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Localization of Pigment Epithelium-Derived Factor in Growing Mouse Bone

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Abstract. Pigment epithelium-derived factor (PEDF) is a potent anti-angiogenic factor found in a wide range of fetal and adult tissues, where it is thought to play a role in the regulation of angiogenesis during development. The temporal expression of PEDF during endochondral bone formation has not previously been reported. In this study, we analysed the expression pattern of PEDF in growing mouse hindlimbs from newborn day one through to maturation at week 9, using immunohistochemistry and *in situ* hybridization. PEDF expression was demonstrated in chondrocytes within the resting, proliferative and upper hypertrophic zones of the epiphyseal growth plate. The pattern of expression was consistent throughout the developmental stages of the mouse. In addition, PEDF was expressed by osteoblasts lining the bone spicules in the ossification zone of metaphyseal bone, as well as by osteoblasts lining cortical periosteum. These novel results demonstrate that PEDF is developmentally expressed in both cartilage and bone cells during endochondral bone formation, and strongly suggest that it may play a regulatory role in the processes of chondrocyte and osteoblast differentiation, endochondral ossification, and bone remodelling during growth and development of long bones.

Key words: PEDF — Chondrocyte — Osteoblast — Growth plate — Cartilage — Endochondral ossification — Bone

Pigment epithelium-derived factor (PEDF) is a 50-kDa secreted glycoprotein of 418 amino-acid residues, which was originally isolated from conditioned media of cultured primary human fetal retinal pigment epithelial cells [1], PEDF shares high sequence and structural homology with the serine protease inhibitor family, but does not inhibit proteases [2]. It has multiple and varied biological properties. It promotes neuronal development, differentiation and survival [2–4], and plays regulatory roles in the cell cycle and in growth arrest and senescence of fibroblasts in culture [5]. PEDF is also an exceptionally potent inhibitor of angiogenesis, both *in vitro* [6] and *in vivo* [7–9], due to suppression of endothelial cell migration and proliferation and induction of endothelial cell apoptosis.

Secreted PEDF binds with high affinity to collagens of the extracellular matrix, which determines its localization in specific tissues [10]. Northern blot analysis has demonstrated the presence of PEDF transcript in a wide range of fetal and adult tissues, including bone marrow, liver, heart, lung and brain [11], PEDF is thought to play a role in the regulation of physiological angiogenesis during development of these tissues. Recent studies on PEDF-deficient mice have confirmed that PEDF is a key regulator of vasculature in the pancreas and prostate, with excessive stromal vasculature occurring in the absence of PEDF [8]. Extensive studies have also demonstrated that PEDF plays a major role in eye development and in maintaining the homeostasis of angiogenesis in the vitreous humour, cornea and retina [6, 12]. A shift in the equilibrium between PEDF and pro-angiogenic vascular endothelial growth factor (VEGF) in the eye promotes growth of blood vessels, leading to proliferative vitreoretinopathy [6, 13, 14].

Similar to the vitreous and corneal compartments of the eye, where PEDF is expressed in high amounts, epiphyseal cartilage lacks an intrinsic vascular supply. However, during the physiological process of endochondral ossification, the microenvironment of the growth plate changes from an angiostatic to an angiogenic one. This process is co-ordinated by a complex balance between specific pro-angiogenic factors such as VEGF, fibroblast growth factors and transferrin [15–17] and anti-angiogenic factors such as chondromodulin-I and transforming growth factor-β [18, 19] expressed at various levels of the growth plate. It culminates in the vascular invasion of the lowermost hypertrophic chondrocytes that is essential for endochondral ossification.

In a previous immunohistochemical study, we showed the expression pattern of the potent anti-angiogenic factor PEDF within mouse epiphyseal car-

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tilage [20]. Following this study, we now demonstrate the chronological expression of PEDF mRNA and protein during the progression of endochondral ossification in postnatal growing mouse long bone. PEDF is expressed in epiphyseal cartilage, in actively remodelling bone, and in various connective tissues. On the basis of these findings, together with its anti-angiogenic and collagen-binding properties, we propose that PEDF may play a regulatory role during endochondral bone formation and skeletal development

Materials and Methods

Animals and Tissue Preparation

Long bones from the hindlimbs of newborn (day one) C57B1/6 J male mice and five-week-old and nine-week-old BALB/c nude male mice were used. All tissues were fixed in 4% (w/v) paraformaldehyde in diethyl ester pyrocarbonic acid (DEPC)-treated phosphate buffered solution (PBS), pH 8.0, at room temperature for 48 hours, before being decalcified for 2 weeks in 15% (w/v) ethylenediaminetetraacetic acid (EDTA) and 0.5% (w/v) paraformaldehyde in DEPC-PBS, pH 8.0, at 4°C. Tissue processing was performed using standard protocols, and hindlimbs were then embedded in paraffin. Longitudinal sections (5 μm wide) were obtained from tissue blocks using a rotary microtome (Leica).

Synthesis of Riboprobes

PEDF riboprobe corresponding to the full-length cDNA was generated by subcloning the 1251 bp fragment obtained using reverse-transcription PCR from mouse liver total RNA, into the HindIII-XbaI sites of pcDNA3 (Invitrogen). The plasmid was linearized with XbaI or HindIII and transcribed with T7 or Sp6 RNA polymerases (Invitrogen) into sense and antisense orientations, respectively. The riboprobes were labelled with digoxigenin (DIG) using an RNA labelling kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

In Situ Hybridization

In situ hybridization was performed as described previously [21], with minor modifications. Paraffin sections were dewaxed in xylene and rehydrated in ethanol before rinsing in DEPC-treated water. Deproteinization was performed with 0.2 M HCl for 20 min at room temperature, followed by digestion with proteinase K at 5 μg/ml in 0.1 M Tris buffer (pH 8.0) and 50 mM EDTA for 30 min at 37°C, followed by 2 mg/ml of glycine in DEPC-PBS for 5 min. The tissues were then fixed in 4% paraformaldehyde in DEPC-PBS for 15 min. Slides were rinsed in DEPC-PBS between each pretreatment, and all procedures were carried out at room temperature unless indicated otherwise. Prehybridization was performed at 37°C for 1 hour in hybridization buffer, which consisted of 50% formamide, 5-fold SSC (SSC contains 0.15 M NaCl and 0.15 M sodium citrate pH 7.0), 2% blocking reagent, 0.1% *N*-lauroylsarcosine, and 0.02% sodium dodecyl sulfate. The DIG-labelled riboprobe was diluted to a final concentration of 1 ng/μl, and 25 μl was applied to each slide. Slides were protected with converslips and incubated for 16-18 hours at 42°C in a humidified chamber. Following overnight hybridization, slides were washed in 2× SSC at 37°C for 15 min. Excess probe was removed by treatment with 25 μg/ml RNase A (Invitrogen) in 2× SSC at 37°C for 30 min, and the slides were washed in decreasing concentrations of SSC at 37°C for 15 min each. The hybridized probes were detected with the antidigoxigenin antibody coupled to alkaline phosphatase (Roche Molecular Biochemicals), used according to the manufacturer's instructions. To ensure reproducibility, each probe was hybridized to at least four serial sections from each sample. The results shown are representative samples from each time point. To ensure specificity of the signal, negative controls were utilized where sections were either hybridized with a sense riboprobe or treated with 200 μg/ml RNase A in 2× SSC (2 h at 37°C) prior to the prehybridization step, then subsequently hybridized with antisense riboprobe as described above. All sections were lightly counterstained with Methyl Green and aqueous mounted before being Depex coverslipped.

Immunohistochemistry

Immunohistochemical studies were performed using the standard indirect immunoperoxidase method, with minor modifications. Briefly, slides were deparaffinized in xylene and rehydrated through a series of ethanol washes. Pretreatment with 0.05% (w/v) trypsin (Invitrogen) in 1× PBS, for 20 min at 37°C was performed to enhance the immunoreactivity for PEDF by reducing the masking of antigens by glycosaminoglycans, followed by 2 washes in 1× PBS. Sections were then incubated for 10 min with 3% (w/v) hydrogen peroxide in methanol to inhibit endogenous peroxidase activity. After two washes in 1× PBS, nonspecific binding was blocked by incubating the slides in 10% (v/v) normal rabbit serum (Dako) in 1x PBS for 60 min at room temperature. Slides were then overlaid with the primary antibody (polyclonal goat antibody, Santa Cruz, sc-16956, cross-reactive with PEDF of mouse origin) diluted in 1× PBS. After incubation overnight at 4°C and 2 washes in 1× PBS, slides were incubated in the secondary antibody (HRP-labelled rabbit anti-goat IgG, Dako) at 1:200 dilution in 1× PBS for 1 hour, followed by a further 2 washes in PBS. Specific antibody binding was visualized using diaminobenzidine (Sigma). Sections were counterstained with hematoxylin, dehydrated through increasing concentrations of ethanol, and cleared in xylene, before being Depex-mounted under coverslips. To confirm the specificity of antibody staining, for each specimen analysed controls included sections in which the primary or secondary antibody was substituted with 1× PBS.

Results

A similar pattern of staining for PEDF protein and mRNA was observed across the different timepoints of growing mouse long bone examined. PEDF expression was observed in epiphyseal cartilage and in areas of active bone remodelling in the primary spongiosa and periosteum of metaphyseal bone. PEDF was also present in the surrounding skeletal muscle and collagen-rich connective tissues around blood vessels and muscle fibers, as well as in tendino-ligamentous structures.

Localization of PEDF in Epiphyseal Cartilage

In situ hybridization demonstrated high levels of PEDF transcripts in areas of endochondral ossification. Strong hybridization signal for PEDF was detected in resting, proliferative and upper hypertrophic chondrocytes of epiphyseal cartilage throughout the chronological development of long bone (Fig. 1A, Fig. 2A, C).

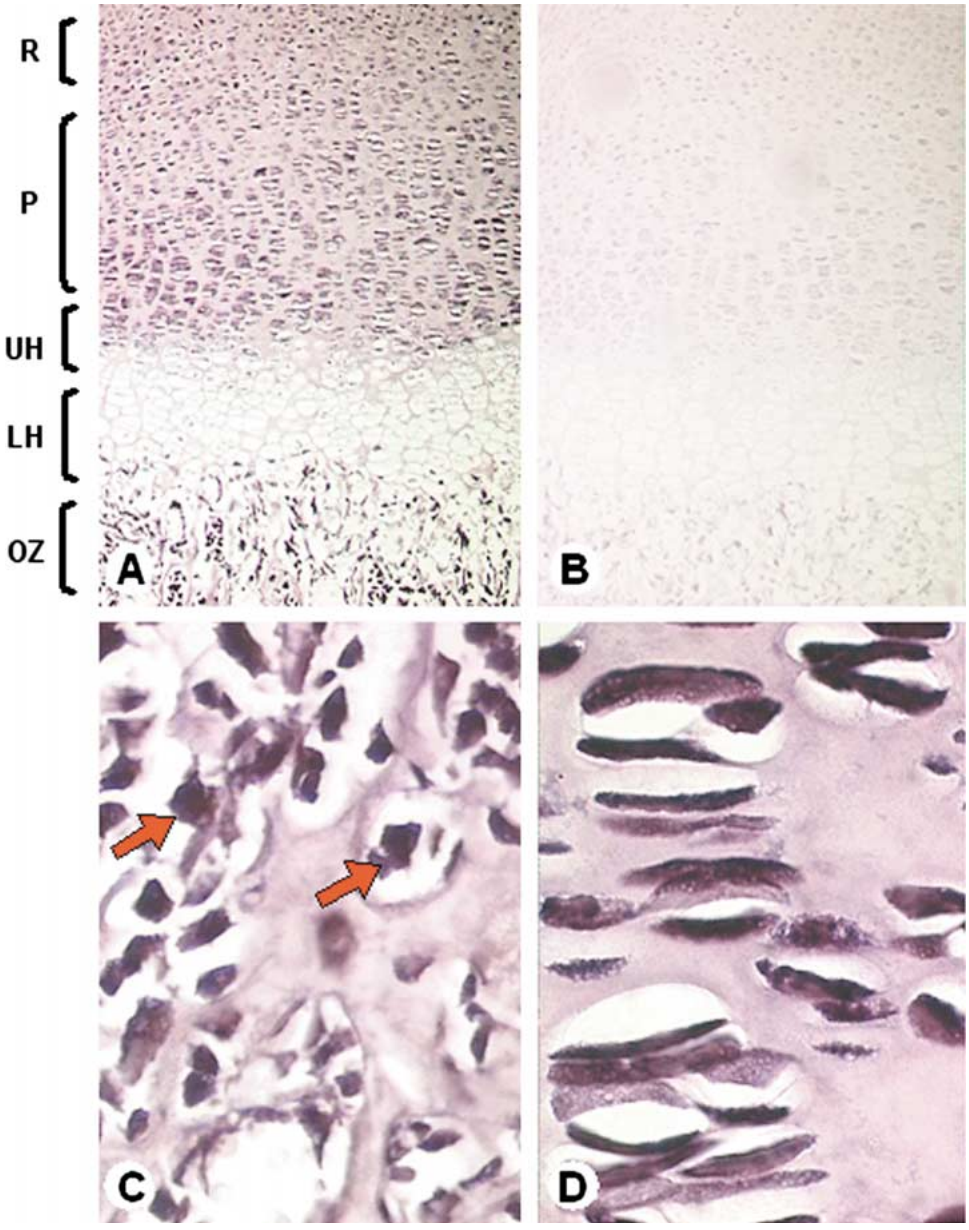

Fig. 1. Localization of PEDF by *in situ* hybridization in newborn mouse tibia. (**A**) Strong hybridization signal for PEDF mRNA (purple color) is detected within resting (R), proliferative (P) and upper hypertrophic (UH) chondrocytes of epiphyseal cartilage, as well as in the ossification zone (OZ) in the proximal tibiae of a newborn (day one) mouse. There is weak-to-absent signal in lower hypertrophic (LH) chondrocytes (×100). (**B**) No hybridization signal is observed with an RNase-pretreated control (×100). Higher magnification views of the ossification zone (**C**) and growth plate (**D**) show strong hybridization signal in active osteoblasts lining bone trabeculae (arrows) and in the cytoplasm of the chondrocytes (×500).

Hybridization signal was restricted mainly to the cytoplasm of the chondrocytes. There was a faint hybridization signal observed in a few lower hypertrophic chondrocytes, but in the majority of these cells hybridization signal was not found. Hybridization signal was absent in the corresponding negative control sections, which included sections incubated with sense probe, and ribonuclease (RNase) pretreatment of sections prior to the addition of the antisense probe (Fig. 1B, Fig. 2B).

Consistent with the *in situ* hybridization results, immunohistochemistry staining showed strong immunoreactivity for PEDF protein within the resting, proliferative and upper hypertrophic zones of the growth plate cartilage (Fig. 3A, D, G). Immunostaining for PEDF was observed mainly in the inter-territorial regions of the matrix in resting, proliferative and upper hypertrophic chondrocytes, and especially around lacunae (Fig. 3 h). Fainter staining was present in the cytoplasm of the majority of the chondrocytes within these zones. This is consistent with PEDF being a secreted protein that binds with high affinity to glycosaminoglycans and collagens in the extracellular matrix [10]. Within the hypertrophic zone, there was a gradual decrease in staining intensity for PEDF as chondrocytes differentiated towards the base of the growth plate. In the lower hypertrophic zone the majority of cells did not stain, although very weak staining was observed in some parts of the niter-territorial matrix around these chondrocytes. No immunostaining was observed in all negative controls in which the anti-PEDF primary antibody was substituted with 1× PBS (Fig. 3).

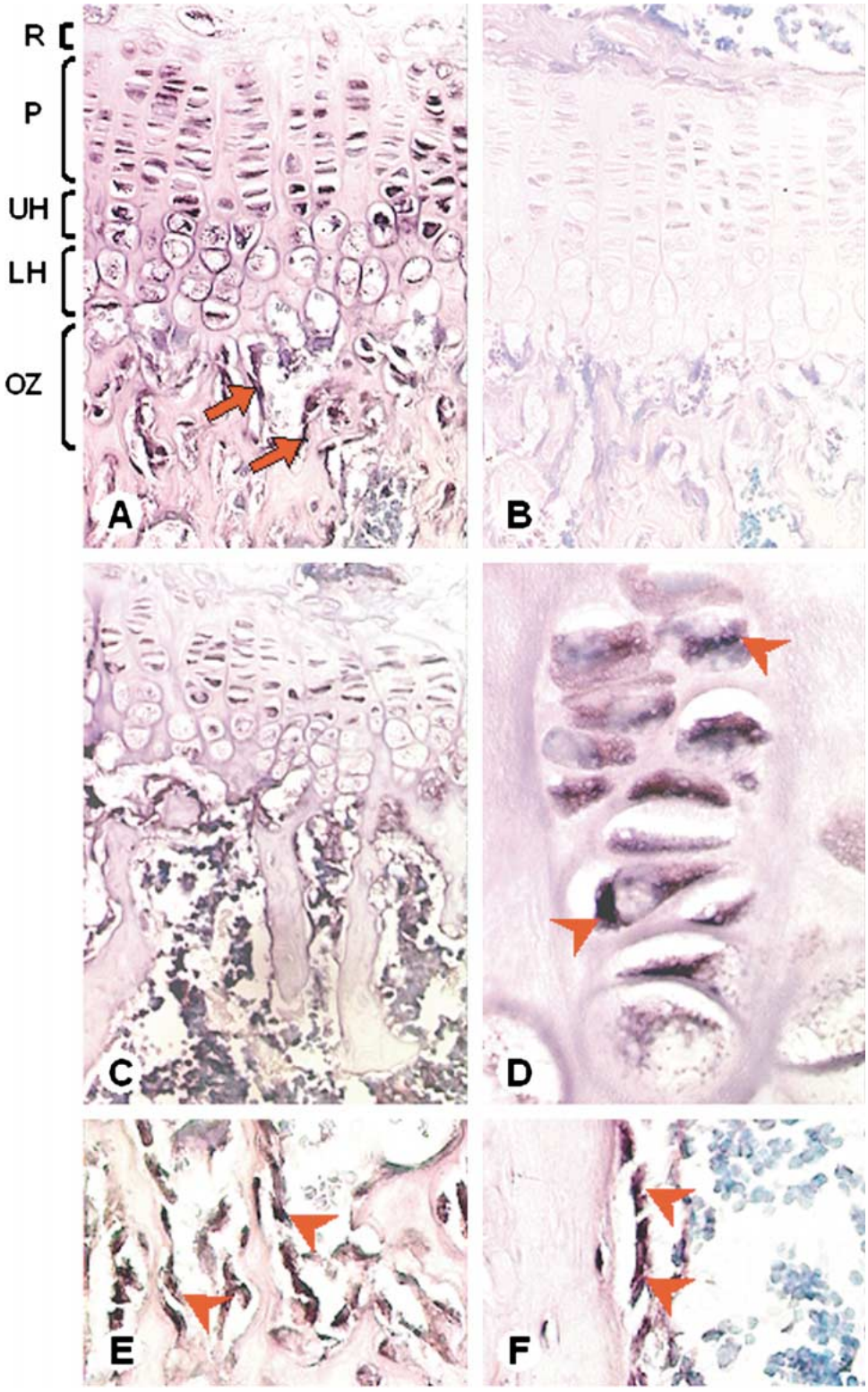

Fig. 2. Localization of PEDF by *in situ* hybridization in 5 and 9-week-old mouse tibia. Photomicrographs shown are of representative sections of the proximal tibia of a 5-week-old (**A, B**) and 9 week-old (**C, D**) mouse. (**A**) Strong hybridization signal for PEDF mRNA is detected in resting (**R**), proliferative (**P**) and upper hypertrophic (**UH**) growth plate chondrocytes, and in osteoblasts within the ossification zone (arrows, OZ). There is weak-to-absent signal in lower hypertrophic (**LH**) with an RNase-pretreated control (×100). (**C**) In older, 9 week-old mice, the expression pattern of PEDF mRNA is similar to that of 5-week-old mice (×100). (**D**) Magnified view of the proliferative zone of the growth plate shows strong hybridization signal, mainly in the cytoplasm of the chondrocytes (arrowheads, ×500). (**E**) Strong hybridization signal for PEDF is also detected in active osteoblasts lining bone spicules in the ossification zone of the primary spongiosa (arrowheads) and in

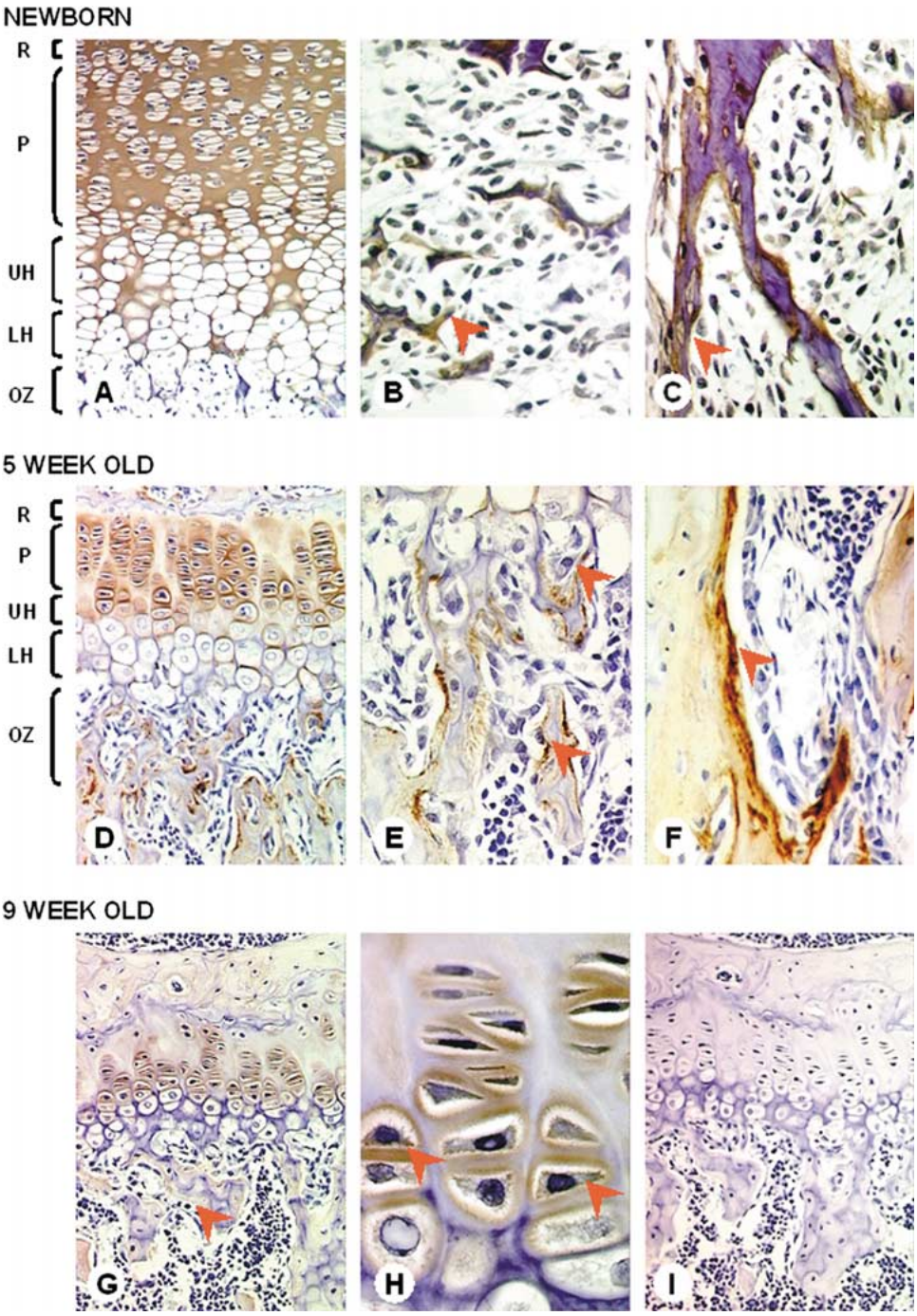

Fig. 3. Temporospatial pattern of PEDF protein expression in developing mouse tibiae. Photomicrographs shown are of representative sections of the proximal tibia of a newborn (**A**–**C**), 5-week-old (**D**–**F**) and 9-week-old (panels **G**–**I**) mouse. PEDF immunoreactivity (brown color) is consistently observed in chondrocytes, around lacunae and in the inter-territorial matrix of the resting (R), proliferative (P) and upper hypertrophic (UH) zones of the growth plate, as well as in the ossification zone (OZ) of the tibia, in newborn (**A**), 5-week-old (**D**) and 9-week-old (**G**) mice. There is weak to absent immunostaining for PEDF in the lower hypertrophic (LH) zone (×100). PEDF protein is also detected in the newly formed bone matrix of the ossification zone, adjacent to metaphyseal osteoblasts (arrowsheads), in newborn (**B**) and 5-week-old (**E**) mice (×200). (**G**) With maturation (9-week-old mice) and fewer active osteoblasts in the ossification zone, there is markedly reduced staining for PEDF in the primary spongiosa (arrowhead, ×100). Strong immunoreactivity for PEDF protein is also observed in the periosteal bone matrix adjacent to metaphyseal osteoblasts (arrowheads, **C** and **F**) (×200). (**H**) Within the epiphyseal cartilage, staining for PEDF is most intense in the matrix adjacent to the chondrocytes, with lighter staining within the cytoplasm of the chondrocytes (arrowheads, ×500). (**I**) Negative control section in which the primary antibody was substituted with 1× PBS.

Localization of PEDF in Remodelling Bone

PEDF protein and mRNA were also detected in areas of active bone remodelling. There was a strong hybridization signal for PEDF mRNA observed in osteoblasts lining the newly formed bone spicules in the ossification zone of the primary spongiosa, adjacent to the growth plate (Fig. 1C, Fig. 2A, E). Hybridization signal for PEDF mRNA was also detected in active osteoblasts localized along the surface of the periosteum of cortical bone (Fig. 2F). Positive immunostaining for PEDF protein was observed predominantly in the bone matrix of the trabeculae of the primary spongiosa and the periosteum, adjacent to osteoblasts, in the presence of only faint staining within the osteoblasts themselves (Fig. 3). After long bone growth had been accomplished (in 9-week-old mice), there were markedly fewer active osteoblasts in the ossification zone of the primary spongiosa. Accordingly, there was reduced immunostaining for PEDF protein in metaphyseal bone trabeculae of the ossification zone in 9-week-old mice compared with 5-week-old mice (Fig. 3D, G). There was absent immunostaining and hybridization signal for PEDF protein and mRNA in the majority of hematopoetic bone marrow cells at all timepoints.

Discussion

Following our novel observation of PEDF in postnatal mouse growth plate cartilage [20], we investigated the temporal expression pattern of PEDF during the progression of endochondral ossification in mouse long bone using immunohistochemistry and *in situ* hybridization. A similar pattern of staining for PEDF protein and mRNA was observed across the different timepoints of developing mouse bone examined PEDF expression was observed in epiphyseal cartilage and in areas of endochondral ossification and active bone remodelling in the primary spongiosa and periosteum of metaphyseal bone. PEDF was also present in the surrounding skeletal muscle and collagen-rich connective tissues around blood vessels and muscle fibers, as well as in tendinoligamentous structures.

Within epiphyseal cartilage, strong staining for PEDF mRNA and protein was observed in the resting, proliferative and upper hypertrophic zones of epiphyseal cartilage. The localization of PEDF to these avascular growth plate layers is compatible with its potent anti-angiogenic properties, and is consistent with studies that have shown the presence of specific factors with angiogenesis inhibitory properties in these layers [22–25]. In the hypertrophic zone of epiphyseal cartilage, there was a decrease in intensity of both immunostaining and *in situ* hybridization signal for PEDF as chondrocytes underwent hypertrophy, terminal differentiation and mineralization towards the base of the growth plate.

The expression pattern of PEDF in the upper layers of epiphyseal cartilage, but not in the lower hypertrophic chondrocyte layers, opposes that of VEGF. VEGF is a potent mitogenic and chemotactic agent for endothelial cells. Immunohistochemical analyses of human neonatal growth plates have shown high expression of VEGF in chondrocytes of the lower hypertrophic and mineralised zones, with absent expression in the resting and upper proliferative chondrocytes [15, 26]. The upregulation of VEGF and other angiogenic factors during chondrocyte hypertrophy has also been demonstrated *in vitro* [17, 27, 28]. The expression of these pro-angiogenic factors by hypertrophic chondrocytes stimulates blood vessel invasion from the perichondral vascular network into the growth plate. These vessels bring with them osteoclast and osteoblast progenitor cells, which degrade the mineralized cartilaginous matrix and replace it with bone. When the cumulative balance between pro- and anti-angiogenic factors dictating the precise transition of epiphyseal cartilage from an angiostatic to an angiogenic phenotype is disturbed, marked disturbances in growth plate architecture and abnormal longitudinal growth ensues [29, 30]. The contrasting expression patterns of PEDF and VEGF within epiphyseal cartilage are akin to their reciprocal expression patterns in the eye, where an underlying cooperative relationship between VEGF and PEDF appears to maintain vascular quiescence. Similar to the eye, where a shift in the equilibrium between PEDF and VEGF promotes growth of blood vessels [13], the opposing expression pattern of PEDF and VEGF in epiphyseal cartilage implies their significance as key modulators of angiogenesis and the vascular invasion that occurs during endochondral bone formation. Indeed, we showed that the balance between PEDF and VEGF in the growth plate dictated tumor growth and invasion in a mouse model of osteosarcoma [20]. Advanced tumors were able to penetrate the VEGF-expressing lower growth plate zones, but were consistently unable to invade the anti-angiogenic PEDF-expressing zones.

Our *in situ* hybridization studies showed a strong signal for PEDF mRNA in the cytoplasm of epiphyseal chondrocytes, whereas immunohistochemical staining showed that PEDF protein was accumulated predominantly in the inter-territorial matrix of mature to hypertrophic epiphyseal chondrocytes, with fainter staining within the chondrocytes themselves. These combined studies are consistent with PEDF being a rapidly secreted protein that subsequently binds with high affinity to collagen-rich extracellular matrix [31, 32]. PEDF has a unique asymmetric charge distribution whereby positively charged basic residues are concentrated around the C-terminal region, and at the opposite surface, negatively charged acidic residues are concentrated in the N-terminal region. The negatively charged

part of the PEDF molecule binds ionically with positively charged areas of collagen [10]. In contrast, the positively charged surface of PEDF binds ionically to negatively charged glycosaminoglycans [33, 34]. The significance of these opposing binding surfaces remains unclear, although it has been proposed that this may determine its surface localization and consequently modulate its biological effects [34, 35], These dual binding sites may explain the abundance of PEDF in matrix-rich substances such as cartilage and bone.

During the preparation of this manuscript, Tombran-Tink and Barnstable [36] describe the expression of PEDF in osteoblast and osteoclast culture from bone marrow preparations. In the present study, we have shown for the first time the expression of PEDF by osteoblasts *in vivo*. Strong hybridization signal for PEDF mRNA was detected within active osteoblasts lining metaphyseal bone trabeculae in the ossification zone and periosteum. There was relatively faint immunostaining for PEDF within osteoblasts, but strong staining for PEDF protein within the osseous matrix directly adjacent to these cells. This staining pattern is consistent with PEDF being rapidly secreted by osteoblasts and subsequently binding with high-affinity to the newly laid type I collagen, and is similar to the immunostaining pattern observed by Kosaki et al. [35] in murine embryonic tissues. With skeletal maturation and subsequent reduction in numbers and activity of osteoblasts in the ossification zone, there was less staining for PEDF mRNA and protein observed within the primary spongiosa of metaphyseal bone.

The anti-angiogenic effects of PEDF have been associated with induction of endothelial cell apoptosis by up-regulation of the Fas ligand mRNA and surface Fas ligand in actively dividing endothelial cells [37, 38], Terminally differentiated chondrocytes at the base of the growth plate undergo apoptosis before being removed by phagocytic cells to provide space for ingress of blood vessels and bone marrow stromal cells. The mechanisms by which chondrocytes undergo apoptosis are unknown, although there is some evidence that this is mediated in part through the Fas system [39, 40]. As such, it is tempting to speculate that PEDF may play a role in the induction of apoptosis in terminally differentiated chondrocytes and thus play multiple regulatory roles in endochondral ossification.

In light of its potent anti-angiogenic activity, widespread tissue distribution and multiple functions, there has been increasing focus on the potential therapeutic application of PEDF in malignant conditions. There has been speculation that PEDF may be a tumor suppressor, as the gene is located in the short arm of chromosome 17 (17p13) [41], a region that contains the tumor suppressor genes p53 and BRCA-1. Consistent with this, PEDF has been shown in animal studies to suppress the growth of neuroblastoma, Wilm's tumor and prostate cancer, by targeting both the tumor cells as well as their associated vasculature [7–9]. High expression of PEDF within epiphyseal cartilage, together with its anti-angiogenic and anti-tumor properties, may explain the resistance of cartilage to malignant invasion [20]. This warrants further investigation into the use of the PEDF in malignancies affecting bone.

In conclusion, results of the present study have shown the expression of PEDF by chondrocytes in the avascular layers of epiphyseal cartilage and by osteoblasts in areas of new bone formation and remodelling in developing mouse bone. Considering its multiple regulatory roles as an anti-angiogenic factor [6], in the cell cycle [5] and in promoting the differentiation of various cell types [4, 7, 42], PEDF may act in both a paracrine and autocrine fashion by modulating function and differentiation of endothelial cells, chondrocytes, osteoclasts and osteoblasts during early skeletal development. Recently, PEDF null mice have been generated [9]. Analysis of the skeletal phenotype of these mice will provide further insight into the role of this protein during endochondral ossification and bone and connective tissue development.

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