Upregulation of the angiogenic factor heparin affin regulatory peptide by progesterone in rat uterus

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Abstract

Heparin affin regulatory peptide (HARP), also named pleiotropin, is a secreted polypeptide that belongs to a new family of heparin-binding growth/differentiation factors. In this study, we investigated the expression and distribution of HARP mRNA and protein in rat uterus. Semi-quantitative reverse transcriptase PCR experiments showed variations in HARP mRNA levels throughout the estrous cycle, with a maximum during diestrus, pointing to hormonal regulation of HARP mRNA expression. Uterine expression of HARP mRNA was studied in ovariectomized animals treated with 17β-estradiol, progesterone alone or progesterone and RU486. In these experiments, progesterone upregulated HARP mRNA expression. Induction was observed 6 h after progesterone injection and was inhibited by RU486 treatment. In contrast, after 17β-estradiol injection, a slight decrease in HARP mRNA expression was observed. *In situ* hybridization studies with digoxigenin-labeled DNA probe revealed that HARP mRNA was present in smooth muscle cells of both myometrium and blood vessels and also in endothelial cells from endometrium. Immunohistochemical studies showed that HARP expression was not limited to cells that expressed HARP mRNA, but also occurred in both the luminal and glandular epithelium even though its transcript was never detected. We conclude that HARP may mediate the effects of progesterone on the homeostasis and vascularization of uterine tissue.

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Introduction

Rat endometrium undergoes cyclic tissue proliferation and differentiation. These changes are mainly under the influence of two ovarian steroids, 17β-estradiol and progesterone. The proliferative phase is characterized by high levels of 17β-estradiol and intense mitotic activity of stromal and epithelial cells. Under the influence of progesterone, uterine tissues differentiate and endothelial cells proliferate and migrate (Zhou *et al.* 1994, Harrison-Woolrych *et al.* 1995). The action of steroids is mediated by growth/differentiation factors that act in autocrine or paracrine fashion (Brigstock *et al.* 1989, Cullinan-Bove & Koos 1993). For example, 17β-estradiol induces overexpression of mRNA for platelet-derived growth factor (Gray *et al.* 1995), epidermal growth factor and their related receptors in both stromal and epithelial uterine cells (Di Augustine *et al.* 1988, Lingham *et al.* 1988, Huet-Hudson *et al.* 1990). Expression of insulin-like growth factor-I is upregulated in human endometrium during the estrogen-dominated proliferative phase (Zhou *et al.* 1994). Growth/differentiation factors are differently expressed in the proliferative and secretory phases. For example, vascular endothelial growth factor is expressed in estrogen-responsive epithelium lining the uterus. Under the influence of progesterone, when maximal development of secretory endometrium and development of new blood vessels take place, the site of expression of vascular endothelial growth factor shifts to the cells of the underlying stroma (Shweiki *et al.* 1993). The same applies to the expression of heparin-binding epidermal growth factor (Zhang *et al.* 1994). Studies indicate that keratinocyte growth factor is a progestomedin expressed by stromal cells, whereas expression of its receptor is estrogen-dependent (Koji *et al.* 1994, Siegfried *et al.* 1995). Recent results suggest that progesterone is also a potent stimulator of basic fibroblast growth factor in the rat uterus (Rider *et al.* 1997).

Heparin affin regulatory peptide (HARP), also named pleiotropin (Courty *et al.* 1991, Li *et al.* 1990), is a secreted growth/differentiation factor that belongs to the heparin-binding growth factor family. HARP displays mitogenic activity on epithelial cells (Fang *et al.* 1992, Delbé *et al.* 1995), fibroblasts (Milner *et al.* 1989), smooth muscle cells (Ohyama *et al.* 1994) and endothelial cells (Courty *et al.* 1991) and also has angiogenic effects (Laaroubi *et al.* 1994, Czubayko *et al.* 1996). Recent studies suggest that, during

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development, HARP is involved in stroma–epithelium interactions (Vanderwinden *et al.* 1992, Mitsiadis *et al.* 1995*b*) and in growth and cell differentiation steps (Peng *et al.* 1995, Szabat & Rauvala 1996). HARP has been isolated from adult brain (Courty *et al.* 1991), follicular fluid (Ohyama *et al.* 1994), mature uterus (Milner *et al.* 1989) and uterine luminal fluid (Brigstock *et al.* 1996). In addition, HARP mRNA is upregulated by androgens in cultured prostatic epithelial cells (Vacherot *et al.* 1995). However, the biological functions of HARP in adulthood are not yet known.

The biological properties of HARP and its distribution in the female genital tract led us to suspect that it may play a role in the cyclic activities of the uterus. We thus investigated expression of HARP mRNA in the rat uterus throughout the estrous cycle by semi-quantitative reverse transcriptase (RT)-PCR, cellular distribution of HARP mRNA by *in situ* hybridization and HARP protein distribution by immunohistochemistry. The effects of 17β-estradiol and progesterone on HARP mRNA expression were then studied in ovariectomized animals.

Materials and Methods

Animals

Virgin mature female Crl:CD (SD) BR rats aged 6–8 weeks (Charles River, St Aubin lès Elbeuf, France) were housed in standardized conditions. In studies of HARP mRNA expression on specific days of the estrous cycle, animals with at least two consecutive 4-day cycles (determined by daily vaginal smears) were used. For direct analysis of the effects of sex steroids on HARP mRNA expression, 6-week-old females were ovariectomized. Two weeks after surgery, the animals (n=3 for each group) were injected i.p. (0·2 ml) with vehicle (PBS/ethanol, 10:1, v/v), 17β-estradiol (5 μg/kg) or progesterone (250 μg/kg) and killed 6 h later. For time-course experiments, ovariectomized rats were injected with progesterone and killed various times after the treatment. RU486 (a gift from Professor E E Baulieu) was suspended in sesame oil, and 2 mg/rat in a volume of 200 μl was administered s.c. in two injections, 12 h and 1 h before progesterone or vehicle injection. The uterus was removed and small pieces were frozen in OCT compound (JAKURA Finetek, Zoeterwoude, Netherlands) while other parts were immediately frozen in liquid nitrogen. All rat husbandry and experiments were conducted in accordance with the European guide for care and use of laboratory animals.

Probe and labeling

For *in situ* hybridization, 1·2 kb HARP cDNA was used as a PCR template to generate a 463 bp fragment with sense (5′-GAAAATTTGCAGCTGCCT-3′) and antisense (5′-TTCTCCTGTTTCTTGCCT-3′) primers. The first PCR mixture contained (final concentrations) 100 ng cDNA, 50 mM KCl, 10 mM Tris–HCl, pH 9, 0·1% Triton X-100, 2 mM $MgCl_2$, 0·2 mM each dATP, dGTP, dCTP, dTTP and 1 μM each primer. *Taq* DNA polymerase (5 U) was added to a final reaction volume of 100 μl and the amplification was run in a Thermojet thermocycler (Eurogentec, Seraing, Belgium) for 4 min at 95 °C for 35 cycles (1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C). The final product was purified by low-melting-point agarose gel electrophoresis. A second unidirectional PCR with digoxigenin-11-dUTP (Boehringer Mannheim, Heyfan, France) generated single-stranded cDNA. In the unidirectional amplification, 50 ng of the double-stranded stock was used with a primer ratio of 50:1 and 40 pM limited primer. The experimental conditions were the same as above, except that digoxigenin-11-dUTP was added at a ratio of 0·13 mM dTTP/0·07 mM digoxigenin-11-dUTP. The probe was quantified by ELISA with anti-digoxigenin as primary antibody and adjusted to a concentration of 0·5 μg/ml.

RT-PCR

mRNA concentration was determined using semi-quantitative RT-PCR, by comparison with the internal control transcription factor IID (TFIID) (Peterson *et al.* 1990). cDNA was prepared from 1 μg total RNA, purified using the RNeasy Midi kit (Qiagen, Countaboeuf, France), as described by Kandel *et al.* (1991). PCR was performed as described by Radvanyi *et al.* (1993), using 2 μl (1/50) of the reverse transcription product in a final volume of 50 μl containing all four dNTPs (each at 100 μM) with 1 μM each primer (a pair for TFIID with a pair for HARP) and 2 μCi [α-^{32}P]dCTP. The sequences of the primers were: CGTCTAGTGGCCCAGATC TGT and CGGTAGGCATCCTGATTGTCA (TFIID) and GAAAATTTGCAGCTGCCT and TTCTCCTGT TTCTTGCCT (HARP). All primers are given in the 5′ to 3′ direction. The number of cycles was 20 and chosen to be in the exponential phase of the two PCRs.

The amplification reactions were performed using a Thermojet thermocycler with an initial cycle of 95 °C for 5 min before the addition of Hi-*Taq* DNA thermostable polymerase (Bioprobe, Montreuil, France). Each cycle was then as follows: 94 °C for 1 min, 57 °C for 1 min and 72 °C for 80 s. These 20 cycles were followed by a 72 °C incubation step for 15 min. The PCR-amplified products were loaded in duplicate and electrophoresed in 8% polyacrylamide gels, fixed in 7% acetic acid and vacuum-dried. Co-amplified radioactive fragments were quantified using a Molecular Dynamics 300 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Each measurement was repeated for three independent PCRs.

For each sample, a negative control including RT reagents without RT was performed and showed no signal after the PCR experiment.

In situ *hybridization*

Cryosections (10 μm thick) of uterine tissue were collected on SuperFrost/Plus glass slides (Menzel-Glaser, Freiburg, Germany), air-dried and fixed in 4% paraformaldehyde buffer for 30 min at room temperature. After being washed, sections were dehydrated in graded ethanol solutions and stored with silica gel at −80 °C. Before use, sections were rehydrated and pretreated with Proteinase K (10 μg/ml diluted in 2 mM $CaCl_2$–20 mM Tris–HCl, pH 7·4, at 37 °C for 30 min) and 4% paraformaldehyde for 20 min successively before prehybridization (3 h at 37 °C) in a humidity chamber with solution containing 50% (v/v) formamide, 4 × SSC, 5% dextran sulfate (w/v), 1 × Denhardt's solution, 0·25 mg/ml tRNA and heat-denatured salmon sperm DNA (0·5 mg/ml).

Hybridization was carried out at 37 °C overnight in prehybridization buffer supplemented with 0·5 μg/ml digoxigenin-labeled sense or antisense probe. The slides were washed twice for 15 min at 37 °C in 2 × SSC, 1 × SSC and 0·2 × SSC. After saturation of non-specific binding sites with saturation buffer (150 mM NaCl, 100 mM Tris–HCl, pH 7·4) containing 1% (w/v) Blocking Reagent (Boehringer Mannheim) and 3% normal sheep serum, alkaline phosphatase–anti-digoxigenin conjugate (Boehringer Mannheim), diluted in saturation buffer containing 0·3% Triton X-100, was added. After several washes, antibody complexes were revealed by adding alkaline phosphatase substrate (337·5 mg/ml nitroblue tetrazolium and 175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in buffer containing 0·1 M NaCl, 50 mM $MgCl_2$ and 0·1 M Tris–HCl, pH 9·5) and 1 mM levamisol. Color precipitate development was monitored at room temperature.

Western blotting procedure

Uterus in diestrus (0·5 g) was homogenized, using an Ultra-Turax homogenizer, in lysis buffer (20 mM Hepes, pH 7·4, 1 μg/ml each aprotinin, leupeptin and pepstatin, 0·1 mM phenylmethylsulphonyl fluoride, 3 mM EDTA) containing 2 M NaCl. After centrifugation (12 000 ***g*** for 15 min), the supernatant was diluted 5-fold with lysis buffer and incubated overnight at 4 °C with heparin–Sepharose CL-6B beads (Pharmacia, Uppsala, Sweden) on a rotating rack. The beads were washed twice with 20 mM Hepes (pH 7·4) 0·5 M NaCl and once with 20 mM Hepes, pH 7·4. Heparin–Sepharose-bound molecules were then eluted with electrophoresis sample buffer (50 mM Tris–HCl, pH 6·8, 10% glycerol, 0·02% bromophenol blue, 2% SDS and 5% β-mercaptoethanol) at 95 °C for 5 min.

Uterus samples, human recombinant HARP (50 ng) and human recombinant midkine (MK) (50 ng) expressed in bacteria were run on SDS–15% polyacrylamide gel then electrotransferred to Immobilon-P membranes in 10 mM CAPS buffer (3-(cyclohexylamino)-1-propanesulfonic acid), pH 11, containing 10% methanol. Non-specific binding was prevented by incubating the membrane in PBS–0·2% Tween-20 (PBS-T) containing 3% gelatin. The membrane was then incubated overnight at 4 °C with specific anti-HARP antibodies (1 μg/ml) diluted in PBS-T containing 0·1% gelatin and 1·5% normal goat serum. These antibodies were raised in rabbits and isolated from immune serum by Protein A–Sepharose affinity chromatography, then purified by HARP–HiTrap affinity chromatography according to the manufacturer's instructions (Pharmacia, Bois d'Arcy, France). After being washed with PBS-T, the membrane was incubated in peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sanofi, Marnes-La-Croquette, France). Detection was performed using the enhanced chemiluminescence method (Amersham, Les Ullis, France).

Immunohistochemistry

Serial 8 μm cryosections were fixed with acetone for 15 min at 4 °C. The sections were then incubated for 30 min at room temperature with saturation buffer (2% normal goat or rat serum in PBS). Endogenous biotin was blocked by using the Vector blocking kit (Vector Laboratories, Burlingame, CA, USA). Incubations with rabbit polyclonal anti-HARP immunoglobulins (2 μg/ml) or mouse monoclonal anti-von Willebrand factor, anti-α-actin (Boehringer) or anti-cytokeratin (Dako, Copenhagen, Denmark) immunoglobulins were performed for 1 h at 37 °C in saturation buffer. Sections were incubated, according to the primary antibody, with biotinylated goat anti-rabbit antibody or with biotinylated rat anti-mouse antibody (Jackson, West Groove, PA, USA; dilution 1:1000) for 30 min at room temperature. After amplification with avidin–biotinylated alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA), antigen–antibody complexes were revealed using fast red substrate of alkaline phosphatase in the presence of 1 mM levamisol. Negative controls were run with 2 μg/ml unbound immunoglobulins from HARP affinity chromatography or without secondary antibody. The specificity of the staining was controlled using unbound immunoglobulins from HARP–HiTrap affinity chromatography or by omitting the primary or secondary antibody or ABC reagent.

Statistical analysis

All data were assessed using the GraphPad Instat program (San Diego, CA, USA). Effects of hormones on HARP mRNA, expressed as HARP mRNA:TFIID mRNA ratio, were analyzed by one-way ANOVA combined with the Student–Newman–Keuls test.

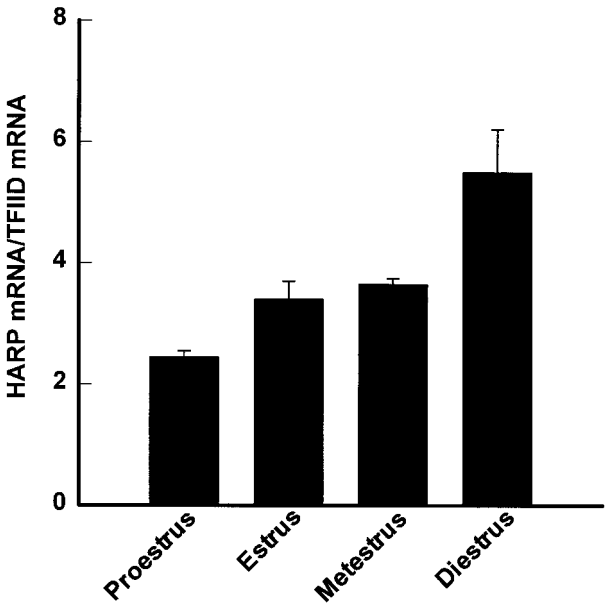

Figure 1 HARP mRNA expression in rat uterus during the estrous cycle. HARP mRNA expression was analyzed using semi-quantitative RT-PCR experiments with TFIID as an internal control (see Materials and Methods section). The results are means ± S.D. of triplicate samples from three animals.

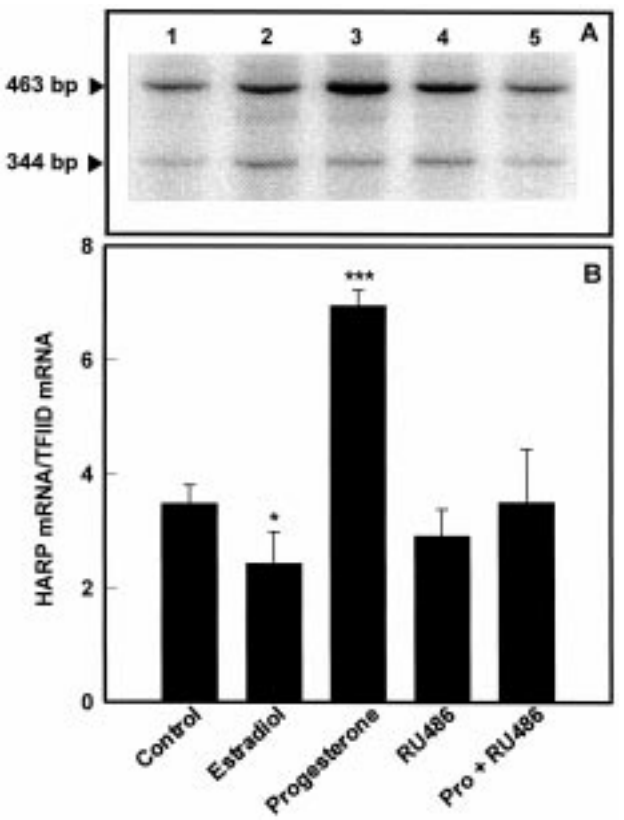

Figure 2 Effect of progesterone and 17β-estradiol on steady-state HARP mRNA levels in uterus from ovariectomized rats using semi-quantitative RT-PCR. (A) Representative gels of electrophoresis of HARP and TFIID transcripts analyzed by RT-PCR method. Ovariectomized animals were treated with PBS/ethanol (lane 1), 17β-estradiol (5 μg/kg; lane 2), progesterone (250 μg/kg; lane 3), RU486 (lane 4) or progesterone+RU486 (lane 5) and killed 6 h later. Radioactivity of HARP gene fragment (463 bp) and TFIID gene fragment (344 bp) was measured using a PhosphorImager. (B) Quantitative analysis of HARP mRNA using TFIID as an internal control. The results are means ± S.D. of triplicate samples from three animals. *P<0·05, ***P<0·001 as compared with control using the Student–Newman–Keuls test.

Results

HARP mRNA expression is dependent on the estrous cycle and upregulated by progesterone

During each of the four phases of the estrous cycle, HARP mRNA expression in rat uterus was compared using semi-quantitative RT-PCR. For each sample, the amount of HARP mRNA was quantified relative to the respective level of TFIID, a ubiquitous transcription factor. Results differed during the estrous cycle (Fig. 1). The highest level of HARP mRNA was detected in diestrus; it then decreased during proestrus and began to increase during estrus and metestrus. Similar results were obtained using RNase protection assays (data not shown).

To study possible hormonal modulation of HARP mRNA expression and whether the strong HARP mRNA expression during diestrus was related to the elevated levels of circulating progesterone, rats were ovariectomized and treated 2 weeks later with progesterone, 17β-estradiol or PBS/ethanol as control. Uteri were removed 6 h after the last steroid injection. Total RNA was purified from three animals for each treatment and analyzed by semi-quantitative RT-PCR. A representative gel from electrophoresis of RT-PCR products is shown in Fig. 2A. The 344 and 463 bp bands correspond to the amplification of TFIID and HARP respectively. PhosphorImager quantification and statistical analysis showed that progesterone treatment significantly increased HARP mRNA expression in rat uterus (Fig. 2B). In order to investigate the specificity of this effect, animals were pretreated with mifepristone (RU486), a progesterone receptor antagonist (Baulieu 1989). Progesterone effects were completely abolished by RU486, and no modulation of HARP mRNA expression was observed when the inhibitor was administered alone. In addition, HARP mRNA levels were slightly decreased by 17β-estradiol.

To study the time course of progesterone stimulation of HARP mRNA, ovariectomized rats were killed at various times after the progesterone injection. As shown in Fig. 3, 6 h after a single progesterone injection, HARP PCR product was increased and the maximal yield was obtained at 10 h (about × 2 and × 3 respectively).

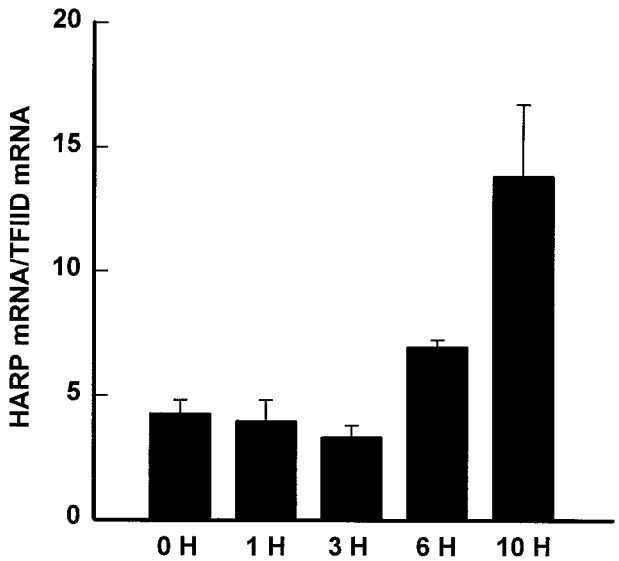

Figure 3 Time course of the effect of progesterone on the expression of HARP mRNA in the uterus of ovariectomized rats. Ovariectomized animals were killed 0, 1, 3, 6 or 10 h after injection of progesterone. HARP mRNA expression was measured using semi-quantitative RT-PCR with TFIID as an internal control.

HARP distribution in rat uterus

The semi-quantitative RT-PCR described above clearly established that HARP mRNA expression was under the control of progesterone. To understand the physiological role of HARP in the uterus, we used *in situ* hybridization to identify HARP-expressing cell types. In good agreement with the results obtained using the RT-PCR method, HARP mRNA staining showed marked differences according to the phase of the estrous cycle (Fig. 4). An increase in staining was observed from proestrus (Fig. 4A) to estrus (Fig. 4C and D) and diestrus (Fig. 4E and F). As shown in Fig. 4A, C and E, HARP mRNA was localized in cells expressing α-actin, i.e. in smooth muscle cells of the myometrium, including artery walls from myometrium and basal endometrium (Fig. 4A and C). In the central endometrium, HARP mRNA was localized in capillary endothelial cells (Fig. 4D and F). Therefore immunohistochemical examination with an anti-α-actin antibody gave no labeling in this central zone (data not shown). Whatever the stage of the estrous cycle, luminal and glandular epithelial cells, positively stained with anti-cytokeratin in serial sections (data not shown), were negative for HARP mRNA (Fig. 4D and F). Interestingly, stroma cells underlying luminal epithelial cells, which were negative in proestrus and estrus (Fig. 4D), expressed HARP mRNA from metestrus to diestrus (Fig. 4F). Sections from proestrus hybridized with the sense probe exhibited no staining (Fig. 4B).

Immunochemical studies with anti-HARP antibodies were used to locate HARP protein in the uterus. The specificity of the antibodies was first tested by Western blot. As shown in Fig. 5A (lane 3), the purified recombinant anti-human HARP immunoglobulins recognized rat uterus HARP as a single 18 kDa band. No signal was detected using MK, a protein that belongs to the HARP family and shows 50% sequence homology with HARP (lane 1), compared with a positive control with human recombinant HARP (lane 2). HARP immunoreactivity was detected throughout the estrous cycle, but immunostaining increased from proestrus to diestrus. Smooth muscle cells derived from the myometrium and blood vessels were strongly stained in diestrus (Fig. 5C). Diffuse staining was observed in the endometrium, especially in the collagen-rich basal zone near the myometrium, and in the luminal (Fig. 5D and F) and glandular (Fig. 5E) epithelium. Only during metestrus and diestrus did the stroma cells in the pericellular regions lining the luminal epithelium show a positive signal (Fig. 5F). Capillary endothelial cells from the central endometrium were also clearly labeled (Fig. 5F). A similarly localized weak signal was seen during proestrus (not shown). Unbound immunoglobulins from HARP–HiTrap affinity chromatography were used as non-specific immunoglobulins (Fig. 5B).

In situ hybridization studies of uterine sections showed cell-specific HARP mRNA expression after ovariectomy followed by hormone treatment. Analysis of transverse uterine sections from ovariectomized rats showed low HARP mRNA expression (Fig. 6A). Similar results were observed after 17β-estradiol treatment (Fig. 6B). In contrast, progesterone treatment strongly increased HARP transcript levels in smooth muscle cells from myometrium and blood vessels (Fig. 6C) and in endometrial endothelial cells (Fig. 6D and E) positively stained with anti-von Willebrand factor (Fig. 6F). Moreover, within 3 h of progesterone injection, HARP mRNA was already detectable in endothelial cells (data not shown). Immunohistochemical studies of ovariectomized hormone-treated animals showed that progesterone significantly increased the amount of HARP protein. In progesterone-treated ovariectomized animals, HARP immunostaining was clearly observed in vascular and myometrial smooth muscle cells (Fig. 7E) and also in endothelial endometrial cells (Fig. 7F). As compared with progesterone-treated ovariectomized animals, weak staining was observed in these cells in PBS/ethanol-treated rats (Fig. 7A and B) and 17β-estradiol-treated rats (Fig. 7C and D).

Discussion

Throughout the life span of mature females, the uterus goes through waves of tissue growth and decline which are coupled to the cyclic secretion of hormones such as ovarian steroids. In all mammalian species, these ovarian steroid secretions are essential to maintain physiological uterine functions. However, little is known about the precise

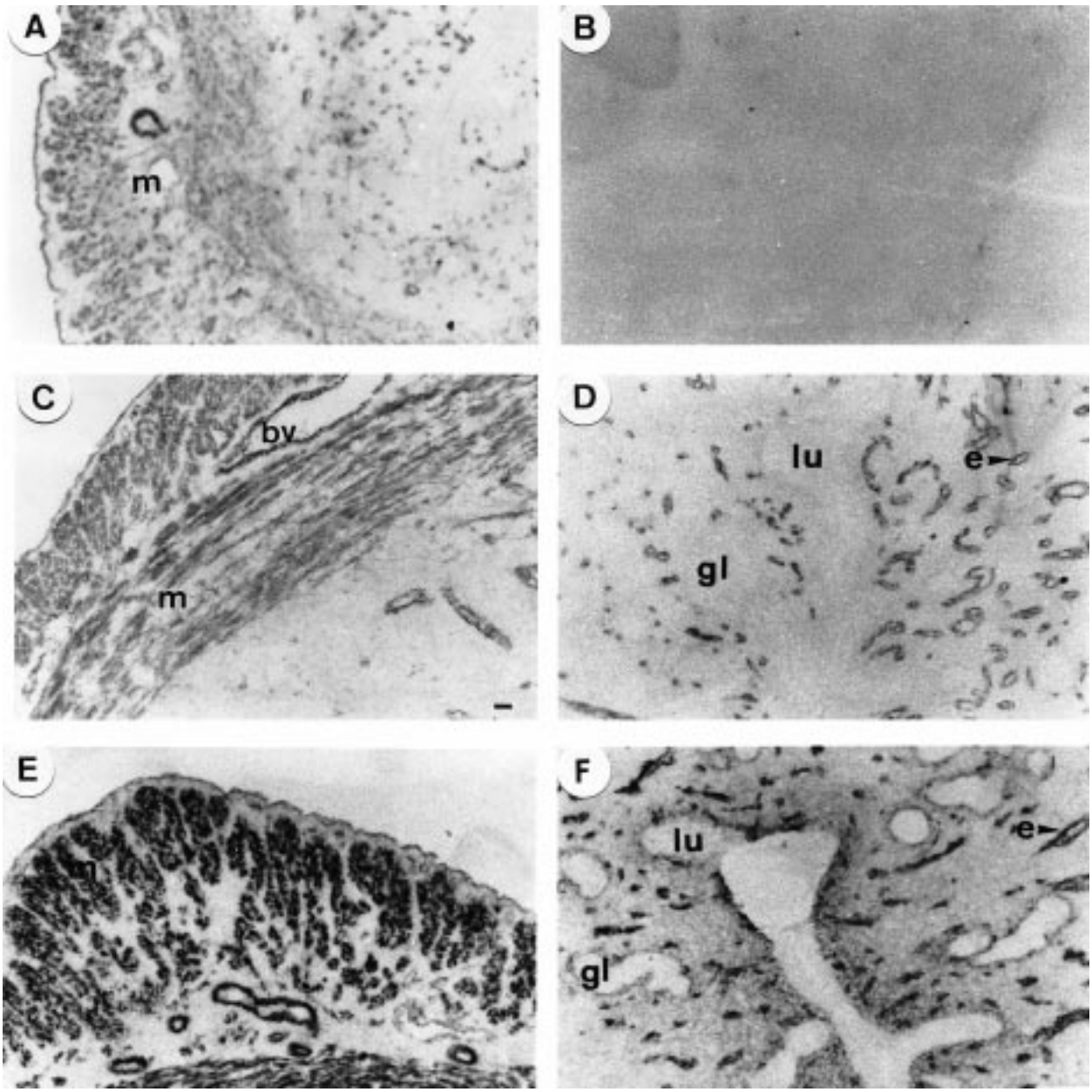

Figure 4 Localization of HARP mRNA in the rat uterus at various stages of the estrous cycle. *In situ* hybridization of HARP mRNA was performed with digoxigenin-labeled single-stranded cDNA. Photomicrographs of transverse sections of rat uterus collected during proestrus (A), estrus (C and D) and diestrus (E and F). Sections presented in A, C, D, E and F were treated with antisense probe. The section in B was treated with sense probe as negative control. m, Myometrium; bv, blood vessels; e, endothelial capillary cells; lu, luminal epithelium; gl, glandular epithelium. Magnification, × 90. Scale bar in C represents 10 μm.

molecular mechanisms by which these hormones regulate both the growth and differentiation of uterine cells. Tissue changes in the uterus require the expression of growth/differentiation molecules acting directly or indirectly on uterine homeostasis. Here, we studied estrous cycle-dependent variations in HARP mRNA in the rat uterus. HARP mRNA expression was clearly dependent on the estrous cycle, the highest levels being associated with the progesterone-dominated secretory phase of the cycle. This increase in HARP mRNA expression was also observed in ovariectomized rats treated with progesterone, demonstrating that HARP mRNA expression is under the control of progesterone. In addition, RU486 inhibited progesterone

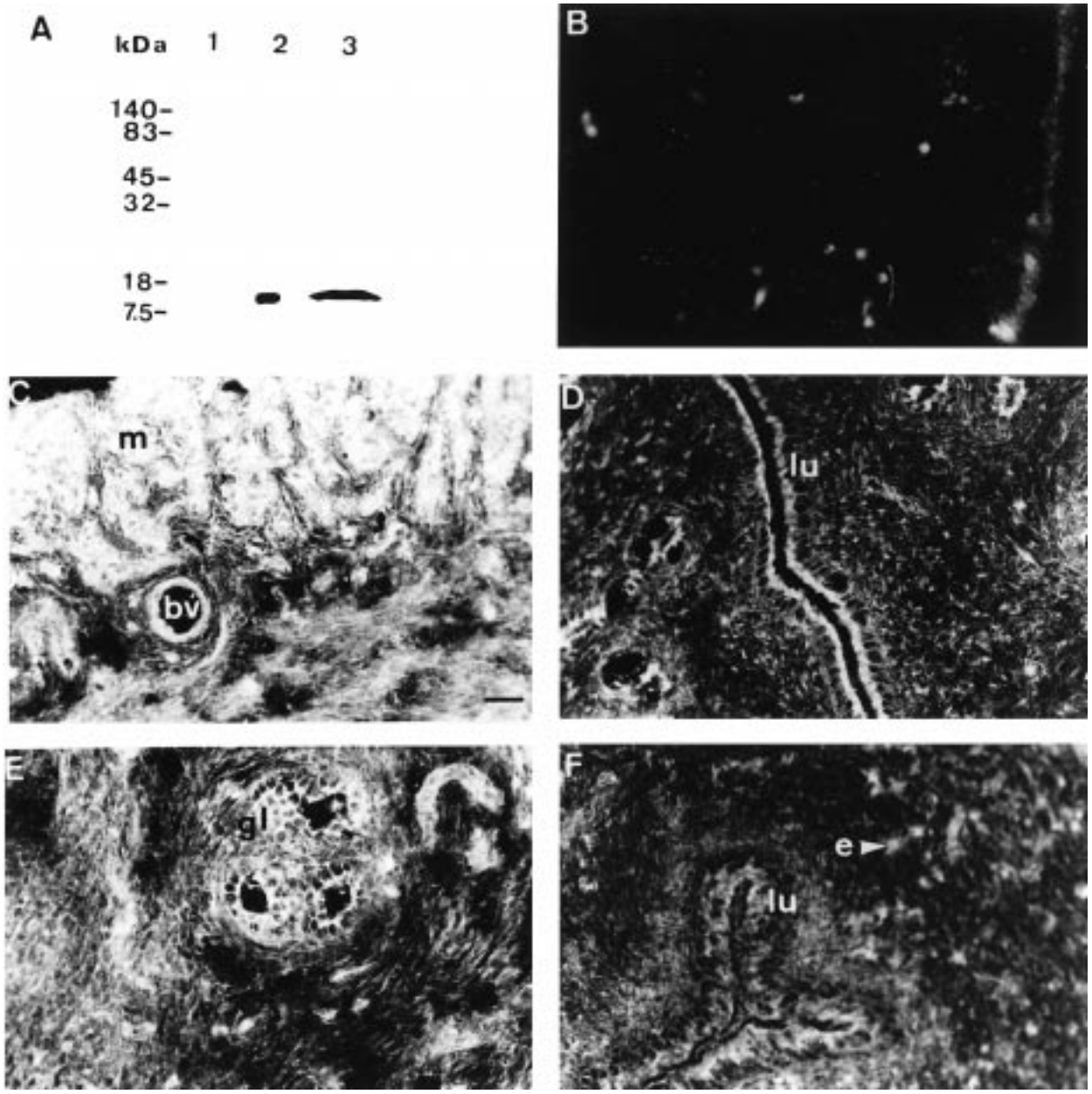

Figure 5 Characterization of anti-HARP antibodies and cellular distribution of HARP protein in the rat uterus. The specificity of the anti-HARP immunoglobulins was tested by Western blotting (A) with purified human recombinant MK (lane 1, 50 ng), human recombinant HARP (lane 2, 50 ng) and a crude extract from rat uterus (lane 3). Transverse sections of uterus collected during diestrus (C, E and F) and estrus (D) were used for immunodetection with anti-HARP immunoglobulins. Non-specific staining with unbound immunoglobulins from HARP–HiTrap affinity chromatography is presented in (B). Immunostaining was observed using fluorescence microscopy. m, Myometrium; bv, blood vessels; e, endothelial capillary cells; lu luminal epithelium; gl, glandular epithelium. Magnification × 225. Scale bar in C represents 10 µm.

upregulation of HARP mRNA, indicating that this effect is progesterone receptor-mediated. This is the first *in vivo* demonstration that HARP mRNA expression is hormone-dependent. Previously, we investigated the hormonal regulation of HARP mRNA in the epithelial cell line PNT 1A, derived from prostatic secretory luminal cells, and found that it was upregulated by dihydrotestosterone and testosterone. This induction of expression was inhibited by Anandron, a specific androgen inhibitor (Vacherot *et al.* 1995).

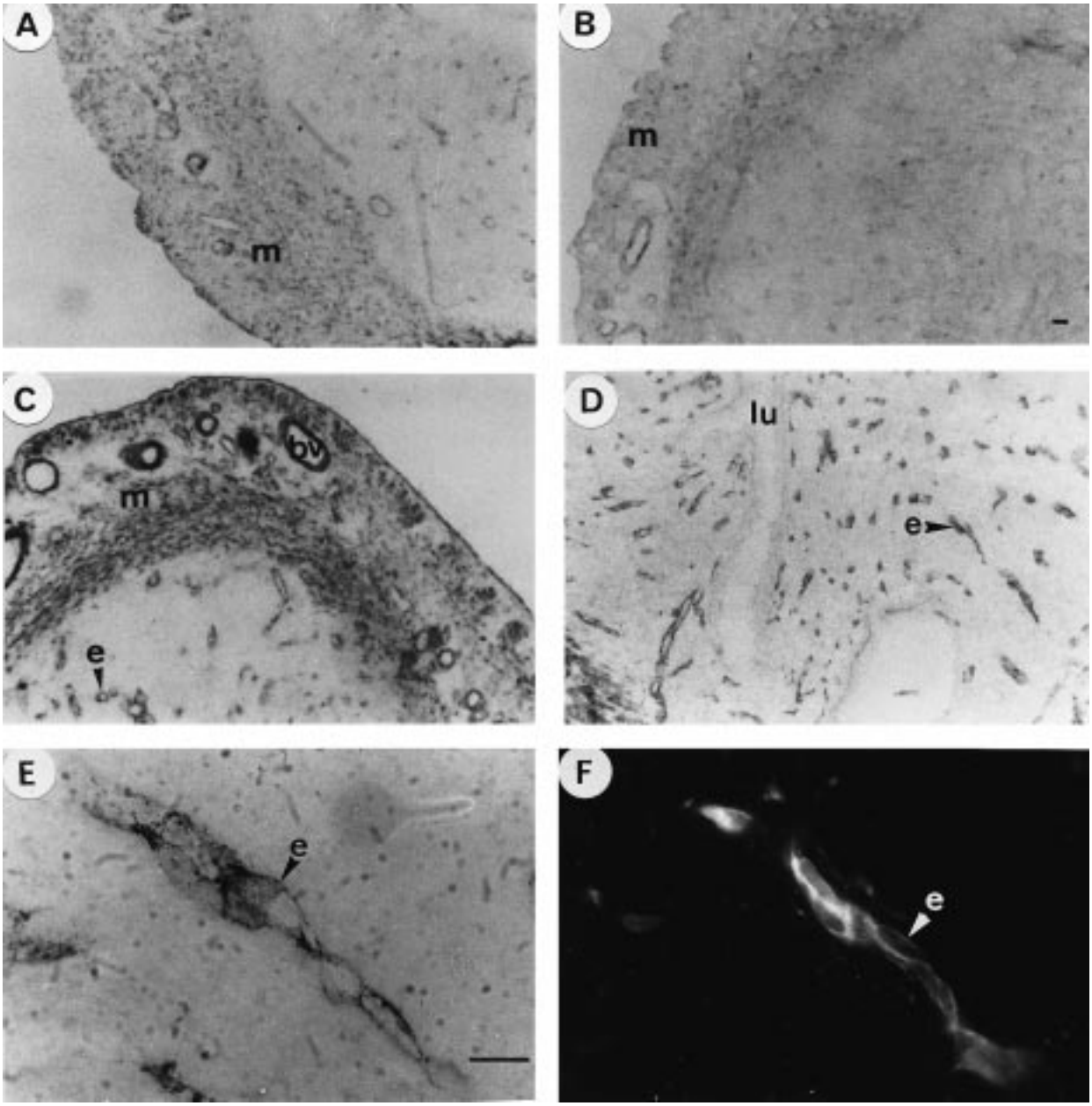

Figure 6 Progesterone upregulation of HARP mRNA expression in rat uterus assessed by *in situ* hybridization. Transverse sections of rat uterus collected from ovariectomized animals 6 h after injection with PBS/ethanol (A), 17β-estradiol (B) or progesterone (C and D) were hybridized with digoxigenin-labeled antisense probe. m, Myometrium; bv, blood vessels; e, endothelial capillary cells; lu, luminal epithelium. Magnification × 90. Bar in B, 10 µm. A high magnification of a capillary blood vessel from section D is presented in E to show that in serial section this structure was immunostained using an anti-von Willebrand factor (F). Magnification × 360. Scale bar in E represents 10 µm.

During adulthood, HARP mRNA expression in smooth muscle cells is restricted to organs displaying hormonal regulation of growth/differentiation, including rat uterus, human prostate and human breast. The presence of HARP mRNA in smooth muscle cells has been observed in myometrium from gravid and non-gravid mice (Vanderwinden *et al*. 1992) and also in myoepithelial cells and smooth muscle cells of blood vessels of human breast (Ledoux *et al*. 1997). In addition, HARP mRNA is expressed in fibromuscular stroma of the normal human prostate gland (F Vacherot, D Caruelle, D Chopin, S Gil-Diez, D Barritault, J P Caruelle & J Courty,

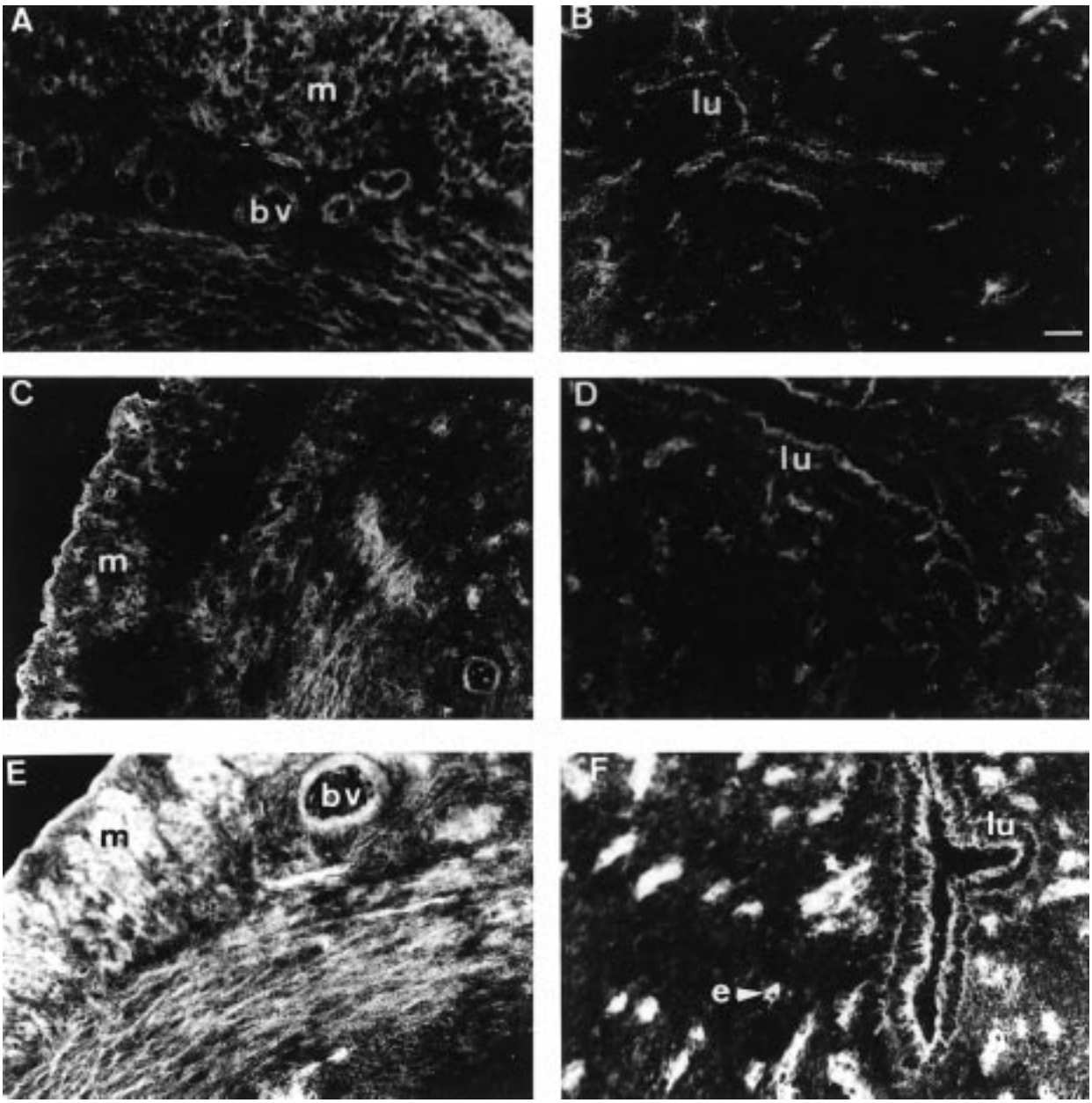

Figure 7 Immunostaining for HARP in the uterus of ovariectomized animals injected with PBS/ethanol (A and B), 17β-estradiol (C and D) or progesterone (E and F). m, myometrium; bv, blood vessels; e, endothelial capillary cells; lu, luminal epithelium. Magnification × 225. Scale bar in B represents 10 μm.

unpublished observation). In contrast, HARP mRNA is not expressed in smooth muscle cells of adult rat viscera (Vanderwinden *et al.* 1992) or adult rat aorta (D Caruelle & J Courty, unpublished observation). Since different smooth muscle cell functions require different cell phenotypes (Frid *et al.* 1994), the results of our study suggest that HARP expression in uterine smooth muscle cells may play a role in the control and maintenance of cell differentiation through an autocrine or juxtacrine mechanism.

Our study shows that HARP mRNA and protein are also found in endometrial capillary endothelial cells. This localization of HARP is supported by its biological activities. Therefore purified HARP has angiogenic activity *in vivo* (Kurtz *et al.* 1995). The C-terminal part of the molecule enhances plasminogen activator levels and downregulates plasminogen activator inhibitor-1 expression in bovine aortic endothelial cells (Kojima *et al.* 1995*a*). Similar results have been reported with MK (Kojima *et al.*

1995*b*). It is noteworthy that ribozyme targeting of HARP reduces angiogenesis in melanoma and subsequent growth of tumors *in vivo* (Czubayko *et al.* 1996). In addition, HARP belongs to the family of heparin-releasable proteins (Novotny *et al.* 1993). This family, which includes lipoprotein lipase, tissue factor pathway inhibitor, extracellular superoxide dismutase, histaminase, platelet factor 4 and tumor necrosis factor-binding protein-1, contributes to vascular homeostasis. Although the precise physiological role of HARP localized in endothelial cells remains to be clarified, it is tempting to speculate that the protein may, in concert with other angiogenic factors, influence angiogenesis during the cyclic growth and decline of the rat uterus.

Whatever the hormonal status of the rat uterus, HARP protein was found in both the luminal and glandular epithelium, despite the absence of its transcript, suggesting that HARP may act by a paracrine mechanism. Similar observations have been reported in human mammary gland (Ledoux *et al.* 1997). During diestrus, the pericellular region around the luminal and glandular epithelium expressed HARP protein. As HARP is a secreted polypeptide (Li *et al.* 1990), it could rapidly diffuse towards luminal and glandular epithelial cells. A similar mechanism has been proposed for MK protein, with the same opposition between mRNA and protein distribution during organogenesis (Mitsiadis *et al.* 1995*b*). Moreover, other groups also reported that, during embryogenesis and adulthood, no HARP transcripts were detected along the entire urogenital rat system in any epithelial structure (Vanderwinden *et al.* 1992), although the protein has been localized on the surface of epithelial cells during mouse development (Mitsiadis *et al.* 1995*a*). Hence, HARP could regulate the proliferation and/or differentiation of epithelial cells, implying the existence of a cell-surface receptor mediating its biological activity. Given its high affinity for heparin, HARP probably binds to heparan sulfate chains. It has been reported that a detergent-solubilized component from neuronal cells and perinatal rat brain binds HARP. Immunological analysis indicated that this cell-surface component was N-syndecan (Raulo *et al.* 1994) but, as fibroblast growth factor-2 competes for HARP binding (Raulo *et al.* 1994), N-syndecan does not seem to be specific for HARP. In many epitheliomesenchymal organs, during mouse embryonic development, both HARP and MK are co-distributed with syndecan-1 (Mitsiadis *et al.* 1995*b*), a proteoglycan also expressed in endometrium (Inki 1997). Although the function of syndecan in the mitogenic activity of HARP is unknown, a syndecan-like molecule could regulate the binding of HARP to high-affinity binding sites mediating the mitogenic activity. Recently, *in vitro* experiments on bovine epithelial lens cells demonstrated that HARP stimulated cell division through a tyrosine kinase pathway, suggesting the presence of high-affinity binding sites on these cells (Souttou *et al.* 1997). The identification of such high-affinity binding sites is an important challenge in elucidating the biological function of this molecule in the uterus and other hormone-dependent tissues, during both physiological and pathophysiological processes.

In summary, this study demonstrates that progesterone upregulates HARP mRNA steady-state levels in rat uterus. However, nothing is known about the regulatory elements of the HARP rat gene, and the mechanisms by which progesterone can affect the expression of this gene remain to be determined. Further *in vitro* studies are necessary to elucidate the precise mechanism underlying steroid regulation of HARP expression.

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