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Expression of Vascular Endothelial Growth Factors and Their Receptors during Osteoblast Differentiation

MARTINE M. L. DECKERS, MARCEL KARPERIEN, CHRIS VAN DER BENT, TAKEYOSHI YAMASHITA, SOCRATES E. PAPAPOULOS, AND CLEMENS W. G. M. LÖWIK

Departments of Endocrinology and Metabolic Diseases (M.M.L.D., M.K., C.v.d.B., S.E.P., C.W.G.M.L.) and Pediatrics (M.K.), Leiden University Medical Center, 2333ZA Leiden, The Netherlands; and Pharmaceutical Research Laboratory, Kirin Brewery Co. Ltd. (T.Y.), 3 Miyahara, Takasaki, Gunma 370-12, Japan

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ABSTRACT

Endochondral bone formation is regulated by systemically and locally acting growth factors. A role for vascular endothelial growth factor (VEGF) in this process has recently been proposed, because inactivation of VEGF inhibits endochondral bone formation via inhibition of angiogenesis. Despite the known effect of VEGF as specific endothelial growth factor, its effects on osteoblast differentiation have not been studied. We, therefore, examined the expression of VEGF-A, -B, -C, and -D and their receptors in a model of osteoblast differentiation using the mouse preosteoblast-like cell line KS483. Early in differentiation, KS483 cells express low levels VEGF-A, -B, and -D messenger RNA, whereas during mineralization, KS483 cells express high levels.

In addition, expression of the VEGF receptors, VEGFR1, VEGFR2, and VEGF165R/neuropilin, coincided with expression of their ligands, being maximally expressed during mineralization. VEGF-A production during osteoblast differentiation was stimulated by insulin-like growth factor I that enhances osteoblast differentiation and was inhibited by PTH-related peptide that inhibits osteoblast differentiation. Furthermore, continuous treatment of KS483 cells with recombinant human VEGF-A stimulated nodule formation.

Although treatment of KS483 cells with soluble FLT1, an agent that blocks binding of VEGF-A and -B to VEGFR1, did not inhibit nodule formation, this observation does not exclude involvement of VEGFR2 in the regulation of osteoblast differentiation. As it is known that VEGF-A, -C, and -D can act through activation of VEGFR2, other isoforms might compensate for VEGF-A loss. The expression pattern of VEGFs and their receptors shown here suggests that VEGFs play an important role in the regulation of bone remodeling by attracting endothelial cells and osteoclasts and by stimulating osteoblast differentiation. (*Endocrinology* **141:** 1667–1674, 2000)

IN BONE, COMMUNICATION between osteoblasts and osteoclasts is well recognized (1, 2). Besides osteoclasts, osteoblasts are located in proximity to endothelial cells. Vascular invasion is a prerequisite for endochondral bone formation and fracture healing (3). Factors produced by endothelial cells may therefore affect osteoblast function or differentiation and *vice versa* (4–7). Osteoblasts are also able to produce paracrine factors that influence endothelial cell function (8, 9). In this cross-talk between endothelial cells and osteoblasts, vascular endothelial growth factor (VEGF), which acts as an angiogenic factor, may play an important role (10). A recent study by Gerber *et al.* showed that blocking the action of endogenous VEGF inhibited both bone formation and resorption in juvenile mice (11).

VEGF is a glycoprotein that shares homology to platelet-derived growth factor (12). It acts as both a permeability and an angiogenic factor (13–15). The VEGF gene can be alternatively spliced, resulting in three distinct gene products corresponding to 120, 164, or 188 amino acids in the mouse, or five distinct gene products, respectively corresponding to 121, 145, 165, 189, and 206 amino acids in humans. Each splice variant has specific characteristics (16, 17). In fetal tissues VEGF messenger RNA (mRNA) expression has been described in various organs, including the skeleton (18, 19).

Recently, other angiogenic factors have been identified that have sequence homology to VEGF, which is now termed VEGF-A. The biological effects of these VEGF family members, named VEGF-B, -C, and -D or c-*fos*-induced growth factor (FIGF) and placenta growth factor-1 (PlGF-1), -2, and -3 are still not completely elucidated. All members of the VEGF family have in common the ability to stimulate endothelial cell proliferation (20). Similar to VEGF-A, VEGF-B can be alternatively spliced into two different splice variants (VEGF-B_{167} and VEGF-B_{186}). VEGF-B is thought to play a role in vascularization of skeletal muscle (21), whereas VEGF-C is most likely involved in lymphangiogenesis (20). VEGF-B and VEGF-C are, like VEGF-A, associated with tumor angiogenesis (22). Recently, expression of VEGF-B and VEGF-D mRNA has been shown during tooth and bone development (23, 24). Expression of PlGF is restricted to the placenta (25).

Homo- or heterodimers of the VEGF family act as paracrine factors through signaling via various type III tyrosine kinase receptors (26): 1) VEGF receptor 1 or *fms*-like tyrosine kinase receptor 1 (FLT-1/VEGFR1), which binds VEGF-A, VEGF-B, and PlGF; 2) VEGFR2 or fetal liver kinase 1 (FLK-1), which binds VEGF-A, -C, and -D with high affinity, but not PlGF (27, 28); 3) VEGFR3 or FLT4, which binds VEGF-C and -D (29, 30). Recently, neuropilin has been identified as a new isoform-specific VEGF receptor ($VEGF_{165}R$). Neuropilin acts as a coreceptor of VEGFR2 and binds PlGF-2 and the isoform

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Address all correspondence and requests for reprints to: Dr. Martine M. L. Deckers, Department of Endocrinology and Metabolic Diseases, Albinusdreef 2, Building 1 C4R-89, 2333 ZA Leiden, The Netherlands. E-mail: m.m.l.deckers@lumc.nl.

$VEGFA_{165}$, but not $VEGFA_{121}$ (31–33). Whether neuropilin is expressed in osteoblasts is at present unknown.

VEGF was reported to induce alkaline phosphatase activity in primary osteoblasts and to enhance their responsiveness to PTH. Furthermore, high affinity sites for VEGF-A have been identified on primary osteoblasts, indicating the presence of functional VEGFRs on osteoblasts (34). This suggests that besides its established effects on endothelial cells, VEGF might play a role in osteoblast differentiation. In addition, osteotropic factors, such as vitamin D3, insulin-like growth factor I (IGF-I), and PGs, have been shown to increase VEGF-A mRNA expression and VEGF secretion by osteoblast-like cells (35–38). In this study we examined expression of VEGFs and their receptors in preosteoblast-like cells as well as the potential role of VEGFs during differentiation of KS483 cells, a nontransformed preosteoblast-like cell line.

Materials and Methods

Mineralization experiments

The KS483 cell line is a nontransformed stable subclone of the parental cell line KS4. This preosteoblast-like cell line is derived from fetal mouse calvaria as described earlier and has the ability to form mineralized nodules *in vitro* (39, 40). For differentiation studies KS483 cells were seeded in 6-well plates at a density of 15,000 cells/cm^2 in αMEM (Life Technologies, Inc., Breda, The Netherlands) supplemented with 10% FCS (Integro B.V., Zaandam, The Netherlands) and penicillin/streptomycin (Life Technologies, Inc.). Three days after plating the cells reached confluence and were subsequently cultured in presence of 50 μg/ml ascorbic acid and in the presence or absence of growth factors [PTH-related peptide (PTHrP)-(1–34); Bachem, Heidelberg, Germany], recombinant human (rh) $VEGF_{165}$, rhIGF-I (Santa Cruz Biotechnology, Inc., Breda, The Netherlands) or goat antimouse VEGF neutralizing antibody (R&D Systems, Uithoorn, The Netherlands). Truncated VEGFR1 or soluble fms-like tyrosine kinase receptor 1 (sFLT1) was provided by Dr. H. Weich (National Center for Biotechnology, Braunschweig, Germany). The medium was replaced every 3–4 days. When nodules appeared (between 14–18 days after plating), medium was replaced by medium containing 5 mM β-glycerophosphate and ascorbic acid to further stimulate osteogenic differentiation. At different time points, supernatants and cell layers were collected separately. VEGF-A production and alkaline phosphatase activity were determined in the culture supernatants, whereas mRNA, DNA, and protein expression were determined in the cell layer.

Nodule formation

After 18 days of culture the number of nodules was counted in control cultures and in VEGF-A-, IGF-I-, PTHrP-, or sFLT1-treated cultures by light microscopy at a 40-fold magnification. Cultures were performed in quadruplicate, and three fields per well were counted.

DNA assay

At the end of each culture period the supernatants were aspirated, and the cells were washed twice with PBS. DNA was released from the cells by freeze-thawing in 1 × SSC (standard saline citrate) buffer containing 0.01% SDS. DNA content was determined using Hoechst stain 2495 (ICN Biomedicals, Inc., Zoetermeer, The Netherlands) and calibrated against a DNA standard of 0.5–10 μg/ml DNA. Samples were excited with light (460 nm), after which fluorescent emission was measured at 365 nm.

VEGF-A enzyme-linked immunosorbent assay (ELISA)

VEGF-A levels in culture supernatants were assayed with a quantitative sandwich ELISA (R&D Systems) according to the manufacturer's instructions. Values were assayed in triplicate and corrected for DNA content. The sensitivity of the assay is 3 pg/ml, and it detects both $VEGF_{120}$ and $VEGF_{164}$. Cross-reactivity with human $VEGF_{165}$, murine platelet-derived growth factor, or murine PlGF was less than 0.05%. Cross-reactivity with other members of the VEGF family is not known.

RNA isolation

The cells were lysed in a solution of 4 M guandinium thiocyanate containing 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol, and total RNA was extracted by the addition of phenol and chloroform followed by precipitation with isopropanol as previously described (41). The concentration of the isolated RNA was determined by UV absorption spectrophotometry at a wavelength of 260 nm. RNA quality was checked on an agarose gel containing 0.5 μg ethidium bromide per ml.

Competitive RT-PCR

Total RNA (1 μg) was reversed transcribed into complementary DNA (cDNA) as previously described (42). A RT-PCR mixture was prepared that contained first strand buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$), 5 mM dithiothreitol, 200 ng random hexanucleotides, 2.5 U murine Moloney leukemia virus reverse transcriptase, 0.375 mM deoxy-NTPs (Life Technologies, Inc.), and 1 U RNAsin (Promega Corp., Leiden, The Netherlands). To correct for differences in cDNA levels, 4-fold serial dilutions of an internal standard plasmid pMUS that encode for the housekeeping gene β_2-microglobulin (β_2-MG) were coamplified with 5 ng sample cDNA by competitive RT-PCR. The synthetic internal standard pMUS was provided by Dr. D. Shire (Sanofi Pharmaceuticals, Inc., Labege, France). This method has been described in detail by Van Bezooijen *et al.* (42). The PCR reaction was carried out in a Hybaid Omnigene thermal cycler (Biozym, Landgraaf, The Netherlands). cDNA was denatured at 94 C for 3 min, followed by repeated cycles consisting of 94 C for 30 sec, 56 C for 30 sec, and 72 C for 1 min. For quantification of β_2-MG levels, cDNA was amplified over 33 cycles. Gels were photographed under UV light and analyzed by computerized densitometry. Samples were regarded to contain equal amounts of cDNA at a ratio of cDNA to construct of 1, as described previously (42) (Fig. 1).

RT-PCR

Primers pairs for studying expression of PTH/PTHrP receptor; bone sialoprotein (BSP); osteocalcin (OC); and VEGF-A, -B, -C, and -D and its receptors, FLT1/VEGFR1, FLK1/VEGFR2, FLT4/VEGFR3, and neuropilin, are described in Table 1. Primers for VEGFR1 and VEGFR2 mRNA were used as reported by Fong *et al.* (43), whereas other primers were designed using Primer III Design software (http//www.genome.wi.mit.edu) and checked against GenBank to avoid cross-reactivity with other known sequences. A PCR mixture was prepared using 20 ng cDNA/reaction, PCR buffer [10 mM Tris-HCl (pH 9.6), 50 mM NaCl, and 0.2 mg BSA/ml; Eurogentec, Seraing, Belgium], 0.25 μM oligonucleotide primers (Eurogentec), 1.5 mM $MgCl_2$, 0.2 mM deoxy-NTP (Promega Corp.), and 0.5 U Goldstar DNA polymerase/μl (Eurogentec). The PCR reactions were carried out in a Hybaid Omnigene Thermal cycler (Biozym, Landgraaf, The Netherlands). cDNA was denatured at 94 C for 3 min, followed by repeated cycles to amplify the cDNA consisting of 94 C for 30 sec, 58 or 60 C for 30 sec, and 72 C for 1 min. PTH/PTHrP receptor and VEGF-B were amplified over 30 cycles. BSP, osteocalcin, VEGF-A, VEGF-B, VEGF-D, FLT1, and VEGFR2/FLK1 cDNA were amplified over 35 cycles, VEGFR165/neuropilin over 30 and 35 cycles, and VEGF-C and FLT4 over 38 cycles. Samples and a 100-bp ladder were electrophoresed on a 1.5% agarose gel containing 0.5 μg ethidium bromide/ml. PCR reactions were repeated at least three times. Relative mRNA levels of three separate experiments were determined by densitometry. Bands with the highest intensity were regarded as 100%.

Western blotting

KS483 cells were lysed in 50 mM Tris buffer (pH 7.5) containing 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 20 μg/ml Trasylol, and protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany) on days 3, 7, 10, 14, and 18 after plating. Production of VEGFR2 protein was assessed by Western blotting. Protein content was determined by bicinchoninic acid (Pierce Chemical Co., Breda, The Netherlands) using serum albumin as a standard according to the manufacturer's protocol. Cell lysates (15 μg/lane) were subsequently electrophoresed on a 7.5% polyacrylamide gel and

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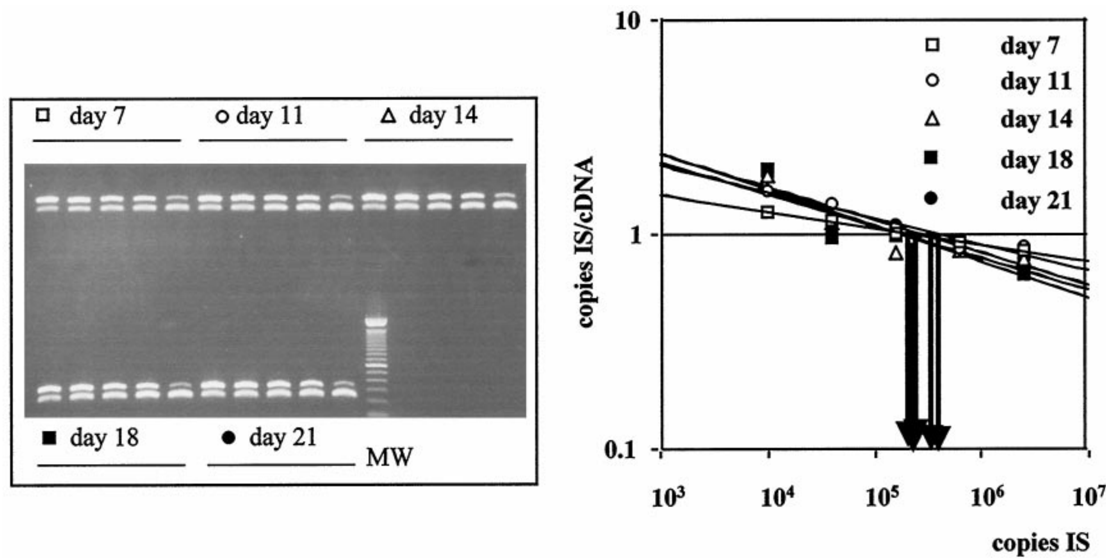

FIG. 1. Quantification of household gene mRNA expression KS483 cells were seeded in 6-well plates at a density of 15,000 cells/cm^2. At confluence, 50 μg/ml ascorbic acid were added. β-Glycerophosphate was added when nodules appeared (day 18 onward). Seven, 10, 14, 18, and 21 days after plating, RNA was isolated and reversed transcribed into cDNA. A, Four-fold serial dilutions of an internal standard plasmid pMUS that encodes for the housekeeping gene β_2-MG were coamplified with 5 ng sample cDNA. After 33 cycles of PCR, mRNA levels were quantified by computerized image analysis. B, Quantification of ratios between construct and sample cDNA from a representative experiment. MW, One hundred-base pair ladder.

TABLE 1. Primers: oligonucleotides used in RT-PCR

Primer			Ref. no.
β_2-Microglobulin	Sense	TGACCGGCTTGTATGCTATC	44
β_2-Microglobulin	Antisense	CAGTGTGAGCCAGGATATAG	
Osteocalcin	Sense	GCAGCTTGGTGCACACCTAG	
Osteocalcin	Antisense	GGAGCTGCTGTGACATCCAT	
VEGF-A	Sense	CTGTGCAGGCTGCTGTAACG	45
VEGF-A	Antisense	GTTCCCGAAACCCTGAGGAG	
VEGF-B	Sense	GATCCAGTACCCGAGCAGTC	21
VEGF-B	Antisense	GCACCTACAGGTGTCTGGGT	
VEGF-C	Sense	CAAGGCTTTTGAAGGCAAAG	29
VEGF-C	Antisense	TGCTGAGGTAACCTGTGCTG	
VEGF-D	Sense	CTCCAGGAACCCACTCTCTG	46
VEGF-D	Antisense	TCCTGGCTGTAGAGTCCCTG	
VEGFR1	Sense	CGCGGTCTTGCCTTACGCGCT	43
VEGFR1	Antisense	CCATTTATGGGCTGCTTCCCCCCTGCA	
VEGFR2	Sense	AGAACACCAAAAGAGAGAGGAACG	43
VEGFR2	Antisense	GCACACAGGCAGAAACCAGTAG	
VEGFR3	Sense	CGAGACTGGAAGGAGGTGAC	47
VEGFR3	Antisense	GTACGTGTAGTTGTCCGCCC	
Neuropilin	Sense	GACTTCCAGCTCACAGGAGG	32
Neuropilin	Antisense	AGAGCCGGACATGTGATACC	

blotted on a Hybond-P membrane (Amersham Pharmacia Biotech, Den Bosch, The Netherlands). Nonspecific binding sites were blocked in 0.1 M Tris-buffered saline (pH 7.5)/0.1% Tween (TTBS) containing 3% BSA for 1 h, followed by incubation with polyclonal rabbit antimouse VEGFR2 antibody (C-20, Santa Cruz Biotechnology, Inc.) diluted in TTBS/0.05% BSA overnight at 4 C. After repeated washing with TTBS, the blots were incubated with peroxidase-conjugated sheep antirabbit secondary antibody (Amersham Pharmacia Biotech) diluted in TTBS/0.05% BSA for 1 h at room temperature. The signal was visualized using the chemiluminescence detection system ECL (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Statistics

Results are depicted as the mean ± SEM. Differences between groups were determined by one-way ANOVA for multiple comparison, followed by Fisher's least significant difference test.

Results

Expression of VEGFs during differentiation of KS cells

In the presence of ascorbic acid and β-glycerophosphate, mouse preosteoblast-like KS483 cells differentiate into mature osteoblasts, as indicated by the formation of alkaline phosphatase positive nodules between days 14 and 18 after plating and their subsequent mineralization. The characterization of this cell line has been described in detail previously (39, 40). Expressions of PTH/PTHrP receptor, BSP, and OC mRNA were studied in cultures of KS483 cells by semiquantitative PCR. Differences in cDNA levels among the samples were first corrected by competitive quantitative RT-PCR as previously described (42). Samples were regarded to contain equal amounts of cDNA at a ratio of construct to cDNA of 1. The quantification

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of a representative experiment is depicted in Fig. 1. As shown in Fig. 2A, PTH/PTHrP was expressed at low levels 7 days after plating, and this expression increased during further differentiation. The differentiation markers BSP and OC were expressed at low levels when nodules appeared (14 days after plating) and increased during further differentiation (Fig. 2, B and C). In the same experiment mRNA expressions of VEGF-A isoforms were compared, with the expression of osteoblast differentiation markers. For this, primers were designed that can hybridize to three different splice variants of the mouse VEGF-A gene (VEGF-A_{120}, VEGF-A_{164}, and VEGF-A_{188}), resulting in the amplification of VEGF-A_{120} (230 bp) and VEGF-A_{164} (360 bp) mRNA levels. In parallel with PTH/PTHrP receptor mRNA expression, VEGF-A_{120} and VEGF-A_{164} mRNA were expressed at low levels early in differentiation (7 days after plating) and were strongly increased during mineralization, when BSP and OC were abundantly expressed (Fig. 3A). The matrix-bound isoform VEGF-A_{188}, was not expressed by KS483 cells, but was expressed by fetal liver, which was used as a positive control (data not shown).

VEGF-B, -C, and -D mRNA expression during differentiation of KS483 cells

The expression of other members of the VEGF family (VEGF-B, -C, and -D) during osteoblast differentiation was examined by RT-PCR. Two separate bands of VEGF-B mRNA expression were detected, namely VEGF-B_{167} and VEGF-B_{186} (Fig. 3B). In line with VEGF-A mRNA expression, VEGF-B mRNA expression was expressed at low levels 7 days after plating; then its expression increased, and it was maximally expressed 21 days after plating (Fig. 3B). VEGF-D mRNA expression paralleled VEGF-B mRNA expression; it was expressed at low levels early in differentiation and highly expressed during mineralization (Fig. 3D). In contrast, expression of VEGF-C was only detected at very low levels after 21 days of culture. This was not due to suboptimal PCR conditions, because in fetal lung, which was used as a positive control, VEGF-C was abundantly expressed (Fig. 3C).

Expression of VEGF receptors during differentiation of KS483 cells

VEGFR1, VEGFR2, and VEGF165R/neuropilin, but not VEGFR3, were expressed in a differentiation-dependent manner (Fig. 3, E–H). Low levels of VEGFR1 were only detected in differentiating osteoblasts during the mineralization phase (18 and 21 days after plating). VEGFR2 mRNA expression was low from days 7–14 and was strongly increased at the start of mineralization (Fig. 3F). $VEGF_{165}R$/neuropilin mRNA was maximally expressed during the mineralization phase (Fig. 3G). The gradual increase in $VEGF_{165}R$/neuropilin mRNA expression was confirmed using 30 cycles of amplification (data not shown). VEGFR3 mRNA expression was not detected in these cultures even after 38 cycles of amplification, although in fetal lung, which was used as a positive control, VEGFR3 mRNA expression was found (Fig. 3H).

VEGF-A production and VEGFR2 protein expression during differentiation of KS cells

Concurrently with the rise in mRNA expression, VEGF-A protein, measured by ELISA, accumulated in the culture medium (Fig. 4A). The levels significantly rose from 245 pg/ml in confluent cultures (3 days after plating) to 750 pg/ml during the mineralization phase (21 days after plating; Fig. 4A). It is important to note that the unconditioned culture medium (αMEM containing 10% FCS) did not contain detectable levels of VEGF-A protein (<3.0 pg/ml). In contrast to VEGF-A, there are no ELISAs commercially available to measure VEGF-B and D levels.

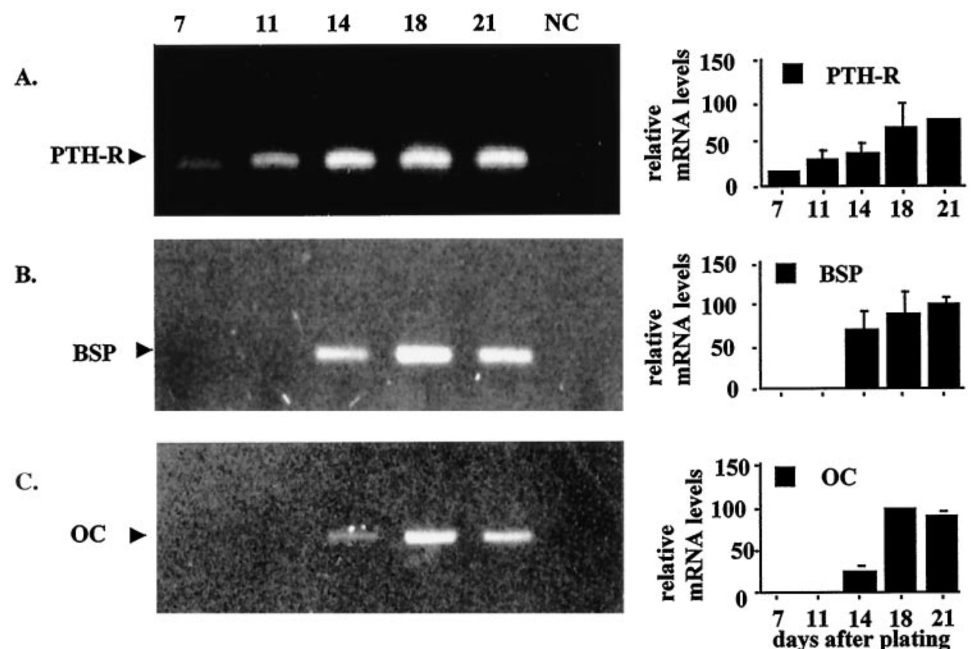

FIG. 2. Expression of PTH/PTHrP receptor, BSP, and OC during differentiation of KS483 cells. KS483 cells were cultured as described in Fig. 1. After standardization for household gene expression, equal amounts of cDNA from each sample were subjected to 30 cycles of PCR to amplify PTH/PTHrP receptor mRNA expression (A), BSP mRNA expression (B), and OC mRNA expression (C). Lane 1, Day 7; lane 2, day 11; lane 3, day 14; lane 4, day 18; lane 5, day 21 after plating; lane 6, water control. The sizes of the products are indicated by *arrows*. NC, Negative control. Representative pictures from three experiments are depicted in the *left panel*, and quantifications of three independent experiments are depicted in the *right panel* as relative mRNA levels ± SEM

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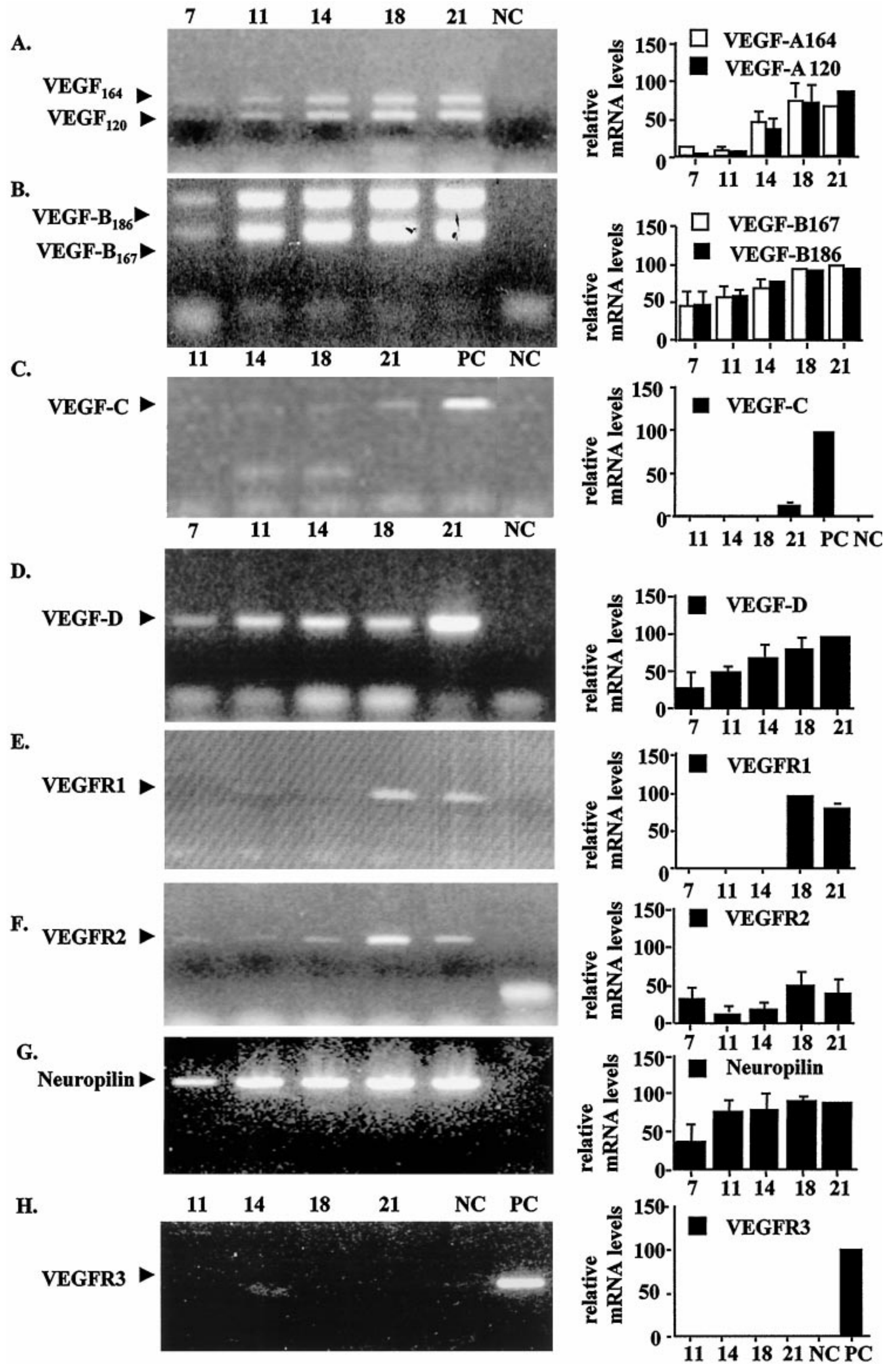

FIG. 3. VEGF and VEGFR mRNA expression during differentiation of KS483 cells. KS483 cells were cultured as described in Fig. 1. After standardization for household gene expression, equal amounts of cDNA from each sample were subjected to PCR using specific primers to amplify the splice variants of murine VEGF-A over 35 cycles yielding products of 360 ($VEGF_{164}$) and 230 bp ($VEGF_{120}$), respectively (A), the splice variants of VEGF-B over 30 cycles (B), and VEGF-C mRNA levels after 38 cycles of PCR (C). Lane 1, Day 11; lane 2, day 14; lane 3, day 18; lane 4, day 21; lane 5, fetal lung (E17); lane 6, water control. D, VEGF-D mRNA over 30 cycles; E, mouse VEGFR1/FLT1 over 35 cycles of PCR; F, VEGFR2/FLK1 over 35 cycles of PCR; G, $VEGF_{165}R$/neuropilin over 35 cycles of PCR. The products were separated on a 1.5% agarose gel. Lane 1, Day 7; lane 2, day 11; lane 3, day 14; lane 4, day 18; lane 5, day 21 after plating; lane 6, water control. H, VEGFR3 mRNA levels after 38 cycles of PCR. Lane 1, Day 11; lane 2, day 14; lane 3, day 18; lane 4, day 21; lane 5, water control; lane 6, fetal lung (E17). NC, Negative control; PC, positive control. The sizes of the products are indicated by *arrows*. Representative pictures from three experiments are depicted in the *left panel*, and quantifications of three independent experiments are depicted in the *right panel* as relative mRNA levels ± SEM.

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In addition, VEGFR2 protein expression was examined by Western blotting (Fig. 4B). In line with the PCR data, VEGFR2 expression was low at the beginning of the culture and increased, with differentiation being maximally expressed during the mineralization phase. Under reducing conditions VEGFR2 migrated as two bands of approximately 220 and 230 kDa (Fig. 4B). Using fetal lung as a positive control, proteins of identical size were detected, which most likely represent differentially glycosylated VEGFR2s. In contrast to VEGFR2 protein levels, we did not analyze VEGFR1 and VEGFR3 protein expression, because PCR data showed that VEGFR1 mRNA and 3 mRNA levels are very low or below the detection limit of PCR, whereas VEGFR2 increased with differentiation.

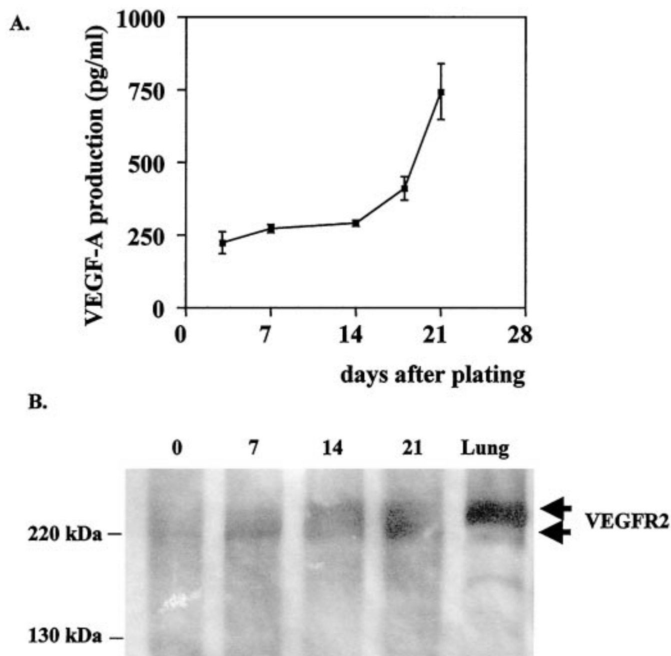

FIG. 4. VEGF-A and VEGFR2 protein production during differentiation of KS483 cells. KS483 cells were cultured as described in Fig. 1. A, VEGF-A protein content was assayed in culture supernatants by VEGF-A ELISA at the time points indicated. Mean values from triplicate experiments are depicted ± SEM. B, Protein was extracted and subjected to SDS-PAGE, blotted, and stained with anti-VEGFR2 antibody. Lane 1, Day 3; lane 2, day 7; lane 3, day 14; lane 4, day 21 after plating; lane 5, fetal lung (E17). VEGFR2 protein is indicated by *arrows*.

Effects of IGF-I and PTHrP on VEGF-A production by KS483 cells

Continuous treatment of KS cells with IGF-I stimulated osteoblast differentiation dose dependently, as shown by a significant increase in nodule formation after 21 days of culture (Fig. 5A). In contrast, continuous treatment with PTHrP significantly inhibited nodule formation after 21 days of culture (Fig. 5A). VEGF levels were determined in the culture medium after 21 days of culture. IGF-I treatment significantly increased VEGF-A levels, whereas PTHrP treatment significantly decreased VEGF-A levels (Fig. 5B).

Effects of VEGF-A, anti-VEGF, and sFLT1 on differentiation during differentiation of KS483 cells

KS483 cells were continuously treated with 1, 2.5, 10, or 25 ng/ml rhVEGF-A. As depicted in Fig. 5, A and B, treatment with 10 and 25 ng/ml rhVEGF-A stimulated nodule formation to a similar extent as IGF-I. Treatment of cultures with a functional blocking antibody directed against murine VEGF-A in the presence or absence of IGF-I did not have an effect on alkaline phosphatase activity (data not shown). Furthermore, treatment of KS483 cells with 50, 100, and 200 ng/ml truncated VEGFR1 (sFLT1) did not affect nodule formation 21 days after plating (Fig. 5C). It should be noted that sFLT1 is able to block endothelial proliferation and endothelial sprouting *in vitro* at the concentrations used (our unpublished results).

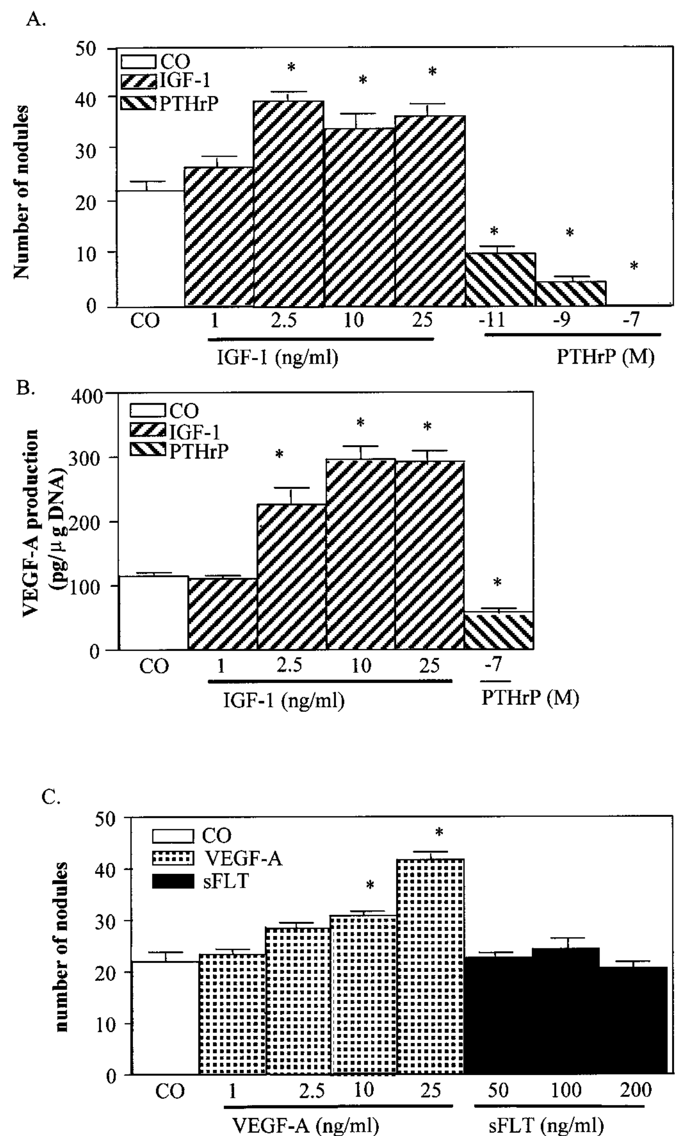

FIG. 5. Effects of IGF-I, PTHrP, VEGF-A, and sFLT1 on nodule formation by KS483 cells. KS483 cells were plated in 24-well plates at a density of 15,000 cells/cm^2. At confluence, 50 μg/ml ascorbic acid and various concentrations of rhIGF-I and PTHrP-(1–34) were added. β-Glycerophosphate was added when nodules appeared (day 15 after plating). The number of mineralized nodules was determined 21 days after plating by light microscopy. Cultures were performed six times, and nodules were counted in triplicate. Values were significantly different from controls as indicated (#, $P < 0.05$). B, In the supernatant, VEGF-A production was measured by ELISA after 21 days of culture. Values represent the mean protein concentration ± SEM corrected for DNA content calculated from triplicate experiments. Values were significantly different from controls as indicated (#, $P < 0.05$). C, At confluence, various concentrations of rhVEGF$_{165}$ and sFLT1 were added to the culture medium for 21 days. The number of mineralized nodules was determined as described above.

Discussion

In the present study using an *in vitro* model of osteoblast differentiation we demonstrate the following: 1) the expression and production of VEGFs and their receptors by nonmalignant untransformed osteoblast-like cells depend on the

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state of differentiation of these cells; 2) the production of VEGF-A increases with differentiation, and this can be modulated by factors that alter the differentiation state of the cells; and 3) exogenous VEGF-A stimulates osteoblast differentiation.

Osteoblast differentiation *in vitro* is characterized by temporal and sequential gene expression, resulting finally in calcium deposition (39, 49). We show here that VEGF-A, -B, and -D are expressed at low levels at the beginning of osteoblast differentiation before BSP and OC mRNA expression. VEGF-A, -B, and -D mRNA expression strongly increased during terminal differentiation in parallel with BSP and OC mRNA expression and were maximally expressed during mineralization. In line with the mRNA expression of VEGF-A, maximum protein levels were found by ELISA during the mineralization phase. When KS483 cells were treated with the osteotropic factors IGF-I and PTHrP, a correlation between osteoblast differentiation and VEGF production was found. Continuous treatment of KS483 cells with PTHrP, a condition that is known to inhibit osteoblast differentiation (50, 51), also inhibited VEGF-A production significantly. In contrast, IGF-I, which stimulated differentiation of KS483 cells, as shown by enhanced nodule formation, significantly and dose dependently increased VEGF production.

It is well known that members of the VEGF family act as paracrine factors through activation of VEGFRs present on endothelial cells. Early observations by Midy and Ploet (34) showed binding of labeled VEGF to high affinity sites on bovine calvarial osteoblasts. Recently, VEGFR1 mRNA expression was reported in murine trabecular osteoblasts (11). In agreement with these studies we demonstrated VEGFR1 mRNA expression by murine KS483 cells. Moreover, this expression was dependent on the differentiation state of the cells. VEGFR2 mRNA expression has been shown in intersegmental regions of vertebrae by *in situ* hybridization (19, 52, 53). We found that VEGFR2 mRNA and protein were expressed in a differentiation-dependent manner. VEGFR2 protein was detected, by immunoblotting, as a 230-kDa mature protein that corresponds to the molecular mass of the mature form of VEGFR2 that is involved in receptor signaling (54). Other studies, however, failed to demonstrate VEGFR2 mRNA in postnatal human trabecular osteoblasts (36) or showed very low VEGFR2 mRNA expression in postnatal mouse trabecular osteoblasts *in vivo* (11). Possible explanations for the differences between our and the other observations include 1) age difference (fetal *vs.* postnatal), as VEGFR2 mRNA levels are described to decline postnatally (53); 2) difference of origin (calvarial bone *vs.* trabecular bone); and 3) cell line-specific properties (cell line *vs.* primary cells). The most recently identified VEGF receptor, $VEGF_{165}R$/neuropilin, was also expressed in a differentiation-dependent manner. To our knowledge we are the first to report expression of this receptor in osteoblast-like cells. This receptor has been identified on endothelial cells and tumor-derived cell lines and acts as an isoform specific coreceptor of VEGFR2 by specifically binding the isoform VEGF-A_{165}, but not VEGF-A_{121} (31, 32). These findings combined with the observation of limb deformities in mice overexpressing neuropilin support a role for this receptor in bone physiology (55).

Treatment of KS483 cells with rhVEGF-A stimulated nodule formation. Interestingly, the effects of rhVEGF-A on differentiation were noted during mineralization at the time when VEGFRs are maximally expressed. These findings, therefore, suggest that VEGF-A might stimulate osteoblast differentiation in an autocrine manner. However, treatment of KS cells with a neutralizing antibody directed against murine VEGF-A or with a truncated VEGF receptor (sFLT1) that can bind both VEGF-A and –B, thereby neutralizing their action, did not affect nodule formation in unstimulated cultures, although both agents were able to inhibit angiogenesis in an *in vitro* model using fetal mouse bone explants (our unpublished results). This suggests that enhanced VEGF-A production by terminal differentiated osteoblasts is not an essential step in the regulation of nodule formation *in vitro*, but that it is instead a marker of osteoblast differentiation. However, we cannot exclude involvement of other VEGF family members in osteoblast differentiation, because VEGF-A loss might be compensated by both VEGF-B and VEGF-D, which are also expressed in a differentiation-dependent manner, as shown here. Observations made in sFLT1-treated cultures make it less likely that VEGF-B can compensate for VEGF-A loss, because both VEGF-A and VEGF-B act through the same receptor (VEGFR1). As it is known that VEGF-A can act not only through VEGFR1 but also through VEGR2, VEGF-A loss might be compensated by VEGF-C or VEGF-D, as these isoforms act through binding of VEGFR2.

The expression pattern of VEGFs and their receptors by KS483 cells before mineralization shown here combined with the recent observation that VEGF substitution can rescue osteoclast formation in mice deficient in osteoclasts due to the absence of macrophage colony-stimulating factor (56) support a novel role for VEGFs in the regulation of bone remodeling. Osteoblast-derived VEGFs may not only act as chemotactic factors on endothelial cells, but may also act on osteoclasts to stimulate their formation and activity.

In conclusion, we show here for the first time that VEGF-A, -B, and -D mRNA and, to a lesser extent, VEGF-C are expressed by the murine osteoblast-like KS483 cell line in a differentiation-dependent manner. Furthermore, exogenously added VEGF-A enhances osteoblast differentiation. VEGF-A may act through various VEGFRs (VEGFR1, VEGFR2, and neuropilin) that are also expressed by osteoblasts in a differentiation-dependent fashion. Continuous treatment of KS483 cells with IGF-I and PTHrP, which modulate osteoblast differentiation, is associated with a change in VEGF-A production. We therefore propose that osteoblast-derived VEGFs may act as paracrine factors, modulating endothelial and osteoclast functions, but may also act as autocrine factors, modulating osteoblast differentiation. These observations might be of importance in processes such as endochondral bone formation and fracture healing, where a close association between angiogenesis and bone formation exists.

References

1. **Rodan GA, Martin TJ** 1981 Role of osteoblasts in hormonal control of osteoblast resorption: a hypothesis. Calcif Tissue Int 33:349–351

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2. **McSheeny PMJ, Chambers TJ** 1986 Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. Endocrinology 118:824–828
3. **Bolander ME** 1992 Regulation of fracture repair by growth factors. Proc Soc Exp Biol Med 200:165–170
4. **Guenther HL, Fleisch H, Sorgente N** 1986 Endothelial cells in culture secrete a potent bone cell active mitogen. Endocrinology 119:193–201
5. **Sato Y, Rifkin DB** 1988 Autocrine activities of basic FGF: regulation of cell movement, plasminogen activator synthesis and DNA synthesis. J Cell Biol 107:1199–1205
6. **Villenueva JE, Nimni ME** 1997 Promotion of calvarial cell osteogenesis by endothelial cells. J Bone Miner Res 5:733–739
7. **Kasperk CH, Börcsök I, Schairer HU, Schneider U, Nawroth PP, Niethard FU, Ziegler R** 1997 Endothelin-1 is a potent regulator of human bone cell metabolism *in vitro*. Calcif Tissue Int 60:368–374
8. **Folkman J, Shing Y** 1992 Angiogenesis. J Biol Chem, 267:10931–10934
9. **Fiorelli G, Orlando C, Benvenuti S, Franceschelli F, Bianchi S, Pioli P, Tanini A, Serio M, Bartucci F, Brandi ML** 1994 Characterization, regulation, and function of specific cell membrane receptors for insulin-like growth factor I on bone endothelial cells. J Bone Miner Res 9:329–337
10. **Wang DS, Miura M, Demura H, Sato K** 1997 Anabolic effects of 1,25-dihydroxyvitamin D_3 on osteoblasts are enhanced by vascular endothelial growth factor produced by osteoblasts and by growth factors produced by endothelial cells. Endocrinology 138:2953–2962
11. **Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N** 1999 VEGF couples hypertrophic cartilage remodeling, ossifiaction and angiogenesis during endochondral bone formation. Nat Med 5:623–628
12. **Keck PJ, HAuser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT** 1989 Vascular permeability factor and endothelial cell mitogen related to PDGF. Science 246:1309–1312
13. **Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF** 1983 Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985
14. **Gospodarowicz D, Abraham JA, Schilling J** 1989 Isolation and characterisation of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc Natl Acad Sci USA 86:7311–7315
15. **Ferrara N, Davis-Smyth T** 1997 The biology of VEGF. Endocr Rev 18:4–25
16. **Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, Keshet E, Neufeld G** 1995 VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. J Biol Chem 272:7151–7158
17. **Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW** 1991 The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 5:1806–1814
18. **Breier G, Albrecht U, Sterrer S, Risau W** 1992 Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development 114:521–532
19. **Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML** 1995 Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. Dev Dyn 203:80–92
20. **Nicosia RF** 1998 VEGF-related molecules in tumours. Am J Pathol 153:11–15
21. **Olofsson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, Orpana A, Pettersson RF, Alitalo K, Eriksson U** 1996 Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA 93:2576–2581
22. **Mori S, Ueda T, Kuratsu S, Hosono N, Izawa K, Uchida A** 1995 Suppression of pulmonary metastasis by angiogenesis inhibtor TNP1470 in murine osteosarcoma. Int J Cancer 61:148–152
23. **Avantaggiato V, Orlandini M, Acampora D, Oliviera S, Simeone A** 1998 Embryonic expression pattern of the murine *figf* gene, a growth factor belonging to platelet-derived growth factor/vascular endothelial grwoth factor family. Mech Dev 73:221–224
24. **Aase K, Lymboussaki A, Kaipainen A, Olofsson B, Alitalo K, Eriksson U** 1999 Localization of VEGF-B in the mouse embryo suggests a paracrine role of the growth factor in the developing vasculature. Dev Dyn 215:12–25
25. **Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG** 1991 Isolation of human placental cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci USA 88:9267–9271
26. **Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z** 1999 Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 13:9–22
27. **Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB** 1991 Identification of a new endothelial growth receptor tyrosine kinase. Oncogene 6:1677–1683
28. **Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Böhlen P** 1992 Identification of the KDR tyrosine kinase as a receptor for vascular endothelial growth factor. Biochem Biophys Res Commun 187:1579–1586
29. **Kukk E, Lymboussaki K, Taira S, Kaipanen A, Jeltsch M, Joukov V, Alitalo K** 1996 VEGF-C receptor binding and pattern of expression with VEGFR3 suggests a role in lymphatic vascular development. Development 122:3829–3837
30. **Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K** 1996 A novel vascular endothelial growth factor, VEGF-C, is a ligand for the FLT4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:290–298
31. **Soker S, Fidder H, Neufeld G, Klagsbrun M** 1996 Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. J Biol Chem 271:5761–5767
32. **Soker S, Takashima S, Miao H-Q, Neufeld G, Klagsbrun M** 1998 Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:735–745
33. **Migdal M, Huppertz B, Tessler S, Comforti A, Shibuya M, Baumann H, Neufeld G** 1998 Neuropilin-1 is a placenta growth factor-2 receptor. J Biol Chem 273:22272–22278
34. **Midy V, Plouet J** 1994 Vasculotropin/vascular endothelial growth factor induces differentiation in cultured osteoblasts. Biochem Biophys Res Commun 199:380–386
35. **Schlaeppi JM, Gutzwiller S, Finkenzeller G, Fournier B** 1997 1,25-Dihydroxyvitamin D_3 induces the expression of vascular endothelial growth factor in osteoblastic cells. Endocr Res 23:213–229
36. **Wang DS, Yamazaki K, Nohtomi K, Shizume K, Ohsumi K, Shibuya M, Demura H, Sato K** 1996 Increase of vascular endothelial growth factor mRNA expression by 1,25-dihydroxyvitamin D_3 in human osteoblast-like cells. J Bone Miner Res 11:472–479
37. **Goad DL, Rubin J, Wang H, Tashjian AH,Jr., Patterson C** 1996 Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology 137:2262–2268
38. **Harada S, Rodan SB, Rodan GA** 1995 Expression and regulation of vascular endothelial growth factor in osteoblasts. Clin Orthop 313:76–80
39. **Yamashita T, Ishii H, Shimoda K, Sampath TK, Katagiri T, Wada M, Osawa T, Suda T** 1996 Subcloning of three osteoblastic cell lines with distict differentiation phenotypes from the mouse osteoblastic cell line KS-4. Bone 19:429–436
40. **Yamashita T, Asano K, Takahashi N, Akatsu T, Udagwa N, Sasaki T, Martin J, Suda T** 1990 cloning of an osteoblastic cell line involved in the formation of osteoclast-like cells. J Cell Physiol 145:587–595
41. **Chomzcynski P, Sacchi N** 1987 Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
42. **Van Bezooijen RL, Farih-Sips HCM, Papapoulos SE, Löwik CWGM** 1998 IL-1α, IL-1β, IL-6 and TNF-α steady state mRNA levels analyzed by RT-competitive-PCR in bone marrow of gonadectomized mice. J Bone Miner Res 13:185–194
43. **Fong G-H, Rossant J, Gertsenstein M, Breitman ML** 1995 Role of the FLT-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66–69
44. **Parnes JR, Seidman JG** 1982 Structure of a wildtype and mutant mouse β2-microglobulin genes. Cell 29:661–669
45. **Claffey KP, Wilkison WO, Spiegelman BM** 1992 Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathway. J Biol Chem 267:16317–16322
46. **Orlandini SZ, Marconini L, Ferruzzi R, Olivieri S** 1996 Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. Proc Natl Acad Sci USA 93:11675–11680
47. **Finnerty H, Kelleher K, Morris G, Bean K, Merberg D, Kriz R, Morris J, Sookdeo H, Turner K, Wood CR** 1993 Molecular cloning of murine Flt and Flt4. Oncogene 8:2293–2298
48. **Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z** 1998 MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of growth plate chondrocytes. Cell 93:411–422
49. **Stein G, Lian J** 1993 Molecular mechanisms mediating proliferation and differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 14:424–441
50. **Bellows AG, Ishida H, Aubin JF, Heersche JN** 1990 Parathyroid hormone reversibly suppresses the differentiation of osteoprogenitor cells into functional osteoblasts. Endocrinology 127:3111–3116
51. **Ishizuya T, Yokose S, Hori M, Noda T, Suda T, Toshiki S, Yamaguchi A** 1997 Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells. J Clin Invest 12:2961–2970
52. **Jakeman LB, Armanini M, Phillips HS, Ferrara N** 1993 Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology 133:848–859
53. **Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A** 1993 High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72:835–846
54. **Takahashi T, Shibuya M** 1997 The 230 kDa form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-γ pathway and partially induces mitotic signals in NIH3T3 fibroblasts. Oncogene 14:2079–2089
55. **Kitsukawa T, Akhihiko S, Kawakami A, Kondoh K, Fujisawa H** 1995 Overexpression of a membrane protein, neuropilin, in chimeri mice causes anomalies in the cardiovascular system, nervous system and limbs. Dev Dyn 121:4309–4318
56. **Niida S, Kaku M, Yoshida H, Kataoka H, Nishikawa S, Tanne K, Maeda N, Kodama H** 1999 VEGF can substitute for MCSF in the support of osteoclastic bone resorption. J Exp Med 190:293–298

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