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Neuropilin-1 Expression in Osteogenic Cells: Down-Regulation During Differentiation of Osteoblasts into Osteocytes

Jay Harper,[1] Louis C. Gerstenfeld,[3] and Michael Klagsbrun[1,2]*

[1]Department of Surgical Research, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115
[2]Department of Pathology, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115
[3]Department of Orthopedic Surgery, Boston University School of Medicine, Boston, Massachusetts 02118

Abstract The expression of neuropilin-1 (NRP1), a recently described VEGF and semaphorin receptor expressed by endothelial cells (EC) but some non-EC types as well, was analyzed in osteoblasts in vitro and in vivo. Cultured MC3T3-E1 osteoblasts expressed NRP1 mRNA and bound $VEGF_{165}$ but not $VEGF_{121}$, characteristic of the VEGF isoform-specific binding of NRP1. These cells did not express VEGFR-1 or VEGFR-2 so that VEGF binding to osteoblasts was strictly NRP1-dependent. In a chick osteocyte differentiation system, NRP1 was expressed by osteoblasts but its expression was absent as the cells matured into osteocytes. Immunohistochemical localization of NRP1 within the developing bones of 36-day-old mice and embryonic Day 17 chicks demonstrated that NRP1 was expressed by osteoblasts migrating alongside invading blood vessels within the metaphysis of the growth plate, as well as by osteoblasts at the developing edge of trabeculae within the marrow cavity. On the other hand, NRP1 was not expressed by osteocytes in either species, consistent with the in vitro results. In addition to osteogenic cells, NRP1 expression by EC was observed throughout the bone. Together these results suggest that NRP1 might have a dual function in bone by mediating osteoblast function directly as well as angiogenesis. J. Cell. Biochem. 81:82–92, 2001. © 2001 Wiley-Liss, Inc.

Key words: VEGF; VEGF receptors; angiogenesis; semaphorins; endochondral ossification

During the development of the long bones, the cartilage of the growth plate is replaced by bone via endochondral ossification. Osteoclasts degrade the calcified cartilage in the metaphysis, the cartilage/bone interface, and the hypertrophic zone of the growth plate. This allows angiogenesis to occur as metaphyseal capillaries invade the normally avascular growth plate [Brighton, 1978; Skawina et al., 1994]. Concurrently, osteoblasts migrate adjacent to these advancing blood vessels to replace the apoptotic hypertrophic chondrocytes, and to replace the degraded cartilage matrix with a bone matrix [Trueta and Little, 1960; Decker et al., 1995]. While it is known that vascular invasion and migration of osteoblasts into the growth plate are critical steps of endochondral ossification, little is known about how these processes are regulated [Trueta and Little, 1960; Brighton, 1978; Skawina et al., 1994; Decker et al., 1995].

Osteoblasts progress through various stages of maturation/differentiation. Mesenchymal cells differentiate into immature osteoblasts that are mitotic and nonsecretory [Caplan, 1990]. These then undergo maturation to become mature osteoblasts, which are highly motile cells that rarely proliferate. They secrete osteoid, an unmineralized bone matrix composed of type I collagen, osteocalcin, and osteopontin that eventually becomes mineralized to form a mature bone matrix [Price et al., 1994]. Both immature and mature osteoblasts express alkaline phosphatase (ALP) activity, but once mature osteoblasts cease migrating and become embedded in matrix, they differentiate into ALP-negative osteocytes.

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*Correspondence to: Dr. Michael Klagsbrun, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115. E-mail: klagsbrun@al.tch.harvard.edu

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Recent evidence suggests that vascular-endothelial growth factor (VEGF) is involved in bone formation. For example, in mice, when endogenous VEGF was neutralized by administration of either a soluble form of Flt-1 [Gerber et al., 1999], or a recombinant, humanized anti-VEGF monoclonal antibody [Ryan et al., 1999], shortening of the long bones occurred. In addition, the rate of apoptosis of hypertrophic chondrocytes decreased, resulting in an expansion of the zone of hypertrophic chondrocytes. The probable mechanism was that VEGF-mediated angiogenesis into the growth plate was inhibited resulting in delayed endochondral ossification and impaired bone growth. In addition, osteoblasts secrete VEGF in vitro [Reher et al., 1999; Saadeh et al., 1999; Steinbrech et al., 1999] and respond to VEGF [Midy and Plouët, 1994]. Together these results suggest that VEGF may have direct effects on osteoblasts during bone formation in addition to the growth and invasion of blood vessels into cartilage.

Although VEGF is capable of stimulating osteoblasts in vitro, these cells did not express the VEGF receptor tyrosine kinases VEGFR-1 or VEGFR-2 [Midy and Plouët, 1994; Wang et al., 1997]. Accordingly, a possible target for VEGF in osteoblasts could be neuropilin-1 (NRP1), a 130 kDa cell surface protein that binds the neuronal guidance regulator, semaphorin [Takagi et al., 1995; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Fujisawa and Kitsukawa, 1998], and the angiogenesis factor VEGF [Soker et al., 1998]. NRP1 is an isoform-specific receptor for VEGF, binding $VEGF_{165}$ but not $VEGF_{121}$. An important property of NRP1 is that it is not only expressed by endothelial cells (EC), but by non-EC as well, for example, tumor cells. The possibility that bone cells express NRP1 was suggested in studies in which NRP1 was overexpressed in transgenic mice. Not only were there neuronal defects, hypervascularity and hemorrhaging, but there were skeletal defects as well such as extra digits in the hind limbs [Kitsukawa et al., 1995]. Thus, NRP1 expression seems to be associated with development of the skeletal system.

Our primary goal was to analyze NRP1 expression in developing bone as this has not yet been done. In this report, we demonstrate that osteoblasts in culture express NRP1 but not VEGFR-1 or VEGFR-2, and bind $VEGF_{165}$. Furthermore, NRP1 expression is down-regulated in a chick osteocyte differentiation system as osteoblasts differentiate into osteocytes. Consistent with the cell culture studies, NRP1 is expressed in vivo by osteoblasts but not osteocytes, in the metaphysis and trabeculae of developing mouse bones and embryonic chick bones. These results suggest that NRP1 may be a novel regulator of osteoblast activity in developing bone, possibly as a receptor for VEGF.

MATERIALS AND METHODS

RT-PCR

Total RNA (5 μg) from either mouse embryoid bodies (kindly provided by Dr. Patricia D'Amore, Schepens Eye Research Institute, Boston, MA) or MC3T3-E1 were reverse transcribed according to the SuperScript II protocol (Life Technologies). Polymerase chain reaction (PCR) was performed using 2 μl of each RT reaction as template. Primers for mouse NRP1 are as follows: Forward 5′-GGC TGC CGT TGC TGT GCG-3′; and Reverse 5′-ATA GCG GAT GGA AAA CCC-3′ that amplify a 404 bp product in the a/CUB domain. Primers for VEGFR-1 are as follows: Forward 5′-GGC TCA GGG TCG AAG TTA AAA GTG CCT-3′; and Reverse 5′-TAG GAT TGT ATT GGT CTG CCG ATG GGT-3′ that amplify a 625 bp product in the extracellular domain. Primers for VEGFR-2 are as follows: Forward 5′-CTC TGT GGG TTT GCC TGG CGA TTT TCT-3′; and Reverse 5′-GCG GAT CAC CAC AGT TTT GTT CTT GTT-3′ that amplify a 408 bp product in the extracellular domain. Primers for chick NRP1 are as follows: Forward 5′-GAT AAT GCT GAA GGA CGC TTA TGG GGA-3′, Reverse 5′-CCT AGT GGT TCC ATG CAC TGG AAA TCT-3′ and amplify a 562 bp region in the a/CUB domain. Primers for cβ-actin are as follows: Forward 5′-ATG CCC CCC GTG CTG TGT TCC-3′; Reverse 5′-CCA GAT CTT CTC CAT ATC ATC-3′ and amplify a 184 bp product. The following PCR was repeated for 30 cycles: 94°C for 45 s, 56°C for 30 s, 72°C for 45 s.

Northern Blot Analysis

mRNA isolated from MC3T3-E1 cells (1 μg) was separated on a 1% agarose/formaldehyde gel. The RNA was transferred to a GeneScreen-Plus membrane (NEN Life Science Products,

Boston, MA) and cross-linked to the membrane by baking at 80°C for 2 h. The blots were then pre-hybridized for 2 h at 65°C in the presence of denatured, sonicated salmon sperm DNA (Strategene, La Jolla, CA). cDNA probes were labeled with α^{32}P-dCTP (New England Nuclear, Boston, MA) using a Redi-Prime Kit (Amersham Pharmacia Biotech, Buckinghamshire, England) according to the manufacturer's protocol. ^{32}P-labeled probes corresponding to the a/CUB domain of mNRP1 [Takagi et al., 1991; Kawakami et al., 1996], and to regions in the extracellular domains of VEGFR-1 and VEGFR-2 [Neufeld et al., 1999], were denatured and hybridized to the appropriate blots (1×10^6 cpm/ml), which were incubated overnight at 65°C. The blots were washed in $2 \times$ SSC (Research Genetics, Huntsville, AL), 0.1% SDS (Bio-Rad, Hercules, CA) with increasing temperature, and then exposed to Kodak BioMax film (Eastman Kodak Co., Rochester, NY) and developed. Blots were stripped and hybridized with a β-actin probe to confirm normalization of RNA loading.

Cross-Linking Analysis

$VEGF_{165}$ and $VEGF_{121}$ were iodinated using IODO-BEADS Iodination Reagent (Pierce, Rockford, IL) as previously described [Soker et al., 1997]. Cross-linking experiments were carried out as previously described [Soker et al., 1996]. Briefly, MC3T3-E1 and PAE/KDR were incubated with $^{125}I\text{-}VEGF_{165}$ or $^{125}I\text{-}VEGF_{121}$ at final concentrations of 5 and 10–25 ng/ml, respectively, in the presence of heparin. For competition experiments, 100–200-fold excess of unlabeled $VEGF_{165}$ was added in addition to $^{125}I\text{-}VEGF_{165}$. Binding proceeded for 1.5 h on ice, and subsequent ^{125}I-VEGF/receptor complexes were cross-linked using disuccinimidyl suberate (DSS, Pierce). Cell lysates were prepared, and cross-linked ^{125}I-VEGF/receptor complexes were resolved by 6% SDS-PAGE. Gels were dried and exposed to BioMax X-ray film and developed.

Antibodies

Previously characterized polyclonal rabbit anti-mouse NRP1 IgG [Kawakami et al., 1996] was the generous gift of Dr. Hajime Fujisawa (Nagoya University, Japan). Horseradish peroxidase-conjugated $F(ab')_2$ fragment goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Normal rabbit control IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry

Tibiae and femurs from 36-day-old mice were dissected and fixed for 4 h up to overnight in 10% buffered formalin (Sigma) at 4°C. The bones were then decalcified in 0.5 M EDTA, pH 7.4 for 7–10 days at 4°C. After the tibiae were dehydrated and embedded in paraffin, 5 μm sections were prepared and stained for NRP1 as follows. The sections were deparaffinized with xylenes (Fisher, Fair Lawn, NJ) and rehydrated with PBS, pH 7.5 following a graded series of alcohols. Sections were digested with 1 mg/ml trypsin (Sigma) for 10–20 min at 37°C for antigen retrieval. After washing with PBS, they were treated with 3% H_2O_2 (Fisher) in methanol (Fisher) to inhibit endogenous peroxidases. Sections were then washed with PBS and incubated in a protein blocking solution (10% goat serum, 10% sheep serum in PBS) at room temperature. Primary (1°) antibodies were then added and allowed to incubate overnight up to 48 h at 4°C. The sections were washed with PBS, and again incubated in blocking solution at room temperature. Horseradish peroxidase-conjugated secondary (2°) anti-rabbit antibodies were added and the sections were incubated for 4 h up to overnight at 4°C. After washing with PBS, NRP1 immunostaining was visualized by incubating the slides in stable diaminobenzidine (DAB, Research Genetics, Huntsville, AL). The mouse NRP1 antibody was also used in immunohistochemistry of chick tibiae since it recognizes the chick homologue of NRP1 [Takagi et al., 1995]. Chick immunostaining was performed according to the protocol used for mouse tibiae with the following modifications. Tibiae were fixed in either 10% buffered formalin or with Histochoice (Amresco). Antigen retrieval was achieved with Proteinase K (Boehringer Mannheim; Indianapolis, IN) at a concentration of 10–20 μg/ml for 8–15 min at room temperature or 37°C. As negative controls, sections were either incubated with 2° antibody only, or the NRP1 1° antibody was replaced with normal rabbit IgG. Immunostained sections were counterstained with hematoxylin Gill No. 3 (Sigma) where indicated.

Cell Culture

MC3T3-E1 osteoblasts were a generous gift from Dr. Peter Hauschka (Children's Hospital, Boston, MA). MC3T3-E1 is an immortalized cell line produced by transforming primary cultures of mouse calvarial osteoblasts via the same method used to immortalize 3T3 fibroblasts [Sudo et al., 1983]. They have been well characterized as an osteoblast cell line, and provide an excellent model of the osteoblastic phenotype in vitro [Natsume et al., 1997]. These cells were cultured as previously described [Damoulis and Hauschka, 1997] in minimum essential media-alpha modification (α-MEM, Life Technologies, Rockville, MD) containing 10% fetal calf serum (FCS) and glutamine, penicillin, and streptomycin (GPS). Porcine aortic EC expressing KDR (PAE/KDR) [Waltenberger et al., 1994] was the generous gift of Dr. Shay Soker (Children's Hospital, Boston, MA) and were grown in F12 media (Life Technologies) containing 10% FCS and GPS.

Osteoblast Differentiation

Calvaria were dissected from 12 or 17-day-old chicken embryos and subjected to three sequential trypsin/collagenase digestions to release osteoblasts as previously described [Gerstenfeld et al., 1996]. Briefly, cell populations collected after the third digestion only were used in order to decrease the possibility of contaminating cell types. Cells were seeded at densities of 1.25×10^5 cells per 60-mm culture dish, or 5×10^5 cells per 10-cm dish in the presence of MEM supplemented with 10% FBS (Sigma) and 1% GPS. After 24 h, cultures were washed with PBS and the media changed to MEM with 10% FBS and 1% GPS. The cells were grown for 2–3 weeks with medium changes every 2 days until the cells reached confluence, at which point the media was changed to BGJ_b (Fitton-Jackson modification, Life Technologies) with 10% FBS plus 1% GPS. After 48 h, the medium was supplemented with 10 mM βGPO_4. After an additional 48 h, the media was replaced with "mineralizing" media (MEM, 10% FBS, 1% GPS, 10mM βGPO_4, and 12.5 μg/ml ascorbic acid). The day on which this mineralization medium was first added is denoted as Day 0. The media was changed every 3 days until the experiments were terminated.

RESULTS

MC3T3-E1 Osteoblasts Express NRP1 but not VEGFR-1 or VEGFR-2

To characterize the expression of NRP1 in osteoblasts, murine MC3T3-E1 osteoblasts were analyzed for expression of VEGF receptors. RT-PCR analysis demonstrated that MC3T3-E1 expressed NRP1, while neither VEGFR-1 nor VEGFR-2 was detected (Fig. 1A). As a positive control, it was shown that murine embryoid

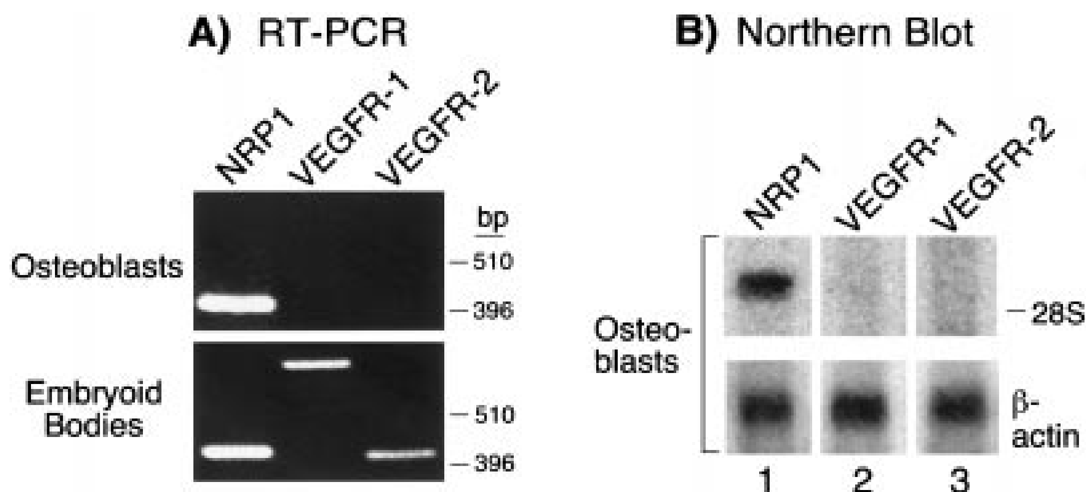

Fig. 1. MC3T3-E1 express NRP1, but not VEGFR-1 or VEGFR-2. (**A**) RT-PCR was performed on RNA purified from MC3T3-E1 osteoblasts and mouse embryoid bodies using primer sets corresponding to mouse homologues of NRP1, VEGFR-1, and VEGFR-2. (**B**) Northern blot analysis of mRNA purified from MC3T3-E1 cells. A message of ≈7 kb hybridized to the mouse NRP1 probe (**lane 1**), but no message hybridized to VEGFR-1 (**lane 2**) or VEGFR-2 (**lane 3**) probes. Blots were stripped and probed for β-actin as a control for loading.

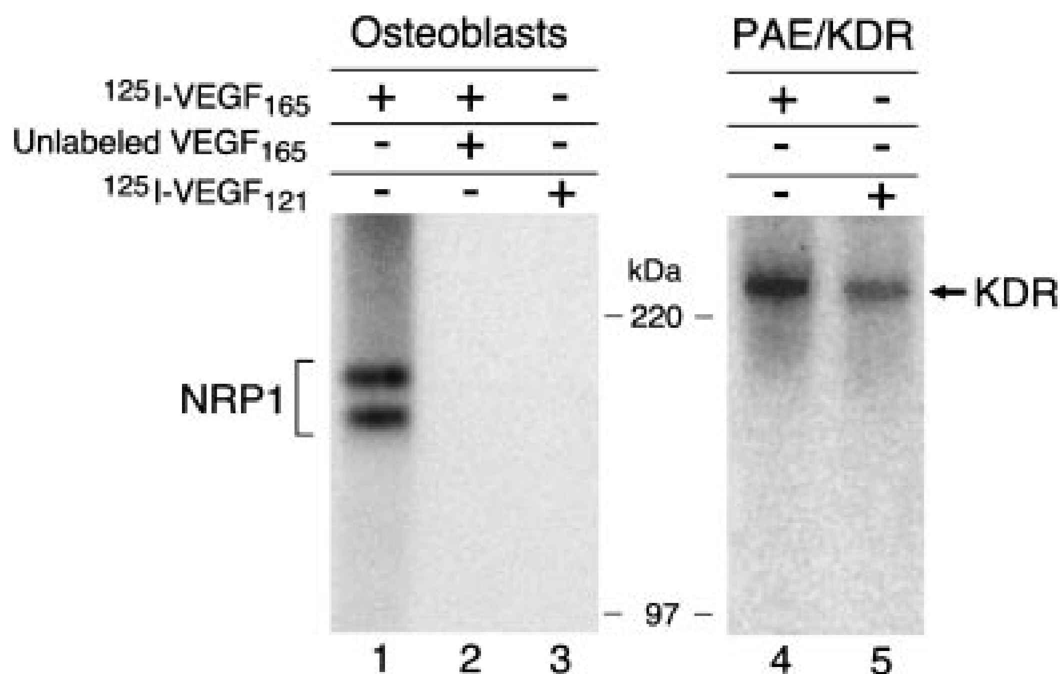

Fig. 2. MC3T3-E1 osteoblasts bind $VEGF_{165}$, but not $VEGF_{121}$. $^{125}I-VEGF_{165}$ (5 ng/ml) or $^{125}I-VEGF_{121}$ (25 ng/ml) was cross-linked to MC3T3-E1 cells (**lanes 1–3**) or to PAE/KDR cells (**lanes 4 and 5**). ^{125}I-VEGF/VEGF receptor complexes were analyzed by SDS-PAGE. $^{125}I-VEGF_{165}$ formed a complex that migrated as a doublet at $\approx$180 kDa (**lane 1**) that could be competed with excess unlabeled $VEGF_{165}$ (**lane 2**). $^{125}I-VEGF_{121}$ did not bind MC3T3-E1 (**lane 3**). As a positive control, PAE/KDR are able to bind both $^{125}I-VEGF_{165}$ (**lane 4**) and $^{125}I-VEGF_{121}$ (**lane 5**).

bodies express VEGFR-1 and VEGFR-2, confirming previous results [Doi et al., 1997]. Expression of NRP1 in embryoid bodies has not been previously reported. NRP1, but not VEGFR-1 or VEGFR-2 expression by MC3T3-E1 cells was also demonstrated by Northern blot analysis (Fig. 1B). A single band of $\approx$7 kb, corresponding to the reported size of NRP1 mRNA [Soker et al., 1998], was detected. It was concluded that MC3T3-E1 osteoblasts express NRP1, but not VEGFR-1 or VEGFR-2 in vitro.

MC3T3-E1 Osteoblasts Bind $VEGF_{165}$ but not $VEGF_{121}$

In order to determine whether the NRP1 mRNA expressed by MC3T3-E1 osteoblasts is translated into protein that can serve as a functional VEGFR, cross-linking analysis with ^{125}I-VEGF was performed (Fig. 2). MC3T3-E1 bound $^{125}I-VEGF_{165}$ to form a VEGF-VEGFR complex that migrated on SDS-PAGE as a doublet with a molecular mass of $\approx$180 kDa (Fig. 2, lane 1). This size corresponds to the sizes of VEGF/NRP1 doublet complexes formed on EC and tumor cells [Soker et al., 1998]. Binding of $^{125}I-VEGF_{165}$ was competed by the addition of a 200-fold excess of unlabeled $VEGF_{165}$ (Fig. 2, lane 2). No complex was formed with $^{125}I-VEGF_{121}$, even when $^{125}I-VEGF_{121}$ was administered at concentrations five times greater than $^{125}I-VEGF_{165}$ (Fig. 2, lane 3). The isoform-specific binding of VEGF is a characteristic of NRP1, which binds $VEGF_{165}$ but not $VEGF_{121}$ [Soker et al., 1998]. As a positive control, PAE/KDR cells formed a $VEGF_{121}$/VEGFR-2 complex of $\approx$240 kDa (Fig. 2, lane 5), corresponding to the reported size of $VEGF_{121}$/VEGFR-2 complexes [Gitay-Goren et al., 1992; Soker et al., 1998]. The lack of a 240 kDa VEGF/VEGFR-2 complex and a 220 kDa VEGF/VEGFR-1 complex in MC3T3-E1 cells was a further indication that these cells do not express VEGFR-1 or VEGFR-2. These results demonstrate that NRP1 is the principal VEGFR in osteoblasts, although it is possible that these cells also express NRP2.

In Vivo Expression of NRP1 in Developing Murine and Avian Bones

NRP1 is expressed by osteoblasts in vitro. However, in vivo analysis is necessary to analyze NRP1 expression spatially in bone.

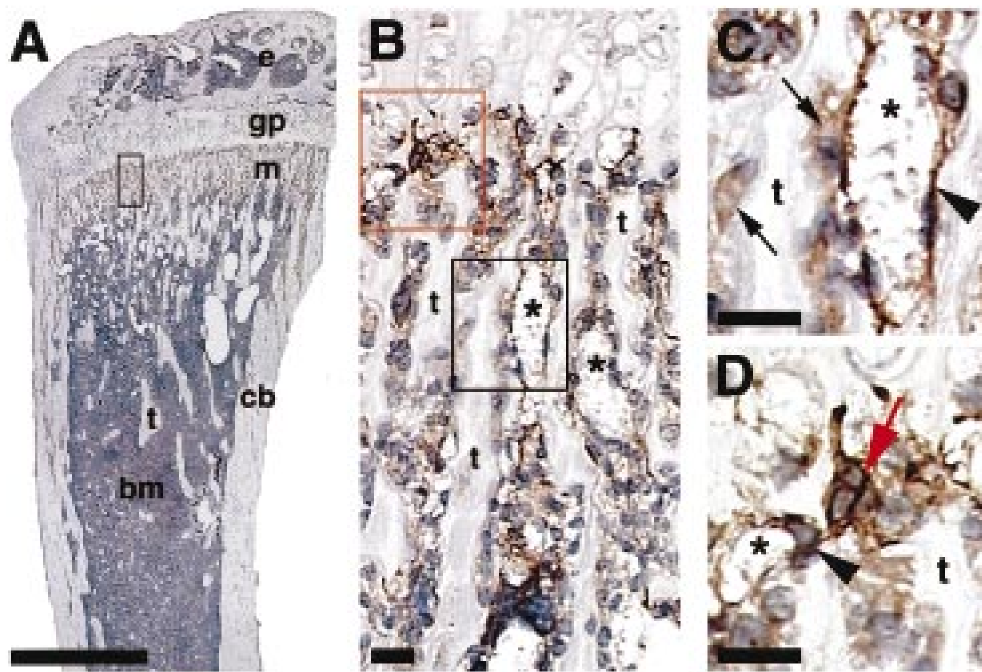

Fig. 3. NRP1 expression in the metaphysis. Fixed and decalcified 36-day-old mouse tibiae immunostained for NRP1 (reddish-brown) and counterstained with hematoxylin (purple color) demonstrated expression of NRP1 in osteoblasts (black arrows), osteoclasts (red arrow), and EC (solid arrowheads) of metaphyseal capillaries (asterisks). (**A**) Low magnification of an NRP1-immunostained tibia. e, epiphysis; gp, growth plate; m, metaphysis; t, trabeculae; bm, bone marrow; and cb, cortical bone. (**B**) Higher magnification of boxed region indicated in panel A showing immunostaining of NRP1 in the metaphyseal region. (**C**) Higher magnification of region outlined by black box in panel B, showing NRP1 immunostaining of osteoblasts located adjacent to an invading metaphyseal capillary. EC (solid arrowheads) comprising the capillary also express NRP1. (**D**) Higher magnification of region outlined by red box in panel B. NRP1 is expressed by an osteoclast at the subchondral border (red arrow), and by the EC (solid arrowhead) of an invading capillary following the osteoclast into the growth plate. Scale bars in A: 1 mm, B, C, and D: 25 μm.

Immunolocalization of NRP1 in the tibiae and femurs of 36-day-old mice was carried out using antibodies that recognize mouse NRP1 [Kawakami et al., 1996]. Mice at this stage of development were used since the long bones are still developing and the growth plate is still intact. Positive NRP1 immunostaining (reddish-brown) was observed primarily in the metaphysis of developing bones (Fig. 3A,B). Figure 3B shows a higher magnification of the area within the black box in Figure 3A and capillaries can be detected (asterisks). At yet a higher magnification, NRP1 immunostaining was observed clearly in osteoblasts (arrows) migrating alongside metaphyseal capillaries (asterisks) invading the growth plate (Fig. 3C). NRP1 immunostaining was observed in multinucleated osteoclasts in regions of bone or mineralized cartilage resorption, for example, at the subchondral border (red arrow in Fig. 3D) and in Howship's lacunae of trabeculae (data not shown). Osteoblasts lining trabeculae within the marrow cavity (Fig. 4A) also expressed NRP1. On the other hand, osteocytes (open arrowheads) located within the trabeculae (Fig. 4A), or cortical bone (Fig 4B) did not express NRP1.

EC (solid arrowheads) in the blood vessels within the developing bone, also express NRP1. NRP1 was expressed by EC of metaphyseal blood vessels invading the growth plate (Fig. 3B,C,D), cortical capillaries in the cortex (Fig. 4B), and medullary capillaries found in the bone marrow (data not shown). Tibiae immunostained with either control rabbit IgG (Fig. 4C) or with secondary antibody only were negative (data not shown). Together these results indicate that NRP1 is expressed by EC and by osteoblasts and osteoclasts, but not osteocytes in the developing bone.

Immunostaining of embryonic chick tibiae demonstrated NRP1 in vivo expression similar to the pattern seen in mouse tibiae. NRP1 expression was observed in the metaphysis of the chick growth plate (Fig. 5A,B). The most intense immunostaining was seen in

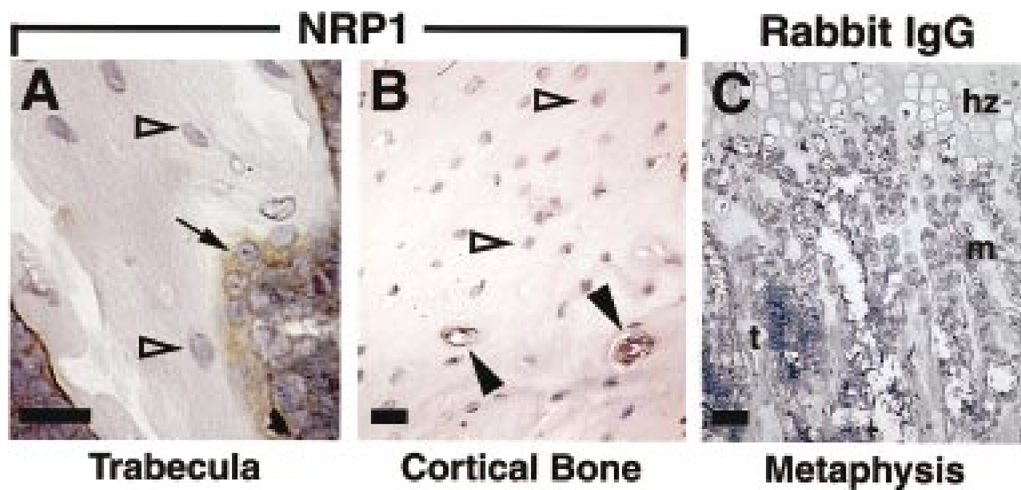

Fig. 4. NRP1 expression in trabeculae and cortical bone. (**A**) NRP1 is expressed on osteoblasts (arrow) lining the edge of a trabecula, while osteocytes (open arrowheads) embedded in the trabecula are negative. (**B**) Osteocytes in cortical bone do not express NRP1 but EC in capillaries (solid arrowheads) are positively immunostained for NRP1. (**C**) Negative control for NRP1 immunostaining in Figures 3 and 4, in which the anti-NRP1 antibody is replaced with normal rabbit IgG. All sections are counterstained with hematoxylin (purple-blue). hz, hypertrophic zone; m, metaphysis; and t, trabeculae. Scale bars in A,B: 25 μm, C: 50 μm.

osteoblasts at the subchondral border while osteocytes located in the cortical bone of the tibia did not express NRP1.

NRP1 is Downregulated as Osteoblasts Differentiate into Osteocytes

Immunostaining of both murine and avian bones demonstrated that osteoblasts express NRP1 but that osteocytes do not. The expression of NRP1 during differentiation of osteoblasts into osteocytes was analyzed using an in vitro system of osteoblast differentiation. Primary calvarial osteoblasts were harvested from E17 chick embryos and grown in culture conditions that promoted differentiation of osteoblasts into osteocytes. A temporal study

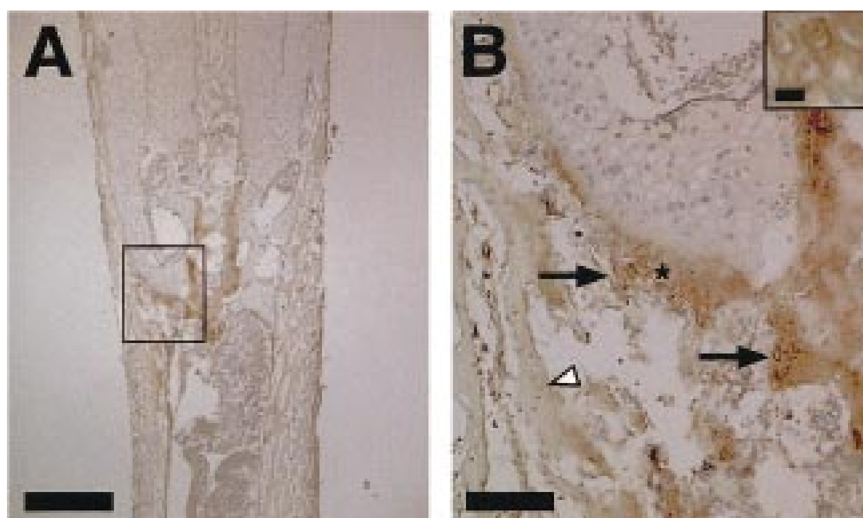

Fig. 5. NRP1 expression in the developing chick bone. Tibiae from E17 chicken embryos were immunostained for NRP1 and counterstained with hematoxylin. (**A**) NRP1 is localized in the metaphyseal region. (**B**) Higher magnification of region in black box indicated in panel A. NRP1 is expressed by osteoblasts (arrows) in the metaphysis adjacent to the hypertrophic cartilage. Osteocytes (open arrowheads) in the cortex do not express NRP1. (B Inset) Higher magnification of region marked by asterisk in panel B, showing osteoblasts expressing NRP1. Scale bars in A: 500 μm, B: 100 μm, B Inset: 10 μm.

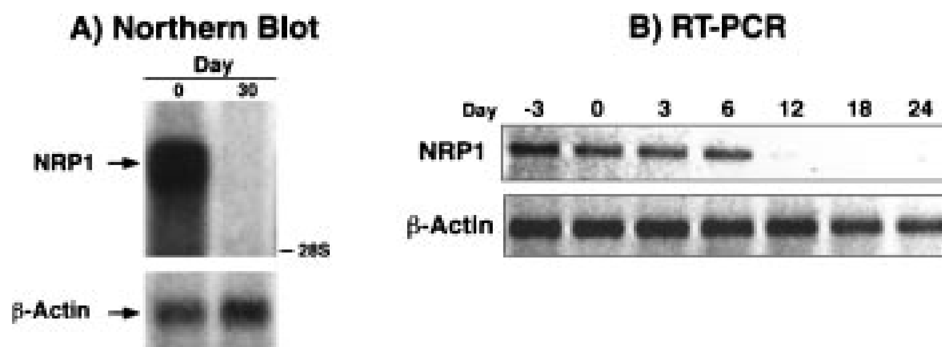

Fig. 6. NRP1 is down-regulated as osteoblasts differentiate into osteocytes. Calvarial osteoblasts were obtained from E17 chicken embryos and cultured in conditions that promote the differentiation into osteocytes. (**A**) Northern blot analysis of RNA levels during osteoblast differentiation. RNA was collected from cells at time zero when differentiation was initiated and at 30 days in culture. β-actin was used to control for loading. (**B**) RT-PCR of osteoblast RNA during differentiation with additional time points utilizing primers designed against the chick homologues of NRP1 and β-actin.

of NRP1 expression in differentiating osteoblasts was performed using Northern blot analysis and semiquantitative RT-PCR. The differentiation of osteoblasts into osteocytes was accompanied by down-regulation of NRP1 mRNA expression. By Northern blot analysis NRP1 expression was not detectable 30 days after addition of differentiation media (Fig. 6A). The experiment was repeated with more time points and RT-PCR analysis (Fig. 6B). By Day 12 after addition of differentiation medium, NRP1 expression was not detectable. These in vitro results confirm the lack of NRP1 expression in osteocytes in vivo shown above.

DISCUSSION

Our results indicate that NRP1 is expressed by osteoblasts, that NRP1 expression is down-regulated as osteoblasts differentiate into osteocytes and that osteoblasts are novel targets for $VEGF_{165}$. Thus, VEGF might be a direct mediator of osteoblast activity in addition to contributing to bone development as an angiogenesis factor. These results were obtained both in vitro and in vivo and in two species, (36-day old) mouse and (embryonic Day 17) chick. NRP1 was detected in vivo in developing murine and chick bones by immunostaining. In both the mouse and the chick, NRP1 was expressed by osteoblasts migrating into the growth plate adjacent to invading metaphyseal blood vessels and by osteoblasts at the developing edge of trabeculae within the marrow cavity. As mature osteoblasts cease migrating, they embed themselves in bone matrix and differentiate into osteocytes. In both species there was a lack of NRP1 expression in osteocytes within the metaphysis, trabeculae, or in cortical bone. These in vivo results were confirmed in culture using an in vitro primary chick osteocyte differentiation model. RT-PCR and Northern blot analysis indicated that differentiation of avian osteoblasts into osteocytes, as determined by the initiation of expression of osteocyte markers, was accompanied by down-regulation of NRP1 expression. Within 2 weeks after addition of differentiation media NRP1 expression was not detectable.

In addition to osteogenic cells, NRP1 expression by EC was observed throughout the bone. Immunostaining of NRP1 in developing bone demonstrated that NRP1 expression occurred in EC of capillaries in the metaphysis, cortical bone, and bone marrow. Since NRP1 is expressed on both osteoblasts and EC within the developing bone, this receptors may play a yet undetermined role in osteoblast function as well as angiogenesis during endochondral ossification.

NRP1 appears to be the only functional VEGF receptor expressed by osteoblasts. VEGF activates two RTKs on EC, VEGFR-1 and VEGFR-2. While MC3T3-E1 osteoblasts express NRP1 and cross-link $VEGF_{165}$ (but not $VEGF_{121}$) to NRP1, these osteoblasts do not express VEGFR-1 or VEGFR-2, or cross-link $VEGF_{165}$ to these VEGF RTKs. Lack of VEGFR-1 and VEGFR-2 expression by osteoblasts is consistent with previous results using primary cultures [Midy and Plouët, 1994;

Wang et al., 1997]. There have been some reports, however, of VEGF RTK expression by osteoblasts. For example, expression of VEGFR-1 and VEGFR-2 in addition to NRP1 has recently been reported in an osteoblast cell line (KS483) using RT-PCR [Deckers et al., 2000] and VEGFR-1 expression in osteoblasts was reported in osteoblasts using in situ hybridization [Gerber et al., 1999]. However, these two studies did not demonstrate VEGFR protein production or functionality.

The function of NRP-1 in osteoblasts is not yet known. NRP1 has a short 40 amino acid cytoplasmic tail that lacks any discernible tyrosine kinase domain and therefore does not appear to be a signaling receptor [Takagi et al., 1995]. The lack of VEGF RTKs in osteoblasts seems to rule out the possibility that it acts as a co-receptor for these RTKs as is the case for EC [Soker et al., 1998]. However, the expression pattern of NRP1 in osteoblasts but not osteocytes in vitro and in vivo suggests a possible role for NRP1 in osteoblast migration. NRP1 is expressed by osteoblasts, motile cells that migrate to areas requiring deposition of mineralized bone matrix during bone development and repair. On the other hand, NRP1 is not expressed in osteocytes, cells that are embedded in the matrix of developing bone, and no longer migrate. A role for NRP1 in migration would be consistent with previous results showing that NRP1 mediates the migration of axons [He and Tessier-Lavigne, 1997; Kolodkin et al., 1997], and EC [Soker et al., 1998; Miao et al., 1999]. In addition, it has been reported that $VEGF_{165}$ ($VEGF_{121}$ was not tested) was able to bind and stimulate migration, and maturation of primary cultures of osteoblasts despite the lack of VEGFR-1 or VEGFR-2 expression [Midy and Plouët, 1994]. In retrospect, it may be that NRP1, then unknown as a VEGF receptor, might have been the unidentified VEGFR receptor that mediated VEGF-induced osteoblast migration. Alternatively, NRP1 might have a role as a carrier of $VEGF_{165}$ that makes this angiogenesis factor available to EC in contact with osteoblasts. Particularly in the metaphysis of the growth plate, osteoblasts are in close proximity to EC of capillaries invading the growth plate.

NRP1 expression is down-regulated when osteoblasts differentiate into osteocytes in vitro and in vivo. The mechanisms regulating the differentiation of osteoblasts into osteocytes are poorly understood. Recent evidence suggests that TGF-β is involved in osteocyte differentiation in vivo [Filvaroff et al., 1999]. Additionally, the full expression of the osteogenic phenotype and differentiated function is regulated via the extracellular matrix through integrins that promote terminal osteoblast differentiation [Xiao et al., 2000]. The results presented in this report demonstrate that perhaps NRP1 is involved in functions specific to the osteoblastic phenotype. Whether loss of NRP1 expression leads to osteocyte differentiation or simply that NRP1 expression is down-regulated as a function of osteocyte differentiation is currently under investigation. Additionally, as osteoblasts differentiate into osteocytes, they no longer have direct communication with the vasculature. The expression of the VEGFR NRP1 may be down-regulated due to the lack of direct contact between osteocytes and the vasculature.

A possible source of VEGF in developing bone could be cartilage. In transgenic mice that colocalize VEGF and LacZ expression, resting and proliferating chondrocytes in the epiphyseal cartilage expressed very low levels of VEGF, while VEGF expression was significantly up-regulated as these chondrocytes underwent maturation, with the highest VEGF expression seen in late maturing and hypertrophic chondrocytes [Harper and Klagsbrun, unpublished]. This observation is consistent with previous reports using either in situ hybridization or immunostaining demonstrating up-regulation of VEGF expression in hypertrophic chondrocytes [Gerber et al., 1999; Horner et al., 1999; Carlevaro et al., 2000]. VEGF secreted by hypertrophic chondrocytes could modulate osteoblast activity during endochondral ossification.

Aside from its function as a VEGFR, NRP1 was previously characterized as a neuronal receptor for the semaphorins, a family of proteins that repel neurons and inhibit axonal outgrowth in dorsal root ganglia [Takagi et al., 1995; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997]. Mice mutant in semaphorin III (Sema3A) display abnormalities in the bone and cartilage (fused vertebrae, partial duplication of ribs, and anomalous development of the sternum), suggesting that Sema3A is involved with the patterning of skeletal elements [Behar et al., 1996]. The semaphorins may also play a role in angiogenesis since binding of Sema3A

to NRP1 inhibits the migration of EC and the outgrowth of capillary tubes from rat aortic segments [Miao et al., 1999]. Preliminary results indicate that MC3T3-E1 osteoblasts express Sema3A and Sema3F [Harper and Klagsbrun, unpublished data]. The function of these NRP1 ligands in the bone is currently under investigation.

In summary, we have established NRP1 as a VEGFR expressed by osteoblasts, but not osteocytes. Understanding the role of NRP1 in bone could yield insights into angiogenesis-dependent pathologies in the bone, such as osteoarthritis, chondrosarcoma, and osteosarcoma, and certain diseases that are a result of aberrant osteogenesis, such as the skeletal dysplasias.

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