Vascular endothelial growth factor-D Activates VEGFR-3
Expressed in Osteoblasts Inducing Their Differentiation*

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Vascular endothelial growth factor (VEGF)-D is a member of the VEGF family of angiogenic growth factors that recognizes and activates the vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3 on blood and/or lymphatic vessels. We show that in the long bones of newborn mice, VEGF-D and VEGF-3 are expressed in the osteoblasts of the growing plate. The treatment of primary human osteoblasts with recombinant VEGF-D induces the expression of osteocalcin and the formation of mineralized nodules in a dose-dependent manner. A monoclonal neutralizing antibody, anti-VEGF-D, or silencing of VEGF-3 by lentiviral-mediated expression of VEGF-D small hairpin RNA affects VEGF-D-dependent osteocalcin expression and nodule formation. Moreover, in primary human osteoblasts, VEGF-D expression is under the control of VEGF, and inhibition of VEGF-D/VEGFR-3 signaling, by monoclonal antibodies or VEGFR-3 silencing, affects VEGF-dependent osteoblast differentiation. These experiments establish that VEGF-D/VEGFR-3 signaling plays a critical role in osteoblast maturation and suggest that VEGF-D is a downstream effector of VEGF in osteogenesis.

During endochondral bone formation, mesenchymal cells differentiate into chondrocytes, which secrete a cartilage template. Chondrocytes in the centers of the cartilage templates become hypertrophic and produce vascular endothelial growth factor (VEGF) that stimulates vascular invasion of the cartilage template. Upon this process, the hypertrophic chondrocytes die through apoptosis and are replaced by osteoblasts brought in from the bone collar (1, 2). The interplay between chondrocytes and osteoblasts at the growth plate determines the longitudinal growth of long bones. Osteoblasts are responsible for matrix deposition and bone mineralization. Early during their differentiation, osteoblasts express RUNX2 (also known as CBFA1), which is held inactive by Twist proteins (3). Later, osteoblasts express the specific marker osteocalcin, which is required for bone mineralization (4).

Several growth factors expressed in the growth plate including epidermal growth factor, members of the transforming growth factor-beta family, Indian hedgehog, and VEGF are involved in cell proliferation and differentiation during bone formation (1, 2). Among these, VEGF has been shown to play a critical role. VEGF was first identified as an angiogenic factor that is expressed in different splicing forms. It binds and activates VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as KDR or Flk1) on endothelial cells, and their expression is required for vascular development and adult angiogenesis (6, 7). During skeletal development, VEGF has been shown to play a role in blood vessel invasion that is essential for coupling resorption of cartilage with mineralization of the extracellular matrix and bone formation (8–10) and synergizes with BMP2 to promote bone formation and bone healing via modulation of angiogenesis (11). VEGF is essential not only for normal angiogenesis but also to allow normal differentiation of hypertrophic chondrocytes, osteoblasts, and osteoclasts (5, 12–18). VEGF plays a role in bone repair. In mouse femur fractures, treatment with exogenous VEGF enhanced not only blood vessel formation but also ossification and new bone maturation, whereas VEGF inhibition decreased bone formation and callus mineralization (19). Although experiments in osteoblasts demonstrated an increase of cell migration in response to VEGF (14), no functional data of the activity of VEGFRs in osteoblasts has been reported.

During mouse development, the expression of another member of the VEGF family, VEGF-D, was detected in the periosteum/osteoblast layer of the developing vertebral column, the limb buds, and the dental mesenchyme close to the enamel epithelium (20). Because in mouse VEGF-D only recognizes murine VEGFR-3 (21), its pattern of expression suggests that VEGF-D/VEGFR-3 signaling plays a role in bone development. VEGF-D has been previously shown to be involved in vascular development, lymphatic maintenance, and tumor angiogenesis (22–28).

We investigated the involvement of the angiogenic growth factor VEGF-D and its receptor VEGFR-3 in osteoblasts. We show that osteoblasts of the long bones of newborn mice and primary human osteoblasts express VEGFR-3. Osteoblasts treated with recombinant VEGF-D respond with VEGFR-3 autophosphorylation, osteocalcin expression, and nodule formation. Moreover, VEGF treatment induces VEGF-D expression in these cells. Accordingly, the inactivation of VEGF-D activity by neutralizing antibodies or VEGFR-3 silencing inhibited both VEGF- and VEGF-D-dependent nodule formation in osteoblasts. Our data demonstrate the involvement of VEGF-D in maturation and regulation of osteoblastic activity via VEGFR-3.

EXPERIMENTAL PROCEDURES

Primary Human Osteoblast Cultures—Bone samples were obtained from 10 women and 4 men (aged 56–78 years, with a mean age of 66 years) who underwent total hip replacement surgery for degenera-
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TABLE 1

| Gene       | Sequence                                                                 |
|------------|--------------------------------------------------------------------------|
| VEGFR-2    | P 5′-ACCAACCCAGGAGGATATTTA-3′                                           |
| VEGFR-3    | R 5′-CTTTTCTCCGCTTCCCTGTAC-3′                                           |
| Osteocalcin| F 5′-GAGTTGAGATGTTGAGATGTTG-3′                                          |
| RUNX2      | G 5′-CTCAGAGAGAGAGAGAGAGAG-3′                                           |
| GAPDH      | R 5′-CTCAGAGAGAGAGAGAGAGAG-3′                                           |
| Lentiviral vector | F 5′-GAGTTGAGATGTTGAGATGTTG-3′                                          |
| VEGF-D     | R 5′-CAGAGAGAGAGAGAGAGAGAG-3′                                           |

Cells were grown on glass coverslips and, after treatment, fixed in 3% Tissue-Tek® OCT™ compound. 10-μm cryostat sections were cut and fixed in 3% paraformaldehyde for 20 min at room temperature. Human osteoblasts were grown on glass coverslips and, after treatment, fixed in 3% paraformaldehyde for 15 min at room temperature. For permeabilization, cryostat sections were incubated with 0.5% Triton X-100 in PBS for 5 min at 4°C. For the staining of cultured human osteoblasts, cells were not permeabilized. Specimens 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 the following primary antibodies: rabbit polyclonal anti-VEGF-D (30), goat polyclonal anti-PECAM-1 (Santa Cruz Biotechnology), rat monoclonal anti-VEGFR-3 (eBioscience), goat polyclonal anti-osteocalcin (Santa Cruz Biotechnology), and goat polyclonal anti-VEGFR-3 (R&D Systems), diluted in 1% bovine serum albumin/PBS. After washing, specimens were incubated for 1 h at 37°C with Alexa Fluor® 568 or Alexa Fluor® 488 secondary antibodies (Molecular Probes), and mounted in Mowiol 4-88 (Calbiochem). Fluorescent images were captured using a Leica TCS SP2 laser scanning confocal microscope.

Cloning, Purification, and Folding of Recombinant Human VEGF-D—To generate the His6-epitope-tagged human VEGF-D (amino acids 90–203, GenBankTM/EBI Data Bank accession number NM_004469), a cDNA clone containing the complete sequence of the VEGF-D gene (31) was PCR-amplified with a forward primer containing an Ndel restriction site and a reverse primer containing a Sall site (Table 1). The PCR fragment was then cloned into the Ndel Sall sites of the bacterial expression vector pET-22b (Novagen). The construct was checked by automated sequencing. VEGF-D-transformed BL21-DE3 Escherichia coli cells were grown for 3 h at 37°C after isopropyl-1-thio-β-D-galactopyranoside induction, pelleted, and solubilized in 6 M guanidium chloride. VEGF-D was purified by immobilized metal affinity chromatography under denaturing conditions (8 M urea) in the presence of 1 mM Tris-(2-carboxyethyl)phosphine-HCl using an AKTA purifier (Amersham Biosciences). To obtain a VEGF-D dimer, the monomer (0.25 mg/ml) was dialyzed against: (a) 6 M urea, 0.1 M Na2PO4, 10 mM Tris-HCl, 5 mM GSH, pH 8.5; (b) 50 mM Tris-HCl, 5 mM NaCl, 0.5 M l-arginine, 5 mM GSH, 1 mM GSSG, 2 mM urea, pH 8.5; (c) in the same buffer as b without l-urea; (d) 50 mM Tris-HCl, pH 8. Each dialysis was performed for 16 h. To eliminate aggregate forms, VEGF-D was loaded onto a His-TRAP affinity column in non-denaturing conditions and eluted with 250 mM imidazole. Imidazole was removed on a HitTrap desalting column, and the dimer formation was checked by gel filtration using a Superdex 75 HR column. All the columns were from Amersham Biosciences.

Monoclonal Antibody Production and Characterization—mAbs against human recombinant VEGF-D were generated using a standard fusion protocol (32). Hybridomas were screened by enzyme-linked immunosorbent assay. Antibody-secreting hybridomas were cloned and inoculated into pristane-primed BALB/c mice for production of ascitic fluid. The isotypes were determined using the mouse monoclonal antibody isotyping kit (Amersham Biosciences). Antibodies were purified by affinity chromatography and characterized by immunoprecipitation and cell proliferation assays.

Cell Proliferation and Viability Assays—Human umbilical vein endothelial cells were grown in M199 culture medium containing 20% fetal bovine serum and growth supplements. 5 × 104 cells were plated in a 96-well plate and starved for 24 h in Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum and 1% bovine serum albumin. Cells were treated with 25 ng/ml VEGF-D in the presence of different concentrations of an anti-VEGF-D mAb and 0.5 μCi/well [methyl-3H]thymidine (Amersham Biosciences). After 20 h, cell proliferation was measured as thymidine uptake by a β-counter. The assays for cell viability were performed by using thiazolyl blue tetrazolium bromide (Sigma) according to manufacturer’s instructions.

Immunoprecipitation and Immunoblotting Assays—For osteoblast immunoprecipitation analysis, cultures were starved in serum-free Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin for 24 h. Before stimulation, cells were incubated with 0.1 mM Na3VO4 for 10 min to inhibit phosphatase activity. Cells were stimulated for 30 min with 25 ng/ml VEGF-D at 37°C, washed with ice-cold PBS containing 0.1 mM Na3VO4, and lysed in 1 ml of lysis buffer (50 mM Heps, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM NaH2PO4, 100 mM NaF, 10 mM diethiothreitol, 1 mM Na3VO4 protease inhibitors, Sigma). Cell lysates were incubated on ice for 10 min and centrifuged at 10,000 × g for 15 min, and the supernatants were incubated with anti-VEGFR-3 antibodies. For immunoprecipitation analysis of VEGF family growth factors, HEK293 stable clones expressing human VEGF164, VEGF-C, and VEGF-D were generated.3 400 μl of supernatant from serum-starved cells were immunoprecipitated with 2 μg of mAb 3.11A25 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40. Immunoprecipitates were analyzed by 6–15% SDS-PAGE. Immunoblot analyses were performed as described previously (33). The following primary antibodies were used: mouse monoclonal anti-VEGFR-3, anti-phosphotyrosine, anti-VEGF, and anti-β-tubulin (Santa Cruz Biotechnology); anti-phospho-p44 MAPK and anti-p44/42 MAPK (Cell Signaling); anti-VEGF-C (R&D Systems); and anti-VEGF-D (mAb 197). The blots were washed, incubated with horseradish peroxidase-la-

3 M. Bardelli, unpublished data.
beled secondary antibodies, and developed by using the enhanced chemiluminescence substrate (Amersham Biosciences).

**VEGFR-3 RNA Interference**—Small hairpin (shRNA) cassette was cloned, and the recombinant lentiviruses were produced as described previously (34). Briefly, oligonucleotides coding for human VEGFR-3 and unrelated shRNA were designed to contain a sense strand 5'-H11032-GAGAC-AAGGACAGCGAGGACA-3' (VEGFR-3 D clone), 5'-GTACATCAAG-GCACGCATCGA-3' (VEGFR-3 F clone), and 5'-GCCACAAGTTC-AGCGTGTC-3' (unrelated) followed by a spacer (5'-TTCAAGAGA-3') and their reverse complementary strand followed by five thymidines as an RNA polymerase III transcriptional stop signal. The complementary oligonucleotides were phosphorylated, annealed, and cloned into the lentiviral vector. HEK293 cells were transiently transfected using PolyFect transfection reagent (Qiagen) according to the manufacturer’s instructions. The lentiviruses were harvested 24 and 48 h later and filtered through 0.22-μm pore cellulose acetate filters. Recombinant lentiviruses were concentrated by ultracentrifugation for 2 h at 50,000 × g. Vector infectivity was evaluated by infecting cells with a green fluorescent protein vector and titrating shRNA-expressing virus by real-time quantitative RT-PCR of a common lentiviral genome region when compared with the green fluorescent protein vector.

**Real-time Quantitative RT-PCR Analysis**—Total RNA was isolated from cells by the guanidinium thiocyanate method, quantified, and integrity was tested by gel electrophoresis. The gene expression analysis was performed using a LightCycler apparatus, and data were analyzed with the LightCycler software version 3.5 (Roche Applied Science). The RT-PCR reactions were set up in microcapillary tubes using the LightCycler RNA amplification kit SYBR Green I (Roche Applied Science) following the manufacturer’s instructions. For each sample, triplicate determinations were made, and the gene expression was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase on the same sample. The primer pairs used are reported in Table 1.

**RESULTS**

**Comparison of VEGF-D and VEGFR-3 Expression in Newborn Mice**—Immunohistochemical staining for murine VEGF-D performed on neonatal radius showed the expression of VEGF-D corresponding to osteoblasts adjacent to hypertrophic chondrocytes (Fig. 1C). To verify whether VEGF-D expression was compatible with a role in bone formation, we analyzed the expression of its receptor VEGFR-3, as in mouse VEGF-D only recognizes VEGFR-3 (21). VEGFR-3 showed expression in the growth plate at the interface between the forming bone and the terminal hypertrophic chondrocytes (Fig. 1). This is a bone-developing region characterized by new blood vessel formation and differentiating osteoblasts. VEGFR-3 is transiently expressed in endothelial cells during active angiogenesis (22, 27, 35). Consistent with this, we observed low levels of VEGFR-3 expression in endothelial cells in the radius of new born mice, reflecting the fact that a peak of angiogenesis in the growing bone takes place before birth. Double staining between VEGFR-3 and PECAM1 (CD31), a marker of endothelial cells, revealed...
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FIGURE 2. VEGF-R3 is expressed in primary human osteoblasts and is activated in response to stimulation with recombinant VEGF-D. A and B, human osteoblasts were grown in complete medium and analyzed by immunofluorescence using anti-VEGFR-3 and anti-osteocalcin antibodies. C, transmitted light interference contrast of the images shown in A and B. Scale bar, 25 μm. D, cell extracts from serum-starved osteoblasts treated with VEGF-D and immunoprecipitated with anti-VEGFR-3 antibodies. The immunoprecipitation was analyzed by Western blotting with anti-phospho-Tyr (P-Tyr) antibodies and with anti-VEGFR-3 antibodies to confirm equal loading. NT, not treated. E, cell extracts from serum-starved osteoblasts treated with VEGF-D were analyzed by Western blotting using anti-phospho-ERK1/2 (P-ERK) and anti-ERK1/2 antibodies to confirm equal loading.

a partial overlapping of these signals, demonstrating that VEGF-3 is still expressed in endothelial cells at this stage (Fig. 1, D–F). A more consistent VEGF-3 signal was observed in osteocalcin positive cells (Fig. 1, G–L). These data demonstrate that VEGF-3 is expressed in mouse osteoblasts.

VEGF-D Activates VEGFR-3 on Primary Human Osteoblasts—To address whether VEGF-D/VEGFR-3 signaling plays a functional role in osteoblasts, we obtained from trabecular bone explants primary human osteoblasts and analyzed VEGF-D response on these cells. We detected VEGF-3 expression in primary human osteoblasts by immunofluorescence analysis and by Western blot (Fig. 2, A–D), whereas we could not detect VEGF-2 in these cells or VEGF-3 in fibroblasts (data not shown). To analyze VEGF-3 autophosphorylation, human osteoblasts were treated with recombinant VEGF-D, and whole cell lysates were immunoprecipitated with an antibody recognizing VEGF-3 C terminus and immunoblotted with anti-phosphotyrosine antibodies. This analysis revealed that VEGF-D treatment induced VEGF-3 autophosphorylation in osteoblasts (Fig. 2D). VEGF-3 activation was leading to an intracellular signaling cascade as we observed that VEGF-D treatment was followed by an increase of the phosphorylation of the intracellular protein ERK1/2 (Fig. 2E). These experiments demonstrate that VEGF-D recognizes and activates VEGF-3 expressed in primary human osteoblasts.

VEGF-D Induces Nodule Formation and Osteocalcin Expression—To investigate whether VEGF-D affects osteoblast differentiation, primary human osteoblasts were grown in complete medium for 14 days in the presence of different concentrations of VEGF-D. As shown in Fig. 3, VEGF-D significantly increased the number of mineralized nodules in a dose-dependent manner (Fig. 3A). In addition, the nodules grew bigger and better mineralized than those of the control cultures that showed less and poorly mineralized nodules (Fig. 3B). To verify whether the neutralization of VEGF-D activity affects osteoblast differentiation, we used the mAb 3.11A25 (isotype IgG2a) able to selectively immunoprecipitate VEGF-D (Fig. 3C) and to inhibit VEGF-D-dependent proliferation of endothelial cells (Fig. 3D). The treatment of osteoblast cultures with mAb 3.11A25 decreased nodule formation to values of untreated cells, whereas a non-correlated antibody did not influence nodule formation even at high concentrations (Fig. 3E). Importantly, the treatment of osteoblasts for 14 days with high concentrations of mAb 3.11A25 did not alter osteoblast viability (Fig. 3F). Taken together, these data demonstrated that VEGF-D induces osteoblasts to form mineralization nodules. Quantitative real-time RT-PCR analysis of the expression of RUNX2, a marker of early differentiation of osteoblast, and osteocalcin, a marker of late osteoblast differentiation, showed that VEGF-D treatment did not influence RUNX2 expression, whereas osteocalcin mRNA was significantly increased in a dose-dependent manner (Fig. 4A). Moreover, osteocalcin induction was inhibited by the anti-VEGF-D-neutralizing antibody (Fig. 4B). Immunofluorescence analysis also showed that VEGF-D treatment increased osteocalcin secretion with respect to untreated cells (Fig. 4, C–F), demonstrating that VEGF-D plays a role in osteoblast differentiation.

Inhibition of VEGF-R3 Signaling Reduces Nodule Formation—To investigate whether VEGF-D-dependent osteoblast differentiation acts via VEGFR-3 signaling, we generated two lentiviral vectors (clones D and F) expressing shRNA designed to inhibit VEGF-R3. Primary human osteoblasts infected with either lentivirus expressing VEGF-R3 shRNA, but not an unrelated shRNA, showed a reduced VEGF-D- and VEGF-D-dependent ERK1/2 activation (Fig. 5A). Osteoblast knockdown for VEGF-R3 showed significant impairment of nodule formation and osteocalcin production following VEGF-D treatment (Fig. 5, B and C).

As VEGF plays a direct role in osteoblast migration, probably via the activation of VEGFR-1 (14), to provide a functional link between VEGF- and VEGF-D-dependent osteoblast differentiation, we analyzed whether VEGF-D expression in osteoblasts depends on VEGF signaling. Quantitative real-time RT-PCR analysis revealed that following VEGF treatment, osteoblasts responded with an increased expression of VEGF-D, demonstrating that in osteoblasts, VEGF-D expression is under the control of VEGF (Fig. 6A). To analyze whether VEGF-D plays a role in VEGF-dependent osteoblast differentiation, we treated osteoblasts with VEGF in the presence of the monoclonal antibody inhibiting VEGF-D activity and measured the
nodule formation induced by VEGF. VEGF treatment of primary human osteoblasts induced a significant number of mineralization nodules. The pretreatment of these cultures with VEGF-D-neutralizing antibodies efficiently inhibited VEGF-dependent nodule formation (Fig. 6B). This inhibition specifically affected the VEGFR-3 signaling as we also observed inhibition of VEGF-dependent nodules formation in cells silenced for VEGFR-3 (Fig. 6, C and D). Together, these results demonstrate that VEGF induces nodule formation in osteoblasts via the activation of the VEGF-D/VEGFR-3 signaling.

DISCUSSION

This study reveals that VEGF-D/VEGFR-3 signaling induces primary human osteoblast differentiation. Examination of long bones of newborn mice showed that VEGF-D is expressed together with VEGFR-3 in the osteoblasts of the growth plate, suggesting that VEGF-D/VEGFR-3 signaling might contribute to the bone formation in vivo by an autocrine activation of osteoblasts during their maturation. Accordingly, we found that VEGFR-3 is expressed in primary human osteoblasts and VEGF-D stimulates their differentiation measured as osteocalcin induction and mineralized nodule formation. Furthermore, we demonstrated, by VEGFR-3 silencing, that VEGF-D signaling plays a functional role in VEGF-D-dependent osteoblast maturation. Because in mouse VEGFR-3 is expressed in osteoblasts and VEGF-D binds only this receptor (21), these results suggest that VEGF-D/VEGFR-3 signaling in bone formation is a common function between mouse and human.

VEGF-D induces angiogenesis, lymphangiogenesis, as well as metastatic spread of tumors via lymphatic vessels activating VEGF-3 on vascular and lymphatic endothelial cells (30, 36, 37). Besides playing a role in lymphatic vessel homeostasis, VEGFR-3 is implicated in the remodeling of the primary vascular network, and in reorganizing the integrity of endothelial vessels during angiogenesis (22, 25–27). On endothelial cells, VEGFR-3 signaling activates proliferation, migration, and survival (38). The data presented in this report demonstrate that, in addition to playing a biological function in endothelial cells, VEGF-D/VEGFR-3 signaling is also implicated in osteoblast differentiation. Expression analysis on primary human osteoblasts demonstrated that VEGF-D induces the expression of osteocalcin, a late marker of differentiation, whereas it has no effect on the early marker RUNX2. RT-PCR analysis in these cells revealed that the inhibitors of RUNX2, Twist 1 and Twist 2, expressed early during osteoblast differentiation (3), are already down-regulated in osteoblasts before VEGF-D treatment. These data therefore suggest that VEGF-D/VEGFR-3 signaling is not involved in the activation of RUNX2 function but must be involved in the activation of other yet unknown regulator(s) of osteoblast differentiation that act at a later stage of osteoblast maturation. This is also confirmed by the fact that VEGF-D induced mineralization in these cells.

Hypertrophic chondrocytes secrete a number of growth factors including VEGF, which orchestrate blood vessel formation, chondrocyte matura-

4 M. Orlandini, unpublished observation.
tion, the differentiation of osteoblasts forming the mineralized bone collar, as well as the recruitment of osteoclasts into hypertrophic cartilage (Ref. 16 and references therein). In line with these experiments, our results are consistent with a model in which VEGF acts early on bone differentiation by inducing vessel formation, osteoblast recruitment to the growth plate, and also stimulating VEGF-D production in osteoblasts. VEGF-D in turn acts as a downstream effector of VEGF with autocrine activity on osteoblasts. Therefore these two factors contribute to the process of timely coordinated osteoblast differentiation. These results also imply that VEGF, probably acting on VEGFR-1, stimulates a different cellular response than VEGF-D acting on VEGFR-3. A similar conclusion was previously reached by the analysis of sinusoidal endothelial cells in which VEGFR-1 induced these

FIGURE 4. VEGF-D treatment stimulates osteocalcin production, a gene related to osteoblast differentiation. A, analysis of the osteocalcin and RUNX2 mRNA levels by quantitative real-time RT-PCR. RNA was extracted from primary human osteoblasts treated for 14 days with increasing amounts of VEGF-D. B, analysis of the osteocalcin transcripts by quantitative real-time RT-PCR. RNA was collected from osteoblasts treated for 14 days with 25 ng/ml VEGF-D and different amounts of the anti-VEGF-D mAb 3.11A25. NC, non-correlated purified antibodies (40 µg/ml). C and D, human osteoblasts untreated (control) or treated for 12 days with 25 ng/ml VEGF-D and analyzed by immunofluorescence staining using anti-osteocalcin antibodies. E and F, transmitted light interference contrast of the images shown in C and D. Scale bars, 16 µm. Data represent the ± S.E. of three independent experiments each in triplicate.

FIGURE 5. VEGFR-3 silencing reduces nodule formation and osteocalcin expression in primary human osteoblasts. A, Western blot analysis of cell extracts from cells infected with a lentiviral vector expressing unrelated (unr) or VEGFR-3 shRNA clone D (D) and clone F (F) grown for 14 days in complete medium. The membranes were probed with anti-VEGFR-3, anti-phospho-ERK1/2 (p-ERK), anti-β-tubulin, or anti-ERK1/2 antibodies as indicated. B, nodule formation in control (unr) and VEGFR-3 silenced osteoblast cultures treated for 14 days with 25 ng/ml VEGF-D. C, analysis of the osteocalcin transcripts by quantitative real-time RT-PCR, using RNA collected from osteoblasts treated as in B. Data represent the ± S.E. of three independent experiments each in triplicate.
cells to produce the hepatocyte growth factor, whereas VEGFR-2 inhibited their proliferation (39). Further analysis of the downstream signaling from these two receptors might enlighten the physiological differences between these two receptors in osteoblasts.

Our data suggest that VEGF-D plays a role in the development of long bones. During mouse development, VEGF-D expression was also detected in the periosteum layer of the developing vertebral column and in the dental mesenchyme close to the enamel epithelium, where dentin and enamel matrices are deposited (20), suggesting that VEGF-D might also play a role in intramembranous bone formation.

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