHYNNRDRMTVTKSKNQL
hMEGF11	(833)	NPYKISPAIGERHSVGAVTGMLLFTLVVLLGLLAWRRQKEGRDLAPRVSYTPAMRMTSTDYISLQACGMDR-----HYXSNPSYHTLQCAIYSPHYNNRDRMTVTKSKNQL
mJedi	(738)	CESEFTIMFTSFTVTHNSGAVIGIAVIGTIVVALIALLIGYKQKH-EHEHLAVYS-TGTLDGSDYVMPDVSYS-----HYXSNPSYHTLQCAIYSPHYNNRDRMTVTKSKNQL
Consensus	(841)	N-----S-ALPA---S-GAV-GI--L--LVV-LLALFI-YR--QKKE-E--PAV-YEPAMR---D---SG--P-----H---NPSYHTLQCAIYSPHYNNRDRMTVTKSKNQL
hMEGF10	(959)	FVALKNVFKGCPVGCCTGTLPADWKHGGYLNELGAFGLDRSY-NKSLKDLKNSRYN-----SNCSLSSSENRYATIKNPPVLPKSSFCGAVEMKSPARRDSYAEVENS
hMEGF11	(914)	-----NYIMDKGFDYKESVGS-----SSTCLNSENRYATIKNPPVLPKSSFCGAVEMKSPARRDSYAEVENS
mJedi	(843)	FVSSQAPERPSAHRNHVTLPADWKHRRPHERGASHLDRSYCSYSYHN-EGPFGHKGPISERGLGASVMSLI--SENRYATIKNPPVLPKSSFCGAVEMKSPARRDSYAEVENS
Consensus	(961)	FV-----R-----TLPADWKH-----E-GA--LDRSY-M-KS-KD-GK-S-C-----SS-CSI--SENRYATIKNPPVLPKSSFCGAVEMKSPARRDSYAEVENS
hMEGF10	(1068)	TSANR-----NVLEVEPTVSVVQGVFSNNG-----RLSDPQYILKNSHIPCHYDILPVARDSSSPKQDSDSGSSNSSSSSE
hMEGF11	(982)	STNR-----NIVEVEPTVSVVQGVGCHNS-----SYIQNAVYLRFRNSHIPCHYDILPVARDSSSPKQDSDSGSSNSSSSSE
mJedi	(961)	DRQQLOLPQRDSTVHEQPSLTHNSESLSPTLPPCPQYDSKNSHIPCHYDILPVARDSSSPKQDSDSGSSNSSSSSE
Consensus	(1081)	---NR-----N-VEVEPTVSVVQGV-----L-Q--YDLPKNHIPGHYDILLPVR-SPS-----GFS-----S

population of small cells (R1 on Fig. 4B) only (Fig. 4C). The cells expressing Jedi were CD3-negative (data not shown), which implies that these cells likely belong to the thymus stromal environment.

Expression of Jedi in Bone Marrow Progenitor/Stem Cells Leads to Reduction of Committed Myeloid Progenitor Cells

To assess the potential role of Jedi in hematopoiesis, we expressed FL, extracellular membrane-tethered (MT) or IC forms of Jedi in bone marrow MNC using retrovirally mediated gene transfer. Transfected GFP-positive BM MNCs were sorted and their CFU-c content was analyzed in methylcellulose medium supplemented with growth factors promoting myeloid differentiation. Figure 5 demonstrates that the numbers of CFU-c were significantly (about 6.5-fold) reduced for cells transduced with Jedi-FL compared with control SFG vector, or Jedi-MT. In contrast, BM MNC expressing Jedi-IC produced more CFU-c than control cells.

Survival of Colony-Forming Cells on NIH 3T3 Fibroblasts Expressing Jedi

Expression of Jedi in AFT024 cells, and in bone marrow stromal cells points to a possible role for Jedi in support of hematopoiesis. Therefore, we investigated whether expression of Jedi protein in NIH 3T3 cells may enhance their hematopoietic supporting activity. We used retroviral gene transfer to create NIH 3T3-derived cell lines expressing either FL Jedi—NIH 3T3-FL or the MT variant of Jedi—NIH 3T3-MT. The control cell line, NIH 3T3-SFG, was obtained by transduction with the pSFG vector. Expression of FL and MT forms of Jedi on the cell surface was verified using FACS with anti-Jedi antibody (Fig. 6A). To determine the effect of Jedi on the maintenance of hematopoietic progenitors, mouse bone marrow MNC were cultured on monolayers of retrovirally transduced NIH 3T3 cells in the absence of exogenous hematopoietic growth factors. CFU-c assays performed on non-adherent cells har-

vested on Days 2–12 revealed a substantial reduction in the number of progenitors in co-cultures with cells expressing full-length Jedi, but not with those expressing the MT form of the protein (Fig. 6B). On Days 9 and 12, the total number of colonies in NIH 3T3-FL co-cultures was 3.8- and 4.9-fold lower than in control co-cultures. These results indicate that the expression of the full-length Jedi on stromal cells substantially reduces the number of non-adherent myeloid progenitors.

To assess whether a direct cell-to-cell contact is required for the observed effect, BM MNCs were cultured in Transwells to keep them separated from retrovirally transduced monolayers by a 0.4 μm pore size membrane. Colony assays of cells in the Transwells on Day 8 revealed no substantial difference in the number of colony-forming cells in wells with NIH3T3-SFG and NIH3T3-FL (Fig. 6C). Therefore, the negative effect of Jedi on BM non-adherent colony-forming cells requires a direct cell-to-cell contact. Although it is likely that this effect is mediated by a direct interaction of stroma with BM cells, the role of diffusible factors (such as soluble forms of Jedi) in this process cannot be excluded.

The observed reduction of non-adherent colony-forming cells by the Jedi protein can be a result of suppression of differentiation of the stem/early progenitor cells into late progenitors. To verify this possibility, we quantitated HPP cells and quiescent HPP (HPP-q) cells in the BM MNCs co-cultured with Jedi-expressing NIH 3T3 monolayers. The obtained results (Fig. 6D) indicate that expression of the FL, but not the MT form of Jedi on stromal cells resulted in an approximately threefold decrease in the number of non-adherent HPP cells, whereas the number of quiescent HPP (calculated as difference between HPP-q and HPP cells) was reduced even more dramatically. Therefore, expression of Jedi on stromal cells results in a reduction of the number of both early and late non-adherent myeloid progenitor cells.

Fig. 2. The alignment of amino acid sequences of human MEGF10, MEGF11, and mouse Jedi. Alignment was performed using AlignX module of the Vector NTI 5.0 suite (InforMax, Inc.) on the basis of Clustal W algorithm. Internal dashes indicate gaps introduced for maximal alignment. Amino acids identical in all three sequences are highlighted in red on yellow background; those identical in two sequences only are black on blue

background; blocks of strongly similar amino acids are in white on green background; weakly similar amino acids are in green. TM regions are underlined. The amino acids of the consensus sequence highlighted in red correspond to the tyrosine, serine and threonine residues which are predicted to be phosphorylated in Jedi and are conserved in at least one additional member of the Jedi/MEGF family.

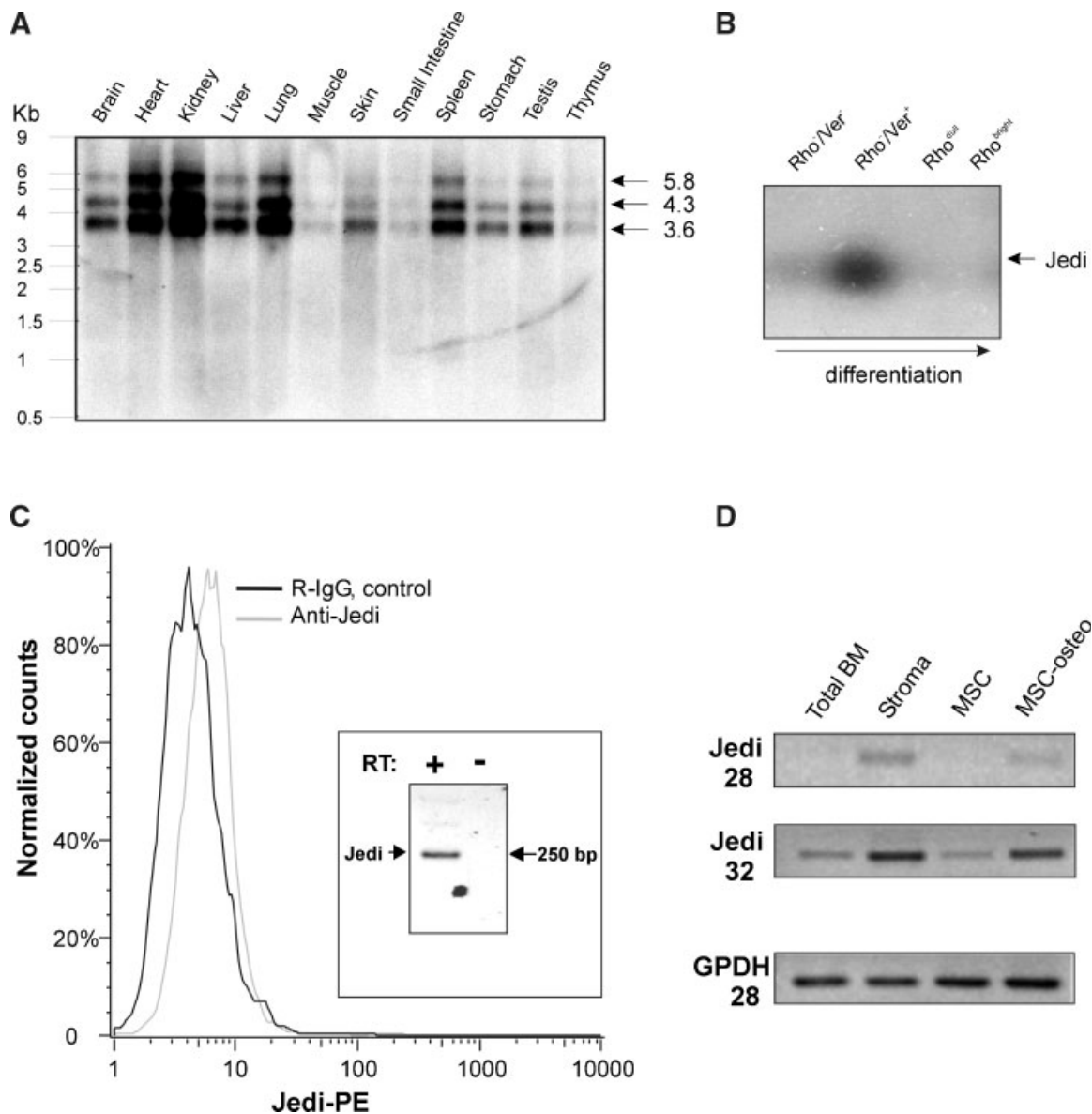


Fig. 3. Expression of Jedi in mouse tissues and cells. **A:** Analysis of Jedi mRNA expression in adult mouse tissues by Northern hybridization. The blot was hybridized with Jedi probe representing the extracellular part of the protein (nucleotides 202–1196). Positions and calculated lengths of Jedi transcripts are indicated on the right. **B:** Expression of Jedi in adult mouse hematopoietic stem/progenitor cell fractions. One microgram of amplified total cDNA from each sorted fraction was electrophoresed on 2% alkaline agarose gels, transferred onto Hybond-N⁺ membrane and hybridized with radioactively labeled probe corresponding to the 800-bp fragment of Jedi cDNA located in the 3' end of Jedi RNA (nt 3444 to 4244). The Rho⁻/Rho(Ver)⁻, Rho⁻/Rho(Ver)⁺, Rho^{duil}, Rho^{bright} sorted bone marrow cell fractions correspond to LTR stem cells, STR stem cells, multipotential progenitor cells and committed progenitor cells, respectively [as described by Zinovyeva et al., 2000]. **C:** Expression of Jedi protein in AFT024 stromal cell line.

Overlaid are FACS plots of AFT024 cells labeled with control rabbit IgG (black) and anti-Jedi-ex (gray). Inset depicts RT-PCR analysis of expression of Jedi RNA in AFT024 cell line. The predicted fragment size is 250 bp. Reverse transcriptase (RT) was added to the experimental sample (+) but not to the control one (-) to exclude false positives from contaminating DNA. **D: Upper panel:** expression of Jedi RNA in bone marrow-derived cells analyzed by RT-PCR. Identical amounts of total RNAs from unfractionated bone marrow, stromal cells from the long-term bone marrow culture, cultured MSCs and MSCs, differentiated along the osteogenic lineage, were used for analysis. Stromal cells and MSCs were prepared using methods described earlier [Dexter et al., 1984; Peister et al., 2004]. **Lower panel:** control amplification of the housekeeping gene glyceraldehyde phosphate dehydrogenase using the same preparations of first strands as in the upper panel.

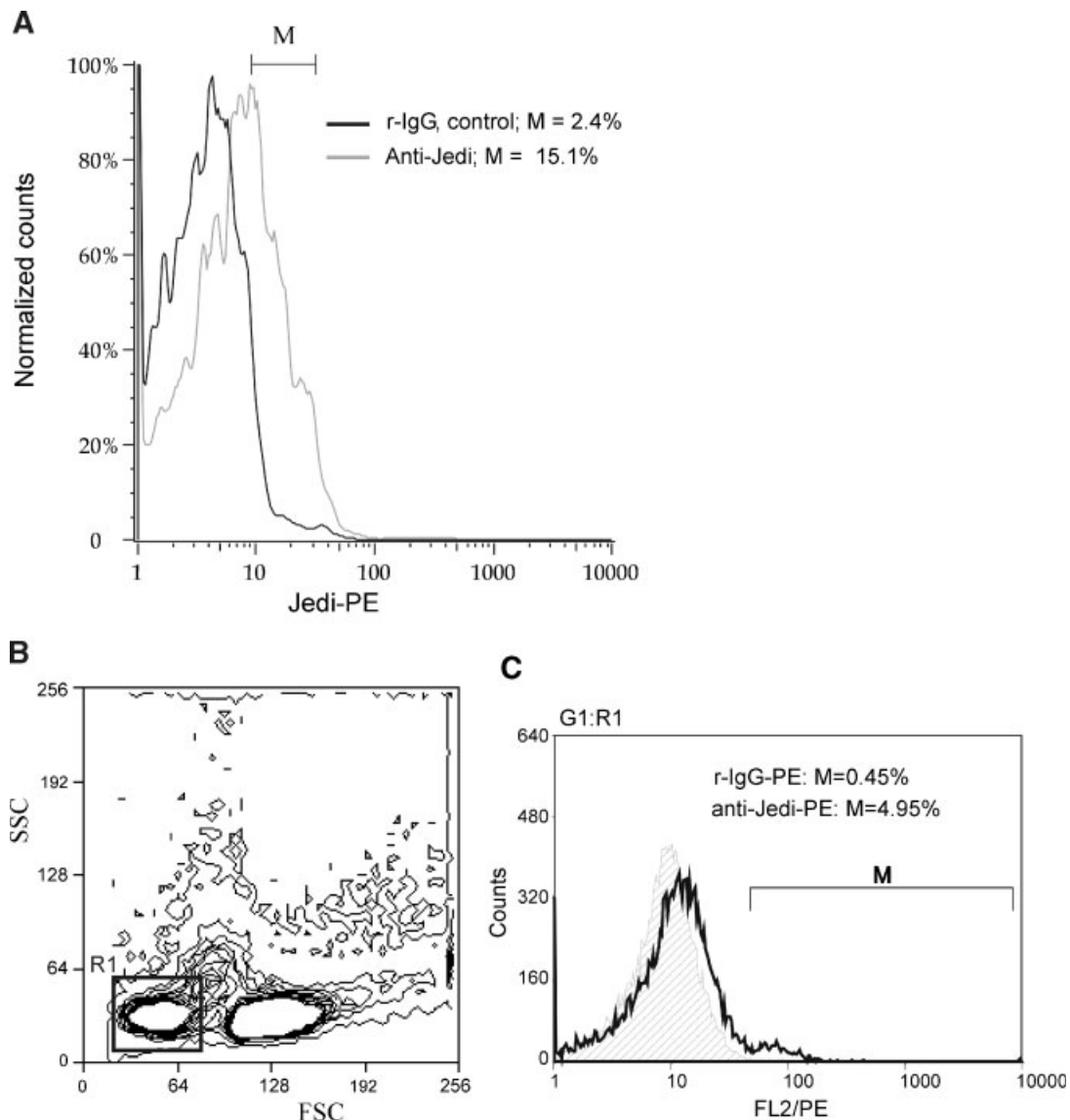


Fig. 4. Expression of Jedi protein in mouse cells. **A:** Expression of Jedi in BM Lin-negative cells. Overlay of FACS plots of gated Lin-negative populations of BM MNC, stained with the cocktail of FITC labeled Lin antibodies along with either control rabbit IgG (black) or anti-Jedi-ex antibody (gray), and detected using secondary goat-anti-rabbit PE labeled antibody. FITC dim cells (5% of total) were gated and plotted for PE fluorescence.

B,C: Expression of Jedi protein in the thymus. A single cell suspension of thymocytes was labeled with either control rabbit IgG or anti-Jedi antibody and developed with secondary goat-anti-rabbit PE labeled antibody. **B:** Smallest cells on the FSC x SSC histogram (R1) were gated and analyzed for PE. **C:** Overlay of FACS plots.

Jedi and Notch Signaling

The general structural similarity of Jedi to the Notch ligands, in particular the presence of a DSL-like domain, suggested that Jedi may be a novel ligand involved in Notch signaling. To verify this possibility, we performed experiments with the extracellular domain of Jedi. It was shown earlier that whereas the expression of the intact Notch ligand Jagged1 stimulates Notch signaling as judged by the reporter

activity in transiently transfected cells, addition of the extracellular domain of Jagged1 to cells produces the opposite effect, namely inhibition of Notch signaling [Small et al., 2003]. We therefore expressed myc-6xHis-tagged extracellular domains of Jedi and Jagged1 (sJedi and sJagged1, respectively) in COS1 cells, and applied the obtained conditioned media to HEK293 cells transiently co-transfected with Notch1, CBF-luc, and pRL-CMV plasmids. The results (Fig. 7A) indicate a

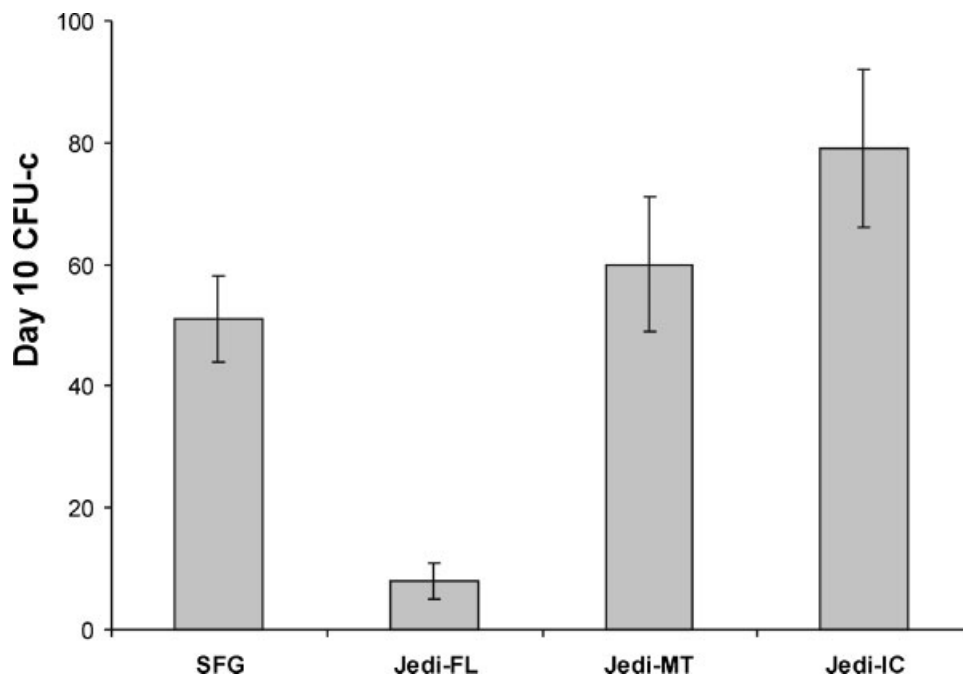


Fig. 5. Effect of Jedi expression in BM MNC on myeloid differentiation. Retroviral vectors encoding full length (FL), extracellular membrane truncated (MT), intracellular (IC) forms of Jedi, or control vector (SFG) were transduced into BM MNC cells. Transfected cells expressing GFP marker were collected using FACS and plated, in triplicate at 800 cells per 2-cm dish, in 3435 methylcellulose media. After 10 days GM-CFC, G-CFC, and M-CFC were scored. The data shown represent mean numbers of colonies for four experiments, with error bars indicating standard deviation ($P=0.005$, Student test).

distinct decrease in Notch signaling activity after addition of media conditioned by sJedi- or sJagged1-expressing cells compared to that of the untransfected COS1 cells.

Since the culture supernatants represent a highly heterogeneous mixture of polypeptides, the observed effects for sJedi might be caused by other protein factor(s). To address this issue, we performed partial purification of sJedi and sJagged1 proteins in culture supernatants using metal ion chromatography. Judging by Western blots (not shown), the enrichment factors was about 1,000-fold for both proteins. When amounts of partially purified of sJedi or sJagged1 proteins equivalent to those used in the previous experiment were applied to responding cells, a similar or more pronounced inhibition in Notch signaling was observed (Fig. 7B). Transfection of responder cells with sJedi or sJagged1 plasmid also resulted in suppression of the Notch pathway. These results provide substantial evidence that Jedi participates in Notch signaling, either directly through interaction with Notch, or indirectly through one of the components of the Notch pathway.

We next sought to establish whether the Jedi role in the Notch signaling was positive, like the established Notch ligands Jagged or Delta, or negative. To this ends, we compared the effects of Jedi and Jagged1 on Notch signaling using the co-culture assay [Moloney et al., 2000]. NIH 3T3 cells expressing Jagged1 induced an approximately fivefold increase of CBF1 reporter expression compared to control cells, whereas for cells expressing Jedi, there was no induction but rather a weak inhibition of reporter signal (Fig. 7C). To verify that the observed lack of positive effect from Jedi expression was not due to a much lower expression of Jedi compared to Jagged1 in NIH 3T3 cells, both proteins fused C-terminally with V5-6xHis tag, were retrovirally expressed in NIH 3T3 cells. Western blotting using anti-V5 antibodies demonstrated that Jedi and Jagged1 were expressed at similar levels (data not shown). Moreover, the fused version of the Jagged1 protein was still able, although weaker, to induce expression of CBF1 reporter in co-culture experiments, whereas the fused variant of Jedi had no effect. We then asked whether the virtual absence of effect of Jedi on

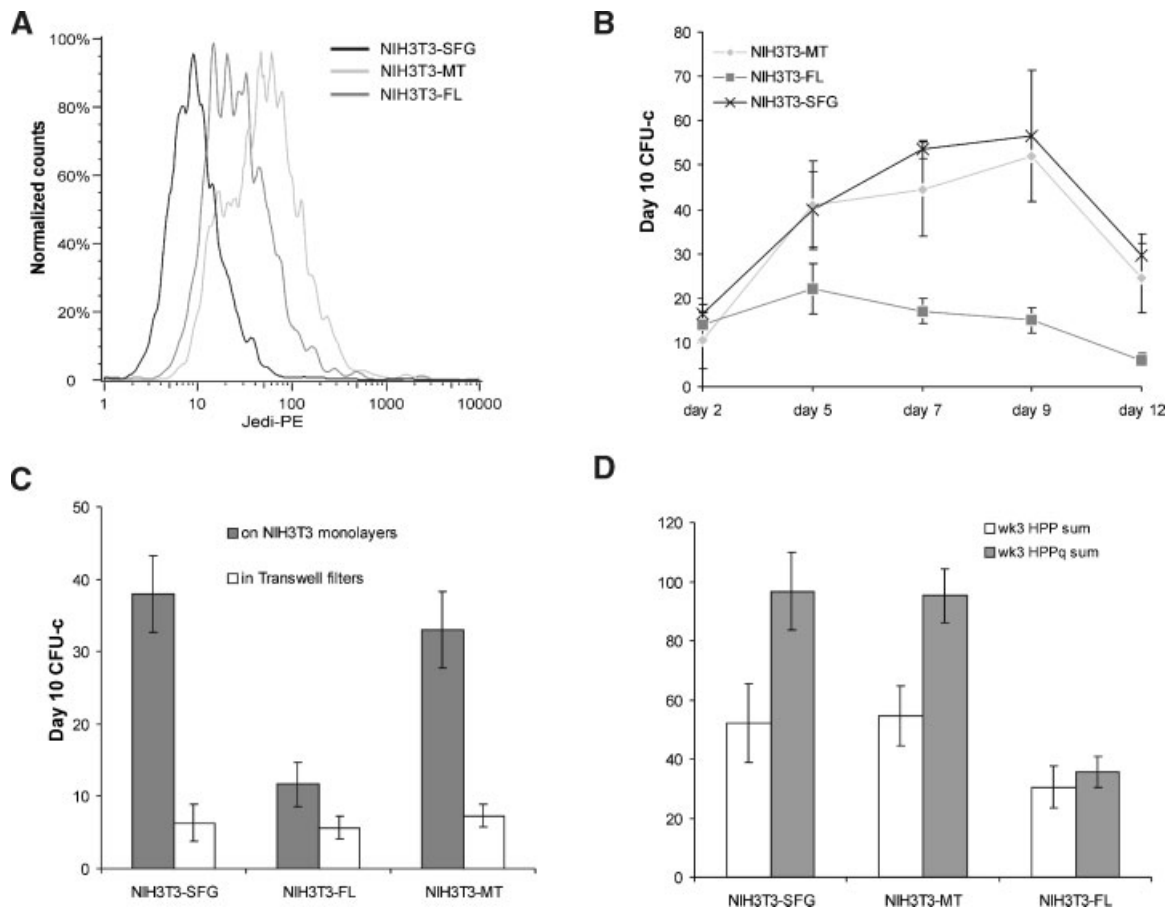


Fig. 6. Effect of Jedi protein expression in NIH-3T3 cells on maintenance of hematopoietic progenitors. **A:** Flow cytometry analysis of NIH-3T3 cells transduced with retroviral vectors expressing full length or extracellular MT forms of Jedi, or with a control vector (NIH-3T3-FL, NIH-3T3-MT or NIH-3T3-SFG cells, respectively). For cell staining, anti-Jedi antibody was used. Overlay of the FACS plots of control NIH-3T3-SFG (black), NIH-3T3-FL (dark gray), and NIH-3T3-MT (light gray) cells is shown. **B:** Co-culture of BM MNCs with control NIH-3T3-SFG (black), NIH-3T3-FL (dark gray) or NIH-3T3-MT (light gray) cells. BM MNCs were placed in 12-well plate (1×10^5 cells per well) containing a monolayer of one of the indicated cell types. After indicated periods, aliquots of non-adherent cells were plated in

triplicate in methylcellulose media 3534 (StemCell Technologies). CFU-c was scored at Day 10. **C:** Co-culture of BM MNCs with NIH3T3-SFG, NIH3T3-FL or NIH3T3-MT cells for 7 days in direct contact (black boxes) or in Transwells (white boxes). CFU-c was scored as in B. **D:** HPP and HPP-q assays for co-cultures of BM MNCs. After 7 days of co-culture of 10^5 BM MNCs with a monolayer of NIH3T3-SFG, NIH3T3-FL or NIH3T3-MT cells, non-adherent cells were plated in MethoCult GF 3434 (Stem Cell Technologies) supplemented with GM-CSF (and anti-TGF-beta antibody for HPP-q assay), and colonies 0.3 mm or larger were scored after 21 days. The data shown represent mean numbers of colonies for three (B) or two (C, D) experiments, with error bars indicating standard deviation ($P=0.005$, Student test).

Notch signaling in this type of assay could be attributed to cell type-specific effects. When we co-cultured CHO cells expressing Jedi-V5-6xHis protein with NIH 3T3 responder cells, a small but reliable inhibition of Notch signaling was observed, whereas expression of Jagged1-V5-6xHis by CHO cells provided a moderate activation of the reporter signal (Fig. 7D).

Tyrosine Phosphorylation of Jedi

The data reported above demonstrate that the IC domain of Jedi is critical for the observed

negative effects of the protein on colony formation by bone marrow cells, indicating the role of the IC domain in the cell signaling. Accordingly, analysis of the Jedi IC region revealed that 5 out of total 14 tyrosine residues, namely the residues 803, 821, 923, 941, and 976, were predicted to be phosphorylated with high probability by the NetPhos 2.0 prediction server (<http://www.cbs.dtu.dk/services/NetPhos>). To assess whether the Jedi protein can be phosphorylated in vivo, we used peroxyvanadate treatment of cells as a model system.

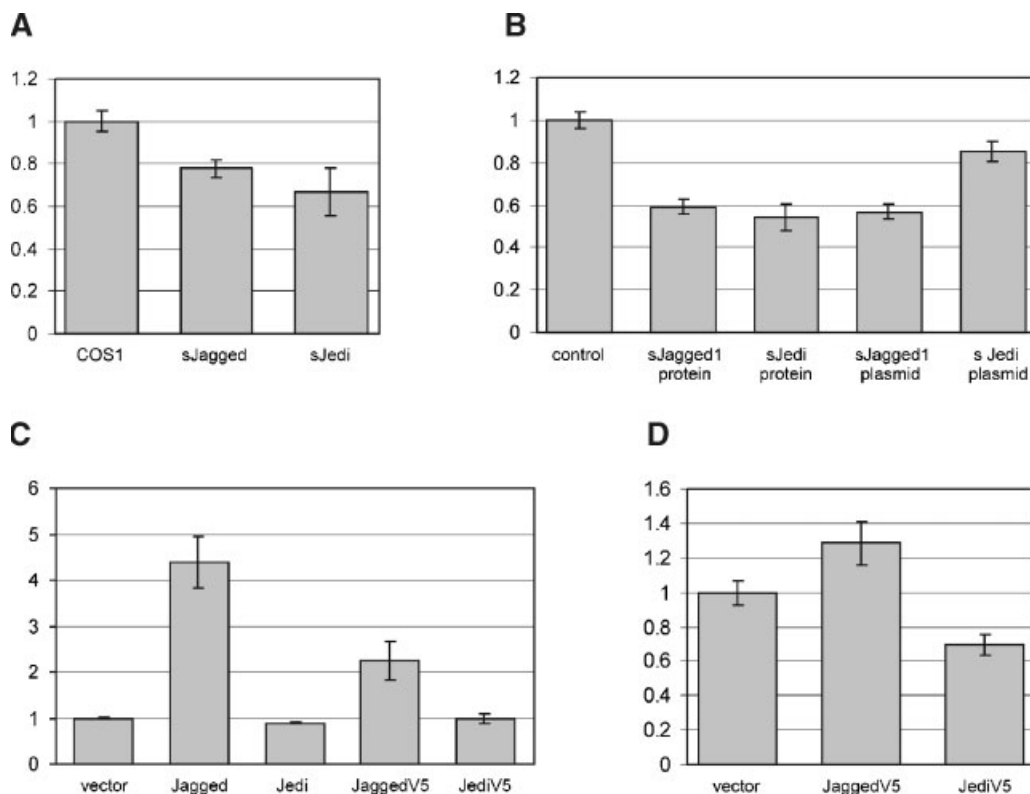


Fig. 7. Analysis of involvement of Jedi in Notch signaling. **A,B:** Analysis of the effects of the extracellular domain of Jedi (sJedi) on Notch signaling. **A:** HEK293 cells co-transfected with plasmids encoding full-length Notch1, CBF1 reporter, and pRL-CMV were incubated with media conditioned by non-transfected COS1 cells (control) and COS1 cells transfected with sJedi- or sJagged1-expressing plasmids. **B:** Same as A, but cells were incubated with partially purified sJedi or sJagged1 proteins (enrichment factor approximately 1,000-fold in both cases). Effects of transfection of cells with sJedi- or sJagged1-expressing plasmids were also examined. **C,D:** Analysis using the cell

co-culture assay. **C:** NIH 3T3 cells co-transfected with plasmids encoding full-length Notch1, CBF1 reporter and pRL-CMV were co-cultured with either control NIH 3T3 cells (transduced with the pBabe vector), or with cells stably expressing untagged or V5-6xHis-tagged variants of Jagged1 and Jedi. **D:** Same as C, but responder cells were co-cultured with CHO cells stably expressing V5-6xHis-tagged variants of Jagged1 and Jedi. In all experiments, the results were plotted as relative luciferase units (RLU), which reflect fold activation induced by experimental samples over those obtained with control samples.

Peroxyvanadate, as an inhibitor of tyrosine phosphatases, shifts the equilibrium between dephosphorylated and phosphorylated state of proteins towards the latter. The IC part of Jedi was expressed in NIH 3T3 cells using plasmids pEGFP-Jedi-IC and pEGFP-Jedi-ICA (Fig. 8A) and liposomal transfection. The cells were then incubated with peroxyvanadate or left untreated. Expressed EGFP-Jedi-IC and pEGFP-Jedi-ICA fusions or control EGFP were precipitated with anti-GFP antibody, and the level of Jedi tyrosine phosphorylation was assayed with anti-phosphotyrosine antibody using Western blotting. Figure 8B demonstrates that EGFP-Jedi fusion protein is phosphorylated in peroxyvanadate-treated cells. The truncated form, EGFP-Jedi-ICA, can also be phosphorylated.

DISCUSSION

The present study describes the identification of a cDNA encoding a transmembrane protein with multiple EGF-like repeats, which is expressed during early stages of hematopoietic differentiation and is likely to be involved in regulation of hematopoiesis. The protein sequence alignment (Fig. 2) indicates that the similarity between Jedi and the MEGF10 and MEGF11 proteins is prominent along their extracellular domains, whereas their IC domains are much more divergent, with the similarity confined largely to several relatively short stretches of conserved amino acids encompassing invariant tyrosines. The tyrosine residues 803, 821, 923, 941, and 976 are potential

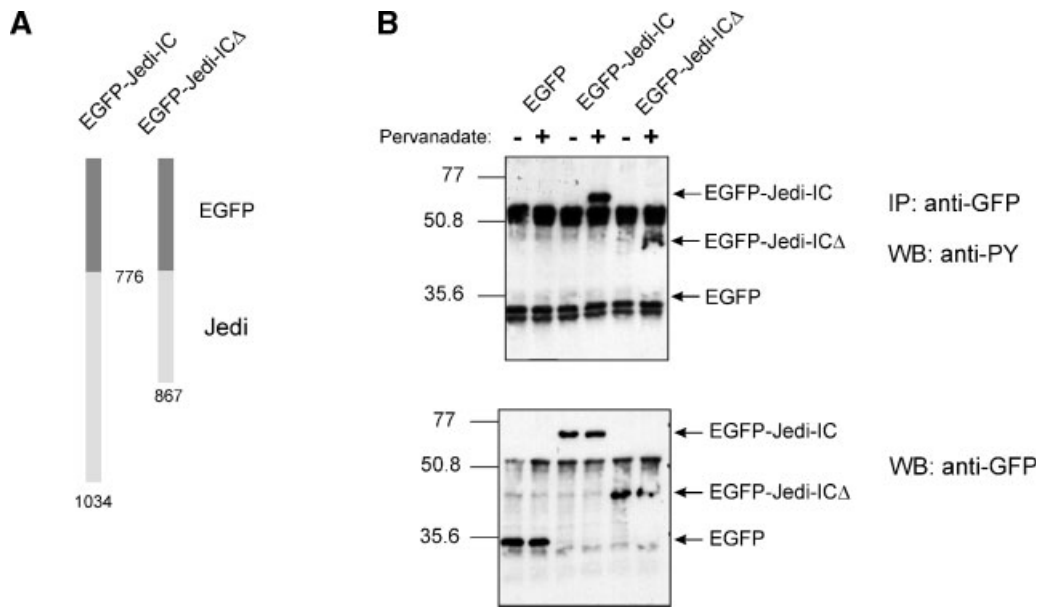


Fig. 8. Jedi can be tyrosine phosphorylated in vivo. **A:** Schematic representation of EGFP-Jedi fusion proteins. Jedi-IC (aa 77-6-1034) and Jedi-ICΔ (aa 776-867) cDNAs were cloned into pEGFP-N2 vector (Clontech) to produce chimeric proteins in which the Jedi part (light gray) was fused N-terminally with the EGFP (dark gray). **B:** Western blot analysis of Jedi tyrosine phosphorylation. Control EGFP, EGFP-Jedi-IC and EGFP-Jedi-ICΔ proteins were expressed in NIH 3T3 fibroblasts.

Cells were treated (+) or not treated (–) with pervanadate. Cell lysates were subjected to immunoprecipitation with polyclonal anti-GFP (Boehringer Mannheim) antibody, followed by Western blot analysis. Blots were probed with anti-phosphotyrosine antibody (4G10, **upper panel**) and reprobed with monoclonal anti-GFP antibody (**lower panel**). Sizes of the marker proteins in kiloDaltons are shown on the left.

candidates for phosphorylation. These tyrosines reside predominantly within the regions highly conserved between all three proteins. Our experiments indicate that the IC domain of Jedi becomes phosphorylated in vivo, at least under conditions when activity of tyrosine phosphatases is inhibited. Additionally, the majority of serine/threonine residues predicted to be phosphorylated with high probability are located in the same amino acid stretches. We therefore postulate that the conserved amino acid stretches in the IC domains of the Jedi/MEGF family function in the IC signaling, primarily through phosphorylation and subsequent docking of signaling proteins to these regions.

The structural similarity between Jedi, MEGF10 and MEGF11 is indicative that all three proteins belong to a distinct protein family. A substantially lower degree of similarity was observed between Jedi and the vertebrate members of the DSL family Delta and Jagged. The similarity to DSL proteins is primarily confined to the domain organization, namely the presence of multiple EGF-like repeats and a DSL-like domain. Jedi and MEGF proteins lack a cysteine-rich stretch preceding

the transmembrane domain which is characteristic of Serrate/Jagged proteins. In addition, the EGF repeats of Jedi/MEGF family bear distinct features separating them apart from Notch receptors and ligands and the majority of EGF-like repeat proteins. Among them are the somewhat longer repeat length and the presence of two additional cysteines at highly conserved positions. Finally, the IC domain of Jedi bears little similarity to the corresponding domains of DSL proteins. Since the Notch ligands bind their receptors via the DSL domain, the question remains whether the DSL-like domain of the Jedi/MEGF family may perform a similar function.

Expression of Jedi mRNA was observed both in early hematopoietic progenitors, and in cells known to support hematopoiesis, namely in bone marrow stromal and osteogenic cells, and in AFT024 cells. This suggested the possibility that Jedi may participate in regulation of hematopoiesis by supporting early progenitors. In contrast to our expectations, retroviral-mediated expression of Jedi both in bone marrow cells and in supporting NIH 3T3 stroma resulted in a clear reduction of the number of clonogenic myeloid cells. This is in a sharp

contrast to the reported action of Notch ligands Jagged and Delta which, when expressed in stroma, increase the number of colony forming cells [Jones et al., 1998; Varnum-Finney et al., 1998; Tsai et al., 2000; Varnum-Finney et al., 2000]. It is obvious that such a complex and important process as hematopoiesis should be tightly regulated by the organism. Therefore, we hypothesize that the observed in vitro activity of Jedi is conserved in vivo, and the role of Jedi involves fine-tuning of hematopoiesis by exerting negative effect on stem/progenitor cell compartment.

At present, it is unclear what mechanisms are used by Jedi to exert its inhibitory action. Given the similarity of Jedi to the Notch ligands, one of the most plausible explanations would be that Jedi antagonizes Notch signaling by competing with Notch ligands for binding to the receptor. Our data reveal that the soluble form of Jedi inhibits Notch signaling, and the degree of inhibition is similar to that of the soluble form of the Notch ligand Jagged1. These results provide evidence for participation of Jedi in regulation of Notch signaling. Co-culture experiments with NIH 3T3 cells expressing intact Jedi or Jagged1 ruled out the positive role of Jedi in Notch signaling, although they failed to provide unequivocal evidence for a negative role, which would explain the observed inhibition of hematopoietic colony formation by Jedi. It is of note in this respect that for Dlk, another Notch ligand-like transmembrane protein with multiple EGF-like repeats, both positive [Moore et al., 1997b] and negative [Ohno et al., 2001] effects on hematopoietic colony formation were reported. Recently, however, Dlk was unequivocally demonstrated to bind to specific Notch EGF-like repeats and inhibit Notch signaling [Baladron et al., 2005]. It is of interest that NIH 3T3 cells used as a responder cell population in our co-culture experiments express high levels of Dlk message [Moore et al., 1997b], which might interfere with analysis of Jedi effects. Considering the high complexity of Notch signaling, it is very likely that the magnitude of effects of Jedi on this pathway depends on the cellular context. Indeed, when we tested CHO cells in the same assay system, we observed a small but reliable inhibition of Notch signaling by Jedi, whereas Jagged1 exerted a moderate positive effect.

During the cloning of full-length Jedi cDNA, we identified a cDNA variant encoding a

potentially secreted form of Jedi. So far similar forms were described for Delta1 protein in *Xenopus* [Chitnis et al., 1995] and Jagged1 [Zimrin et al., 1996; Li et al., 1997]. Although this subject is still a matter of controversy, the majority of studies indicate that soluble forms of DSL ligands can act as antagonists of their intact counterparts [Chitnis et al., 1995; Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997; Franklin et al., 1999]. Whether the secreted forms of Jedi act as antagonists of the intact transmembrane protein remains to be established. However, our experiments with the extracellular domain of Jedi provide strong evidence in favor of the potential role of secreted Jedi proteins in negative regulation of Notch signaling, and through this, hematopoiesis in vivo.

ACKNOWLEDGMENTS

We thank Natalia Ivanova for initial work on Gene Expression Fingerprinting with murine hematopoietic cells and sharing research data, Michel Sadelain and Renier J. Brentjens for providing pSFG vector, assistance and helpful discussions. We also thank Drs. I. Lemischka and Dr. R. Ploemacher for gifts of the AFT024 and FBMD-1 cell lines, respectively, and Dr. D. Small for a gift of hJagged1/pcDNA3.1/Hygro(+), sJagged1 and Notch1 plasmids. We are grateful to Dr. N. Drize for providing us with preparation of bone marrow stromal cells, and Dr. N. Nikulina for a gift of MSCs and MSCs differentiated along osteogenic lineage. We thank Gargy Debnath for assistance with cell cultures and Jill R. Storry and Matthew Stubbs for help in reading and correcting the manuscript.

REFERENCES

- Baladron V, Ruiz-Hidalgo MJ, Nueda ML, Diaz-Guerra MJ, Garcia-Ramirez JJ, Bonvini E, Gubina E, Laborda J. 2005. *dlk* acts as a negative regulator of Notch1 activation through interactions with specific EGF-like repeats. *Exp Cell Res* 303:343–359.
- Beresten SF, Stan R, van Brabant AJ, Ye T, Naureckiene S, Ellis NA. 1999. Purification of overexpressed hexahistidine-tagged BLM N431 as oligomeric complexes. *Protein Expr Purif* 17:239–248.
- Bettenhausen B, Hrabe de Angelis M, Simon D, Guenet J, Gossler A. 1995. Transient and restricted expression during mouse embryogenesis of *Dll1*, a murine gene closely related to *Drosophila* Delta. *Development* 121: 2407–2418.

- Chitnis A, Henrique D, Lewis J, Ish-Horowicz D, Kintner C. 1995. Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature* 375:761–766.
- Dexter TM, Simmons P, Purnell RA, Spooncer E, Schofield R. 1984. The regulation of hemopoietic cell development by the stromal cell environment and diffusible regulatory molecules. *Prog Clin Biol Res.* 148:13–33.
- Dunwoodie SL, Henrique D, Harrison SM, Beddington RSP. 1997. Mouse *Dll3*: A novel divergent *Delta* gene which may complement the function of other *Delta* homologue during early pattern formation in the mouse embryo. *Development* 124:3065–3076.
- Fortunel N, Hatzfeld J, Kisselev S, Monier MN, Ducos K, Cardoso A, Batard P, Hatzfeld A. 2000. Release from quiescence of primitive human hematopoietic stem/progenitor cells by blocking their cell-surface TGF-beta type II receptor in a short-term in vitro assay. *Stem Cells* 18:102–111.
- Franklin JL, Berechid BE, Cutting FB, Presente A, Chambers CB, Foltz DR, Ferreira A, Nye JS. 1999. Autonomous and non-autonomous regulation of mammalian neurite development by Notch1 and Delta1. *Curr Biol* 9:1448–1457.
- Harlow E, Lane D. 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 999 p.
- Hsieh JJ, Henkel T, Salmon P, Robey E, Peterson MG, Hayward SD. 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.
- Hukriede NA, Gu Y, Fleming RJ. 1997. A dominant-negative form of Serrate acts as a general antagonist of Notch activation. *Development* 124:3427–3437.
- Ivanova NB, Belyavsky AV. 1995. Identification of differentially expressed genes by restriction endonuclease-based gene expression fingerprinting. *Nucleic Acids Res* 23:2954–2958.
- Jones P, May G, Healy L, Brown J, Hoyne G, Delassus S, Enver T. 1998. Stromal expression of Jagged 1 promotes colony formation by fetal hematopoietic progenitor cells. *Blood* 92:1505–1511.
- Kojika S, Griffin JD. 2001. Notch receptors and hematopoiesis. *Exp Hematol* 29:1041–1052.
- Lemischka I. 2001. Stem cell dogmas in the genomics era. *Rev Clin Exp Hematol* 5:15–25.
- Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J, Costa T, Pierpont ME, Rand EB, Piccoli DA, Hood L, Spinner NB. 1997. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* 16:243–251.
- Li L, Milner LA, Deng Y, Iwata M, Banta A, Graf L, Marcovina S, Friedman C, Trask BJ, Hood L, Torok-Storb B. 1998. The human homologue of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity* 8: 43–55.
- Lim J, Hao T, Shaw C, Patel AJ, Szabo G, Rual JF, Fisk CJ, Li N, Smolyar A, Hill DE, Barabasi AL, Vidal M, Zoghbi HY. 2006. A protein-protein interaction network for human inherited ataxias and disorders of Purkinje cell degeneration. *Cell* 125:801–814.
- Lindsell CE, Shawber CJ, Boulter J, Weinmaster G. 1995. Jagged: A mammalian ligand that activates Notch1. *Cell* 80:909–917.
- Maher J, Brentjens RJ, Gunset G, Rivière I, Sadelain M. 2002. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCR/CD28 receptor. *Nat Biotechnol* 20:70–75.
- Milner LA, Bigas A. 1999. Notch as a mediator of cell fate determination in hematopoiesis: Evidence and speculation. *Blood* 93:2431–2448.
- Moloney DJ, Panin VM, Johnston SH, Chen J, Shao L, Wilson R, Wang Y, Stanley P, Irvine KD, Haltiwanger RS, Vogt TF. 2000. Fringe is a glycosyltransferase that modifies Notch. *Nature* 406:369–375.
- Moore KA, Ema H, Lemischka IR. 1997a. In vitro maintenance of highly purified, transplantable hematopoietic stem cells. *Blood* 89:4337–4347.
- Moore KA, Pytowski B, Witte L, Hicklin D, Lemischka IR. 1997b. Hematopoietic activity of a stromal cell transmembrane protein containing epidermal growth factor-like repeat motifs. *Proc Natl Acad Sci USA* 94:4011–4016.
- Morrison SJ, Uchida N, Weissman IL. 1995. The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol* 11:35–71.
- Nagase T, Nakayama M, Nakajima D, Kikuno R, Ohara O. 2001. Prediction of the coding sequences of unidentified human genes. XX. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. *DNA Res* 8:85–95.
- Nakayama M, Kikuno R, Ohara O. 2002. Protein-protein interactions between large proteins: Two-hybrid screening using a functionally classified library composed of long cDNAs. *Genome Res* 12:1773–1784.
- Nye JS, Kopan R. 1995. Developmental signaling. Vertebrate ligands for Notch. *Current Biol* 9:966–969.
- Ohno N, Izawa A, Hattori M, Kageyama R, Sudo T. 2001. dlk inhibits stem cell factor-induced colony formation of murine hematopoietic progenitors: Hes-1–8 independent effect. *Stem Cells* 19:71–79.
- Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF, Prockop DJ. 2004. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood* 103:1662–1668.
- Phillips RL, Ernst RE, Brunk B, Ivanova N, Mahan MA, Deanehan JK, Moore KA, Overton GC, Lemischka IR. 2000. The genetic program of hematopoietic stem cells. *Science* 288:1635–1640.
- Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. 2002. “Stemness”: Transcriptional profiling of embryonic and adult stem cells. *Science* 298:597–600.
- Rivière I, Brose K, Mulligan RC. 1995. Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. *Proc Natl Acad Sci USA* 92:6733–6737.
- Rovira A, De Angioletti M, Camacho-Vanegas O, Liu D, Rosti V, Gallardo HF, Notaro R, Sadelain M, Luzzatto L. 2000. Stable in vivo expression of glucose-6-phosphate dehydrogenase (G6PD) and rescue of G6PD deficiency in stem cells by gene transfer. *Blood* 96:4111–4117.
- Schweisguth F. 2004. Notch signaling activity. *Curr Biol* 14:R129–R138.

- Shmelkov SV, Visser JW, Belyavsky AV. 2001. Two-dimensional gene expression fingerprinting. *Anal Biochem* 290:26–35.
- Small D, Kovalenko D, Kacer D, Liaw L, Landriscina M, Di Serio C, Prudovsky I, 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, Kovalenko D, Soldi R, Mandinova A, Kolev V, Trifonova R, Bagala C, Kacer D, Battelli C, Liaw L, Prudovsky I, Maciag T. 2003. Notch activation suppresses fibroblast growth factor-dependent cellular transformation. *J Biol Chem* 278:16405–16413.
- Staal FJ, Clevers HC. 2001. Regulation of lineage commitment during lymphocyte development. *Int Rev Immunol* 20:45–64.
- Sun X, Artavanis-Tsakonas S. 1997. Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in *Drosophila*. *Development* 124:3439–3448.
- Tax FE, Yeagers JJ, Thomas JH. 1994. Sequence of *C. elegans lag-2* reveals a cell-signaling domain shared with Delta and Serrate of *Drosophila*. *Nature* 368:150–154.
- Terskikh AV, Miyamoto T, Chang C, Diatchenko L, Weissman IL. 2003. Gene expression analysis of purified hematopoietic stem cells and committed progenitors. *Blood* 102:94–101.
- Tsai S, Fero J, Bartelmez S. 2000. Mouse Jagged2 is differentially expressed in hematopoietic progenitors and endothelial cells and promotes the survival and proliferation of hematopoietic progenitors by direct cell-to-cell contact. *Blood* 96:950–957.
- Varnum-Finney B, Purton LE, Yu M, Brashem-Stein C, Flowers D, Staats S, Moore KA, Le Roux I, Mann R, Gray G, Artavanis-Tsakonas S, Bernstein ID. 1998. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood* 91:4084–4091.
- Varnum-Finney B, Wu L, Yu M, Brashem-Stein C, Staats S, Flowers D, Griffin JD, Bernstein ID. 2000. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J Cell Sci* 113 Pt23:4313–4318.
- Visser JW, de Vries P, Hogeweg-Platenburg MG, Bayer J, Schoeters G, van den Heuvel R, Mulder DH. 1991. Culture of hematopoietic stem cells purified from murine bone marrow. *Semin Hematol* 28:117–125.
- Yan XQ, Lacey DL, Saris C, Mu S, Hill D, Hawley RG, Fletcher FA. 1999. Ectopic overexpression of c-mpl by retroviral-mediated gene transfer suppressed megakaryopoiesis but enhanced erythropoiesis in mice. *Exp Hematol* 27:1409–1417.
- Zimrin AB, Pepper MS, McMahon GA, Nguyen F, Montesano R, 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.
- Zinovyeva MV, Zijlmans JM, Fibbe WE, Visser JW, Belyavsky AV. 2000. Analysis of gene expression in subpopulations of murine hematopoietic stem and progenitor cells. *Exp Hematol* 28:318–334.