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In Fibroblasts *Vegf-D* Expression Is Induced by Cell-Cell Contact Mediated by Cadherin-11*

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Vascular endothelial growth factors (VEGFs) are a highly conserved family of growth factors all angiogenic *in vivo* with mitogenic and chemotactic activity on endothelial cells. *VEGF*s are expressed in fibroblasts either in hypoxia or in response to growth factors. Here we report that, differently from the other members of the family, *Vegf-D* is induced by cell-cell contact. By *in situ* hybridization we demonstrated that noninteracting fibroblasts express low levels of *Vegf-D* mRNA, whereas contacting cells express high levels of *Vegf-D* transcripts. By immunostaining we observed that the surface protein cadherin-11 is localized at the opposite sites of interacting cell surfaces. Ca^{2+} deprivation from the culture medium determined the loss of cadherin-11 from the cell surfaces and down-regulation of *Vegf-D* mRNA. Moreover, a cadherin-11 antisense RNA construct inhibited *Vegf-D* expression in confluent BALB/c fibroblasts, whereas in NIH 3T3 cells, which express low levels of cadherin-11, *Vegf-D* induction could be obtained by overexpression of cadherin-11. This suggests that cell interaction mediated by cadherin-11 induces the expression of the angiogenic factor *Vegf-D* in fibroblasts.

The VEGF[1] family is composed of several structurally and functionally related growth factors involved in vascular development. This family includes the vascular endothelial growth factor (VEGF), the placental growth factor, VEGF-B, VEGF-C, VEGF-D, and VEGF-E (1–11). All members of this family are angiogenic *in vivo* and able to stimulate proliferation of endothelial cells *in vitro*. Each member of the family recognizes and activates specific receptors on endothelial cells: VEGF recognizes VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1); placental growth factor and VEGF-B recognize VEGFR-1 (12–14); VEGF-C and VEGF-D recognize VEGFR-2 and VEGFR-3 (Flt-4) (7, 15, 16). This latter is almost exclusively expressed in lymphatic vessels, suggesting that these factors, beside playing a role in angiogenesis, are also involved in the formation of lymphatic vessels.

Due to the similarity of structure and promiscuity of receptor recognition, the specific role of each member of the family has not yet been identified. In differentiating tissues, specific regulation of each factor may be required to determine the correct succession and composition of the appropriated angiogenic factors for vessel formation. *VEGF* expression has been extensively studied. It responds to low levels of oxygen with induced transcription and increasing mRNA stability (17–19). Moreover, *VEGF* mRNA expression is up-regulated by epidermal growth factor, transforming growth factor-β, IL-6 in several cell types, and by IL-1β in smooth muscle cells (20–22). *VEGF-C* expression in cultured fibroblasts is induced by serum, phorbol 12-myristate 13-acetate, and several factors, including IL-1β and tumor necrosis factor α (23, 24). *Vegf-D* appeared to be differently regulated, because it was expressed in cells grown in low serum conditions (9).

Analyzing *Vegf-D* mRNA expression in mouse fibroblasts we observed that this growth factor, differently from the other members of the VEGF family, was induced by calcium-dependent cell-cell interactions. Cell-cell adhesion is mediated by cadherins, a large family of transmembrane calcium-dependent adhesive glycoproteins that form homotypic binding with their extracellular domain on adjacent cells (25–27). Although it is generally thought that cadherin expression results in a tight cell association, this is not a general principle and mesenchymal cells, which are loosely associated, express mesenchyme-specific cadherins like cadherin-11 (28–31).

The data presented in this report demonstrate that *Vegf-D* messenger is strongly induced by direct cell-cell contact. This induction can be inhibited by depletion of extracellular Ca^{2+} from the culture medium. Inhibition of cadherin-11 expression in contacting fibroblasts reduces *Vegf-D* mRNA induction, whereas cadherin-11 expression in fibroblasts, that do not express cadherin-11, restores *Vegf-D* induction. These results identify cadherin-11 as a surface molecule involved in *Vegf-D* regulation by cell-cell interaction.

EXPERIMENTAL PROCEDURES

Cloning and Cell Culture—The mouse *cadherin-11* full-length cDNA was amplified from a mouse fibroblast cDNA library (9), using the primers 5′-GAGAGGATCCACCACCATGAAGGAGAACTACTG-3′ and 5′-GAGACTCGAGTTAAGAGTCATCATCAAAAGTG-3′. The polymerase chain reaction product was cloned into the plasmid pcDNA3 (Invitrogen Corp.) in the sense (giving MO447) and antisense (giving MO334) orientation under the control of the cytomegalovirus promoter. The oligonucleotide sequences were obtained from the EBI Nucleotide Sequence Data Base under accession numbers D21253 (OB-cadherin) and D31963 (cadherin-11). All constructs were checked by automated sequencing.

Mouse embryo fibroblasts were isolated from 14-day CD1 mouse embryos as described previously (32). Unless otherwise stated, mouse embryo, mouse 3T3-type, NIH 3T3, and BALB/c 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified, 5% CO_2 atmosphere. Stable clones expressing mouse cadherin-11 were obtained from NIH 3T3 cells transfected with the *cadherin-11* expression vector MO447 by standard $CaPO_4$ precipitation procedures (32). Transfectants were selected using 1 mg/ml G418 (Life Technologies, Inc.). Stable clones expressing mouse *cadherin-11* in the antisense orientation were obtained

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; IL, interleukin; PBS, phosphate-buffered saline; poly-HEME, poly-2-hydroxyethyl methacrylate.

from BALB/c 3T3 fibroblasts transfected with the plasmid MO334, and transfectants were selected using 0.4 mg/ml G418. The same empty vector was used to generate mock stable clones.

Cell-Cell Contact and Induction Experiments—Mouse fibroblasts were plated 14–16 h before day 0 on 10-cm tissue culture dishes at different density, and starting from day 0, culture medium was changed every 2 days. Low, medium, and high cell densities corresponded to cells plated from about 20% to about 70% confluence. The degree of cell confluence was monitored under an inverted microscope. The cell cycle was arrested by adding cyclosporin A (0.95 μg/ml), colchicine (0.11 μg/ml), or tunicamycin (0.5 μg/ml) in the culture medium of subconfluent fibroblasts for 24 h. Conditioned medium was obtained from the culture medium of fibroblasts growing at high cell confluence, diluted 1:1 (v/v) with complete medium, and used to stimulate subconfluent fibroblasts for 33 h. The heparin wash of confluent fibroblasts was performed by using a solution of heparin (100 μg/ml, Sigma-Aldrich) in PBS. After taking off the medium, the heparin solution was left on confluent cells for 2 h at room temperature, collected, centrifuged, diluted in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, and used to stimulate subconfluent fibroblasts for 33 h. As negative control the heparin solution not left on the cells was used. To chelate Ca^{2+} in the culture medium, confluent fibroblasts were grown for 24 h in the presence of 2.2 mM EGTA. Poly-HEME (Sigma-Aldrich) was used to inhibit cell adhesion to growth surface in culture dishes. Culture plates were coated with 6 mg/ml poly-HEME in 95% ethanol and allowed to air dry in a sterile environment. Fibroblast cells were seeded at high cell density in plates precoated with poly-HEME and after 48 h cells were collected by centrifugation and RNA was extracted. To block or stimulate Ca^{2+} flux through calcium channels, fibroblasts were grown either for 18 h at high cell density in the presence of Ca^{2+} channel antagonists (10 μM diltiazem, 50 μM amiloride, 20 μM nifedipine, 10 μM verapamil, 1 μM ω-conotoxin GVIA) or for 24 h at low cell density in the presence of a Ca^{2+} channel agonist (1.25 μM BAY K-8644). Cyclosporin A, colchicine, tunicamycin, diltiazem, amiloride, and ω-conotoxin GVIA were purchased from BioMol Research Laboratories, and nifedipine, verapamil, BAY K-8644 were from Sigma-Aldrich. Cell synchronization agents and calcium channel modulators were used at concentrations established from the literature to have maximal effects on their targets.

Northern Blot Analysis—Total cellular RNA was extracted from cells by the guanidinium thiocyanate method (33). Total RNA (10 μg) was run on denaturing formaldehyde-agarose gel, transferred onto nylon membranes, and cross-linked by UV irradiation using a Stratalinker (Stratagene). Filters were hybridized with ^{32}P-labeled probes, washed as described (9), and analyzed by using a PhosphorImager (Molecular Dynamics). Rat glyceraldheyde-3-phosphate dehydrogenase (*gapdh*) was used as a control for RNA loading.

In Situ Hybridization—Digoxigenin-labeled *Vegf-D* sense and antisense RNA probes were generated from a cDNA fragment corresponding to the complete coding sequence of the mouse *Vegf-D* gene. Mouse fibroblasts were grown on microscopic slides at different degrees of confluence and fixed for 20 min with 4% paraformaldehyde in PBS. *In situ* hybridization was performed as described previously (34) with minor modifications. Briefly, to increase permeability cells were treated for 10 min with 0.2 N HCl and for 25 min at 37 °C with 1 μg/ml proteinase K (Sigma-Aldrich) in 50 mM Tris-HCl, pH 8. Then cells were washed in PBS and post-fixed for 10 min with 4% paraformaldehyde in PBS. Cells were hybridized overnight in a humidified chamber at 37 °C with the digoxigenin-labeled probes diluted at 1 μg/ml in hybridization buffer (60% deionized formamide, 2× SSC buffer, 50 mM sodium phosphate, 5% dextran sulfate, 250 μg/ml yeast RNA, and 250 μg/ml salmon sperm DNA). The slides were washed, and the hybridized digoxigenin-conjugated probes were detected by using the fluorescent antibody enhancer set (Roche Diagnostics) according to standard procedures. Slides were counterstained with propidium iodide (Sigma-Aldrich) at 100 ng/ml, mounted in PBS containing 2% 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) and 50% glycerol, and examined under a Leica TCS confocal laser-scanning microscope. The sense strand gave no signal.

Immunoblotting—Whole cell extracts were prepared by rinsing cultures with cold buffer (20 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM EGTA). Cells were harvested with a rubber policeman, centrifuged, and lysed in 0.5% Nonidet P-40 buffer (20 mM HEPES, pH 7.4, and 2 mM EDTA) containing Complete protease inhibitors (Roche Diagnostic). Protein concentration of cell extracts were determined by using the BCA protein assay reagent (Pierce). The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Equal loading was confirmed by staining in Ponceau S solution (Sigma-Aldrich). The membranes were blocked for 1 h at room temperature in PBS containing 3% dry milk and 0.1% Triton X-100 and incubated with goat polyclonal antibodies against OB-cadherin (Santa Cruz Biotechnology, Inc.) at 0.4 μg/ml for 2 h at room temperature. The blots were washed, incubated with horseradish peroxidase-labeled donkey anti-goat IgG (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature and washed in PBS, and finally the bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Immunofluorescence—Mouse fibroblasts were seeded onto glass coverslips, cultured overnight, and fixed with 3% paraformaldehyde in PBS for 15 min. Cells were then permeabilized in 0.5% Triton X-100 in PBS for 3 min and blocked for 1 h with 1% bovine serum albumin in PBS. Coverslips were incubated for 1 h at 37 °C with goat polyclonal anti-OB-cadherin antibodies. After washing, the coverslips were incubated for 45 min at 37 °C in the presence of donkey anti-goat IgG labeled with tetramethylrhodamine isothiocyanate (Jackson ImmunoResearch Laboratories). To localize actin filaments, fluorescein isothiocyanate-labeled phalloidin (Sigma-Aldrich) was added along with secondary antibodies at 2 μg/ml. Coverslips were then mounted in Mowiol 4-88 (Calbiochem) and examined under a Leica TCS confocal laser-scanning microscope.

RESULTS

Vegf-D mRNA Is Expressed in Confluent Cells—Unlike *VEGF-C*, whose expression is induced by several growth factors (23, 24), *Vegf-D* is not induced by cell treatment with platelet-derived growth factor, epidermal growth factor, fibroblast growth factor 4, basic fibroblast growth factor, or transforming growth factor β (data not shown). Moreover, we previously observed that in low serum conditions fibroblasts expressed high levels of *Vegf-D* transcripts (9).

To test whether *Vegf-D* mRNA induction may require high cell density, RNA was collected at various time points from fibroblasts plated at different densities. At day 0, when cells were plated at low (20% confluence) or medium (40% confluence) density, the expression of *Vegf-D* was barely detectable, whereas some *Vegf-D* expression could be detected in cells plated at the highest density (70% confluence) (Fig. 1*A*, *lanes 1–3*). Two days later, after the cells reached a higher confluence, we observed a correspondent induction of *Vegf-D* transcripts. In particular, *Vegf-D* expression was increased in the cells originally plated at medium and high density (*lanes 5* and *6*). In fact, after 2 days, these cells reached about 90 and 98% confluence, respectively, with elevated cell-cell interactions. At day 2, cells that were originally plated at low density (*lane 4*) were at about 30% confluence and showed still low *Vegf-D* expression. Quantitative analysis revealed that cells plated at higher density reached the highest expression of *Vegf-D* between days 4 and 6 from plating, cells plated at medium density at day 6, and cells plated at low density at about day 8 (Fig. 1*B*). Thus, the levels of *Vegf-D* transcripts and cell density are directly correlated.

Extracellular Ca^{2+} Is Required for Vegf-D Expression—Next we examined whether cell cycle arrest or soluble autocrine growth factor(s) accumulating in the culture medium, or on the surface of cells growing at high cell density, could be responsible for *Vegf-D* induction. The treatment of subconfluent cells with the cell cycle inhibitors cyclosporin A, colchicine, or tunicamycin did not lead to *Vegf-D* induction (Fig. 2*A*). Neither the treatment of subconfluent cells with conditioned medium nor with a heparin wash from confluent cells could induce *Vegf-D* expression, suggesting that autocrine-soluble factors were not involved in *Vegf-D* induction (Fig. 2*B*, compare *lane 1* with *lanes 2* and *4*). To test whether cell-cell interaction and/or cell-plate contacts would play a role in *Vegf-D* induction, we analyzed *Vegf-D* mRNA levels in cells growing either in the presence of EGTA or in plates precoated with poly-HEME. In the presence of EGTA, cells grew at a normal rate, acquired a round shape, and lost interactions with each other and with the culture plate. Deprivation of Ca^{2+} from the culture medium

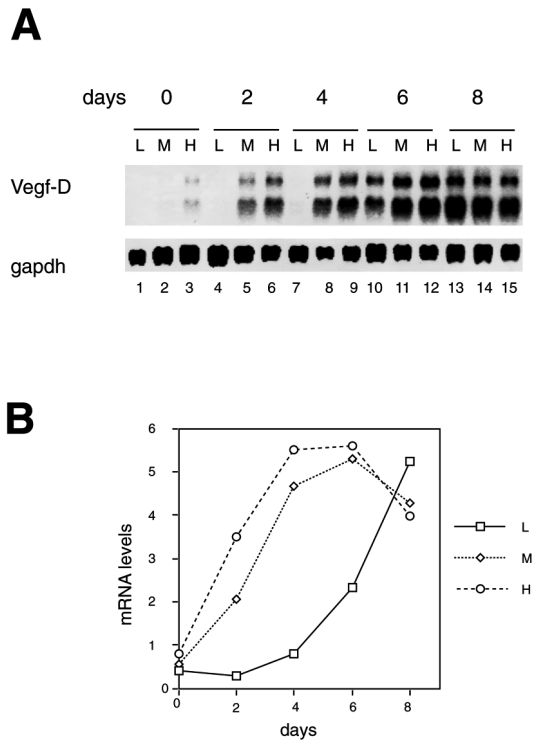

FIG. 1. **Analysis of *Vegf-D* expression in correlation with cell density in cultured mouse fibroblasts.** *A*, Northern blot analysis using *Vegf-D* and *gapdh* cDNA probes. Fibroblasts were plated at low (*L*), medium (*M*), and high (*H*) cell density. After 15 h, corresponding to day 0, cell confluence was monitored and resulted about 20%, 40%, and 70%, respectively, for plates L, M, and H. Starting from day 0, every 2 days the culture medium was changed, the degree of confluence was monitored, and the RNA was collected. Above the lanes are indicated the days from the time of plating. *Vegf-D* transcripts were induced when cells reached confluence at day 2 for M and H, and at day 6 for L (*lanes 5*, *6*, and *10*, respectively). *B*, quantitative analysis of *Vegf-D* mRNA levels from a representative experiment with cells plated as in *A*. The blots were analyzed by using a PhosphorImager, and the values, normalized to the *gapdh* mRNA levels, are expressed as arbitrary units.

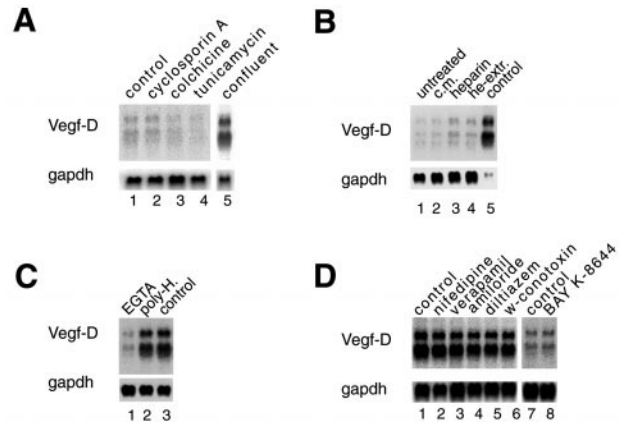

FIG. 2. ***Vegf-D* expression is Ca^{2+}-dependent in cultured fibroblasts.** *A*, Northern blot analysis of RNA extracted from subconfluent fibroblasts treated for 24 h with drugs as indicated. Cell cycle arrest did not lead to *Vegf-D* expression in sparse cells. Total RNA from untreated subconfluent fibroblasts (*lane 1*). *B*, Northern blot analysis of RNA extracted from subconfluent fibroblasts stimulated for 33 h with conditioned media obtained from fibroblasts grown at high cell density. *Vegf-D* was not induced by autocrine growth factor(s). Subconfluent cells not treated with conditioned medium (*untreated*); subconfluent cells treated with conditioned medium obtained from fibroblasts at confluence (*c.m.*); subconfluent cells treated with a solution of heparin (*heparin*); subconfluent cells treated with a heparin wash of fibroblasts at confluence (*he-extr.*); fibroblasts at confluence (*control*). *C*, Northern blot analysis of RNA extracted from fibroblasts grown for 24 h in the presence of 2.2 mM EGTA or for 48 h in culture plates precoated with poly-HEME (*poly-H*), which inhibits cell adhesion to the culture plate. Deprivation of Ca^{2+} from the culture medium inhibited *Vegf-D* mRNA accumulation. Total RNA from untreated confluent cells (*control*). *D*, Northern blot analysis of RNA extracted from fibroblasts grown in the presence of calcium channel modulators. Fibroblasts were grown either at high cell density for 18 h with calcium channel antagonists (diltiazem, amiloride, nifedipine, verapamil, ω-conotoxin GVIA) or at low cell density for 24 h with a calcium channel agonist (BAY K-8644). *Vegf-D* expression was not induced by calcium flux through calcium channels. Control of untreated fibroblasts confluent (*lane 1*) and subconfluent (*lane 7*).

strongly inhibited *Vegf-D* mRNA accumulation (Fig. 2*C*, *lane 1*). On the contrary, culture plates precoated with poly-HEME, which inhibited cell adhesion to the plate, did not affect *Vegf-D* induction (Fig. 2*C*, *lane 2*), suggesting that cell-matrix interactions are not involved in *Vegf-D* expression. Because Ca^{2+} depletion from the culture medium might affect calcium influx into the cells, we tested the expression of *Vegf-D* mRNA in cells treated with different Ca^{2+} channel blockers. The treatment of confluent cells with the calcium channel antagonists nifedipine, verapamil, amiloride, diltiazem, or ω-conotoxin GVIA did not inhibit *Vegf-D* mRNA expression, and the treatment of sparse cells with the calcium channel agonist BAY K-8644 did not induce *Vegf-D* expression (Fig. 2*D*). Therefore, this excluded that calcium influx plays a role in the *Vegf-D* up-regulation.

Vegf-D mRNA Is Strongly Induced by Direct Cell-Cell Interaction—To directly observe *Vegf-D* mRNA expression in contacting cells we performed *in situ* experiments with cultured fibroblasts plated at various degrees of confluence. Hybridization with *Vegf-D* antisense probe showed that single cells expressed very poor levels of *Vegf-D* mRNA, whereas contacting fibroblasts did express high levels of *Vegf-D* messenger. This could be observed even at the level of two interacting cells (Fig. 3, *A* and *B*).

Taken together, the above experiments demonstrated that both cell-cell interaction and extracellular calcium ions are required for *Vegf-D* up-regulation in mouse fibroblasts. Homophilic calcium-dependent cell-cell interactions are mediated by cadherins (25). We therefore tested the hypothesis that direct interaction between contacting fibroblasts, mediated by cadherins, could be responsible for *Vegf-D* up-regulation.

First we examined by Northern blot analysis the expression of cadherins in sparse and confluent fibroblasts with different cadherin probes. We observed that, in fibroblasts, grown at different degrees of confluence, *cadherin-11*, a mesenchymal-specific cadherin, was strongly induced by cell-cell contact (Fig. 4). Importantly, *cadherin-11* mRNA induction was preceding *Vegf-D* mRNA of 8–12 h, suggesting that cadherin-11 could be involved in *Vegf-D* regulation.

Immunostaining of contacting fibroblasts using anti-cadherin-11 antibodies revealed a positive staining at the cell surfaces (Fig. 5). In sparse cells cadherin-11 was localized at intercellular contacts, whereas it was mostly absent from surfaces free of cell contact (Fig. 5*A*). At confluence cadherin-11 staining was observed at the level of the whole cell membrane (Fig. 5*B*). To examine whether the localization of cadherin in fibroblasts depends on Ca^{2+}, contacting cells were treated with EGTA. As expected, within the first hour cadherin-11 signal disappeared from the cell surface and became mostly cytoplasmic (Fig. 5*C*). Addition of Ca^{2+} to the media restored cell-cell contacts, with reappearance of cadherin-11 at the intercellular contacts and induction of *Vegf-D* mRNA in the cells (not shown).

Cadherin-11 Is Required for Vegf-D Induction in Interacting Cells—In our study, mouse 3T3 type fibroblasts, derived from mice strain 129/SvJ × C57BL/6J (129-B6) (35), were used for

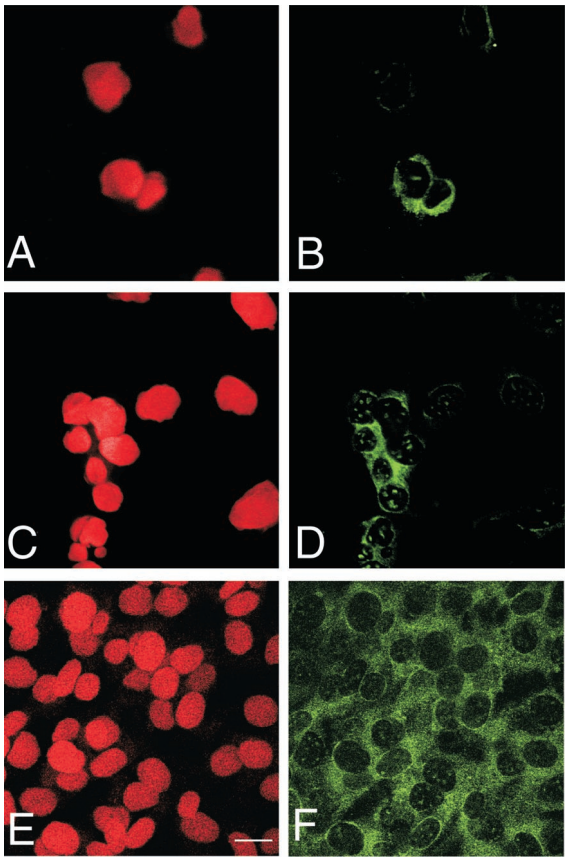

FIG. 3. **Direct cell-cell contacts induce *Vegf-D* mRNA.** *B*, *D*, and *F*, mouse fibroblasts, grown on microscopic slides at different degrees of confluence, were analyzed by *in situ* hybridization with a digoxigenin-labeled probe specific for *Vegf-D* transcripts (*green*). *A*, *C*, and *E*, nuclei of the same cells as in *B*, *D*, and *F* were visualized with the nuclear dye propidium iodide (*red*). Stainings were analyzed by confocal microscopy. Single cells expressed very poor levels of *Vegf-D* mRNA, whereas contacting fibroblasts expressed high levels of *Vegf-D* messengers (*E* and *F*), even at the level of two interacting cells (*A* and *B*). The *scale bar* represents 30 μm.

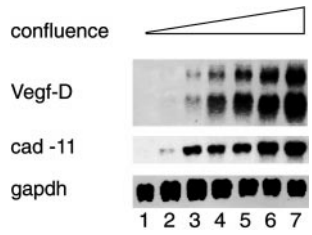

FIG. 4. ***Cadherin-11* mRNA is up-regulated in contacting fibroblasts.** Northern blot analysis using *Vegf-D, cadherin-11*, and *gapdh* probes. Fibroblasts were plated at low cell density, and every 2 days the culture medium was changed, the degree of confluence was monitored, and the RNA was collected. *Cadherin-11*, a mesenchyme-specific cadherin, was induced when cells established cell-cell contacts (*lane 3*).

comparative analysis of gene expression, because *Vegf-D* was strongly expressed in these cells. We tested whether other fibroblasts showed the same *Vegf-D* mRNA regulation. Primary embryo fibroblasts obtained from CD1 mice and BALB/c 3T3 fibroblasts revealed a *Vegf-D* mRNA strong induction that correlated with *cadherin-11* high expression in confluent cells. Instead, NIH 3T3 fibroblasts expressed barely detectable levels of both *cadherin-11* and *Vegf-D* mRNAs (Fig. 6). Thus, extending the correlation between *Vegf-D* mRNA induction and *cadherin 11* expression in the same cells.

To directly evidence that cadherin-11 is required for *Vegf-D* expression, we generated, from BALB/c 3T3 cells, stable cell

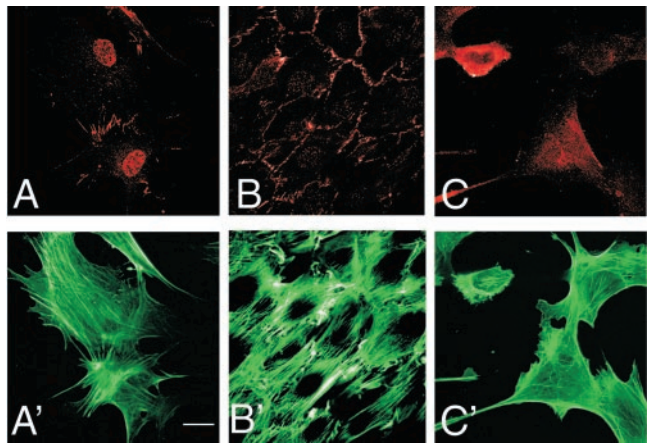

FIG. 5. **Cadherin-11 is localized at cell-cell interaction surfaces.** *A–C*, immunofluorescence analysis using anti-cadherin-11 primary antibodies (*red*). Mouse fibroblasts were seeded at different degrees of confluence, grown in the absence (*A* and *B*) or in the presence (*C*) of 2.2 mM EGTA in the culture medium and processed for immunostaining. *A′*, *B′*, and *C′*, actin filaments of the same cells as in *A–C* were stained using fluorescein isothiocyanate-labeled phalloidin (*green*). Stainings were analyzed by confocal microscopy. Cadherin-11 is localized on the cell surface at intercellular contacts (*A*), and in the presence of EGTA cadherin-11 signal disappeared from the cell surface and became mainly cytoplasmic (*C*). The *scale bar* represents 30 μm.

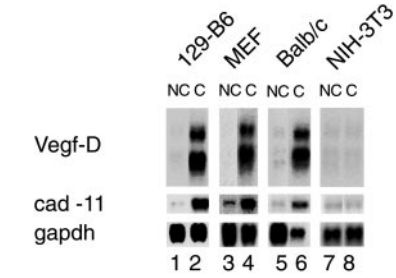

FIG. 6. ***Vegf-D* and *cadherin-11* mRNA expression correlates in different fibroblasts.** RNA was collected from 3T3-type (129-B6), mouse embryo fibroblasts, BALB/c, or NIH 3T3 fibroblasts grown at low (*NC*) and high (*C*) cell confluence. *Vegf-D* and *cadherin-11* expression correlated in confluent fibroblasts from different origin, whereas in NIH 3T3 fibroblasts low levels of *Vegf-D* and *cadherin-11* mRNAs were detected.

lines overexpressing *cadherin-11* in the antisense orientation. Analysis of several stable clones revealed a variable level of cadherin-11 measured by Western blot of cell lysates. Two clones expressing low levels of cadherin-11 were analyzed for *Vegf-D* expression (Fig. 7*A*). By Northern blot analysis of the *cadherin-11* antisense clones using *Vegf-D* probe, we observed that inhibition of cadherin-11 resulted in a strong reduction of *Vegf-D* expression (Fig. 7, compare *A* and *B*).

The converse experiment was performed in NIH 3T3 fibroblasts, because these cells expressed low levels of cadherin-11. From NIH 3T3 we generated stable clones expressing, under the control of a constitutive promoter, cadherin-11 and analyzed *Vegf-D* mRNA expression levels in contacting cells. Two clones expressing higher levels of cadherin-11 were chosen for *Vegf-D* expression analysis (Fig. 7*C*). In these cells the ectopic expression of cadherin-11 induced a significant increase of *Vegf-D* transcripts (Fig. 7, compare *C* and *D*).

DISCUSSION

In multicellular organisms, intercellular interactions and inductive signals play a major role in cell fate during development. Cell-cell adhesion, dictated by homophilic surface molecules like cadherins, determine cell patterning establishing the tissue architecture; however, secreted growth factors, which

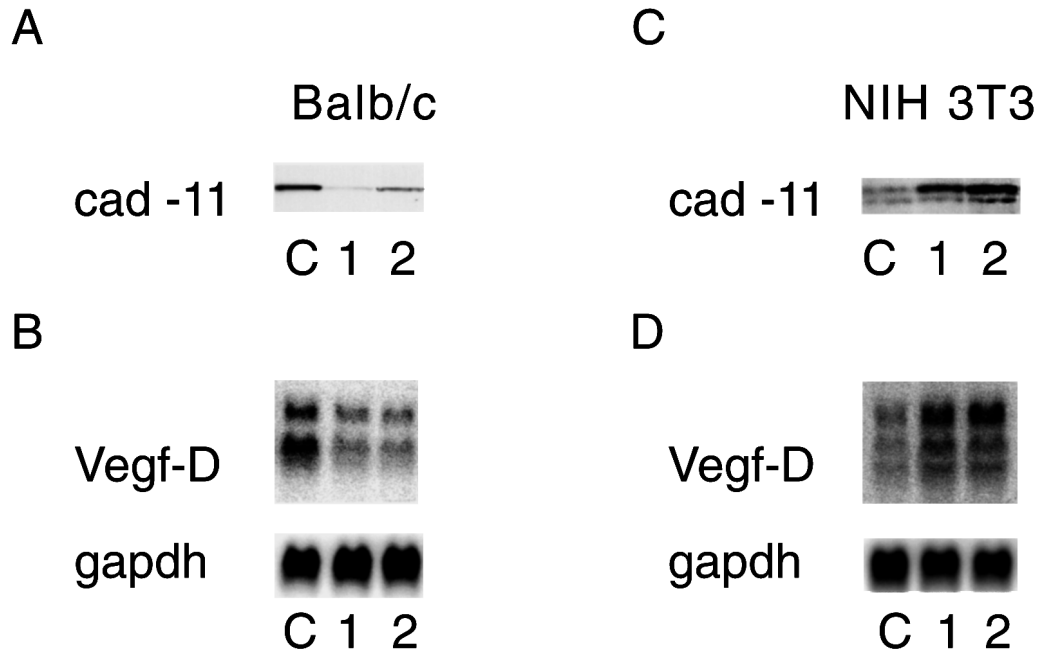

FIG. 7. **Cadherin-11 is required for *Vegf-D* induction.** *A* and *B*, inhibition of cadherin-11 affects *Vegf-D* expression in fibroblasts. Stable cell lines, overexpressing *cadherin-11* in the antisense orientation, were generated in BALB/c 3T3 cells and grown at high cell confluence for 4 days. *A*, Western blot analysis of cell lysates from *cadherin-11* antisense clones revealed with anti-cadherin-11 antibodies. *B*, Northern blot analysis of RNA extracted from the same antisense clones as in *A*, using *Vegf-D* and *gapdh* probes. *C* and *D*, overexpression of cadherin-11 induces *Vegf-D* in NIH 3T3 fibroblasts. Stable cell lines, overexpressing cadherin-11, were generated in NIH 3T3 cells and grown at high cell confluence for 4 days. *C*, immunoblotting analysis of cell lysates from cadherin-11 stable clones with anti-cadherin-11 antibodies. *D*, Northern blot analysis of RNA extracted from the same clones as in *C*, using *Vegf-D* and *gapdh* probes. Mock clones are in *lanes C*.

act at a few cell diameters, modify the expression pattern of neighboring target cells. Here we demonstrate that in cultured fibroblasts direct cell-cell interaction, mediated by the mesenchyme-specific cadherin-11, triggers *Vegf-D* mRNA induction, suggesting cross-talk occurs between cell adhesion and growth factor signaling.

Several lines of evidence support the model that *Vegf-D* expression is regulated by direct cell-cell interactions via cadherin-11. First, *Vegf-D* is not induced in subconfluent cells under diverse culture conditions, whereas its expression is dramatically increased in cells that reach confluence. Second, the addition of conditioned media from cells highly expressing *Vegf-D* does not induce its expression in cells grown at low density, excluding the possibility that autocrine-diffusible factors are instrumental in this activation. Third, depletion of extracellular calcium ions, but not inhibition of cells to signal through calcium flux, blocks *Vegf-D* expression, demonstrating that direct calcium-dependent cell-cell interactions are required. Fourth, *Vegf-D* expression directly correlates with cadherin-11 localization on the cell-interacting surfaces. Fifth, down-modulation or overexpression of cadherin-11 in fibroblasts affects *Vegf-D* expression in a negative and positive manner, respectively. Thus, the experiments described in this report provide evidence that cadherin-11 mediates a cell interaction signaling that leads to the regulation of Vegf-D in contacting fibroblasts.

Cadherins play an important role in cell recognition and sorting during development (36, 37; and references therein). Their function has been perceived to link and stabilize connections between cells through interaction with the cytoskeleton. However, cytoplasmic domains of cadherins are highly diversified, and examples of cadherins have been found associated with signal transduction molecules and/or able to induce intracellular messengers, suggesting that cadherins mediate signal transduction pathways upon ligand binding (for review see Ref. 38). During development *Vegf-D* mRNA expression appears to be restricted to cadherin-11-positive mesenchymal cells. In the developing mouse embryo *cadherin-11* is expressed in migratory cells derived from neural crest cells and in cells involved in mesenchymal condensation (30, 31). *Vegf-D* expression shows a striking overlap with *cadherin-11* (30, 31, 39).

VEGFs are a family of angiogenic factors that are known to induce vessels sprouting *in vivo* and activation of endothelial specific receptors *in vitro* which are in part overlapping (7, 12–16). Although it is difficult to assign a specific role to each member of the family, it is likely that a coordinated regulation of each factor is required for the correct three-dimensional formation of new vessels. Therefore, the pattern of expression of each factor may contribute to explain its role in the angiogenic process. Interestingly, VEGF is strongly induced by hypoxia whereas VEGF-B, VEGF-C, and VEGF-D are not (17, 19, 23, 40). In lung fibroblasts VEGF-C is up-regulated by growth factors (23, 24). By contrast, in mouse fibroblasts Vegf-D is not induced by growth factor treatment,[2] whereas it is up-regulated by cadherin-dependent cell-cell interaction. Vegf-D regulation by cell-cell contact is consistent with a model in which aggregating fibroblasts switch on the expression of a factor that is chemotactic to endothelial cells and thus could recruit them to the forming tissue. The cell-cell contact mediated by cadherin-11 *in vitro* may mimic, in part, the angiogenic process and suggests that Vegf-D expression might be involved in the initial steps of the angiogenic process.

The neovascularization and/or formation of lymphatic vessels in solid tumors is induced by tumor cells that express inductive angiogenic factors, which promote vessels sprouting with the recruitment of endothelial cells to the growing tumor mass (10, 41, 42). However, tumor vasculature is highly disorganized with vessels being dilated, tortuous, and fenestrated and having excessive branching and uneven diameters (43).

[2] M. Orlandini and S. Oliviero, unpublished observations.

This could be the result of the imbalance of angiogenic factors. The fine regulation of VEGF-D by cadherin signaling is probably compromised in tumor cells especially considering that cadherin expression is altered during the malignant progression of tumors (44–49). In this respect it is interesting that the expression of VEGF-D has been found to correlate inversely with tumor size and node positivity in lung adenocarcinoma (50).

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