

## $\beta$ -Catenin Inversely Regulates Vascular Endothelial Growth Factor-D mRNA Stability\*

Received for publication, April 23, 2003, and in revised form, July 31, 2003  
Published, JBC Papers in Press, August 13, 2003, DOI 10.1074/jbc.M304255200

Maurizio Orlandini, Serena Semboloni, and Salvatore Oliviero‡

From the Dipartimento di Biologia Molecolare, Università degli Studi di Siena, via Fiorentina 1-53100 Siena, Italy

**The angiogenic and lymphangiogenic vascular endothelial growth factor (VEGF)-D is the only member of the VEGF family that is not induced by hypoxia or by serum factors, but its induction is mediated by direct cell-cell contact. Here we show that VEGF-D mRNA is down-modulated either by  $\beta$ -catenin mobilization from the cell membrane, by activation of the Wnt signaling pathway, or by transfection with the  $\beta$ -catenin stable mutant. Down-modulation of  $\beta$ -catenin by means of RNA interference showed an increase of VEGF-D mRNA steady state in fibroblasts. The  $\beta$ -catenin-dependent decrease of VEGF-D mRNA is indirect and mainly due to reduced VEGF-D mRNA stability, as demonstrated by experiments of mRNA decay in the presence of transcription or translation inhibitors. By transient transfection of chimeric constructs carrying fusion of VEGF-D sequences under the control of the cytomegalovirus early promoter, we demonstrated that  $\beta$ -catenin negative regulation is on the VEGF-D mRNA 3'-untranslated region. We mapped the VEGF-D mRNA destabilizing element to a sequence, conserved between mouse and human VEGF-D, which contains an AU-rich element of group I. These results unveiled a new regulatory pathway for VEGF-D, which explains, at least in part, VEGF-D regulation in tumor progression.**

During development and in the vascularization of tumors, the newly forming tissues produce inductive signaling that leads to the formation of capillaries throughout. In particular, progression of tumor malignancy is characterized by the acquisition by tumor cells of angiogenic and lymphangiogenic ability (1, 2). This is obtained by the expression of secreted angiogenic factors, which induce endothelial cell differentiation (3–5). The VEGF<sup>1</sup> family of angiogenic factors includes VEGF-A, the placental growth factor, VEGF-B, VEGF-C, VEGF-D, and VEGF-E (6–16). VEGF-A is a potent angiogenic factor able to induce vessel sprouting. It recognizes the receptors VEGFR-1

and VEGFR-2 on endothelial cells. It is strongly expressed in tumors as it is induced by hypoxia with induced transcription and increasing mRNA stability (17–19). VEGF-A is also up-regulated by epidermal growth factor, transforming growth factor- $\beta$ , and interleukin-6 in several cell types and by interleukin-1 $\beta$  in smooth muscle cells (20–22).

VEGF-D, like VEGF-C, shows some structural and functional differences from VEGF-A (14, 23–26). They bind and activate VEGFR-2 and VEGFR-3. This latter is a tyrosine kinase receptor specifically found in adult lymphatic endothelium. In cell culture, VEGF-C is induced by serum, phorbol 12-myristate 13-acetate, and several factors including interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  (27, 28). In contrast, VEGF-D is highly expressed in fibroblasts grown under low serum conditions and is induced by direct cell-cell contact mediated by the surface molecule cadherin-11 (14, 29).

VEGF-D was brought to attention with respect to tumor development, as in mice models tumors expressing this factor showed development of intra-tumor lymphatic vessels and dissemination of tumor cells in lymph nodes (2, 30). Importantly, recent data show (31–35) that in several tumors the expression of VEGF-D is associated with lymph node metastasis and may be used as a novel prognostic factor in cancer development. However, some authors (Refs. 36 and 37 and our results on lung tumors)<sup>2</sup> reported that VEGF-D was underexpressed in tumors when compared with normal tissues.

To understand VEGF-D regulation by cell-cell contact, we analyzed VEGF-D mRNA levels by altering cytoplasmic  $\beta$ -catenin expression in fibroblasts.  $\beta$ -Catenin is present in the cell in two fractions, a membrane-associated fraction linked to cadherins with a structural function, and an unstable cytoplasmic fraction that acts as a transcriptional regulator (38, 39). Our data indicate that cytoplasmic  $\beta$ -catenin down-modulates VEGF-D inducing the degradation of VEGF-D mRNA. This regulation requires new protein synthesis suggesting an indirect regulation. These results might explain VEGF-D regulation in tumors.

### EXPERIMENTAL PROCEDURES

**Cloning and Plasmid Constructs**—To obtain the luciferase reporter gene under the control of the CMV early promoter (FG470), a 878-bp *Bgl*III blunt-ended/*Hind*III fragment that contains the CMV early promoter was cut from the pcDNA3 vector (Invitrogen) and subcloned into the *Sac*I blunt-ended/*Hind*III sites of the pGL3-Basic vector (Promega). To generate the entire mouse VEGF-D 3'-UTR (nucleotides 1360–1875; the nucleotide sequence of mouse VEGF-D is numbered according to the sequence with GenBank<sup>TM</sup> accession number NM010216) and the deletion mutants of this region (nt 1360–1612, nt 1616–1875, and nt 1757–1875) fused to the luciferase reporter gene, or to generate the entire human VEGF-D 3'-UTR (nt 1575–2110; GenBank<sup>TM</sup> accession number NM004469) and the deletion mutant of this region (nt 1989–2110) fused to the luciferase reporter gene, DNA fragments were PCR-

\* This work was supported by Associazione Italiana per la Ricerca sul Cancro, Ministero Italiano dell'Istruzione, dell'Università e della Ricerca, and EC-TMR Grant HPRN-CT-2002-00256. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dipartimento di Biologia Molecolare, Università degli Studi di Siena, via Fiorentina 1-53100 Siena, Italy. Tel.: 39-0577-234931; Fax: 39-0577-234929; E-mail: oliviero@unisi.it.

<sup>1</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; HEK, human embryonic kidney; UTR, untranslated region; Ab, antibody; mAb, monoclonal antibody; CMV, cytomegalovirus; HA, hemagglutinin; siRNA, small interfering RNA; ARE, AU-rich element; nt, nucleotides; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; PBS, phosphate-buffered saline; RNAi, RNA interference; TCF, T-cell factor; CAT, chloramphenicol acetyltransferase; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase.

<sup>2</sup> M. Rocchigiani and S. Oliviero, unpublished results.

amplified from a cDNA clone corresponding to the complete sequence of the mouse (14) or the human (26) *VEGF-D* gene. The following pairs of primers containing an *Xba*I restriction site in the sense primer and a *Bam*HI restriction site in the antisense primer were used: H192, 5'-GAGATCTAGATTCAACTTCCTTTCAAGTCC-3', and H193, 5'-GAGAGGATCCGCTGGGCTGCATAACAGTG-3' (1360–1875); H192 and H403, 5'-GAGAGGATCCAAACAGTGTATTTCCTTCCCTTTCTGAGTG-3' containing the *VEGF-D* polyadenylation sequence (1360–1612, underlined); H401, 5'-GAGATCTAGAGAGGAGCCCTTGTTTTG-3' and H193 (1616–1875); H402, 5'-GAGATCTAGATGTATGATCAGCACTGATCA-3' and H193 (1757–1875); J180, 5'-GAGATCTAGATTCAGCGTTCACAGTCC-3', and J181, 5'-GAGAGGATCCGGCTTGAATAATGGTGTATTTC-3' (1575–2110); J182, 5'-GAGATCTAGATCGTATGATCAGTACTGAC-3' and J181 (1989–2110). To generate the mouse (nt 1833–1875) and the human (nt 2070–2110) deletion mutants fused to the luciferase reporter gene, the following pairs of primers containing an *Xba*I restriction site in the sense primer and a *Bam*HI restriction site in the antisense primer were annealed and blunted with Klenow enzyme: J178, 5'-GAGATCTAGATGTAAATGTATTTAAAGGAAATAACAC-3', and J179, 5'-GAGAGGATCCGCTGGGCTGCATAACAGTGTATTTC-3' (1833–1875); J183, 5'-GAGATCTAGATTTAAGTGTATTTAAAGGAAATAACAC-3' and J181 (2070–2110). The PCR products and the DNA fragments were then digested and subcloned into the *Xba*I/*Bam*HI sites of FG470 after removal of the polyadenylation signal of SV40. All constructs were checked by automated sequencing. Vector pCS2<sup>+</sup>MMBCmyc tag ( $\beta$ .myc) encoding mouse wild-type  $\beta$ -catenin and vector pCS2<sup>+</sup>MMBCS33Amyc tag ( $\beta$ .4SA) encoding a mutant  $\beta$ -catenin were kindly provided by Dr. Rolf Kemler (see Ref. 40). Plasmid HA-Dvl-2 for the overexpression of the recombinant mouse HA-tagged Dvl-2 and plasmid mFLAGax-(501–560) expressing a small FLAG-tagged region of mouse axin, which prevents  $\beta$ -catenin turnover, were generously provided by Dr. Trevor C. Dale (41). Plasmid PLNC-W1HA encoding murine HA-tagged *Wnt-1* was generously provided by Dr. Jan Kitajewski (see Ref. 42). The reporter plasmids pTOPCAT and pFOPCAT (43) were kindly provided by Dr. Claudio Schneider (see Ref. 44).

**Cell Culture and Transfections**—Mouse 3T3-type fibroblasts, murine BALB/c 3T3, and human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

Stable clones expressing murine *Wnt-1* were obtained from BALB/c 3T3 fibroblasts transfected with the *Wnt-1* expression vector PLNC-W1HA by standard CaPO<sub>4</sub> precipitation procedures (29). Selection was performed by culturing for 2 weeks in the presence of 1 mg/ml G418 (Invitrogen).

Transient transfection experiments were performed by using Polyfect transfection reagent (Qiagen) according to instructions from the manufacturer.

Degradation of the mRNAs was studied in mouse 3T3-type fibroblasts. The cells were grown in complete medium until they reached confluence, and then actinomycin D (5  $\mu$ g/ml; Sigma) as an inhibitor of transcription or cycloheximide (10  $\mu$ g/ml; Sigma) as an inhibitor of protein synthesis, with or without EGTA (2.2 mM), was added to the cell cultures for 2, 4, and 6 h.

**Northern Blot Analysis**—Analysis of RNA was performed as described previously (14). Briefly, total cellular RNA was isolated from cells by the guanidinium thiocyanate method (45). 10  $\mu$ g of total RNA were size-fractionated in a denaturing formaldehyde-agarose gel and stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was transferred onto nylon membranes and cross-linked by UV irradiation. Filters were hybridized with <sup>32</sup>P-labeled probes, washed, and analyzed by using a PhosphorImager (Amersham Biosciences). Rat glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was used as a control for RNA loading.

**Immunoblotting**—Cells were washed twice with cold phosphate-buffered saline (PBS), harvested in TEN (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl), pelleted, and lysed in 0.5% Nonidet P-40 buffer (20 mM HEPES, pH 7.4, and 2 mM EDTA) containing Complete protease inhibitors (Roche Diagnostics). The protein concentrations were determined by using the BCA protein assay reagent (Pierce). The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Equal loading was confirmed by staining in Ponceau S solution (Sigma). Filters were blocked for 1 h at room temperature in PBS containing 3% dry milk and 0.1% Triton X-100, followed by 2 h at room temperature with the primary antibodies in the same buffer. The following primary antibodies were used at the indicated concentrations: mouse monoclonal anti- $\beta$ -catenin (0.2  $\mu$ g/ml; Transduction Laboratories), rat monoclonal anti-HA (0.1  $\mu$ g/ml; Roche Diagnostics), mouse

monoclonal Abs specific for Myc tag (0.2  $\mu$ g/ml; Santa Cruz Biotechnology), and mouse monoclonal anti-FLAG (5  $\mu$ g/ml; Sigma). The blots were washed and incubated with horseradish peroxidase-labeled sheep anti-mouse (Amersham Biosciences) or horseradish peroxidase-conjugated goat anti-rat (Chemicon International) for 1 h at room temperature, washed, and finally developed by using the enhanced chemiluminescence substrate (Amersham Biosciences).

**Immunofluorescent Microscopy**—Mouse fibroblasts were grown on glass coverslips and after treatment fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. For permeabilization, cells were incubated with 0.5% Triton X-100 in PBS for 5 min at 4 °C. Cells were washed twice in PBS, blocked with 1% bovine serum albumin in PBS for 1 h at room temperature, and incubated for 1 h at 37 °C with primary antibodies diluted in 1% bovine serum albumin/PBS (15  $\mu$ g/ml for  $\beta$ -catenin and 15  $\mu$ g/ml for anti-HA Abs). After washing, cells were incubated for 1 h at 37 °C in the presence of Alexa Fluor-568 goat anti-mouse or Alexa Fluor-488 goat anti-rat secondary antibodies (Molecular Probes). To localize actin filaments, fluorescein isothiocyanate-labeled phalloidin (Sigma) was added along with secondary antibodies at 2  $\mu$ g/ml. Coverslips were counterstained with 0.1  $\mu$ g/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) and mounted in Mowiol 4-88 (Calbiochem). Fluorescent images were captured using a Nikon DXM1200 digital camera from a Nikon Eclipse E600 microscope.

**RNA Interference**—siRNA duplexes were synthesized by Dharmacon Research Inc. (Lafayette, CO). The sequence of the  $\beta$ -catenin oligonucleotides was 5'-GUAGCUGAUUUGACGGGCTT-3' and 5'-GCCGCUCAAUUCAGCUACTT-3'. Cells were transfected twice, 48 h apart, with the siRNA duplex by using OligofectAMINE reagent (Invitrogen) according to the manufacturer's directions. As a negative control the single-stranded sense siRNA was used. 96 h after the first transfection cells were analyzed for Northern blot, immunoblotting, and immunofluorescent microscopy.

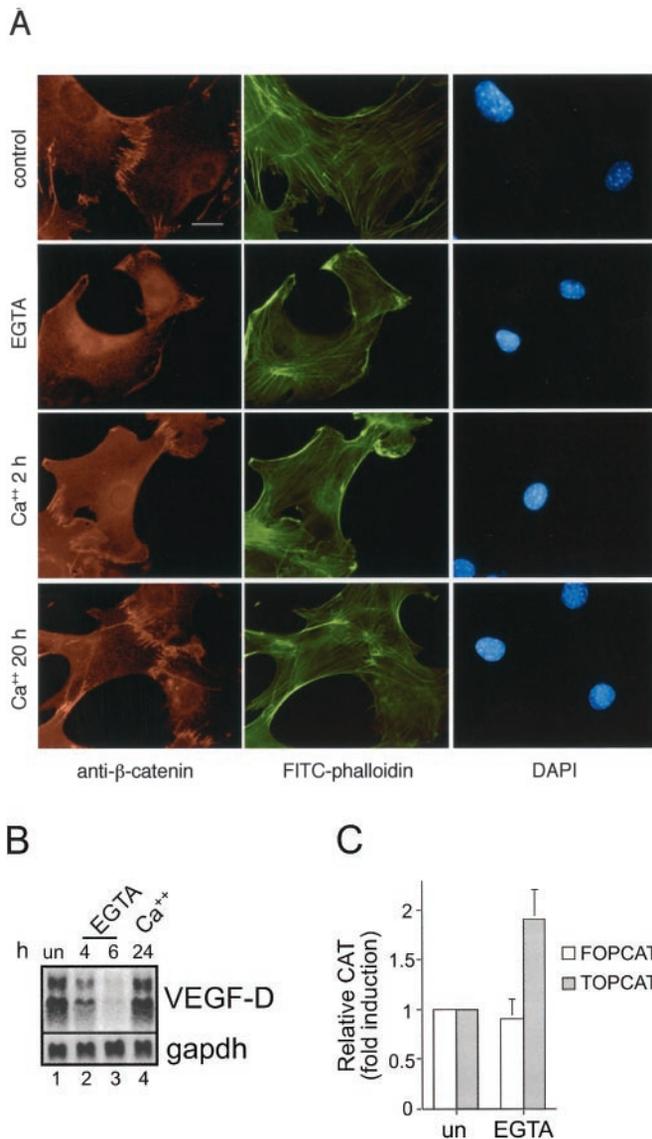
**Reporter Assays**—In chloramphenicol acetyltransferase (CAT) assay, mouse 3T3-type and BALB/c 3T3 fibroblasts were seeded in 6-well dishes and transiently transfected. Typically a transfection experiment included 0.4  $\mu$ g of reporter plasmid (TOPCAT or FOPCAT) and 0.1  $\mu$ g of pRSV- $\beta$ -galactosidase plasmid for the transfection efficiency control. The expression plasmids were added to the reporter as indicated in the figure legends, and the total amount of transfected plasmids was maintained constant at 2  $\mu$ g by the addition of empty vectors. CAT activity was determined as described previously (46). Acetylated forms of chloramphenicol were quantified by using the ImageQuant radio analytic system from Amersham Biosciences.

In luciferase assay, mouse 3T3-type fibroblasts and HEK 293 cells were seeded in 6-well dishes and transiently transfected with 0.1  $\mu$ g of the reporter plasmid containing the luciferase gene under the control of the CMV early promoter either fused to the full-length *VEGF-D* 3'-UTR or to its deletion mutants. As control the polyadenylation signal of SV40 was used. 0.05  $\mu$ g of pRSV- $\beta$ -galactosidase plasmid were used for the transfection efficiency control, and the expression plasmids were co-transfected as indicated in Figs. 6 and 7, maintaining constant at 2  $\mu$ g the total amount of transfected plasmids by the addition of empty vectors. 36–48 h after transfection cells were washed once with PBS and lysed in 250 mM Tris-HCl, pH 7.8. Luciferase activities in cell lysates were determined using the Luciferase Assay System (Promega) in a TD-20/20 luminometer (Turner Designs). Luciferase activities were normalized to  $\beta$ -galactosidase activities and presented as relative luciferase units.

**Statistical Analysis**—All results are presented as means  $\pm$  S.D. of at least three independent experiments. Statistical analysis was performed by using Student's test, and a value of  $p < 0.05$  was considered to be significant.

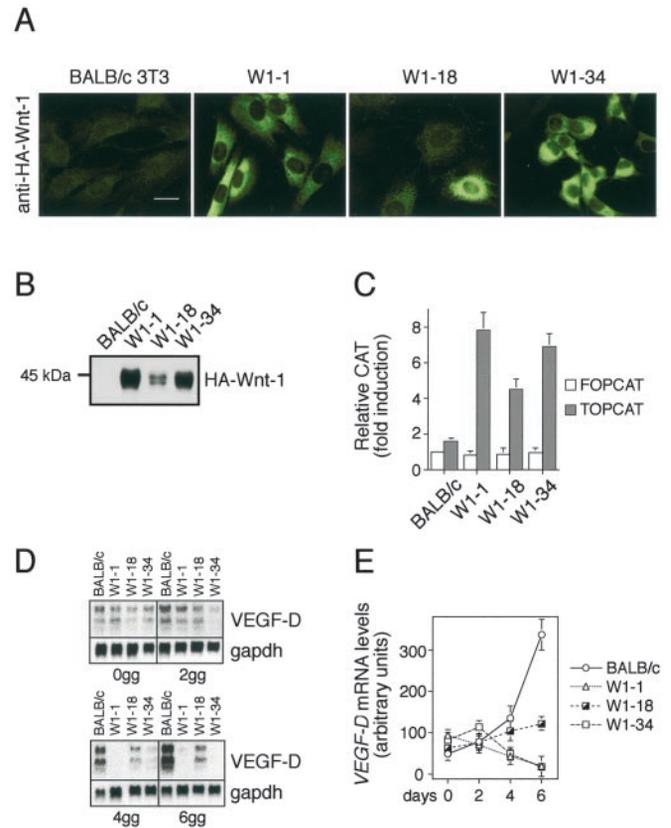
## RESULTS

**The Presence of Cellular Free  $\beta$ -Catenin Correlates with *VEGF-D* mRNA Down-regulation**—*VEGF-D* expression is not induced by growth factors which is different from the other members of the VEGF family. We demonstrated previously in mouse fibroblasts that *VEGF-D* mRNA is induced by cell-cell contact mediated by cadherin-11, a calcium-dependent adhesive protein (29). Depletion of calcium from the culture medium induced cadherin-11 re-localization from the cell surface to the cytoplasm and *VEGF-D* mRNA degradation. In contacting cells the majority of  $\beta$ -catenin is localized at cell-cell contacts where it links the cytoplasmic tail of cadherins to the cytoskeleton. In epithelial cells a direct consequence of cadherin disassembly is



**FIG. 1. Calcium depletion from the culture medium induced  $\beta$ -catenin re-localization into the cytoplasm and *VEGF-D* mRNA degradation.** *A*, immunofluorescence analysis using anti- $\beta$ -catenin Abs (red), fluorescein (FITC)-labeled phalloidin (green) for actin filaments staining, and DAPI (blue) for nuclei staining. Mouse fibroblasts were cultured in normal growth medium (control). To disrupt cell-cell adhesion, 2.2 mM EGTA was added to the culture medium. To observe  $\beta$ -catenin re-localization after 4 h of EGTA treatment, calcium was restored into the culture medium by adding 2.3 mM  $\text{CaCl}_2$ , and cells were fixed and analyzed after 2 h ( $\text{Ca}^{2+}$  2 h) or 20 h ( $\text{Ca}^{2+}$  20 h). The scale bar represents 20  $\mu\text{m}$ . *B*, Northern blot analysis using *VEGF-D* and *gapdh* probes. Total RNA was extracted at the indicated times from confluent mouse fibroblasts grown in the presence of 2.2 mM EGTA (lanes 2 and 3). To restore cell-cell contacts in 4-h EGTA-treated fibroblasts, 2.3 mM  $\text{CaCl}_2$  was added to the culture medium, and total RNA was isolated after 24 h (lane 4). Total RNA from untreated confluent cells (lane 1). *C*, transcriptional activity of TOPCAT in mouse fibroblasts grown in the presence of EGTA. Fibroblasts were seeded  $4 \times 10^5$  per 10-cm Petri dish, co-transfected with 1  $\mu\text{g}$  of either TOPCAT or FOPCAT reporter plasmids, 0.2  $\mu\text{g}$  of pRSV- $\beta$ -galactosidase, and 3.4  $\mu\text{g}$  of carrier DNA. 36 h post-transfection, cells were treated (EGTA) or not (un) for 4 h with 2.2 mM EGTA. CAT activities were determined and normalized for  $\beta$ -galactosidase activities. These results represent the mean  $\pm$  S.D. of four independent experiments.

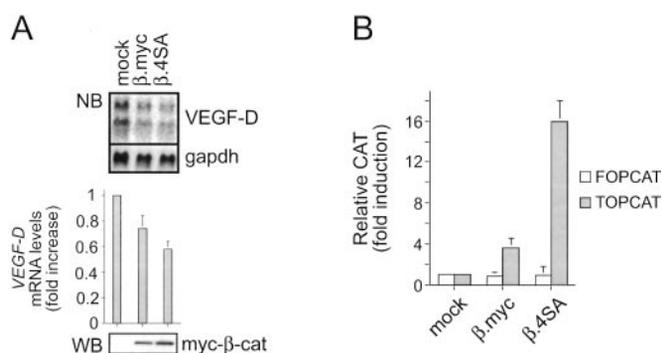
the release of free  $\beta$ -catenin within the cytoplasm (47). We examined the fate of  $\beta$ -catenin in contacting mouse fibroblasts treated with EGTA by using anti- $\beta$ -catenin antibodies (Fig. 1A). In untreated cells, a positive staining was revealed at



**FIG. 2. *VEGF-D* messenger is down-modulated via the Wnt signaling pathway.** Stable clones constitutively expressing Wnt-1 were generated in BALB/c 3T3 fibroblasts. *A*, immunofluorescence analysis of three different clones (W1-1, W1-18, and W1-34) and wild-type cells (BALB/c) using anti-HA mAbs which recognize murine HA-tagged Wnt-1. The scale bar represents 20  $\mu\text{m}$ . *B*, cell extracts from the same stable clones and wild-type cells as in *A* were analyzed by Western blot. Equal amounts of protein were loaded on the gel, and the membranes were probed with anti-HA mAbs. *C*, transcriptional activity of TOPCAT in the same stable clones and wild-type cells as in *A*. TOPCAT or FOPCAT reporter constructs were co-transfected with pRSV- $\beta$ -galactosidase. After 2 days post-transfection CAT activities were measured and normalized for  $\beta$ -galactosidase activities. The result represents the means  $\pm$  S.D. of data from three independent experiments. *D*, Northern blot analysis of RNA extracted from the same stable clones and wild-type cells as in *A*, using *VEGF-D* and *gapdh* probes. Cells were plated at low cell density and starting at day 0 corresponding to 15 h after cell plating; every 2 days the culture medium was changed, and the RNA was collected. *E*, quantitative analysis of *VEGF-D* mRNA levels from three independent experiments with cells plated as in *D*. The mRNA hybridization signals were quantified by densitometric scanning, and the values, normalized to the *gapdh* mRNA levels, were averaged and expressed as arbitrary units.

intercellular contacts. In contrast, within 1 h after cell treatment with EGTA, the  $\beta$ -catenin signal disappeared from the cell surface and re-localized to the cytoplasm. The reintroduction of  $\text{Ca}^{2+}$  into the culture medium restored  $\beta$ -catenin staining at intercellular contacts. After 2 h from  $\text{Ca}^{2+}$  addition, the  $\beta$ -catenin signal was detectable both in the cytoplasm and at the cell-cell contact surfaces, while after 20 h it was mostly present at the intercellular junctions similarly to untreated cells.

The presence of transcriptional active nuclear  $\beta$ -catenin could be demonstrated by transient transfection of a reporter gene containing multimeric responsive TCF-binding sites (TOPCAT) with respect to its control plasmid containing mutated TCF-binding sites (FOPCAT). A significant response to the  $\beta$ -catenin-TCF complex was measured by the TOPCAT construct in fibroblasts grown for 4 h in the presence of EGTA (Fig. 1C). The same EGTA treatment showed reduction of

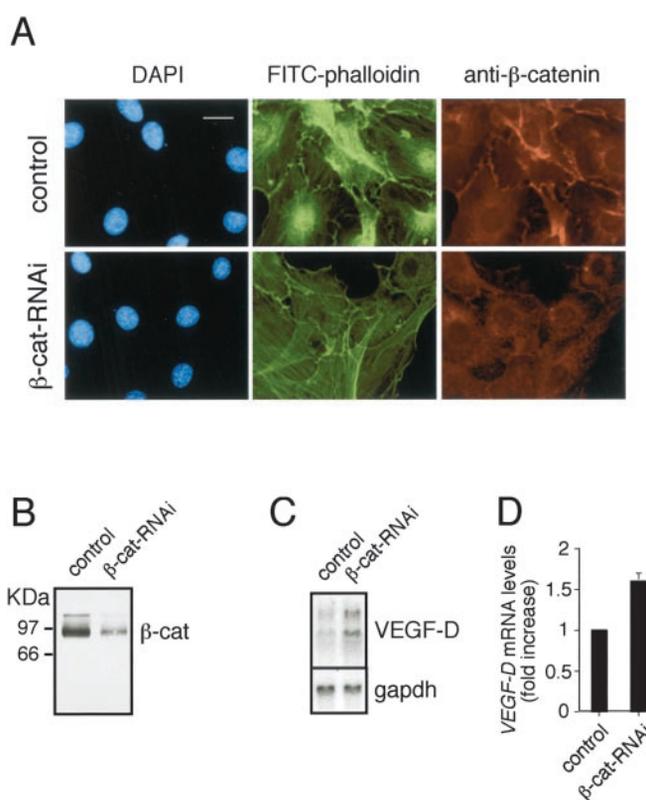


**FIG. 3.  $\beta$ -Catenin is responsible for the decrease of *VEGF-D* mRNA levels.** *A*, mouse fibroblasts were transiently transfected with wild-type or mutant  $\beta$ -catenin. Cells were seeded  $5 \times 10^5$  per 10-cm Petri dish and transfected with  $4 \mu\text{g}$  of wild-type  $\beta$ -catenin ( $\beta.myc$ ), mutant  $\beta$ -catenin ( $\beta.4SA$ ), or empty vector (*mock*). 2 days post-transfection total RNA was isolated, and cell lysates were prepared from different plates. Endogenous *VEGF-D* mRNA expression was followed by Northern blot (*NB*) analysis, and a quantitative analysis of *VEGF-D* mRNA levels, normalized to the *gapdh* mRNA, from three independent experiments is shown. Ectopic  $\beta$ -catenin expression was determined by immunoblotting using mAbs specific for Myc tag. *WB*, Western blot. *B*, transcriptional activity of TOPCAT in mouse fibroblasts transfected with  $\beta$ -catenin wild-type ( $\beta.myc$ ) or  $\beta.4SA$  mutant. TOPCAT or FOPCAT reporter constructs were co-transfected with pRSV- $\beta$ -galactosidase and  $1.5 \mu\text{g}$  of wild-type or mutant  $\beta$ -catenin. 48 h post-transfection triplicate samples were counted, normalized for  $\beta$ -galactosidase activities, and averaged.

*VEGF-D* mRNA with lower levels at 4 and 6 h (Fig. 1*B*). *VEGF-D* mRNA levels were increased to values of the untreated cells at about 24 h after calcium reintroduction in the culture medium, thus suggesting that forced re-localization of free  $\beta$ -catenin to the cytoplasm induced an inhibitory signal on the *VEGF-D* mRNA expression.

**Wnt-1 Signaling Inhibits *VEGF-D* Expression**—In cells, cytoplasmic levels of free  $\beta$ -catenin are maintained low via the proteasome-dependent degradation, as phosphorylated  $\beta$ -catenin is recognized by the F box factor  $\beta$ -TrCP (48–50). Wnt pathway down-regulates  $\beta$ -catenin phosphorylation via the activation of the Frizzled receptor, which induces down-regulation of GSK-3 $\beta$ -adenomatous polyposis coli-axin complex causing the accumulation of cytoplasmic  $\beta$ -catenin (51, 52). To investigate whether the degradation of *VEGF-D* mRNA could be modulated via the Wnt signaling pathway, we generated stable clones constitutively expressing Wnt-1, which acts as an autocrine factor on the Frizzled receptor (42). As shown in Fig. 2, *A* and *B*, clones W1-1 and W1-34 expressed high levels of Wnt-1, whereas clone W1-18 expressed lower levels of the factor. As expected, wild-type cells showed low levels of TOPCAT activity, whereas clones expressing Wnt-1 showed significantly higher expression of TOPCAT promoter (Fig. 2*C*). According to our previous results, wild-type fibroblasts expressed low levels of *VEGF-D* mRNA at low cell density, while it increased after a few days of growth when cells reached confluence (29). In contrast, in clones W1-1 and W1-34, expressing high levels of Wnt-1, *VEGF-D* mRNA did not accumulate, whereas a small *VEGF-D* mRNA increase was observed in clone W1-18, which expressed lower levels of Wnt-1 (Fig. 2, *D* and *E*).

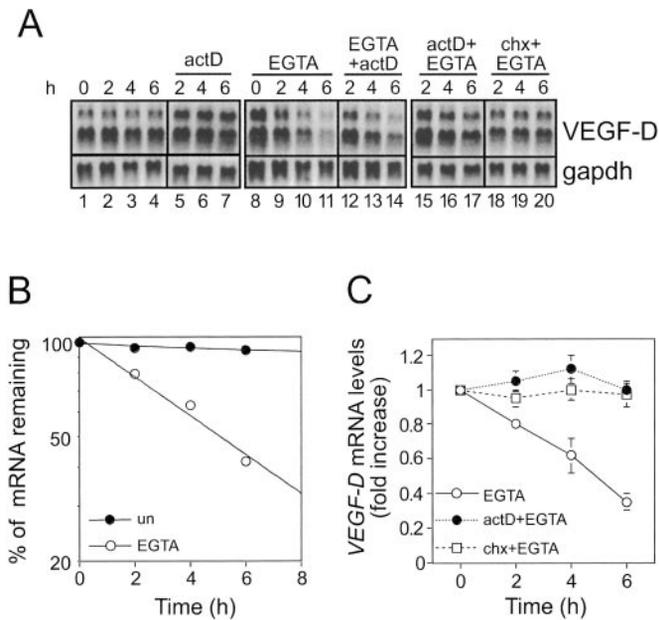
**$\beta$ -Catenin Regulates *VEGF-D* Expression**—To observe directly whether free  $\beta$ -catenin was responsible for the decrease of *VEGF-D* mRNA expression, cells were transiently transfected with  $\beta$ -catenin wild-type or the mutant  $\beta.4SA$ , both carrying a C-terminal Myc tag. In the mutant  $\beta.4SA$ , amino acid residues Ser-33, Ser-37, Thr-41, and Ser-45 have been all substituted with alanine residues to stabilize the protein by blocking its turnover (40). Strong activity of the TOPCAT reporter construct was observed in cells transfected with  $\beta.4SA$



**FIG. 4. Down-regulation of  $\beta$ -catenin raises *VEGF-D* mRNA levels.** *A*, immunofluorescence analysis using anti- $\beta$ -catenin mAbs (*red*), fluorescein (*FITC*)-labeled phalloidin (*green*) for actin filaments staining, and DAPI (*blue*) for nuclei staining. BALB/c 3T3 cells were grown in 24-well Petri dishes and transfected with 60 pmol of single-stranded sense siRNA (*control*) or 60 pmol of siRNA duplex ( $\beta\text{-cat-RNAi}$ ). Scale bar, 20  $\mu\text{m}$ . *B*, Western blot analysis of cell extracts from BALB/c 3T3 cells grown in 6-cm Petri dishes and transfected with 600 pmol of single-stranded sense siRNA (*control*) or 600 pmol of siRNA duplex ( $\beta\text{-cat-RNAi}$ ). The membranes were probed with anti- $\beta$ -catenin mAbs. *C*, Northern blot analysis of total RNA extracted from BALB/c 3T3 transfected as in *B*, using *VEGF-D* and *gapdh* probes. *D*, quantitative analysis of *VEGF-D* mRNA levels normalized to the *gapdh* mRNA. The mean  $\pm$  S.D. of triplicate experiments is shown.

mutant, whereas the wild-type molecule was less effective (Fig. 3*B*). This result was in agreement with Western blot analysis, which revealed higher levels of  $\beta.4SA$  mutant with respect to the wild type as cytoplasmic  $\beta$ -catenin is actively eliminated via the ubiquitin-proteasome pathway. Northern blots analysis of RNA extracted from confluent cells transfected with wild-type or mutant  $\beta$ -catenin showed a significant decrease of the endogenous *VEGF-D* mRNA levels (Fig. 3*A*). The converse experiment, *i.e.*  $\beta$ -catenin depletion, was obtained by means of siRNA. Transfection into BALB/c 3T3 cells of double-stranded modified oligonucleotide obtained from the  $\beta$ -catenin sequence strongly reduced  $\beta$ -catenin both from the cell surface and the cytoplasm (Fig. 4, *A* and *B*). RNAi-dependent reduced  $\beta$ -catenin expression significantly induced *VEGF-D* mRNA (Fig. 4, *C* and *D*) confirming that  $\beta$ -catenin down-regulates *VEGF-D* mRNA expression in fibroblasts.

**$\beta$ -Catenin Affects *VEGF-D* mRNA Stability**—It is generally accepted that  $\beta$ -catenin transduces Wnt signaling by interacting with TCF, and together they translocate into the nucleus where they activate Wnt target genes (39). To investigate whether *VEGF-D* mRNA down-modulation was due to negative regulation of *VEGF-D* transcription, we performed co-transfection experiments of a reporter gene under the control of the *VEGF-D* promoter together with constructs expressing  $\beta$ -catenin. These experiments did not show significant alteration of *VEGF-D* transcriptional rates (not shown). In contrast, meas-

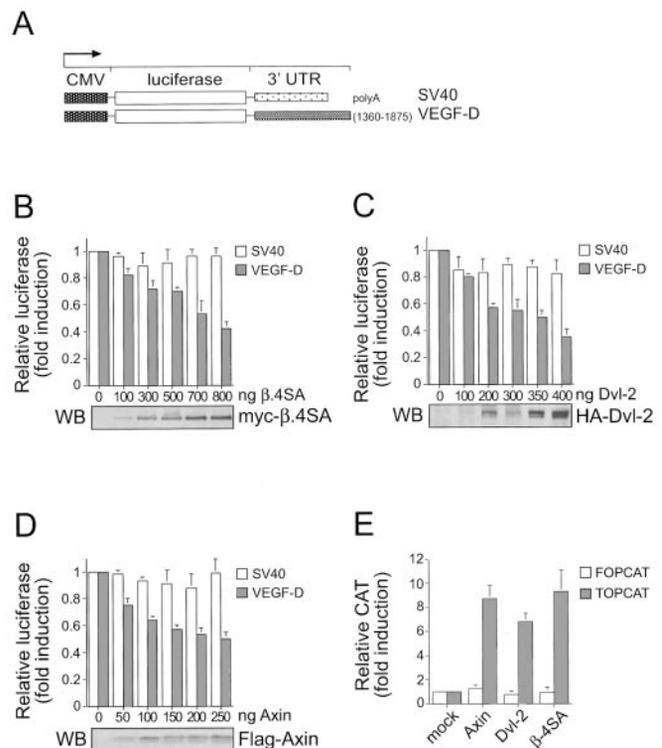


**FIG. 5. *VEGF-D* mRNA stabilization is affected by calcium depletion.** A, Northern blot analysis of total RNA extracted from mouse fibroblasts, using *VEGF-D* and *gapdh* probes. Cells were plated in 6-cm Petri dishes and grown at confluence in normal culture medium (lanes 1–4). Actinomycin D (*actD*) was added to the culture medium at time 0, and RNA levels were determined after 2, 4, and 6 h (lanes 5–7). Confluent cells were treated with EGTA (lanes 8–11), and after 1.30 h of EGTA treatment, actinomycin D was added to the culture medium (lanes 12–14). Alternatively, the inhibitors actinomycin D and cycloheximide (*chx*) were added to confluent cells 30 min before EGTA treatment (lanes 15–20). A representative experiment is shown. B, analysis of the mRNA hybridization signals quantified by densitometric scanning as in A. Values in the graph indicate the percentage of *VEGF-D* mRNA signals remaining either in untreated (*un*; lanes 5–7 of A) or in EGTA-treated cells (*EGTA*; lanes 12–14 of A) after normalization for the *gapdh* signals. mRNA half-lives were deduced from the regression line. C, quantitative analysis of *VEGF-D* mRNA levels normalized to the *gapdh* mRNA from a representative experiment as in A (mean  $\pm$  S.D. of triplicate experiments). mRNA levels from confluent cells treated with EGTA (*EGTA*; lanes 8–11 of A), with the inhibitors actinomycin D (*actD*; lanes 15–17 of A), or cycloheximide (*chx*; lanes 18–20 of A).

urement of the *VEGF-D* half-life in the presence of the inhibitor of transcription actinomycin D showed that *VEGF-D* was a very stable RNA molecule (with a half-life  $>48$  h in confluent cells), while its stability was strongly reduced  $\sim 5$  h after cell treatment with EGTA (Fig. 5, A and B).

Interestingly, if the depletion of calcium was preceded by either the treatment with the inhibitors of transcription (actinomycin D) or protein synthesis (cycloheximide), *VEGF-D* mRNA levels remained elevated (Fig. 5, A and C), thus suggesting that *VEGF-D* mRNA destabilization induced by cytoplasmic  $\beta$ -catenin requires new protein synthesis.

**$\beta$ -Catenin Plays a Key Role in the Destabilization of the *VEGF-D* mRNA**—As the stability of the messenger RNA of many genes is regulated through their 3'-UTR, which contains gene-specific destabilization signals (53, 54), *VEGF-D* mRNA 3'-UTR was fused to the luciferase reporter gene. As a control, the luciferase reporter gene containing the SV40 polyadenylation signal was used (Fig. 6A). Because transcription of these chimeric constructs was driven by the vector containing the CMV early promoter, differences of the luciferase activity could be attributed to differences in RNA stability of the two constructs. The chimeric plasmid containing *VEGF-D* 3'-UTR was transfected alone or together with the mutant  $\beta$ .4SA in mouse fibroblasts. After transfection, cell extracts were analyzed by Western blotting and analyzed for luciferase activity. Under these conditions the construct carrying the *VEGF-D* 3'-UTR



**FIG. 6.  $\beta$ -Catenin destabilizes *VEGF-D* messenger.** A, schematic representation of plasmids used for co-transfection experiments. The CMV early promoter was inserted into the pGL3-Basic vector containing the luciferase reporter gene and the SV40 polyadenylation signal (SV40). The SV40 polyadenylation signal was replaced by the entire *VEGF-D* 3'-UTR (*VEGF-D*). B–D, luciferase activities of the constructs shown in A co-transfected with increasing amounts of either mutant  $\beta$ -catenin (B), Dvl-2 (C), or axin-(501–560) (D). Values of luciferase activities were normalized for  $\beta$ -galactosidase activities. The results represent the means  $\pm$  S.D. of four independent experiments. Expression of mutant  $\beta$ -catenin, Dvl-2, and axin-(501–560) was analyzed by immunoblotting using anti-Myc tag, anti-HA tag, and anti-FLAG mAbs, respectively. E, transcriptional activity of TOPCAT in mouse fibroblasts transfected with either mutant  $\beta$ -catenin ( $\beta$ .4SA), Dvl-2 (*Dvl-2*), or axin-(501–560) (*axin*). TOPCAT or FOPCAT reporter constructs were co-transfected with pRSV- $\beta$ -galactosidase, and 48 h post-transfection triplicate samples were counted, normalized for  $\beta$ -galactosidase activities, and averaged. WB, Western blot.

showed a decrease in the luciferase activity in a concentration-dependent manner (Fig. 6B). When the stable mutant  $\beta$ -catenin was co-expressed with luciferase, carrying in its 3'-UTR the SV40 polyadenylation signal, luciferase activity was not affected, suggesting that  $\beta$ -catenin exerts its action specifically on *VEGF-D* mRNA. Similar results were obtained co-transfecting vectors expressing Dvl-2 or axin-(501–560), which stabilize cytoplasmic  $\beta$ -catenin (Fig. 6, C–E), thus suggesting that  $\beta$ -catenin plays a role in *VEGF-D* mRNA degradation through instability elements contained within its 3'-UTR.

**$\beta$ -Catenin-dependent *VEGF-D*-destabilizing Sequence Contains a Conserved AU-rich Element**—To map the element responsible for  $\beta$ -catenin-dependent *VEGF-D* mRNA destabilization, we generated a series of deletion constructs of the *VEGF-D* 3'-UTR fused downstream to the luciferase reporter gene under the control of the CMV early promoter. These deletion constructs were co-transfected with the mutant  $\beta$ .4SA or the empty vector in mouse fibroblasts. Deletion constructs restricted the destabilizing element to the 3' end of *VEGF-D* mRNA (Fig. 7A).

The human orthologue *VEGF-D* was co-transfected with the vector expressing the stable mutant  $\beta$ .4SA in HEK 293 cells. Human *VEGF-D* mRNA was degraded to comparable levels as the mouse mRNA (not shown). Deletion analysis of the human



and its loss could play a significant role in progression of the disease. Further experiments will be necessary to prove the hypothesis that *VEGF-D* expression could be responsible for the nodal metastatic behavior of tumors underexpressing  $\beta$ -catenin. In light of the present results, it will also be important for the future analysis of *VEGF-D* expression in tumors to take into account that *VEGF-D* might be expressed as a late marker of tumor progression and only in cells that have lost or reduced expression of  $\beta$ -catenin. These analyses will probably clarify the apparent discrepancy of *VEGF-D* expression in tumors reported by different authors (31–37).

Although the model of  $\beta$ -catenin-TCF complex as nuclear transcriptional regulator has been recently challenged, this mechanism of action remains the most likely (39, 63). Our experiments with inhibitors of transcription and translation demonstrated that  $\beta$ -catenin-dependent regulation of *VEGF-D* expression is indirect and occurs at the level of *VEGF-D* mRNA stability. This has been confirmed by transient transfection experiments that demonstrated that *VEGF-D* 3'-UTR was the target for  $\beta$ -catenin *VEGF-D* mRNA destabilization. Deletion analysis allowed us to map precisely within both mouse and human *VEGF-D* 3'-UTR the  $\beta$ -catenin-responsive element. This element, conserved in mouse and human, contained the sequence AUUUA, which corresponds to one ARE of group I (54), thus suggesting that *VEGF-D* instability element could be recognized by some members of the ELAV family of RNA-binding proteins (64).

**Acknowledgements**—We thank Rolf Kemler, Trevor C. Dale, Jan Kitajewski, and Claudio Schneider for the generous gift of vectors expressing  $\beta$ -catenin constructs, Dvl-2, axin-(501–560), Wnt-1, TOP-CAT, and FOPCAT. We also thank Beatrice Grandi for technical help.

## REFERENCES

- Hanahan, D., and Weinberg, R. A. (2000) *Cell* **100**, 57–70
- Stacker, S. A., Achen, M. G., Jussila, L., Baldwin, M. E., and Alitalo, K. (2002) *Nat. Rev. Cancer* **2**, 573–583
- Risau, W. (1997) *Nature* **386**, 671–674
- Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kiechens, L., Gertsensein, M., Fahrig, M., Vandenhoeck, A., Harpal, H., Ebherardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. (1996) *Nature* **380**, 435–439
- Ferrara, N., Carver-More, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J., and Moore, M. W. (1996) *Nature* **380**, 439–442
- Bethsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philpott, K., Mellor, A. L., Knott, T. J., and Scott, J. (1986) *Nature* **320**, 695–699
- Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T. (1989) *Science* **246**, 1309–1312
- Leung, D. W., Cachianes, G., Kuang, W., Goeddel, D. V., and Ferrara, N. (1989) *Science* **246**, 1306–1309
- Maglione, D., Guerriero, V., Vignietto, G., Delli-Bovi, P., and Persico, M. G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9267–9271
- Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R. F., Alitalo, K., and Erikson, U. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 2576–2581
- Grimmond, S., Lagerkrantz, J., Drinkwater, C., Silins, G., Towson, S., Pollock, P., Gotley, D., Carson, E., Rakar, S., Nordenskjold, M., Ward, L., Hayard, N., and Weber, G. (1996) *Genome Res.* **6**, 124–131
- Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lathinen, I., Kukku, E., Saksela, O., Kalkkinen, N., and Alitalo, K. (1996) *EMBO J.* **15**, 290–298
- Lee, J., Gray, A., Yan, J., Luoh, S.-M., Avraha, H., and Wood, W. I. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 1988–1992
- Orlandini, M., Marconini, L., Ferruzzi, R., and Oliviero, S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11675–11680
- Nicosia, R. F. (1998) *Am. J. Pathol.* **153**, 11–16
- Ogawa, S., Oku, A., Sawano, A., Yamaguchi, S., Yazaki, Y., and Shibuya, M. (1998) *J. Biol. Chem.* **273**, 31273–31282
- Levy, A. P., Levy, N. S., Wegner, S., and Goldberg, M. A. (1995) *J. Biol. Chem.* **270**, 13333–13340
- Mincheno, A., Bauer, T., Salceda, S., and Caro, J. (1994) *Lab. Invest.* **71**, 374–379
- Ikeda, E., Achen, M. G., Breier, G., and Risau, W. (1995) *J. Biol. Chem.* **270**, 19761–19766
- Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O., and Alitalo, K. (1994) *J. Biol. Chem.* **269**, 6271–6274
- Choen, T., Nadhari, D., Cerem, L. W., Neufeld, G., and Levi, B. Z. (1996) *J. Biol. Chem.* **271**, 736–741
- Li, J., Perrella, M. A., Tsai, J. C., Yet, S. F., Hsieh, C. M., Yoshizumi, M., Patterson, C., Endego, W. O., Zhou, F., and Lee, M. (1995) *J. Biol. Chem.* **270**, 308–312
- Achen, M. G., Jeltsch, M., Kukku, E., Makinen, T., Vitali, A., Wilks, A. F., Alitalo, K., and Stacker, S. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 548–553
- Yamada, Y., Nezu, J., Shimane, M., and Hirata, Y. (1997) *Genomics* **42**, 483–488
- Jenkins, N. A., Woollatt, E., Crawford, J., Gilbert, D. J., Baldwin, M. E., Sutherland, G. R., Copeland, N. G., and Achen, M. G. (1997) *Chromosome Res.* **5**, 502–505
- Rocchigiani, M., Lestingi, M., Luddi, A., Orlandini, M., Franco, B., Rossi, E., Ballabio, A., Zuffardi, O., and Oliviero, S. (1998) *Genomics* **47**, 207–216
- Enholm, B., Paavonen, K., Ristimäki, A., Kumar, V., Gunji, Y., Klefstrom, J., Kivinen, L., Laiho, M., Olofsson, B., Jukov, V., Ericsson, U., and Alitalo, K. (1997) *Oncogene* **14**, 2475–2483
- Ristimäki, A., Narko, K., Enholm, B., Joukov, V., and Alitalo, K. (1998) *J. Biol. Chem.* **273**, 8413–8418
- Orlandini, M., and Oliviero, S. (2001) *J. Biol. Chem.* **276**, 6576–6581
- Stacker, S. A., Caesar, C., Baldwin, M. E., Thornton, G. E., Williams, R. A., Prevo, R., Jackson, D. G., Nishikawa, S., Kubo, H., and Achen, M. G. (2001) *Nat. Med.* **7**, 186–191
- Ishikawa, M., Kitayama, J., Kazama, S., and Nagawa, H. (2003) *Jpn. J. Clin. Oncol.* **33**, 21–27
- Nakamura, Y., Yasuoka, H., Tsujimoto, M., Yang, Q., Imabun, S., Nakahara, M., Nakao, K., Nakamura, M., Mori, I., and Kakudo, K. (2003) *Clin. Cancer Res.* **9**, 716–721
- White, J. D., Hewett, P. W., Kosuge, D., McCulloch, T., Enholm, B. C., Carmichael, J., and Murray, J. C. (2002) *Cancer Res.* **62**, 1669–1675
- Kurebayashi, J., Otsuki, T., Kunisue, H., Mikami, Y., Tanaka, K., Yamamoto, S., and Sonoo, H. (1999) *Jpn. J. Cancer Res.* **90**, 977–981
- Yokoyama, Y., Charnock-Jones, D. S., Licence, D., Yanaihara, A., Hastings, J. M., Holland, C. M., Emoto, M., Umemoto, M., Sakamoto, T., Sato, S., Mizunuma, H., and Smith, S. K. (2003) *Br. J. Cancer* **88**, 237–244
- George, M. L., Tutton, M. G., Janssen, F., Arnaout, A., Abulafi, A. M., Eccles, S. A., and Swift, R. I. (2001) *Neoplasia* **3**, 420–427
- O-Chareonrat, P., Rhys-Evans, P., and Eccles, S. A. (2001) *Cancer (Phila.)* **92**, 556–568
- Henderson, B. R., and Fagotto, F. (2002) *EMBO Rep.* **3**, 834–839
- Bien, M., and Clevers, H. (2003) *Nat. Cell Biol.* **5**, 179–182
- Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) *EMBO J.* **16**, 3797–3804
- Smalley, M. J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilake, H., Fryer, L. G., Hutchinson, L., Fry, M. J., and Dale, T. C. (1999) *EMBO J.* **18**, 2823–2835
- Young, C. S., Kitamura, M., Hardy, S., and Kitajewski, J. (1998) *Mol. Cell. Biol.* **18**, 2474–2485
- Korinek, V., Barker, N., Morin, P. J., van Wiken, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) *Science* **275**, 1784–1787
- Goruppi, S., Chiaruttini, C., Ruaro, M. E., Varnum, B., and Schneider, C. (2001) *Mol. Cell. Biol.* **21**, 902–915
- Chomzyzski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051
- Pokutta, S., and Weis, W. I. (2002) *Curr. Opin. Struct. Biol.* **12**, 255–262
- Laney, J. D., and Hochstrasser, M. (1999) *Cell* **97**, 427–430
- Marikawa, Y., and Elinson, R. P. (1998) *Mech. Dev.* **77**, 75–80
- Semplici, F., Pinna, L. A., Meggio, F., and Oliviero, S. (2002) *Oncogene* **21**, 3978–3987
- Barker, N., Morin, P. J., and Clevers, H. (2000) *Adv. Cancer Res.* **77**, 1–24
- Huelsken, J., and Behrens, J. (2002) *J. Cell Sci.* **115**, 3977–3978
- Wilus, C. J., Wormington, M., and Peltz, S. W. (2001) *Nat. Rev. Mol. Cell. Biol.* **2**, 237–246
- Chen, C. Y., and Shyu, A. B. (1995) *Trends Biochem. Sci.* **20**, 465–470
- van der Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A. P., Tjon-Pon-Pong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. (2002) *Cell* **111**, 241–250
- Avantaggiato, V., Acampora, D., Orlandini, M., Oliviero, S., and Simeone, A. (1998) *Mech. Dev.* **73**, 221–224
- Bright-Thomas, R. M., and Hargrett, R. (2003) *Eur. J. Surg. Oncol.* **29**, 107–117
- Niki, T., Iba, S., Tokunou, M., Yamada, T., Matsuno, Y., and Hirohashi, S. (2000) *Clin. Cancer Res.* **6**, 2431–2439
- Easwaran, V., Lee, S. H., Inge, L., Guo, L., Goldbeck, C., Garrett, E., Wiesmann, M., Garcia, P. D., Fuller, J. H., Chan, V., Randazzo, F., Gundel, R., Warren, R. S., Escobedo, J., Aukerman, S. L., Taylor, R. N., and Fantl, W. J. (2003) *Cancer Res.* **63**, 3145–3153
- Kageshita, T., Hamby, C. V., Ishihara, T., Matsumoto, K., Saida, T., and Ono, T. (2001) *Br. J. Dermatol.* **145**, 210–216
- Osborne, J. E. (2002) *Br. J. Dermatol.* **146**, 1104
- Bankfalvi, A., Krassort, M., Buchwalow, I. B., Vegh, A., Felszeghy, E., and Piffko, J. (2002) *J. Pathol.* **198**, 343–351
- Chan, S. K., and Struhl, G. (2002) *Cell* **111**, 265–280
- Ma, W. J., Cheng, S., Campbell, C., Wright, A., and Furneaux, H. (1996) *J. Biol. Chem.* **271**, 8144–8151