Cell Cycle Genes PEDF and CDKN1C in Growing Deer Antlers

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ABSTRACT

Deer antlers are the only mammalian appendage to display an annual cycle of full regeneration. The growth phase in antler involves the rapid proliferation of several tissues types, including epidermis, dermis, cartilage, bone, blood vessels, and nerves. Antlers thus provide an excellent model to study the developmental regulation of these tissues. We describe here the identification of two genes, pigment epithelium-derived factor (PEDF) and cyclin-dependent kinase inhibitor 1C (CDKN1C), both of which are known to be involved in cell proliferation and differentiation. These genes were identified as the result of screening an expressed sequence tag database derived from a cDNA library enriched for sequences from the growing antler tip. PEDF mRNA was detected in developing skin, cartilage, and bone during endochondral ossification. PEDF mRNA was not detected within endothelial cells that exhibited positive immunoreactivity to a CD146 antibody. CDKN1C mRNA was expressed by only the immature chondrocytes within the precartilage region. These results suggested that PEDF and CDKN1C are important genes involved in cell proliferation and differentiation during antler growth. Anat Rec 290:994–1004, 2007.

Key words: antler; deer; cartilage; angiogenesis; PEDF; CDKN1C; CD146

Annual antler renewal is a fascinating process that provides an accessible and useful model for investigating the mechanisms involved in organ regeneration and rapid tissue growth. Antler growth rates vary according to the species, with sika deer reaching rates of 12.5 mm/day (Gao and Li, 1988), moose 27.5 mm/day (Goss, 1970), and red deer approximately 10 mm/day (Fennessy et al., 1992). Antler regeneration is initiated each year from the bony appendage on the head of the deer, known as a pedicle. Histogenesis of regenerating antlers relies on the stem cells resident within the pedicle periosteum (Mount et al., 2006; Li et al., 2007; Li and Suttie, 2006; Rolf et al., 2006). Once antler growth is initiated from a pedicle, several definable layers are evident within the growing tip and include epidermis, dermis, mesenchyme, precartilage, cartilage, and bone (Li et al., 2002). Elongation of the antler is driven from the tip with the mesenchymal layer cells proliferating to contribute chondroblasts, which then progressively differentiate as the antler extends (Li and Suttie, 1994). The proliferation of vascular associated cells primarily occurs within the precartilage region and to a lesser extent in the upper cartilage and dermis with a recent study describing a possible role of the angiogenic factors vascular endothelial growth factor (VEGF) and Pleiotrophin in this process (Clark et al., 2006a,b). The process of antler growth requires precise cell cycle control to ensure that proliferation and differentiation are occurring in a coordinated manner. Understanding such processes can give valuable

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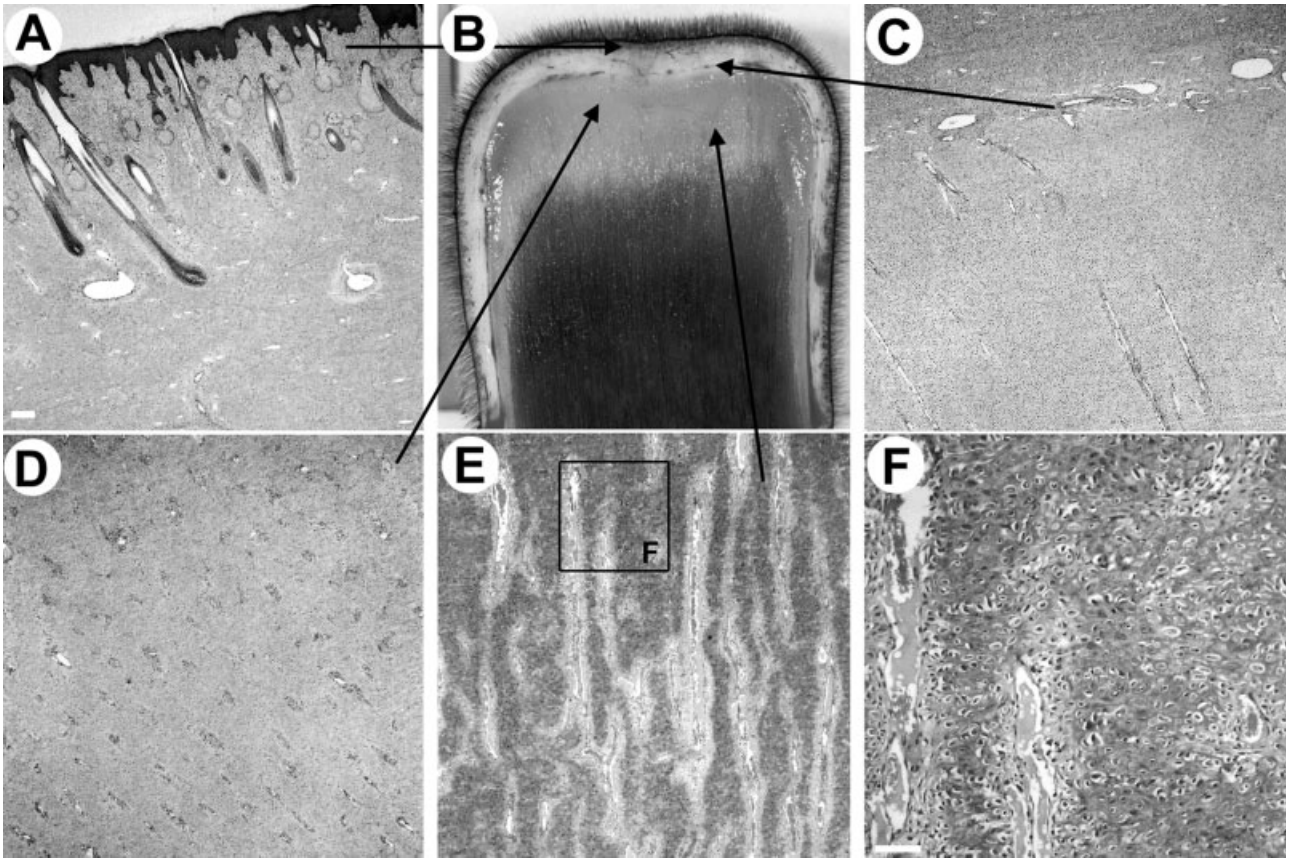

Fig. 1. **B:** A cross-section of the top 5 cm of a growing antler tip. **A,C–E:** Hematoxylin and eosin staining of the upper dermis (A), vascular layer and underlying mesenchyme (C), precartilage (D), and cartilage area (E). **F:** A higher magnification of the cartilage layer shown in E. Scale bars = 200 μm in A (applies to C–E), 100 μm in F.

insights into the control of other rapidly developing tissues such as seen in embryogenesis and pathogenesis.

While both the morphology and histology of antler development have been well studied (Goss, 1995; Kierdorf and Kierdorf, 2001; Kierdorf et al., 2003; Li et al., 2004, 2005; Price et al., 2005), very little is known about the molecular mechanisms involved in regulating the rapid growth of antler. To study gene expression in regenerating antlers we generated an expressed sequence tag (EST) database from a subtracted cDNA library, enriched for transcripts expressed in the growing tip of red deer antlers (Li et al., 2002). Two notable cell cycle control genes represented in the antler EST database were pigment epithelium-derived factor (PEDF) and cyclin-dependent kinase inhibitor 1C (CDKN1C). Expression of these factors is a strong indicator that cells are exiting the cell cycle and are beginning to differentiate.

PEDF has been mostly studied in the retina, where it is a strong inhibitor of angiogenesis (Bouck, 2002), but it is widely expressed in the nervous system where it has both neurotrophic and neuroprotective properties (Tombran-Tink and Barnstable, 2003). A role for PEDF in bone formation was suggested by the expression of PEDF in both cartilage and bone cells during endochondral bone formation in embryonic and newborn mice (Quan et al., 2005). PEDF mRNA has been detected within vascular smooth muscle cells, but not in vascular endothelial cells (Pignolo et al., 1995).

The CDKN1C protein, a cyclin-dependent kinase inhibitor, is a negative regulator of cell proliferation (Lee et al., 1995). Certain cancers originate from underexpression of CDK inhibitors, and the control of cell-cycle proliferation is considered a worthwhile target for cancer research (Lee and Yang, 2001). The CDKN1C gene is located in an imprinted region on human chromosome 11p15.5 and has been implicated in both sporadic cancers and Beckwith-Wiedermann syndrome (Diaz-Meyer et al., 2003), an overgrowth disorder involving tissue and organ hyperplasia and developmental anomalies. CDKN1C is also required for the expression of collagen X during endochondral ossification in bone development (Zhang et al., 1997) and the cell cycle control of hepatocytes during liver development (Awad et al., 2000), and placentas of mice lacking CDKN1C expression show trophoblastic hyperplasia (Matsuura et al., 2002).

We demonstrated the use of CD146 immunoreactivity as a tool to identify endothelial cells in the growing tip of red deer antlers. CD146, also known as MUC18, is a cell adhesion transmembrane molecule of the immunoglobulin super gene family (Johnson, 1991) and is a recognized endothelial cell marker (Bardin et al., 1996). CD146

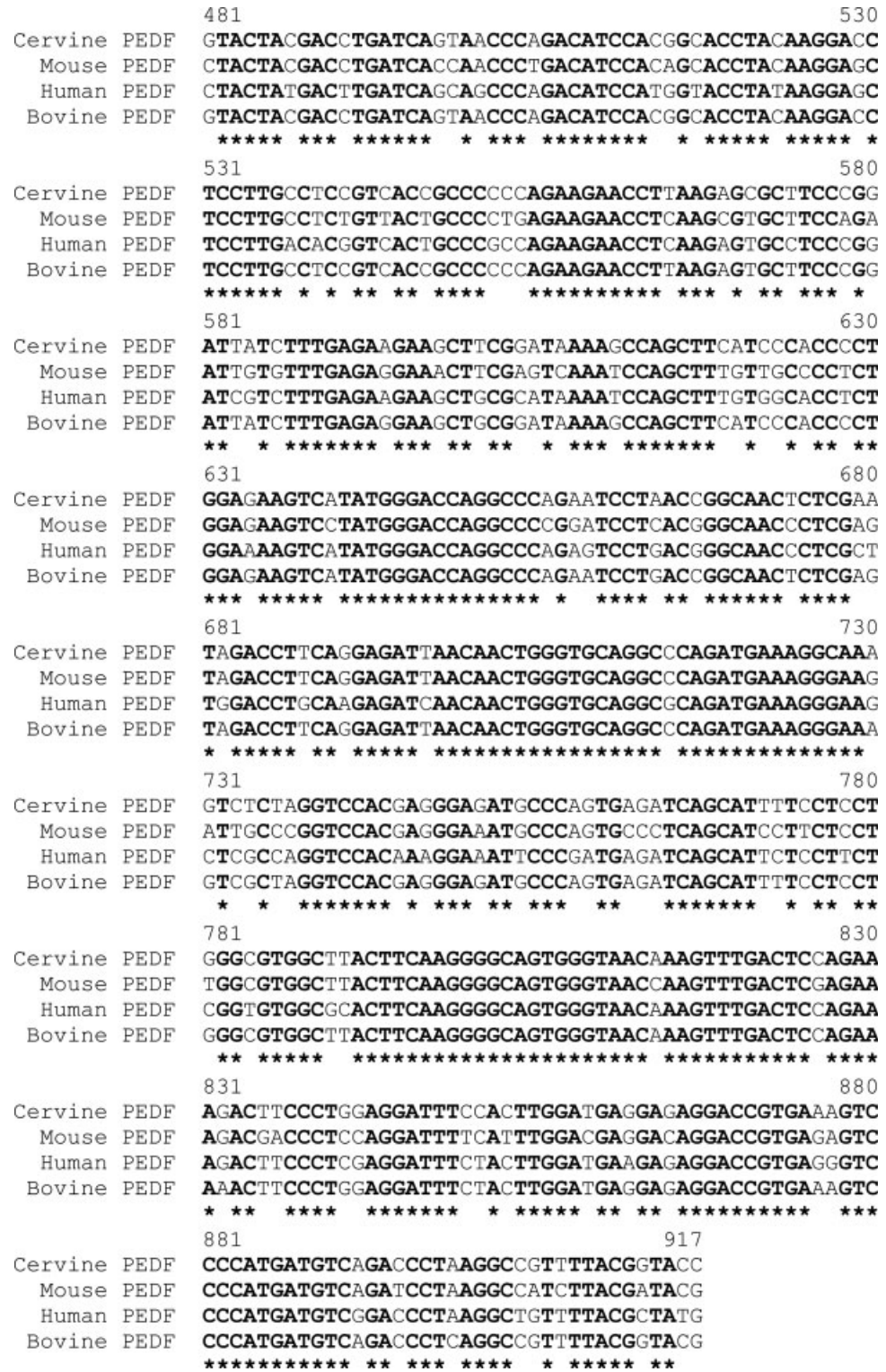

Fig. 2. Alignment of the cervine pigment epithelium-derived factor (PEDF) sequence with mouse, human, and bovine PEDF cDNA sequences (Genbank accession nos. NM_011340, NM_002615, and BTU48229, respectively). Nucleotide numbering shown is based on the human sequence where the coding region runs from 158–1,414 bp. The cervine PEDF sequence aligns within the coding sequence and has 98%, 85%, and 84% identity to the bovine, human, and mouse cDNA sequences, respectively.

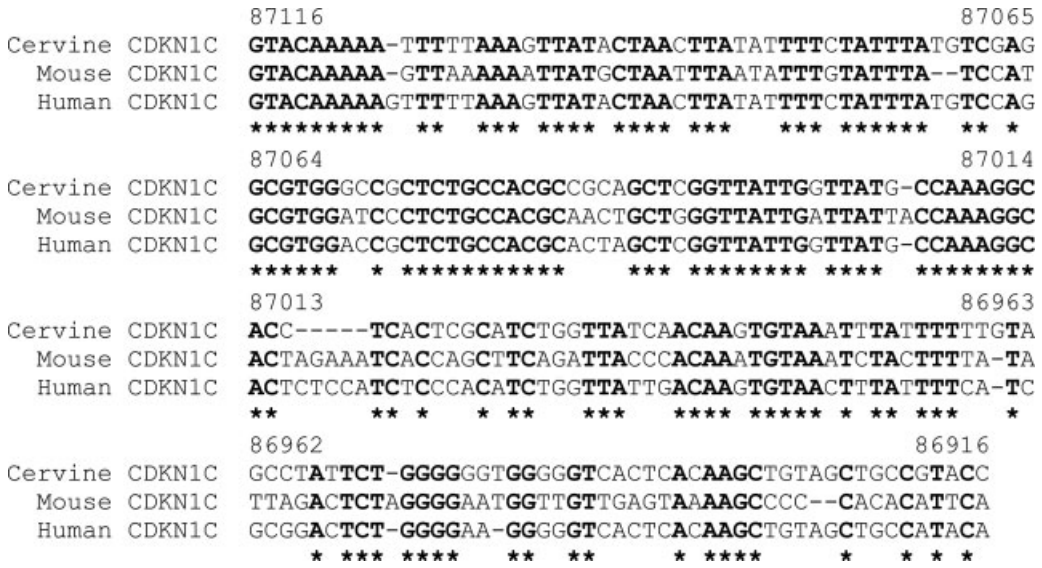

Fig. 3. Alignment of the cervine cyclin-dependent kinase inhibitor 1C (CDKN1C) sequence with mouse and human CDKN1C cDNA sequences (Genbank accession nos. NM_009876 and NM_000076 in the reverse). Nucleotide numbering shown is based on the human sequence. The cervine CDKN1C sequence aligns within the 3′-untranslated region and has 85% and 66% identity to the human and mouse cDNA sequences, respectively.

immunohistochemistry revealed that PEDF mRNA was not found in association with the endothelial cells of the antler. In conclusion, we describe partial cDNA sequences and the expression profiles of mRNA for PEDF and CDKN1C in the growing tip of antler to demonstrate that regenerating antlers are an exciting model to study the molecular aspects of tissue development.

MATERIALS AND METHODS

Antler tissue was collected from red deer 55 to 60 days after casting of the previous hard antler. Antler removal was conducted in accordance with regulations set by the New Zealand National Velveting Standards Board. Local anesthetic (bromocaine) was injected around the junction of the pedicle and the skull before the whole antler was removed above the junction of pedicle and antler. The distal 5 cm of each antler main beam was used for this study. The tissue was rapidly cut into longitudinal strips then stored by one of the following methods: placed directly in liquid nitrogen for RNA extraction, surrounded by Tissue-Tek OCT compound and frozen in liquid nitrogen-cooled isopentane (Sakura, Finetek, USA, Torrance, CA) for immunohistochemistry, or fixed in 10% neutral buffered formalin for 12 hr before wax embedding for in situ hybridization. All deer were supplied and maintained by the AgResearch Invermay Farm.

Details on construction of the subtracted cDNA library have been previously described (Li et al., 2002). Approximately 5,000 randomly selected cDNA clones were sequenced from the 5′-end using an automated sequencer. Vector and cloning primer sequences were removed, and the initial analysis of the resulting cDNA sequences was done by alignment to both known genes and ESTs from other species using the BLAST algorithm (Altschul et al., 1997). ESTs were assembled into contigs (two or more ESTs representing the same gene) or singletons (EST appeared only once) using CAP3 (Huang and Madan, 1999).

The expression of PEDF and CDKN1C within the growing tip of antler was determined by in situ hybridization as previously described (Clark et al., 1996). Antisense and sense cervine RNA probes were generated using ^{33}P[UTP] and hybridized onto antler tissues before development to reveal the silver grains. The probes used for in situ hybridization comprised the partial coding sequence of PEDF and the 3′-untranslated sequence of CDKN1C (Figs. 2, 3). The expression patterns throughout the growing antler tip were related to the antler tissue layers as previously described (Li et al., 2002), namely, dermis, mesenchyme, precartilage, and cartilage.

CD146 immunohistochemistry was conducted on frozen sections. The sections were fixed in 10% neutral buffered formalin for 10 min then washed in Tris buffered saline (TBS; 3 × 5 min). Endogenous peroxidases were blocked by the addition of 3% H_2O_2 in TBS for 30 min followed by washing in TBS. The blocking reagent from the TSA-Indirect kit (NEN Life Science Products, Boston, MA) was applied to the sections for 30 min before addition of either CD146 antibody (Chemicon International, Temecula, CA) or mouse IgG at 10 μg/ml in blocking reagent. The primary antibody was incubated overnight at 4°C and then washed in TBS (3 × 5 min). Sections were then incubated for 30 min with the biotinylated secondary anti-mouse antibody (Zymed, San Francisco, CA) diluted 1:300 in blocking reagent. Washing in TBS (2 × 5 min) was followed by incubation with a streptavidin–horseradish peroxidase (HRP) conjugate (Zymed) at 1:500 for 30 min. Washing in TBS with 0.05% Tween (2 × 5 min) preceded Tyramide Signal Amplification. Slides were incubated in biotinyl tyramide at 1:50 dilu-

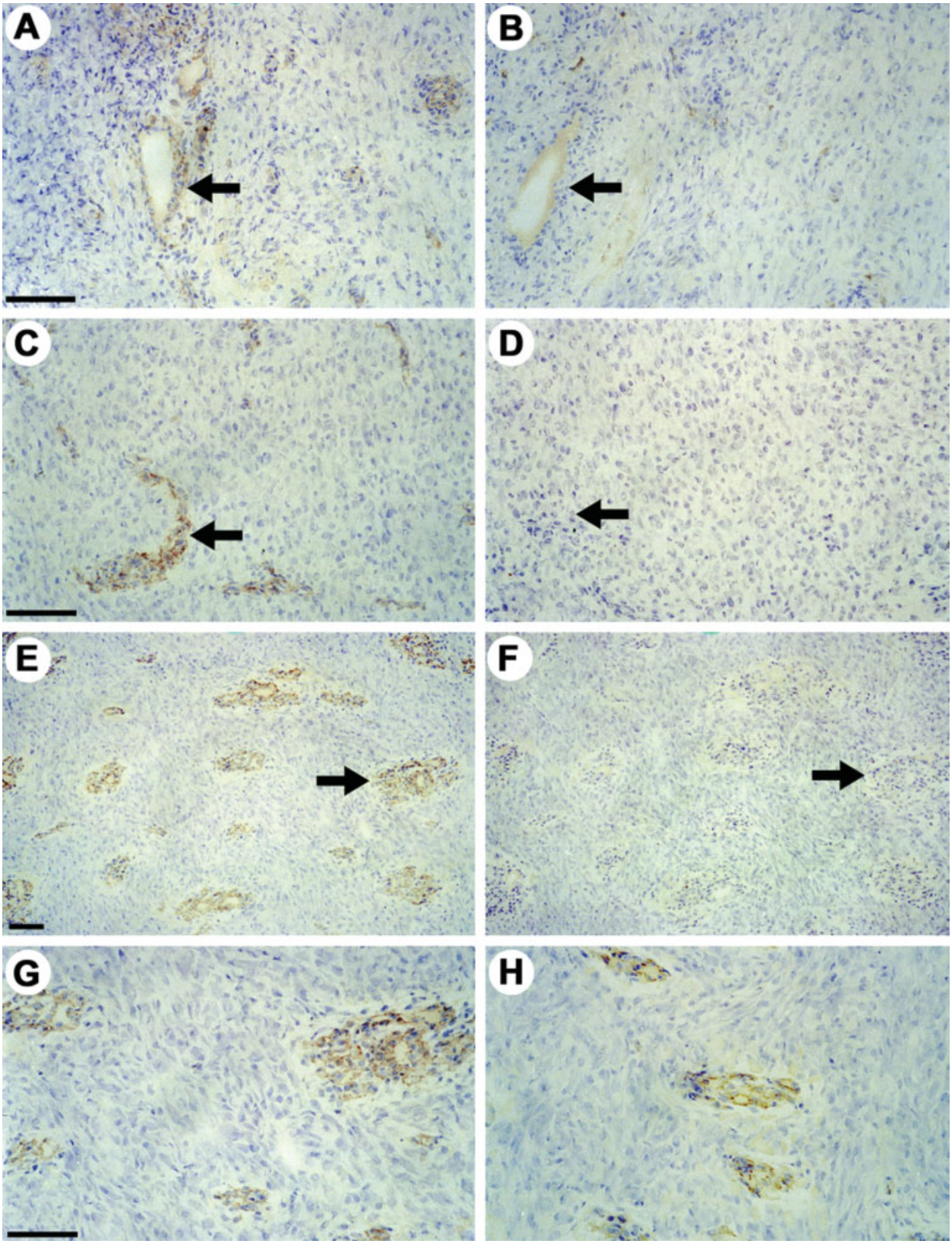

Fig. 4. CD146 immunoreactivity within the growing antler tip. **A,B:** Staining of the vascular layer at the base of the dermis with anti-CD146 (A) and control (B). **C,D:** Vessels of the mesenchyme contained positive anti-CD146 signal (C), which was not in the control (D). **E,F:** Vessels of the precartilage region were clearly labeled for anti-CD146 (E) but not with control IgG (F). **G,H:** The precartilage vessels at higher magnification. Arrows indicate blood vessels. Scale bars = 100 μm in A,C,E,G (applies to B,D,F,H, respectively).

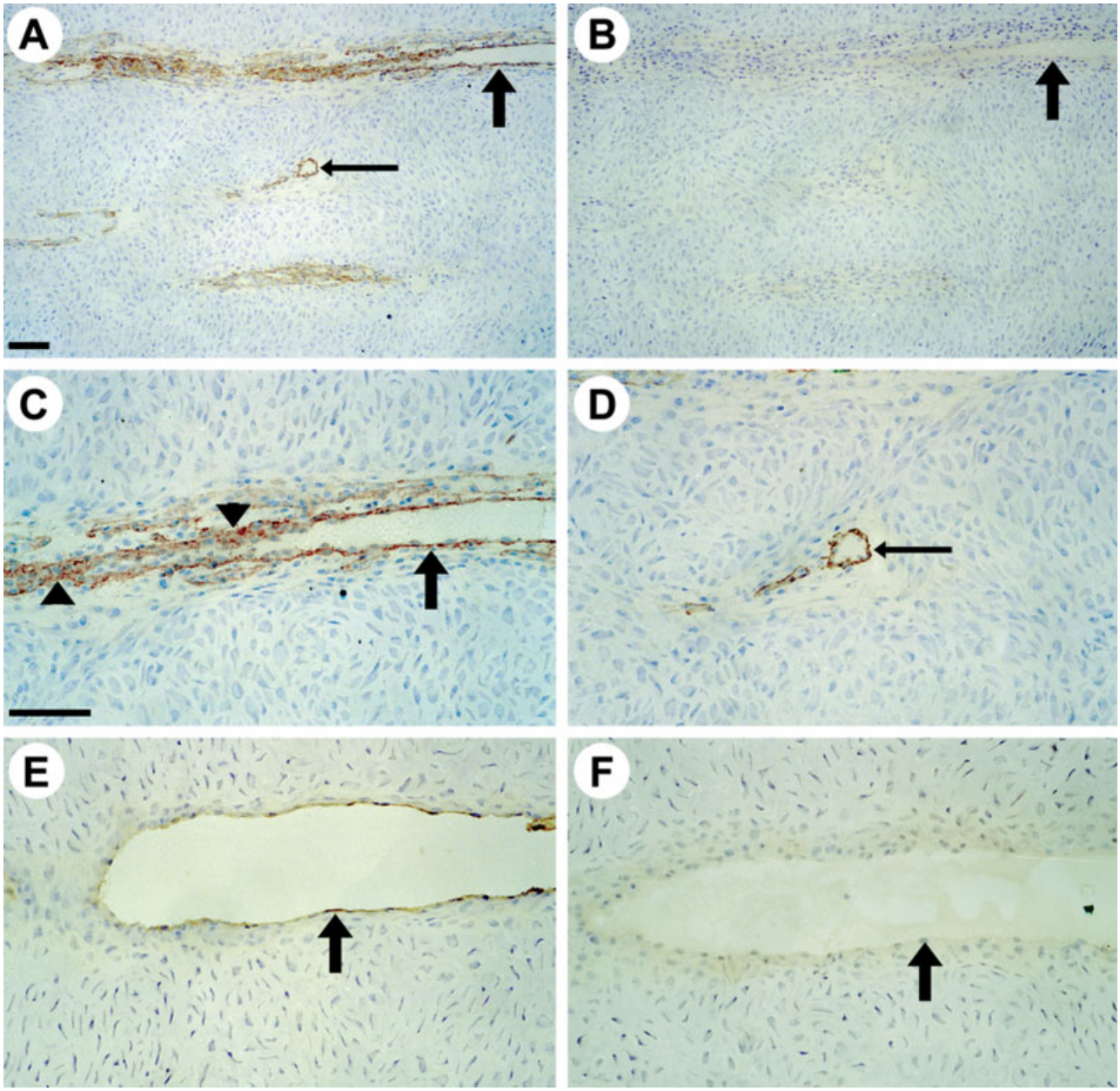

Fig. 5. CD146 immunoreactivity in the cartilage layers of the growing antler. **A:** Within the more proximal cartilage region arrows indicate staining of endothelial cells that line the vascular channels. **C,D:** At a higher magnification, anti-CD146–positive staining appears to be associated with endothelial cells but is at times more than one cell deep (arrowheads). **E:** Vessels within the more mature proximal cartilage have lightly stained anti-CD146–positive endothelial cells. **B,F:** Controls, with very light nonspecific staining inside the vascular channels. Scale bars = 100 μm in A (applies to B), 100 μm in C (applies to C–F).

tion for 10 min then washed in TBS (3 × 5 min) and incubated in streptavidin-HRP as above. Development in diaminobenzidine (Zymed) for 3 min was followed by a TBS wash. Counterstaining with Gills hematoxylin was undertaken before cover-slipping of the slides.

RESULTS

The histology of the antler tip region is shown in Figure 1. The distal 5 cm of the growing antler tip (Fig. 1B) consists of the epidermis and dermis (Fig. 1A) containing hair follicles, sebaceous glands, and blood vessels supplying the dermal fibroblasts. Proximal to the dermal tissue is a layer of blood vessels, and proximal to this is the mesenchymal tissue that contains the progenitor cells for cartilage growth (Fig. 1C). The precartilage tissue (Fig. 1D) is notable for the maturing chondrocytes separated by small evenly arrayed bundles of blood vessels. Within the cartilage region (Fig. 1E,F), the chondrocytes are arranged in columns separated by vascular

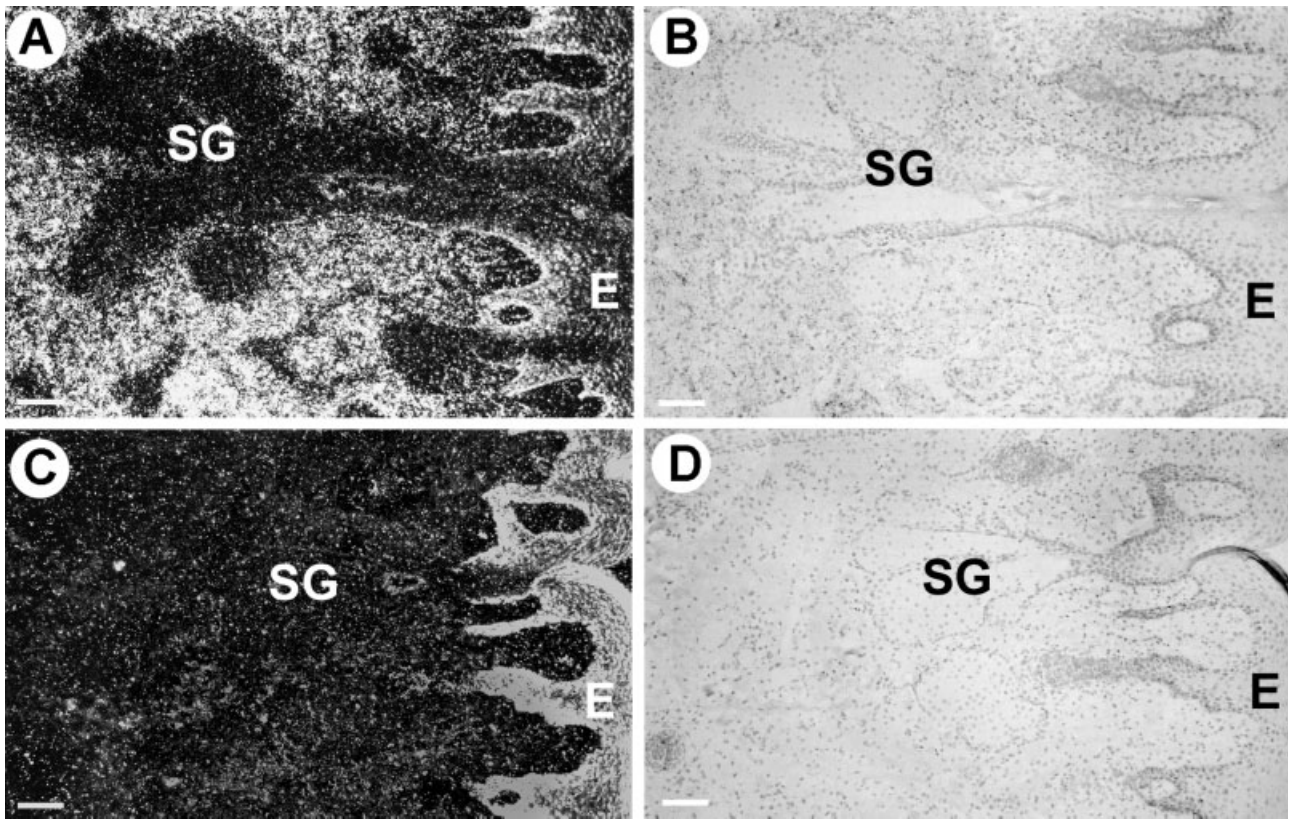

Fig. 6. Expression of pigment epithelium-derived factor (PEDF) mRNA within the growing antler tip as identified by in situ hybridization. Within the dermis, PEDF mRNA was highly expressed within the fibroblasts but not within the sebaceous glands. **A,B:** The antisense probe with darkfield photography and H&E staining, respectively. **C,D:** The sense control probe with darkfield photography and hematoxylin and eosin staining, respectively. The epidermis has auto fluorescence as shown by the control. E, epidermis; SG, sebaceous gland. Scale bars = 100 μm.

channels with associated support cells and immature chondrocytes.

PEDF and CDKN1C were 2 of 930 contigs identified from the cervine antler EST database. PEDF was represented by 20 ESTs and CDKN1C by 26 ESTs of the 5000 sequenced. The 437-bp cervine PEDF sequence aligned within the coding sequence with 98%, 85%, and 84% identity to bovine, human, and mouse PEDF cDNA sequences, respectively (Fig. 2). The cervine PEDF cDNA sequence corresponds to amino acid residues 105 to 253 (GenBank accession no. NM_002615). This amino acid sequence was aligned with the orthologous sequences for the bovine (GenBank accession no. BTU 48229), human (GenBank accession no. NM 002615), and mouse (GenBank accession no. NM011340) PEDF, giving 96.55%, 86.21% and 84.83% identity, respectively.

The 197-bp cervine CDKN1C sequence aligned within the 3′-untranslated region with 85% and 66% identity to human and mouse CDKN1C cDNA sequences, respectively (Fig. 3). The human CDKN1C coding sequence (GenBank accession no. NM_000076) stops 439 bp upstream of the cervine sequence while the mouse coding sequence (GenBank accession no. NM_009876) stops 341 bp upstream.

Immunohistochemistry with the CD146 antibody resulted in staining of the endothelial cells lining the blood vessels within the deer antler (Figs. 4, 5). Within the vascular layer situated proximally to the dermis and the mesenchyme region, anti-CD146–positive immunostaining was detected within both the endothelial and smooth muscle cells of the vessels (Fig. 4A,C). Control tissue (mouse IgG) was either negative or had only light staining within the lumen of the vessels (Fig. 4B,D). The precartilage region had positive anti-CD146 immunostaining of cells within the vascular bundles and associated with both endothelial cells and smooth muscle (Fig. 4E,G,H) and no staining for the IgG-treated control tissue (Fig. 4F). Within the more distal cartilage, CD146 protein was detected in association with the vascular channels (Fig. 5A). No staining was seen within the control tissue (Fig. 5B). At higher magnification, it was evident that the immunostaining was typically only associated with the endothelial cells (Fig. 5C,D). In places, it did appear that the staining was more than one cell thick, which may be an artifact of sectioning where vessels are branching or it may be due to dividing endothelial cells. Within the more mature proximal cartilage, specific anti-CD146 immunostaining was only detected in association with the endothelial cells lining the vascular channels (Fig. 5E). Staining was not detected in the IgG-treated control tissue (Fig. 5F).

PEDF mRNA was readily detectable within the fibroblast cells of the dermis, but was not associated with the vascular cells, sebaceous gland, or hair follicles of the

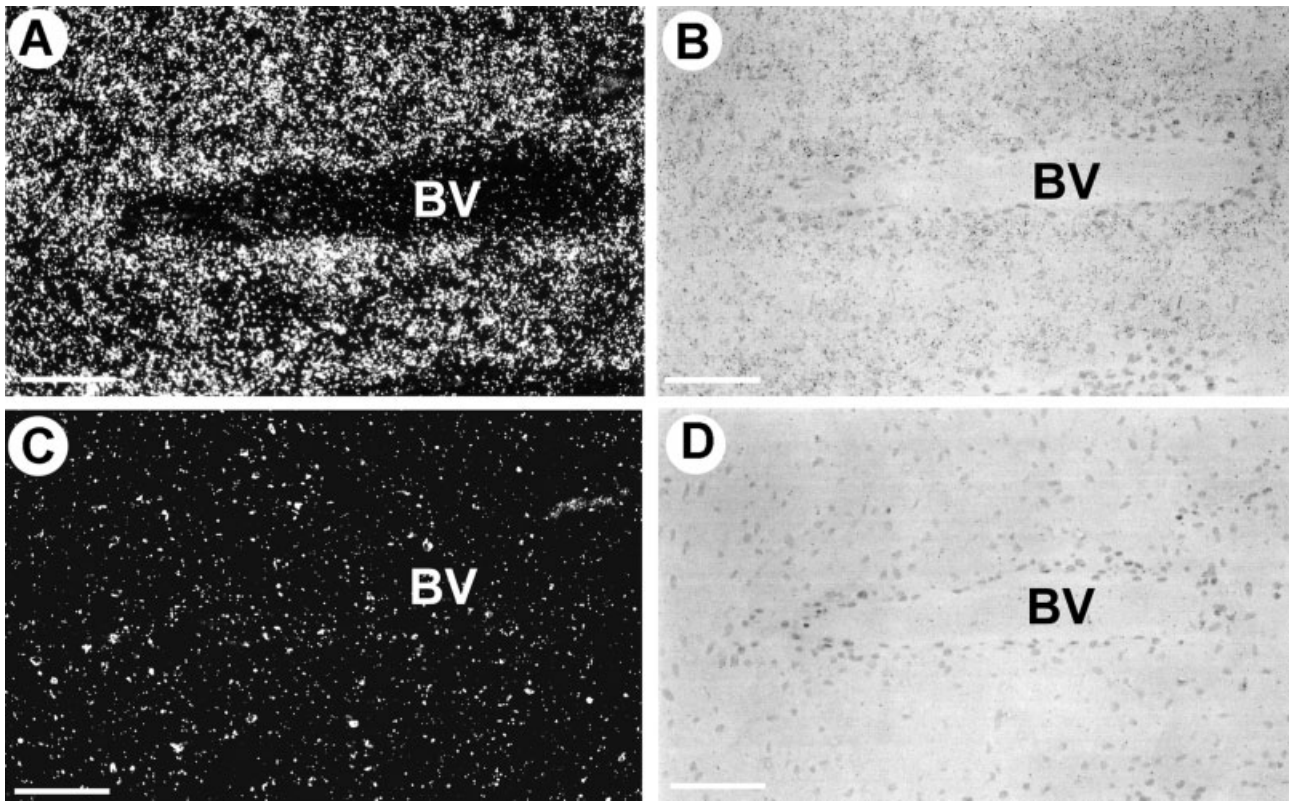

Fig. 7. Pigment epithelium-derived factor (PEDF) mRNA localization within the growing antler tip as identified by in situ hybridization. **A,B:** The distal maturing cartilage region had readily detectable levels of expression (A) but not within the endothelial cells (B). **C,D:** Matching darkfield photography (C) and hematoxylin and eosin stain (D) of the sense control showed only low level background. BV, blood vessel. Scale bars =100 μm.

growing antler tip (Fig. 6). Apparent staining of the epidermis occurs with all probes, including the control and is due to nonspecific autofluorescence. Dermal tissue contained a readily detectable amount of transcript for PEDF. Within the mesenchyme and precartilage and more distal cartilage regions, mRNA for PEDF was detected in association with the chondroblasts and maturing chondrocytes (Fig. 7A,B). Signal was not detected in association with either the endothelial or smooth muscle cells within these regions (Fig. 7). In the more proximal cartilage region, mature chondrocytes form columns separated by blood vessels (Fig. 1E,F). These mature chondrocytes did not produce any detectable PEDF mRNA (Fig. 8). However, cells located just away from the vessels did contain signal for PEDF mRNA. The identity of these cells is unclear, but they may be immature chondrocytes.

The localization of mRNA for CDKN1C was much more confined than for PEDF mRNA. Signal was only detected within the precartilage region (Fig. 9A,B) and only within the maturing chondrocytes and not within the blood vessels of this region (Fig. 9C,D).

DISCUSSION

In this study, we have identified the expression, within the growing tip of antler, of two key genes known to be involved in cell cycle control. These genes are pigment epithelium-derived factor (PEDF) and cyclin-dependent kinase inhibitor 1C (CDKN1C).

PEDF mRNA was detected throughout the growing antler tip in dermal fibroblasts, mesenchymal cells, and maturing chondrocytes (Figs. 6–8), thus suggesting that PEDF may have multiple functions during antler growth. This is the first report of PEDF mRNA expression in dermal cells in situ and may be related to the neurotrophic and neuroprotective roles of PEDF (Tombran-Tink and Barnstable, 2003). Growth rates of 1–2 cm per day require potent stimulation and effective protection of neural development in the rapid growing antler tip. PEDF expression has been associated with the entry into or maintenance of a reversible growth-arrested state in early population doubling level cells (Pignolo et al., 1993). The high expression of PEDF mRNA in dermal and reserve mesenchymal cells may be associated with this activity, as the high growth and differentiation rate in these tissue layers suggests these cells have the characteristics of early population doubling level cells and require PEDF to exclude senescent cells from entering G_o. That PEDF was shown to be highly secreted by 3T3-L1 preadipocytes, but not adipocytes (Kratchmarova et al., 2002), also suggests a role for PEDF in maintaining the high rate of growth and differentiation in the dermal and reserve mesenchymal tissue layers.

PEDF mRNA was not expressed in vascular cells throughout the antler, a finding that is consistent with

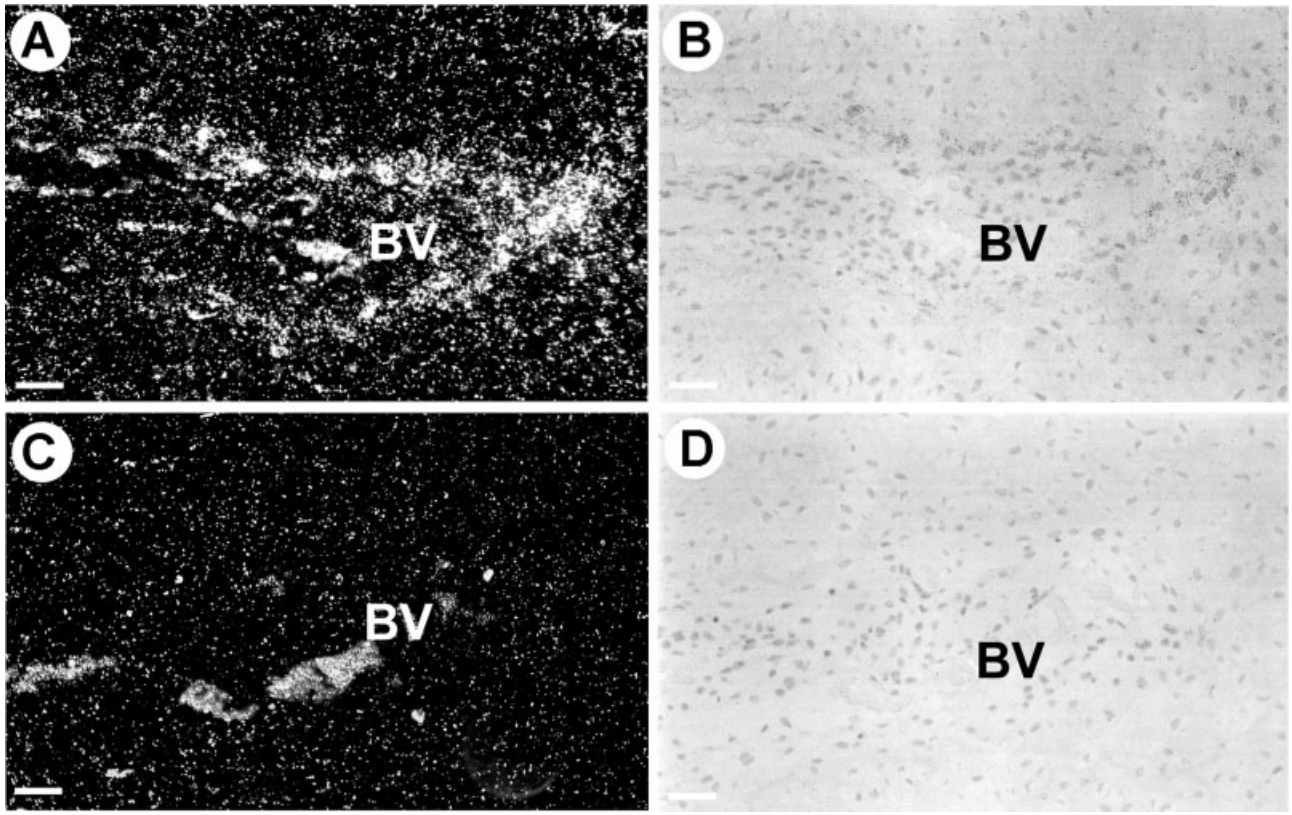

Fig. 8. Pigment epithelium-derived factor (PEDF) mRNA localization within the growing antler tip. The in situ hybridization revealed that, within the more proximal mature cartilage region, mRNA for PEDF could be detected only in association with a population of cells peripheral to the endothelial cells. **A**: Antisense/darkfield. **B:** Hematoxylin and eosin staining. **C,D:** Matching darkfield photography (C) and hematoxylin and eosin staining (D) of the sense control showed only low level background. BV, blood vessel. Scale bars = 100 μm.

the absence of PEDF mRNA in vascular endothelial cells (Pignolo et al., 1995) and indicates that PEDF may not play a direct role in angiogenesis in a growing antler. However, it is reported that PEDF has a synergistic action on cell proliferation in cultured endothelial cells exposed to vascular endothelial growth factor$_{165}$ (VEGF$_{165}$; Hutchings et al., 2002). Therefore, if PEDF has a role in stimulating the growth of vascular channels in antler cartilage, this activity could be dependent on the presence of VEGF$_{165}$ and the VEGF receptors that have been recently described in the precartilage region of the antler tip (Clark et al., 2006b).

PEDF mRNA is present in the region where the remodeling of cartilage to bone begins (Fig. 8). This finding is consistent with the localization of PEDF in regions of active bone formation such as in the embryonic mouse where PEDF is secreted by both osteoblasts and osteoclasts (Tombran-Tink and Barnstable, 2004) and during osteochondral ossification of the developing hindlimb in newborn mice (Quan et al., 2005).

In contrast to PEDF, CDKN1C mRNA was found to be expressed solely by chondrocytes of the precartilage region (Fig. 9). This finding was to be expected as CDKN1C is essential for skeletal growth and development (Takahashi and Nakayama, 2000). In developing long bones of embryonic mice, CDKN1C is expressed at moderate levels in resting chondrocytes, low levels in the proliferative zone, and very high levels in the hypertrophic zone (Zhang et al., 1997). These authors concluded that CDKN1C may have a direct role on chondrocyte differentiation and is required for the expression of collagen Type X and other genes involved with cartilage remodeling to bone. Suppression of CDKN1C mRNA expression during the proliferative phase was shown to be important for enhancing parathyroid hormone-related peptide (PTHrP) expression, which stimulated chondrocyte proliferation during bone development (MacLean et al., 2004).

The use of anti-CD146 immunohistochemistry was crucial for distinguishing endothelial cells within the growing antler tip (Figs. 4, 5). This confirmed PEDF was not expressed by endothelial cells in the dermal and reserve mesenchymal layers. Within the vascular zone at the base of the dermis and the mesenchymal and precartilage regions, the anti-CD146 immunostaining appeared to be associated with both endothelial and smooth muscle cells. Comparison was made with other antibodies, however. Immunohistochemistry for Von Willebrand localized only a subset of endothelial cells within the antler, and alpha smooth muscle actin immunohistochemistry labeled only the vascular smooth muscle (data not shown). Staining of both endothelial and smooth muscle cells with a CD146 antibody has been described by others and the up-regulation of CD146 has

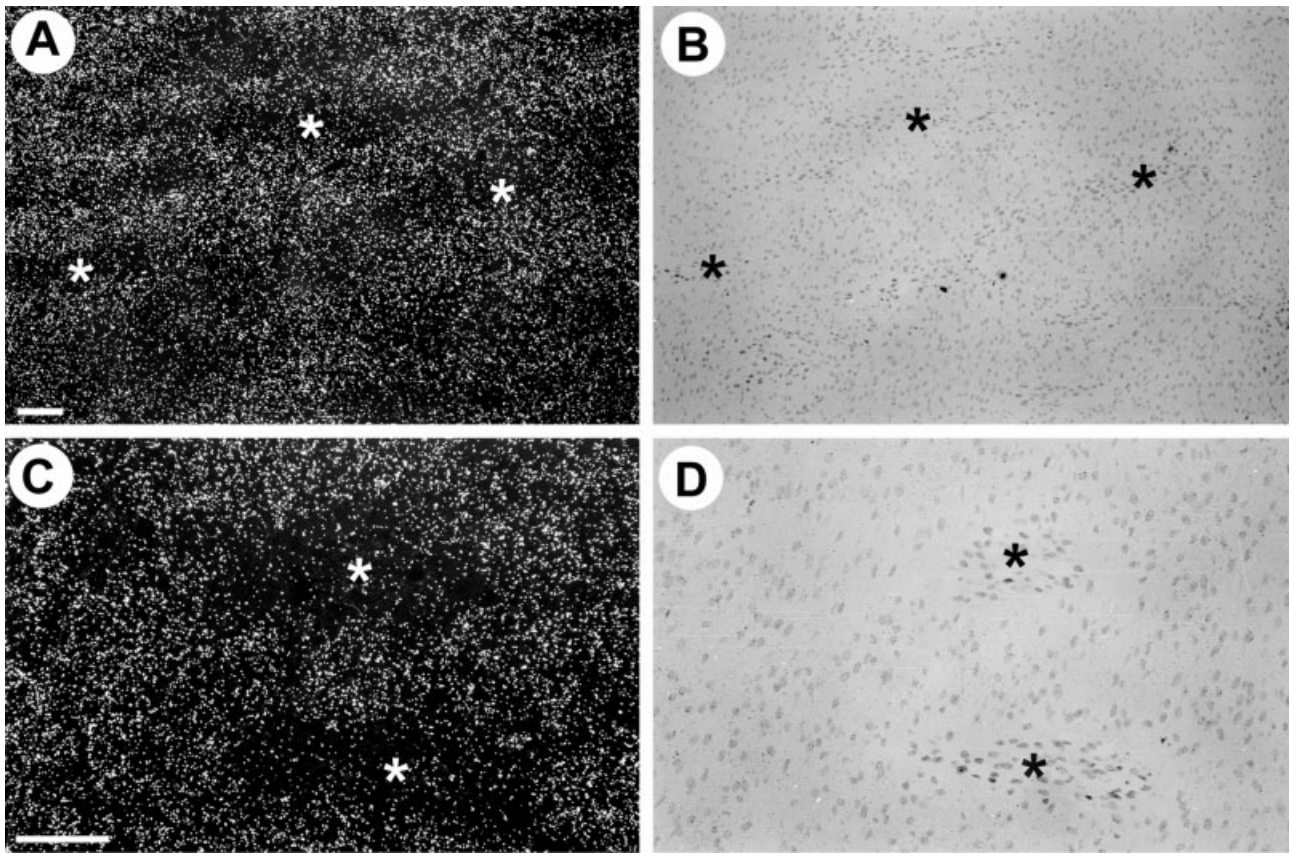

Fig. 9. Expression of cyclin-dependent kinase inhibitor 1C (CDKN1C) mRNA was only detected within the precartilage region of the antler by in situ hybridization. **A,C:** Antisense darkfield. **B,D:** Hematoxylin and eosin staining. C and D are at a higher magnification and show the blood vessels which are not labeled (*). Scale bars = 100 μm in A,C (applies to B,D, respectively).

been reported in response to growth factor induction and cellular proliferation (Sers et al., 1994; Okumura et al., 2004). Cell proliferation is most marked at the upper antler tip regions. In particular, the precartilage and mesenchymal layers are where endothelial cell and chondroblast division occurs, respectively (Clark et al., 2006a). This finding could thus help explain the anti-CD146 immunostaining of both smooth muscle and endothelial cells within these regions.

This first report of PEDF and CDKN1C mRNA expression in the growing antler tip reinforced the use of regenerating antlers as a model for studying the control of skin, nerve, and skeletal tissue development and in aiding the understand the regulatory mechanisms involved in rapid antler growth. Further study of PEDF expression in regenerating antlers will provide valuable information on the various roles of PEDF during tissue growth and development, in particular, defining the opposing antiangiogenic and neurotrophic roles of PEDF, where vascular growth and neural differentiation are occurring together, and understanding the role of PEDF in regulating cartilage development and remodeling to bone. The same applies to further defining the role of CDKN1C in regulating chondrocyte proliferation and differentiation during cartilage development and endochondral ossification.

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