Biochemical and Biophysical Research Communications **257,** 182–186 (1999)
Article ID bbrc.1999.0359, available online at http://www.idealibrary.com on IDEAL®

Angiogenin Is Involved in Morphological Changes and Angiogenesis in the Ovary

Heungshik S. Lee,* In-se Lee,*[,1] Tae-Cheon Kang,* Goo-Bo Jeong,† and Soo-Ik Chang‡[,2]
**Department of Anatomy, College of Veterinary Medicine, Seoul National University, Suwon 441-744, Korea; †Department of Anatomy, College of Medicine, Chungbuk National University, Cheongju 361-763, Korea; and ‡Department of Biochemistry, College of Natural Science, Chungbuk National University, Cheongju 361-763, Korea*

Received January 28, 1999

Angiogenin is a potent angiogenic factor secreted by cultured tumor cells and is found in various normal organs and tissues. The ovary is one of the adult organs in which angiogenesis normally occurs during the female reproductive cycle. In this study, we examined whether angiogenin protein is localized and if angiogenin mRNA is expressed in the normal bovine ovary by immunohistochemistry using polyclonal rabbit anti-bovine angiogenin IgG and by *in situ* hybridization using bovine angiogenin probe, respectively. The localization and mRNA expression of angiogenin in the ovarian follicle and in the corpus luteum were different in their developmental stages. The intensities of immunoreactivities and angiogenin transcripts in the follicle increased from the primordial to the tertiary (or Graafian) follicle. The early corpus luteum contained strong immunoreactivities and mRNA expression of angiogenin but these intensities weakened during regression. The results suggest that angiogenin is involved in morphological changes and angiogenesis in the ovary. © 1999 Academic Press

Key Words: **angiogenin; angiogenesis; ovarian follicles; corpus luteum.**

Angiogenesis, the formation of blood vessels from preexisting vessels, occurs during embryonic and adult life [1]. The molecules and mechanisms involved in both embryonic and adult angiogenesis are similar but the latter may require additional factors and mechanisms [2].

The ovary is one of the adult organs in which angiogenesis occurs normally during the female reproductive cycle [3]. In the ovary, endothelial cell proliferation continues as the corpus luteum grows [3], and angiogenesis is an important mechanism in maintaining the corpus luteum of pregnancy and in secreting progesterone from the corpus luteum [4–5]. Many angiogenic factors and angiogenesis inhibitors have been found in ovarian tissues. These include basic fibroblast growth factor (bFGF) [6, 8], vascular endothelial growth factor (VEGF) [6–7, 9], angiopoietin-1 [10], angiopoietin-2 [10], secreted protein acidic and rich in cysteine (SPARC) [11], and thrombospondin [11]. There, however, has been no direct evidence that any angiogenic factors are essential for ovarian angiogenesis. Recently, Ferrara et al. [12] showed that selective inhibition of VEGF blocks the angiogenesis observed in corpus luteum growth, and suggested that VEGF is essential for corpus luteum angiogenesis.

Angiogenin is a potent angiogenic factor originally purified from the conditioned media of cultured human colon adenocarcinoma cells (HT-29) [13]. It was later detected and purified from normal human serum [14], bovine serum [15–16] and milk [17–18]. These findings suggest that angiogenin is involved both in various normal physiological conditions and in abnormal pathological conditions. In our previous report, we examined the localization of angiogenin in the bovine mammary gland, gallbladder, and liver by immunohistochemical analysis, and our results suggested that epithelial cells and secretory cells are major sites of angiogenin synthesis [19].

In this study, we report for the first time the localization and mRNA expression of angiogenin in the bovine ovary. These results suggest that angiogenin is involved in ovarian follicle and corpus luteum growth, and that angiogenin is involved in angiogenesis in the ovary.

MATERIALS AND METHODS

Tissue processing. Thirty-seven cow ovaries (average age 3–4 years old) were used in this study. All tissues were fixed in 4% paraformaldehyde and embedded in paraffin wax. The sections were cut into 5 μm thicknesses and mounted on Probe-on slides (Fisher, U.S.A.).

[1] To whom correspondence should be addressed. Fax: 82-331-293-6403. E-mail: inselee@plaza.snu.ac.kr.
[2] To whom correspondence should be addressed. Fax: 82-431-267-2306. E-mail: sichang@cbucc.chungbuk.ac.kr.

0006-291X/99 $30.00

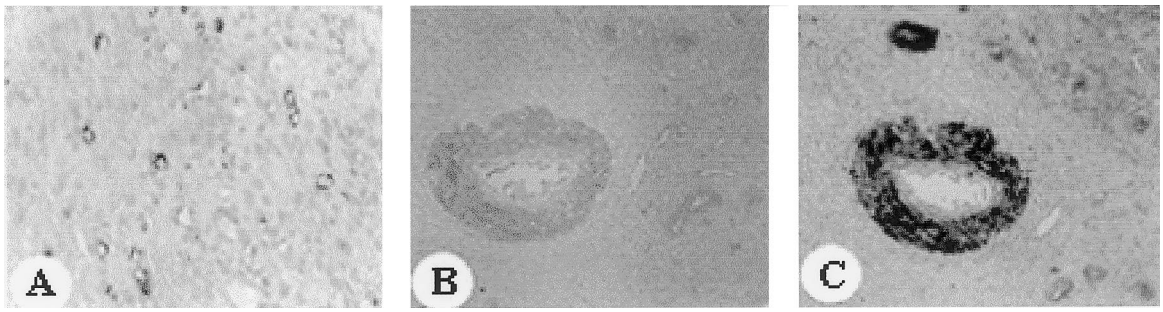

FIG. 1. (A) Immunohistochemical staining for endothelial cells in bovine ovary. The anti-human Factor VIII monoclonal antibody was used as the first antibody, and detection was carried out by biotin-conjugated goat anti-mouse IgG, horseradish peroxidase-conjugated avidin and 3,3′-diaminobenzidine as a chromatogeneous substrate. (B) Immunohistochemical staining for bovine angiogenin (bAng) in bovine ovary. Native polyclonal rabbit anti-bAng IgG was used as the first antibody, and detection was carried out by biotin-conjugated goat anti-rabbit IgG, horseradish peroxidase-conjugated avidin and 3,3′-diaminobenzidine as a chromatogeneous substrate. (C) *In situ* hybridization for bovine angiogenin (bAng) in bovine ovary. Digoxigenin-labeled bAng cDNA probe was used, and detection was carried out by alkaline phosphate-conjugated mouse anti-digoxigenin antibody, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as a chromatogeneous substrate.

Immunohistochemistry. The immunohistochemical studies were performed with the modification of a method as described previously [19]. Briefly, the sections were deparaffinized and hydrated thoroughly in xylene and ethanol. The specimens were washed with phosphate-buffered saline (PBS), and then treated with absolute methanol containing 0.5% hydrogen peroxide for removal of endogenous peroxidase. Thereafter, the sections were blocked with 10% normal goat serum for 2 hours, and then incubated overnight at room temperature with rabbit anti-bovine angiogenin IgG (dilution 1/400 in PBS). The specimens were washed three times with PBS, and then incubated for 2 hours at room temperature with biotinylated goat anti-rabbit IgG (Vector, USA). The sections were washed three times with PBS, and then incubated for 2 hours at room temperature with streptavidin-conjugated horseradish peroxidase (Vector, USA). Localization of angiogenin was visualized with 0.05% 3,3′-diaminobenzidine in Tris buffer containing 0.03% hydrogen peroxide (Sigma, USA). To confirm the specificity of the antibody, the following controls were used; omission of the primary antibody, substitution of normal rabbit serum or incubation of the primary antiserum with bovine angiogenin prior to use. In these cases, no immunoreactivity was detected.

Immunohistochemical staining for endothelial cells in the bovine ovary was carried out in a similar manner. Briefly, anti-human Factor VIII monoclonal antibody (DAKO, U.S.A., dilution 1/400 in PBS) was used as the first antibody, and detection was carried out with biotin-conjugated goat anti-mouse IgG, horseradish peroxidase-conjugated avidin and 3,3′-diaminobenzidine as a chromatogeneous substrate.

In situ hybridization. Consecutive sections were deparaffinized, digested by proteinase K (10 μg/ml, Boehringer Mannheim, Germany) at 37°C for 1 hour and fixed in 0.4% paraformaldehye at 4°C for 20 minutes. Following brief washing in diethyl pyrocarbonate (DEPC)-treated water, sections were incubated in prehybridization buffer (37°C for 1 hour). Hybridization solution buffer containing digoxigenin labeled bovine angiogenin cDNA probe cocktail [32] was applied to sections at a concentration of 1.5 ng/μl, and then parafilms were covered. The slides were incubated in a moist chamber for 16–24 hours at 45°C. After hybridization, the parafilms were removed and the slides were washed sequentially in 4 × SSC, 2 × SSC and 1 × SSC at 37°C. After immersion in Tris buffer, the slides were incubated with 100μl of alkaline phosphatase conjugated anti-digoxigenin antibody (dilution 1/500 in Tris buffer) for 60 minutes at 37°C. Color development was performed in TBS/Triton X-100 [50 mM Tris (pH 7.6), 150 mM NaCl, 0.1% bovine serum albumin, and 0.1% Triton X-100] by adding buffer solution containing 0.33 mg/ml nitro blue tetrazolium (NBT) and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrates with 1 mM levamisole and then slides were left in a dark place for 4 hours. The slides were rinsed in water, and mounted with aqueous mountant. The negative control slides were treated with 200 μg/ml RNase A before hybridization.

RESULTS

Immunohistochemical Staining for Endothelial Cells in Bovine Ovary

Immunohistochemical analysis was used to detect endothelial cells in the bovine ovary. Figure 1A shows immunohistochemical staining of endothelial cells for factor VIII, demonstrating that microvessels are localized in the ovary, and that angiogenesis is involved in the follicle growth and corpus luteum formation.

Immunohistochemical Localization and mRNA Expression of bAng in the Bovine Ovary

The localization of bovine angiogenin (bAng) in the bovine ovary was assessed by immunohistochemical analysis using polyclonal rabbit anti-bAng IgG. Positive reactions were observed in vascular endothelial cells and in vascular smooth muscle cells of the ovarian stroma (Fig. 1B). By *in situ* hybridization, angiogenin transcripts were also detected in vascular endothelial cells and in vascular smooth muscle cells (Fig. 1C).

Immunohistochemical Localization of bAng during Follicle and Corpus Luteum Development

Negative reactions were detected in the oocyte and follicular epithelium in the primordial follicles (Fig. 2A), and in the primary follicles weak positive reactions were detected (Fig. 2B) in the oocyte and granulosa cells. In contrast, strong positive reactions were detected in the oocyte and granulosa cells in the secondary (Fig. 2C) and tertiary (or Graafian) follicles

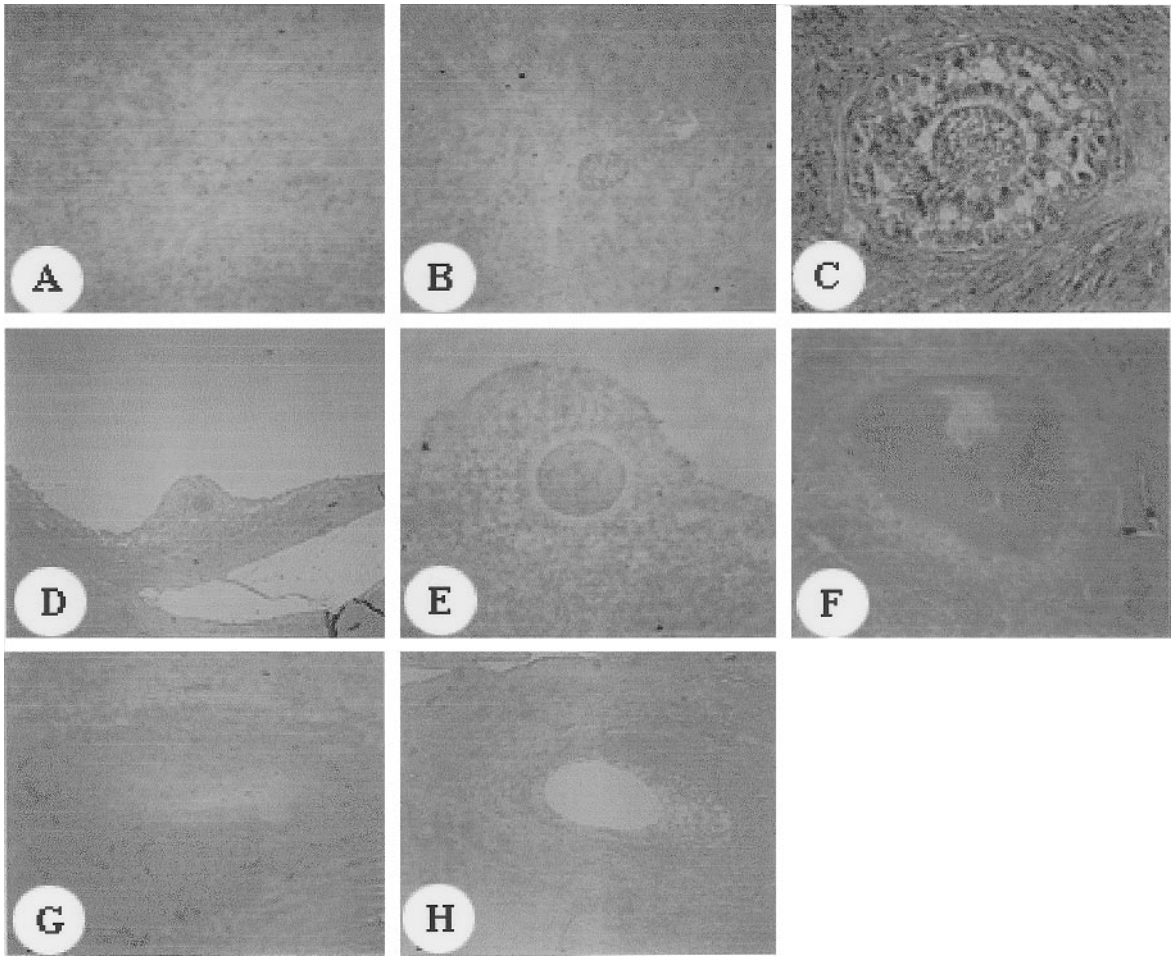

FIG. 2. Immunohistochemical staining for bovine angiogenin (bAng) during follicle and corpus luteum development. Native polyclonal rabbit anti-bAng IgG was used as the first antibody, and detection was carried out by biotin-conjugated goat anti-rabbit IgG, horseradish peroxidase-conjugated avidin and 3,3′-diaminobenzidine as a chromatogeneous substrate. (A) Primodial follicle. (B) Primary follicle. (C) Secondary follicle. (D) The tertiary follicle. (E) High magnification of D. (F) The early corpus luteum. (G) The late corpus luteum. (H) Atretic follicle.

(Figs. 2D and 2E). These immunohistochemical signals, however, were not detected when the specimens were stained with preimmune rabbit IgG or without pre-incubation with rabbit anti-bAng IgG (data not shown). The theca interna and externa in the secondary and tertiary follicles also showed positive reactions (Figs. 2C and 2D). In the corpus luteum, strong positive reactions were detected in the luteal cells during corpus luteum formation (Fig. 2F), but the bAng staining weakened during corpus luteum regression (Fig. 2G). In the atretic follicle, very weak positive reactions were detected in the follicular epithelia, and negative reactions were detected in the theca interna and externa (Fig. 2H).

Expression of mRNA Encoding bAng during Follicle and Corpus Luteum Development

The expression pattern of bAng mRNA is shown in Fig. 3. It was generally similar to the immunolocalization of bAng, in that transcripts were not detected in the oocyte and follicular epithelium in the primordial follicles (Fig. 3A), but were detected in the oocyte and granulosa cells in the tertiary follicles (Figs. 3D and 3E) and in the luteal cells during corpus luteum formation (Fig. 3F). The expression of bAng mRNA decreased during corpus luteum regression (Fig. 3G), and bAng mRNA was not expressed in the theca interna and externa in the atretic follicles (Fig. 3H).

DISCUSSION

Angiogenin is a secreted 14.5-kDa protein that is a potent angiogenic factor [13]. It induces *in vivo* angiogenesis in the chorioallantoic membrane of the chick embryo [13] and in the cornea and meniscus of the knee of the rabbit [20]. It also induces *in vitro* angiogenesis by stimulating the proliferation of human endothelial cells in sparse cultures [21]. In addition, expression of

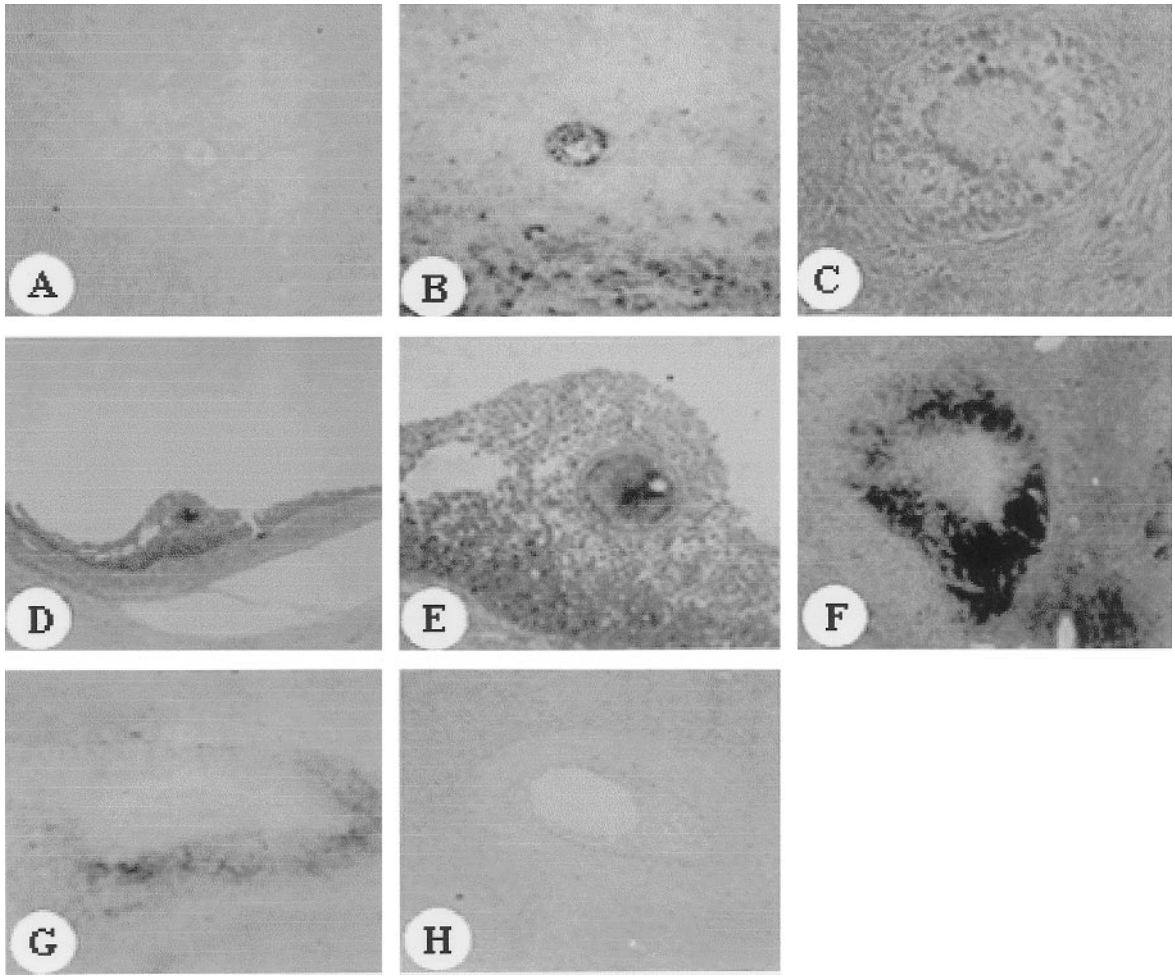

FIG. 3. *In situ* hybridization for bovine angiogenin (bAng) during follicle and corpus luteum development. Digoxigenin-labeled bAng cDNA probe was used, and detection was carried out by alkaline phosphate-conjugated mouse anti-digoxigenin antibody, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as a chromatogeneous substrate. (A) Primodial follicle. (B) Primary follicle. (C) Secondary follicle. (D) The tertiary follicle. (E) High magnification of D. (F) The early corpus luteum. (G) The late corpus luteum. (H) Atretic follicle.

angiogenin mRNA was found in tumor cells, including colonic adenocarcinomas [22], gastric adenocarcinomas [22], pancreatic carcinomas [23] and breast carcinomas [24], and elevated concentrations of angiogenin in serum were found in patients with severe ovarian hyperstimulation syndrome [25], cervical cancer [26], endometrial cancer [27], ovarian cancer [28], and pancreatic cancer [23]. Chimeric anti-angiogenin antibody has been shown to inhibit the formation of human breast xenografts in athymic mice [29].

Ovaries in the female reproduction system exhibit periodic growth and regression, and the ovary is one of the few adult tissues in which angiogenesis occurs as a normal process [4]. Angiogenesis in the ovary is evoked during follicle growth, follicle rupture and ovulation [4]. Thus, vascularization is a prerequisite for follicle growth and corpus luteum formation. As shown in Fig. 1A, the microvessels were identified by immunohistochemical staining of endothelial cells for Factor VIII. This suggests the local release of angiogenic factors in the bovine ovary.

In this study, we firstly examined the localization and mRNA expression of angiogenin in the bovine ovary. Angiogenin was localized and expressed in vascular endothelial cells and vascular smooth muscle cells of the ovarian stroma (Figs. 1B and 1C). Previously, immunolocalization of angiogenin was found in endothelial cells, in smooth muscle cells, and in fibroblasts in bovine mammary gland [19]. These findings demonstrate that angiogenin is expressed in normal tissues, and thus suggest that angiogenin is involved in normal angiogenesis including wound healing and ovarian function.

Next, we examined the localization and mRNA expression of angiogenin in the different growth stages of the bovine ovarian follicle and corpus luteum to study the regulation of ovary angiogenesis by angiogenin. Both the localization and mRNA expression of angiogenin in the ovarian follicle were different in their

developmental stage. The intensities of immunoreactivities and angiogenin transcripts in the follicle increased from the primordial to the tertiary follicles (See Figs. 2A–2E and 3A–3E). These results suggest that angiogenin is involved in the growth of follicles. Kamat et al [7] reported that VEGF mRNA and protein are expressed by human ovarian granulosa and theca cells late in follicle development.

Both the localization and mRNA expression of angiogenin in the corpus luteum were also different in their developmental stages. The early corpus luteum contained strong immunoreactivities and mRNA expression of angiogenin but these intensities weakened during regression. These results suggest that angiogenin is involved in the growth of the corpus luteum.

Strong immunoreactivity and mRNA expression of angiogenin in the oocyte and granulosa cells of tertiary follicles (see Figs. 2E and 3E) and the early corpus luteum (see Figs. 2F and 3F) were found. These results suggest that angiogenin may have biological functions involved in the growth of follicles and the corpus luteum by inducing angiogenesis in the ovary.

It has been suggested that luteinizing hormone (LH) can stimulate the production of angiogenic factors by luteal tissues [30]. Thus, it is tempting to speculate that 1) LH increases production of angiogenin by luteal tissues, 2) LH-induced angiogenin stimulates the growth of endothelial cells in the ovary by regulating VEGF production, and thus 3) corpus luteum mass in the ovary is indirectly regulated by angiogenin. Recently, Franck-Lissbrant et al. [31] showed that growth of the rat prostate gland is regulated by VEGF, which itself is apparently responding to angiogenic activity elaborated by the prostate epithelium under testosterone stimulation.

In conclusion, the observed changes in the localization and mRNA expression of angiogenin during development of the follicle and corpus luteum suggest that angiogenin is involved in morphological changes and angiogenesis in the ovary. In addition, the results of this study will lead to better methods of regulating fertility as well as pathological states in the ovary [28].

ACKNOWLEDGMENT

This work was supported by the Non-Directed Research Fund (96-001-G0101) of Korea Research Foundation, 1996.

REFERENCES

1. Risau, W. (1997) *Nature* **386,** 671–674.
2. Lee, W.-S., Jain, M. K., Arkonac, B. M., Zhang, D., Shaw, S.-Y., Kashiki, S., Maemura, K., Lee, S.-L., Hollenberg, N. K., Lee, M.-E., and Haber, E. (1998) *Circ. Res.* **82,** 845–851.
3. Moses, M. A., and Langer, R. (1991) *Biotechnology* **9,** 630–634.
4. Reynolds, L. P., Killilea, S. D., and Redmer, D. A. (1992) *FASEB J.* **6,** 886–892.
5. Reynolds, L. P., Grazul-Bilska, A. T., Killilea, S. D., and Redmer, D. A. (1994) *Prog. Growth Factor Res.* **5,** 159–175.
6. Reynolds, L. P., and Redmer, D. A. (1998) *J. Anim. Sci.* **76,** 1671–1681.
7. Kamat, B. R., Brown, L. E., Manseau, E. J., Senger, D. R., and Dvorak, H. F. (1995) *Am. J. Pathol.* **146,** 157–165.
8. Gospodarowicz, D., Cheng, I., Lui, G. M., Baird, A., and Bohlen, P. (1985) *Endocrinology* **117,** 2283–2290.
9. Phillips, H. S., Hains, J., Leung, D. W., and Ferrara, N. (1990) *Endocrinology* **127,** 965–967.
10. Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N., Daly, T. J., Davis, S., Sato, T. N., and Yancoopoulos, G. D. (1997) *Science* **277,** 55–60.
11. Bagavandoss, P., Sage, E. H., and Vernon, R. B. (1998) *J. Histochem. Cytochem.* **46,** 1043–1049.
12. Ferrara, N., Chen, H., Davis-Smyth, T., Gerber, H.-P., Nguyen, T.-N., Peers, D., Chisholm, V., Hillan, K. J., and Schwall, R. H. (1998) *Nat. Med.* **4,** 336–340.
13. Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., and Vallee, B. L. (1985) *Biochemistry* **24,** 5480–5486.
14. Shapiro, R., Strydom, D. J., Olson, K. A., and Vallee, B. L. (1987) *Biochemistry* **26,** 5141–5146.
15. Bond, M. D., and Vallee, B. L. (1988) *Biochemistry* **27,** 6282–6287.
16. Bond, M. D., and Strydom, D. J (1989) *Biochemistry* **28,** 6110–6113.
17. Maes, P., Damart, D., Rommens, C., Montreuil, J., Spik, G., and Tartar, A. (1988) *FEBS Lett.* **241,** 41–45.
18. Spik, G., Tartar, A., and Montreuil, J. (19-11-1987) International extension of French Patent No. 8715984.
19. Chang, S.-I., Jeong, G.-B., Park, S.-H., Ahn, B.-C., Choi, J.-D., Chae, Q., Namgoong, S. K., and Chung, S.-I. (1997) *Biochem. Biophys. Res. Commun.* **232,** 323–327.
20. King, T. V., and Vallee, B. L. (1991) *J. Bone Joint Surg.* **73-B,** 587–590.
21. Hu, G.-F., Riordan, J. F., and Vallee, B. L. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2204–2209.
22. Li, D., Bell, J., Brown, A., and Berry, C. L. (1994) *J. Pathol.* **172,** 171–175.
23. Shimoyama, S., Gansauge, F., Gansauge, S., Giovanni, Negri, Oohara, T., and Berger, H. G. (1996) *Cancer Res.* **56,** 2703–2706.
24. Montero, S., Guzman, C., Cortes-Funes, H., and Colomer, R. (1998) *Clin. Cancer Res.* **4,** 2161–2168.
25. Aboulghar, M. A., Mansour, R. T., Serour, G. I., Elhelw, B. A., and Shaarawy, M. (1998) *Hum. Reprod.* **13,** 2068–2071.
26. Chopra, V., Dinh T. V., and Hannigan E. V. (1998) *Cancer Invest.* **16,** 152–159.
27. Chopra, V., Dinh T. V., and Hannigan E. V. (1997) *J. Cancer Res. Clin. Oncol.* **123,** 167–172.
28. Chopra, V., Dinh, T. V., and Hannigan, E. V. (1996) *Cancer J. Sci. Am.* **2,** 279.
29. Piccoli, R., Olson, K. A., Vallee, B. L., and Fett, J. W. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 4579–4583.
30. Redmer, D. A., Kirsch, J. D., and Grazul, A. T. (1987) *in* Regulation of Ovarian and Testicular Function (Mahesh, V. B., Dhindsa, D. S., Anderson, E., and Kalra, S. P., Eds.), Adv. Exp. Med. Biol., Vol. 219, pp. 683–688, Plenum Press, New York.
31. Franck-Lissbrant, I., Haggstrom, S., Damber, J.-E., and Bergh, A. (1998) *Endocrinology* **139,** 451–456.
32. Kim, K.-H. (1996) M.S. thesis. Chungbuk National University, Cheongju, Korea.