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Tissue specific and cyclic expression of insulin-like growth factor binding proteins -1,-2,-3,-4,-5,-6 in the rat oviduct

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Although much is known about the expression insulin-like growth factors (IGF) and their receptors in the murine oviduct, significantly less is known about the expression of IGF binding proteins (IGFBPs). To fill this gap in our knowledge, we identified and characterized the tissue specific expression of IGFBP-1 to -6 in rat oviducts over the estrous cycle by *in situ* hybridization and immunocytochemistry. Tissues were analysed on proestrus (P1000 h, P2000 h), estrus (E0200, E1000 h), and diestrus I and II (DI 1100 h, DII 1100 h). IGFBP-1 was undetectable in the oviduct over the cycle. IGFBP-2 was selectively expressed in the luminal epithelium. The mRNA levels were high between P2000 h and E1000 h but low or undetectable thereafter. Immunoreactive IGFBP-2 was strong to very strong in these cells over most of the cycle. IGFBP-3 mRNA was undetectable in the oviduct; however, strong hybridization and immunoreactive signals were present in the mesosalpinx and mesotubarium, particularly at DI and DII. IGFBP-4 mRNA was not detected in the oviduct. In contrast, immunoreactive IGFBP-4 was observed in the luminal epithelium and the intensity was very strong after ovulation (E1000 h, DI and DII). IGFBP-5 and -6 mRNAs were selectively expressed in circular smooth muscle cells. Hybridization signals were evident over the cycle, but were greatest at estrus. By comparison, IGFBP-5 and -6 proteins were essentially undetectable in these cells except at DII 1100 h when immunostaining was moderate to high. Luminal epithelial cells were weakly positive for IGFBP-5 and -6. However, intense immunostaining was associated with the ciliated border and the luminal fluid juxtaposed to these cells during the cycle. The oocyte-cumulus complexes were immunostained intensely for IGFBP-2, -4, -5 and -6, but their mRNAs were undetectable. The signals were strongest in degenerating cumulus cells suggesting a potential role for these IGFBPs in cumulus apoptosis. These results demonstrate that the estrous cycle is accompanied by major changes in the pattern of expression of IGFBP-2, -4, -5 and -6 in the rat oviduct. We therefore conclude that the regulated production of these particular IGFBPs may be functionally important in modulating IGF activities in the oviduct, oocyte cumulus complexes, and perhaps the preimplantation embryo as well.

Keywords: IGFBPs; oviduct; rat *in situ* hybridization; immunocytochemistry

Introduction

The role of growth factors in the regulation of oviduct function has become a focus of research activity because of their potential involvement in fertilization and early embryonic development (Dey & Paria, 1993). One growth factor family that has received attention is the insulin-like growth factor (IGF) family (Murphy & Barron, 1993; Schultz *et al.*, 1993). The members of the IGF family include IGF-I, IGF-II, the IGF receptors and the six IGF binding proteins, IGFBPs (Shimasaki & Ling, 1991; Rechler, 1993; Jones & Clemmons, 1995).

Three lines of evidence indicate that an intrinsic IGF system may be functionally important in the rat oviduct. First, luminal epithelial cells express IGF-I mRNA and protein, and the levels change during the estrous cycle (Carlsson *et al.*, 1993). A specific role for estradiol in stimulating IGF-I expression in these cells has been established (Carlsson *et al.*, 1993). IGF-II transcripts are also present in the oviducts (Zhang *et al.*, 1994). Together, these data support the concept that rat oviducts synthesize IGF ligands. Second, the IGF-I receptor is expressed in the rat oviduct (Carlsson *et al.*, 1993). This result supports the hypothesis that IGF-I may bind to IGF-I receptors on oviduct target cells and generate cellular responses. And third, IGF-I and II receptors are expressed in rat preimplantation embryos (Zhang *et al.*, 1994). The ability of IGF-I to increase the number of blastomeres in murine embryos suggests the IGF receptors may be physiologically significant (Harvey & Kaye, 1991). Collectively, these lines of evidence have led to the conclusion that locally produced IGFs may regulate oviduct and preimplantation embryo function by autocrine/paracine mechanisms.

The IGF binding proteins (IGFBPs) can modulate, either amplify or attenuate, IGF bioactivity (Rechler, 1993). Consequently, IGFBPs are important components of the IGF system. There are six IGFBPs denoted IGFBP-1 to -6, each is presumed to have a different set of physiological functions (Shimasaki & Ling, 1991). *In situ* hybridization experiments have shown that the IGFBP mRNAs are expressed in a tissue specific manner in both the rat ovary (Erickson *et al.*, 1992a; Erickson *et al.*, 1994a) and uterus, (Girvigian *et al.*, 1994), and the levels of IGFBP expression change during the estrous cycle. These data suggest IGFBPs may be involved in regulating reproduction in the rat. Indeed the concept that IGFBP-2, -3, -4 and -5 are potent inhibitors of FSH action has been established (Bicsak *et al.*, 1990; Liu *et al.*, 1993; Erickson *et al.*, 1994a).

Very little is known about the IGFBPs in the oviduct. In the mouse, the mRNAs for IGFBP-2 and -6 have been identified by RT-PCR in the luminal epithelium of the oviduct, and in the embryos and cumulus cells within the luminal fluid (Hahnel & Schultz, 1994). This result points to the possibility that IGFBPs may be physiologically relevant in the murine oviduct. To gain further insight into the IGFBP system in the oviduct, we have characterized the tissue specific and cyclic expression of the IGFBP mRNAs and proteins in the rat oviduct over the estrous cycle by *in situ* hybridization and immunocytochemistry.

Results

Table 1 and Figures 1 to 5 show the data from *in situ* hybridization experiments and Table 2 and Figure 6 show the results of immunocytochemistry analyses.

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Table 1 Tissue specific expression of IGFBP-2, -3, -4, -5, and -6 mRNAs in the rat oviduct during the estrous cycle

Oviduct tissue / *IGFBPs*	*P 1000 h*	*P 2000 h*	*E 0200 h*	*E 1000 h*	*DI 1100 h*	*DII 1100 h*
Luminal epithelium						
IGFBP-2	–	++	++	+++	–	–
IGFBP-3	–	–	–	–	–	–
IGFBP-4	–	–	–	–	–	–
IGFBP-5	–	–	–	–	–	–
IGFBP-6	–	–	–	–	–	–
Circular smooth muscle						
IGFBP-2	–	–	–	–	–	–
IGFBP-3	–	–	–	–	–	–
IGFBP-4	–	–	–	–	–	–
IGFBP-5	+	++	+++	+++	+++	++
IGFBP-6	++	+	++	++	–	+
Serosa						
IGFBP-2	–	–	–	–	–	–
IGFBP-3	–	–	–	–	–	–
IGFBP-4	–	–	–	–	–	–
IGFBP-5	±	±	±	±	±	±
IGFBP-6	–	–	–	–	–	–

The intensity of hybridization signal indicated above represents a subjective comparison of four sections from each oviduct from two animals hybridized with equal concentrations of sense and antisense cRNAs and exposed for the same time (4 days). The hybridization signal was estimated on a scale of 1+ to 4+, as described previously (Erickson *et al.*, 1992a,b): 1+, weak; 2+, moderate; 3+, strong; 4+, very strong; –, nondetectable; These data were confirmed independently by three investigators. P, proestrus; E, estrus; DI, diestrus day 1; DII, diestrus day 2.

Table 2 IGFBP immunoreactivity in the rat oviduct over the estrous cycle

Oviduct tissue / *IGFBPs*	*P 1000 h*	*P 2000 h*	*E 0200 h*	*E 1000 h*	*DI 1100 h*	*DII 1100 h*
Luminal epithelial cells						
IGFBP-2	+	+++	+++	++++	++++	++++
IGFBP-3	–	–	–	–	–	–
IGFBP-4	+	+	+	+++	+++	+++
IGFBP-5	+	+	+	+	+	+
IGFBP-6	+	+	+	+	+	+
Circular smooth muscle						
IGFBP-2	–	–	–	–	–	–
IGFBP-3	–	–	–	–	–	–
IGFBP-4	–	–	–	–	–	–
IGFBP-5	–	–	–	+	–	++
IGFBP-6	–	–	–	–	+	+++
Serosa						
IGFBP-2	–	–	–	–	–	–
IGFBP-3						
IGFBP-4	–	–	–	–	–	–
IGFBP-5	±	±	±	±	±	±
IGFBP-6	–	–	–	–	–	–

The relative intensity of the immunostaining represents a subjective comparison of four sections from the oviducts of two animals immunostained with indicated specific IGFBP antisera. The grading of the intensity of the immunostaining was as follows: –, non detectable; +, low; ++, moderate, +++, high; ++++, very high. These data were confirmed independently by three investigators.

IGFBP-1

IGFBP-1 mRNA was not detected in the rat oviducts examined.

IGFBP-2

IGFBP-2 mRNA was selectively expressed in the luminal epithelium (Figure 1). The levels were moderate at P2000 h and E0200 h, high at E1000 h, but weak or undetectable at other times in the cycle (Table 1). By comparison, immunoreactive IGFBP-2 was observed in the luminal epithelium throughout the estrous cycle (Table 2 and Figure 6). Low but detectable immunostaining was seen at P1000 h, after which the intensity increased sharply and remained high to very high throughout the rest of the cycle (Table 2). A high level of IGFBP-2 mRNA was also present in the serosal cells located in the bursa region of the ovary during the cycle (Figure 1).

IGFBP-3

IGFBP-3 mRNA was undetectable in the oviduct proper. However, a strong hybridization signal was evident in the region of the smooth muscle cells of the mesosalpinx and mesotubarium, particularly at DI and DII (Figure 2). A similar patterm of IGFBP-3 immunostaining was apparent in these tissues.

IGFBP-4

IGFBP-4 mRNA was not detected in the oviduct (Table 1). By contrast, IGFBP-4 immunoreactivity was found in the luminal epithelium at all stages of the cycle (Table 2, Figure 6). The signal was weak on proestrus and early estrus, but high between E1000 h and DII (Table 2). Strong hybridization and immunoreactive signals were also seen in serosal cells, in the bursa region juxtaposed to the ovary at E1000 h (Figure 3).

IGFBP-5

IGFBP-5 mRNA was selectively expressed in the circular muscle cells (Figure 4, Table 1). The hybridization signals were weak to moderate at proestrus, strong to very strong at estrus and DI and moderate at DII (Table 1). By comparison, immunoreactive IGFBP-5 was detected only at E1000 h and DII, and the intensity was low and mdoerate respectively (Table 2). IGFBP-5 immunostaining was also observed in the cytosplasm of the luminal epithelial cells, but the signal was weak (Table 2). But a strong immunoreactive IGFBP-5 signal was evident at the ciliated border of the these cells and in the luminal fluid (Figure 6). There was detectable IGFBP-5 mRNA and protein in the serosa cells, but the intensities appeared variable (Table 2, Figure 4).

IGFBP-6

IGFBP-6 mRNA was expressed solely in circular smooth muscle cells (Figure 5, Table 1). The hybridization signal was weak to moderate at proestrus and early estrous morning (E0200 h), strong at E1000 h and weak or undetectable during diestrus (Table 1). By comparison, IGFBP-6 immunostaining was limited to DI and DII when the intensity appeared low and high respectively (Table 2). A weak immunoreactive IGFBP-6 signal was apparent in the luminal epithelial cells throughout the estrous cycle (Table 2), but intense immunostaining was apparent at or near the surface of these cells (Figure 6).

Oocyte-cumulus complexes

Expanded oocyte-cumulus complexes were present in the luminal fluid at E1000 h. None of the IGFBP mRNAs was detected in the oocyte-cumulus complexes (see for example Figure 1, panel A). It should be noted that many of the cumulus granulosa cells showed signs of pyknosis e.g., extensively clumped chromatin and cytoplasmic blebbing. Interestingly, thse pyknotic cells were strongly positive for

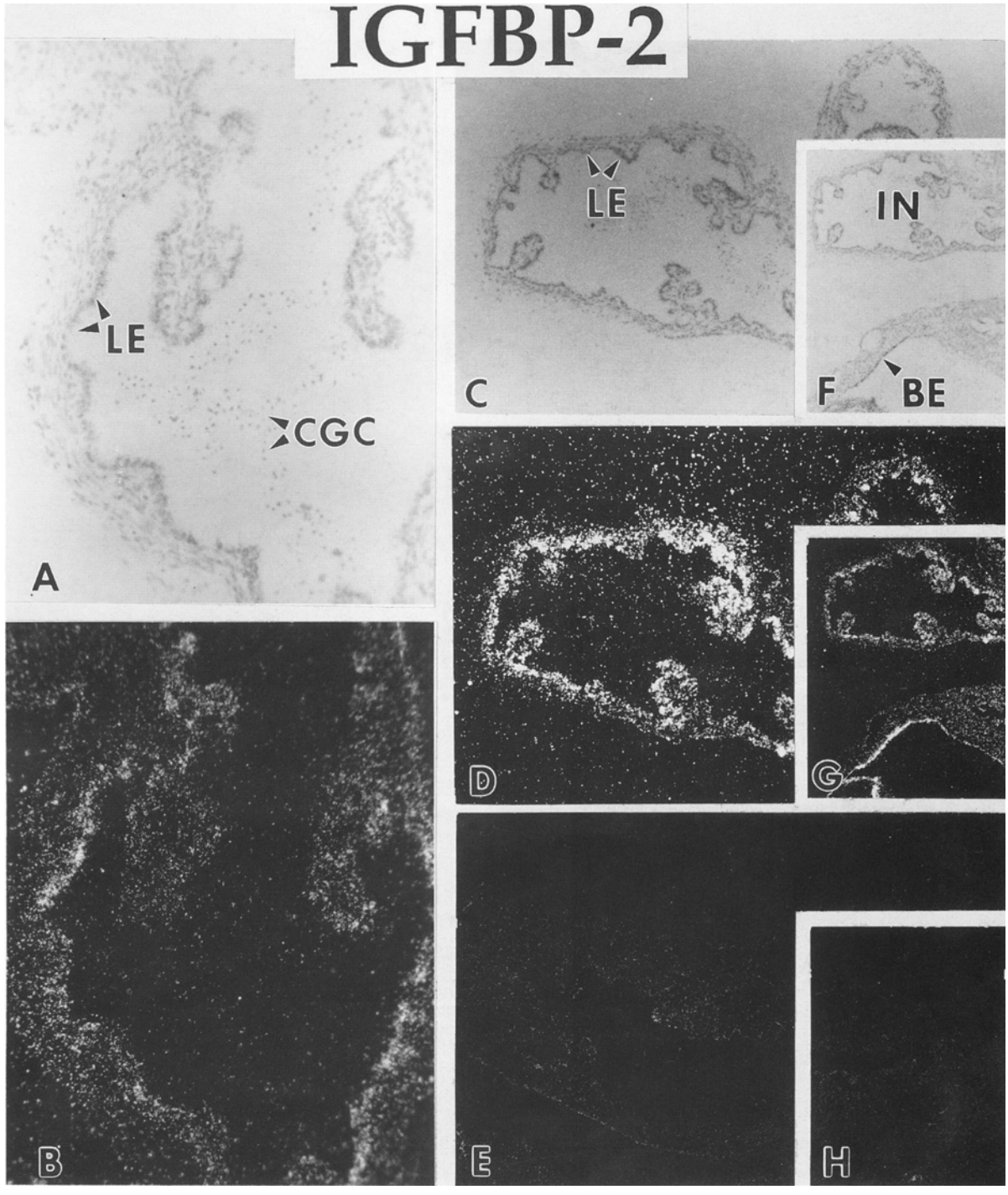

Figure 1 Cellular localization of IGFBP-2 mRNA in the rat oviduct and ovary bursa at 1000 h on estrus. LE, luminal epithelium; CGC, cumulus granulosa cells; IN, infundibulum; BE, bursal epithelium. (**A,C,F**) brightfield photomicrographs. (**B,D,G**) Darkfield photomicrographs after hybridization with the antisense cRNA probe. (**E,H**) Darkfield photomicrographs after hybridization with the sense cRNA probe

immunoreactive IGFBP-2, -4, -5 and -6. Intense immunostaining for these IGFBPs was also evident in occytes (data not shown).

Discussion

These results demonstrate the principle of tissue specific and cycle dependent expression of the IGFBP family in the rat oviduct. It is clear that IGFBP-2 mRNA and protein are expressed in luminal epithelial cells and that IGFBP-5 and -6 mRNAs and proteins are expressed in circular smooth muscle cells. It is also clear that the levels of these IGFBPs change over the estrous cycle. Thus, we conclude that the regulated production of intrinsic IGFBP-2, -5 and -6 may be functionally important in the oviduct, presumably by virtue of their ability to modulate IGF bioactivity. Our experiments also indicate that IGFBP-4, -5, -6 proteins, but not the mRNAs, are detectable in the luminal epithelium and oocyte cumulus complexes. Although these results are more difficult to interpret, the presence or these IGFBPs suggest they also may have role in oviduct regulation.

IGFBP-1 mRNA was not detected in the rat oviducts. The failure to detect a positive signal was not caused by inactivity

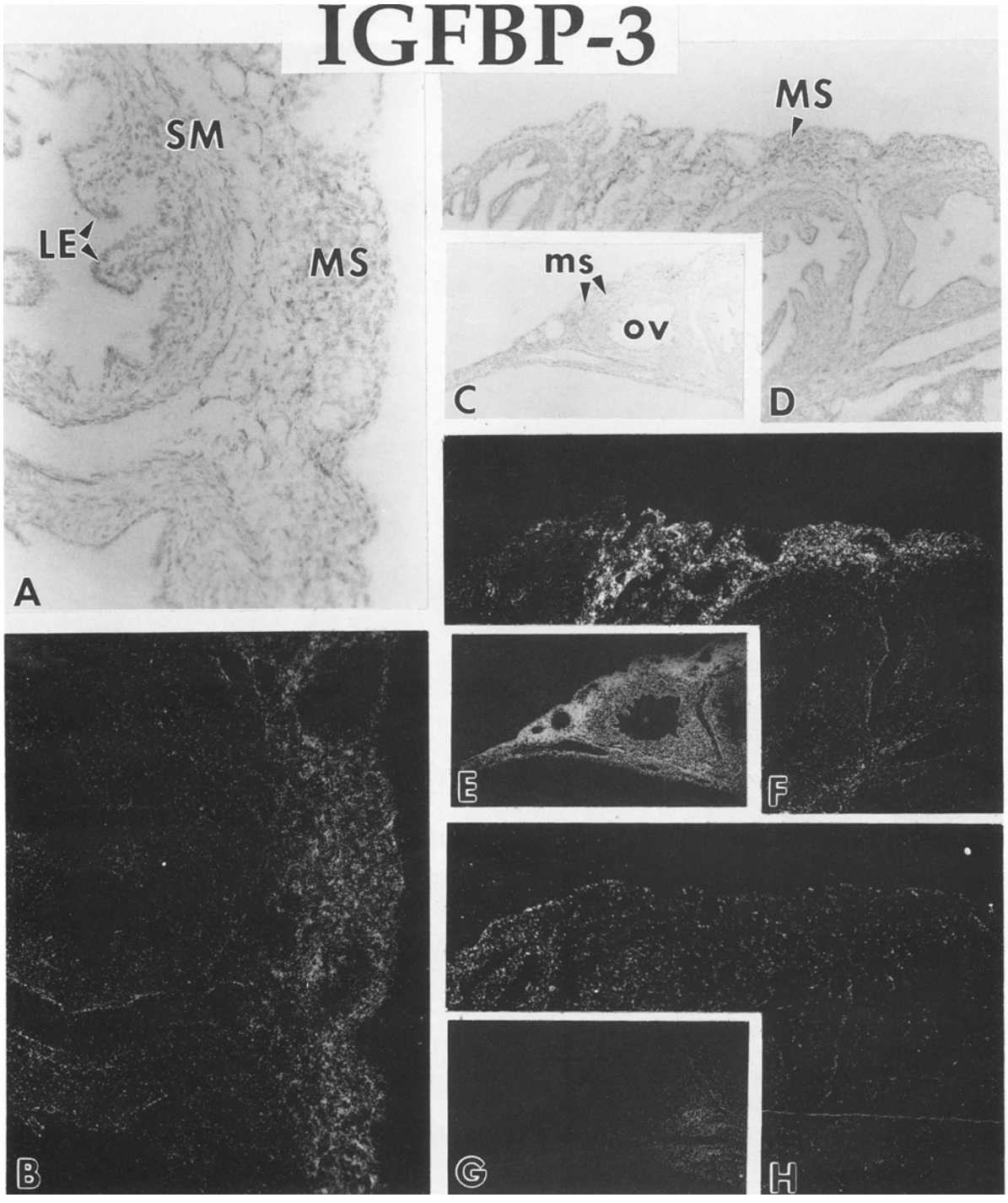

Figure 2 Cellular localization of IGFBP-3 mRNA in the rat oviduct at 1100 h diestrus I. LE, luminal epithelium; SM, smooth muscle; MS, mesosalpinx; OV, oviduct. (**A,C,D**) Brightfield photomicrographs. (**B,E,F**) Darkfield photomicrographs after hybridization with the antisense cRNA probe. (**G,H**) Darkfield photomicrograph after hybridization with the sense cRNA probe

of the IGFBP-1 cRNA probe because it did give a strong hybridization signal in two tissues known to contain the 1.6 kilobase IGFBP-1 mRNA transcript (Ooi *et al.*, 1990) namely, the rat kidney and intestine (Erickson unpublished result). It is possible therefore that intrinsic IGFBP-1 may not play a role in the oviduct of cycling rats. Evidence from studies on the mouse (Hahnel & Schultz, 1994) and human Giudice *et al.*, 1992) oviducts have led to a similar conclusion. The contribution of intrinsic IGFBP-1 in the non pregnant rat ovary (Nakatani *et al.*, 1991) and uterus (Girvigian *et al.*, 1994) has also been questioned.

The presence of IGFBP-2 mRNA and protein in the luminal epithelial cells supports the idea that intrinsic IGFBP-2 might serve as an autocrine/paracrine regulator of oviduct function. A similar conclusion has been reached from studies of IGFBP-2 expression in mouse (Hahnel & Schultz, 1994) and human (Guidice *et al.*, 1992) oviducts. This conclusion was reinforced by our finding that IGFBP-2 expression changes during the estrus cycle. It seems reasonable to propose that these changes are brought about by hormones and growth factors. Estradiol is a potent stimulator of IGFBP-2 mRNA expression in rat pituitary (Michels *et al.*, 1993) and ovary theca interstitial cells (Ricciarelli *et al.*, 1991). Because oviduct luminal epithelial cells contain estrogen receptor (Stumpf, 1969; Jansen, 1984), and because IGFBP-2 mRNA levels are detected after the preovulatory

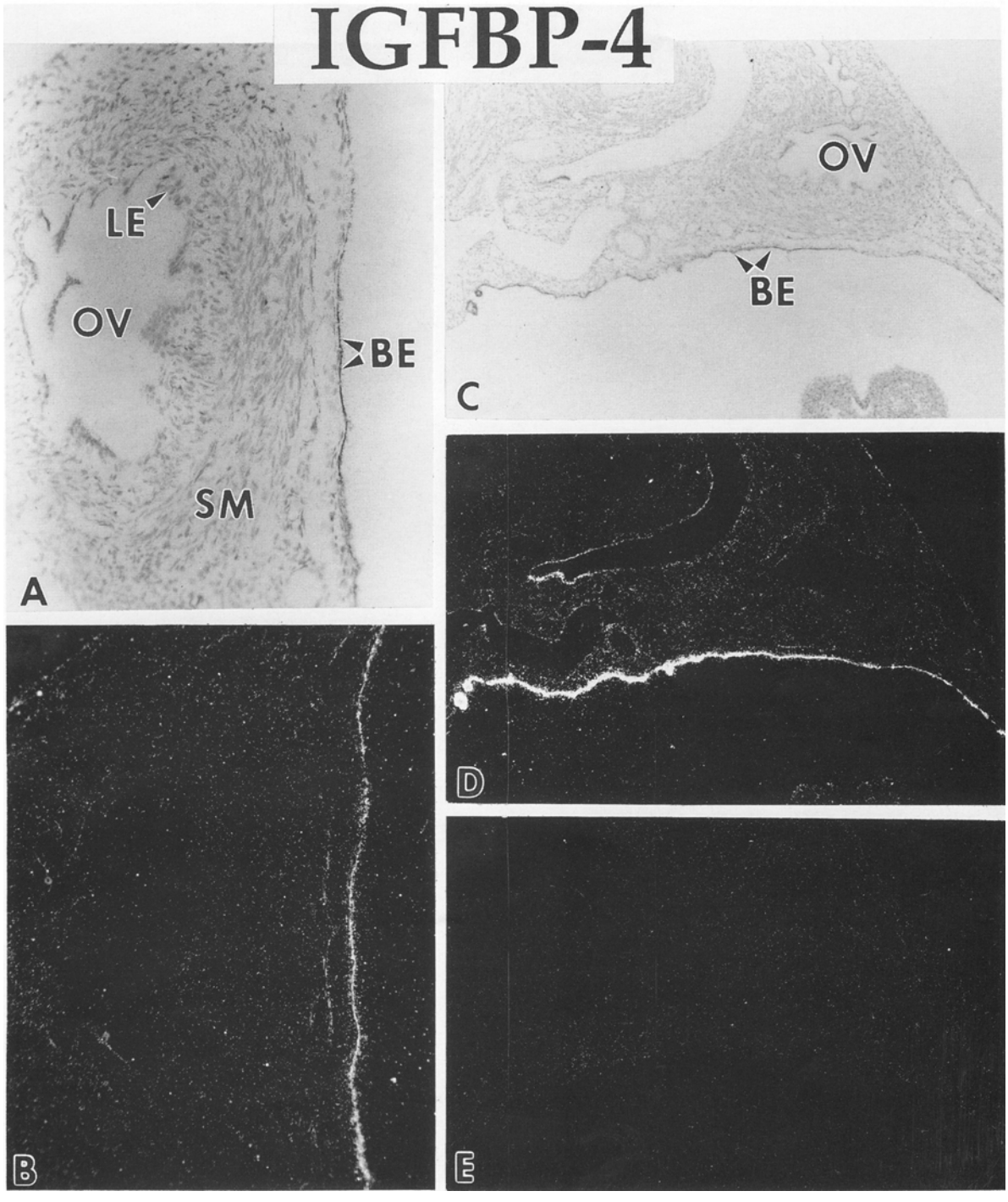

Figure 3 Cellular localization of IGFBP-4 mRNA in the rat oviduct and ovary bursa at 1000 h on estrus. LE, luminal epithelium; OV, oviduct; SM, smooth muscle; BE, bursal epithelium. (**A,C**) Brightfield photomicrographs. (**B,D**) Darkfield photomicrographs after hybridization with the antisense cRNA probe (**E**) Darkfield photomicrograph after hybridization with sense cRNA probe

surge of estradiol on proestrus (Smith *et al.*, 1975), it seems reasonable to propose that IGFBP-2 gene activity in the oviduct may be regulated by estradiol. In rat theca, IGF-I is also a potent stimulator of IGFBP-2 expression (Erickson *et al.*, 1995). Whether IGF-I produced by the luminal epithelium (Carlson *et al.*, 1993) acts as an autocrine stimulus for IGFBP-2 expression remains to be determined.

The ability of the mesosalpinx and mesotubarium to produce IGFBP-3 suggests it may be physiologically relevant. Because the mRNA and protein appeared to be localized to smooth muscle cells, it is possible that intrinsic IGFBP-3 has a regulatory function in the contractile activities of these ligaments; however, further work is necessary to test this possibility.

We identified IGFBP-4 protein in luminal epithelial cells, but the message, was not detectable. This finding is paradoxical and raises questions about the origin of the IGFBP-4. There are two possible explanations to account for the data: an intrinsic origin and/or an extrinsic source. If one assumes an intrinsic origin, then the number of copies of the mRNA must be too low to detect by *in situ* hybridization, perhaps because the IGFBP-4 protein is rapidly being synthesized. Alternatively, the IGFBP-4 proteins could come from ectopic sites. For example, isolated rat granulosa cells have been shown to synthesize and secrete IGFBP-4 spontaneously *in vitro* (Liu *et al.*, 1993; Erickson *et al.*, 1994a,b). This fact, together with the present finding that cumulus granulosa cells contain immunoreactive IGFBP-4, raises the possibility that

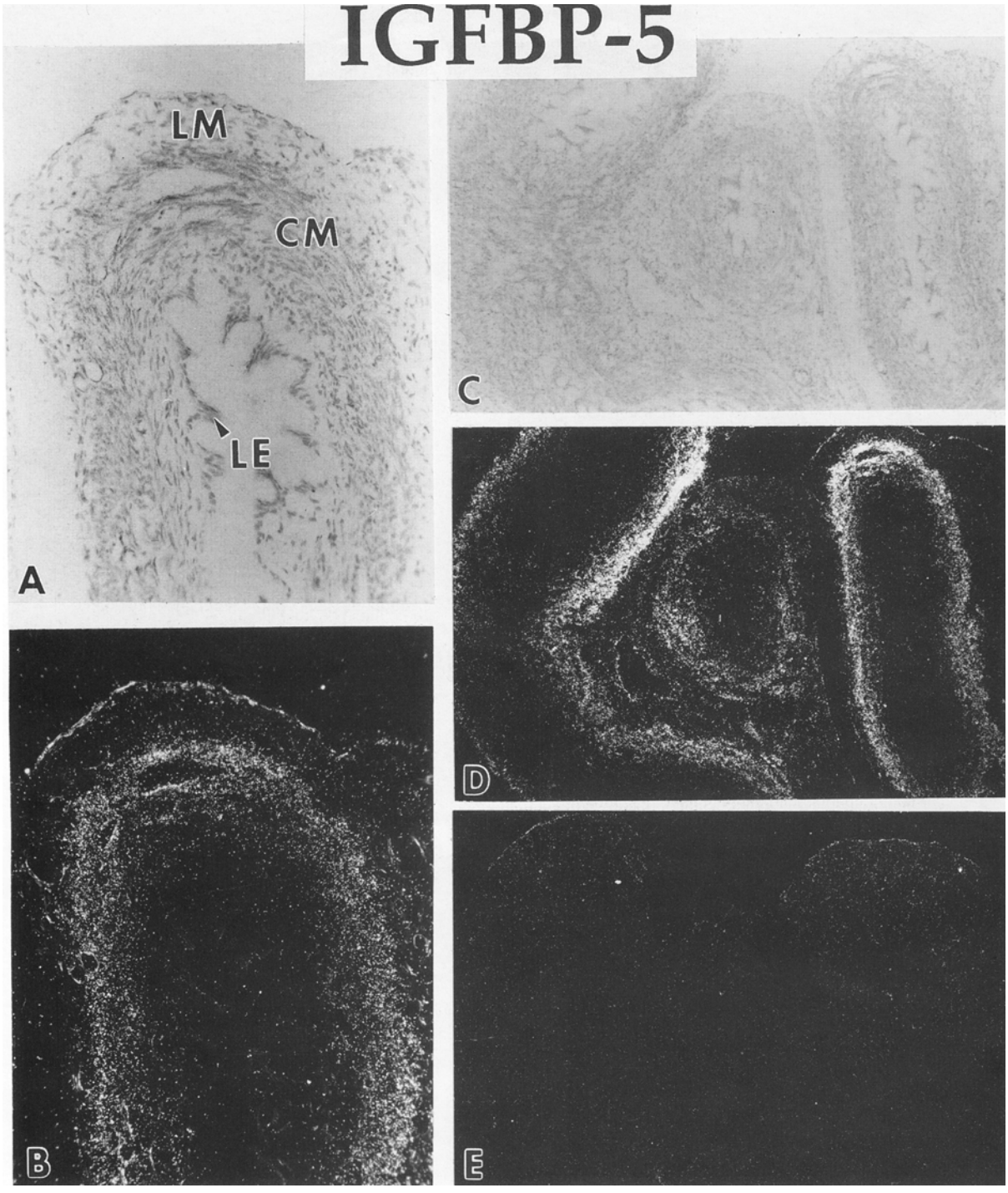

Figure 4 Cellular localization of IGFBP-5 in the rat oviduct at 1000 h on estrus. LE, luminal epithelium; CM, circular smooth muscle; LM, longitudinal muscle. (**A,C**) Brightfield photomicrographs. (**B,D**) Darkfield photomicrographs after hybridization with the antisense cRNA probe. (**E**) Darkfield photomicrograph after hybridization with the sense cRNA probe

these cells might release IGFBP-4 mature proteins and/or fragments that inturn accumulate in the luminal epithelial cells between E1000 h and DII. IGFBP-4 is also expressed in the epithelium of the rat uterus (Girvigian *et al.*, 1994), perhaps some of the IGFBP-4 seen in the oviduct comes from the uterus or possibly the blood. Additional experiments are needed to resolve this paradox.

IGFBP-5 and -6, are expressed in the circular smooth muscle cells and the patterns of expressions are dependent on the stage in the cycle. These data provide evidence that IGFBP-5 and -6 may be physiologically relevant molecules that modulate IGF activity in oviduct smooth muscle. The concept that IGF-I stimulates myogenesis in the rat has been established (Ewton & Florini, 1981). Moreover, it has been demonstrated that rat myoblasts produce IGFBP-4, -5 and -6 *in vitro* and that the expression of these IGFBPs inhibit IGF-I dependent myogenesis (Silverman *et al.*, 1995). Together these data suggest that expression of intrinsic IGFBP-5 and -6 *in vivo* may be an important parameter in modulating the physiological effects of IGF-I in oviduct smooth muscle cells. The question of what controls IGFBP-5 and -6 expression in the oviduct is unknown. Oviduct smooth muscle cells contain estrogen and progesterone receptors (Forcelledo *et al.*, 1986; Fuentealba *et al.*, 1987; Salamonsen & Nancarrow, 1994). Thus, one might speculate that these hormones regulate the differential expression of IGFBP-5

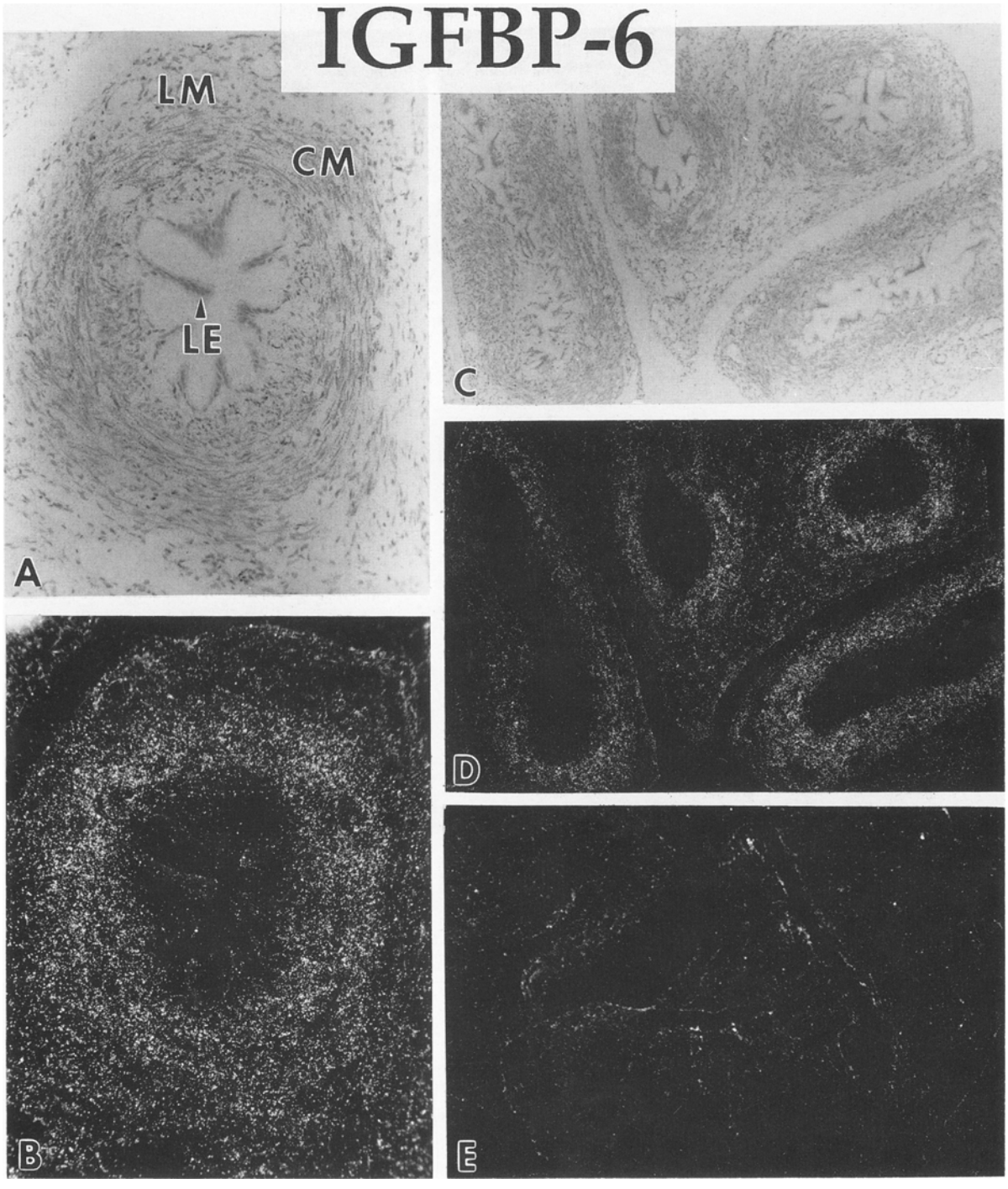

Figure 5 Cellular localization of IGFBP-6 mRNA in the rat oviduct at 1000 h on estrus. LE, luminal epithelium; CM, circular smooth muscle; LM, longitudinal smooth muscle. (**A,C**) Brightfield photomicrographs. (**B,D**) Darkfield photomicrographs after hybridization with the antisense cRNA probe. (**E**) Darkfield photomicrograph after hybridization with the sense cRNA probe

and -6 in these cells. It is noteworthy that even though the mRNAs were present, the IGFBP-5 and -6 proteins appeared to be expressed at only a few stages, most notably at DII. The reason for this apparent discrepancy is not clear; however, it could suggest that IGFBP-5 and -6 expression may be subject to translational and/or pretranslational control mechanisms.

The oocyte-cumulus complexes in the oviduct at E1000 h were found to contain immunoreactive IGFBP-2, -4, -5 and -6. The possibility suggested by these results is that these IGFBPs contribute to the regulation of the oocyte-cumulus cells and/or the oviduct. With respect to the cumulus cells, it has been shown that IGFBP-2, -4 and -5 are potent inhibitors of FSH-induced differentiation in rat granulosa cells (Bicsak *et al.*, 1990; Liu *et al.*, 1993). Furthermore, two of these IGFBPs, namely IGFBP-4 and -5, appear to be involved in granulosa apoptosis during physiological atresia *in situ* (Erickson *et al.*, 1992a,b). Because our data show that the IGFBPs were present in degenerating but not healthy cumulus cells, leads us to hypothesize that IGFBP-2, -4, -5 and possibly -6 may be atretogenic proteins that cause apoptosis in cumulus cells after ovulation. Experiments are now in progress to test this hypothesis. The relationship, if any, between the IGFBPs and the oocyte is unknown. Despite intense immunostaining, no IGFBP mRNAs were detected in the oocyte-cumulus complexes. Again, this is a puzzling

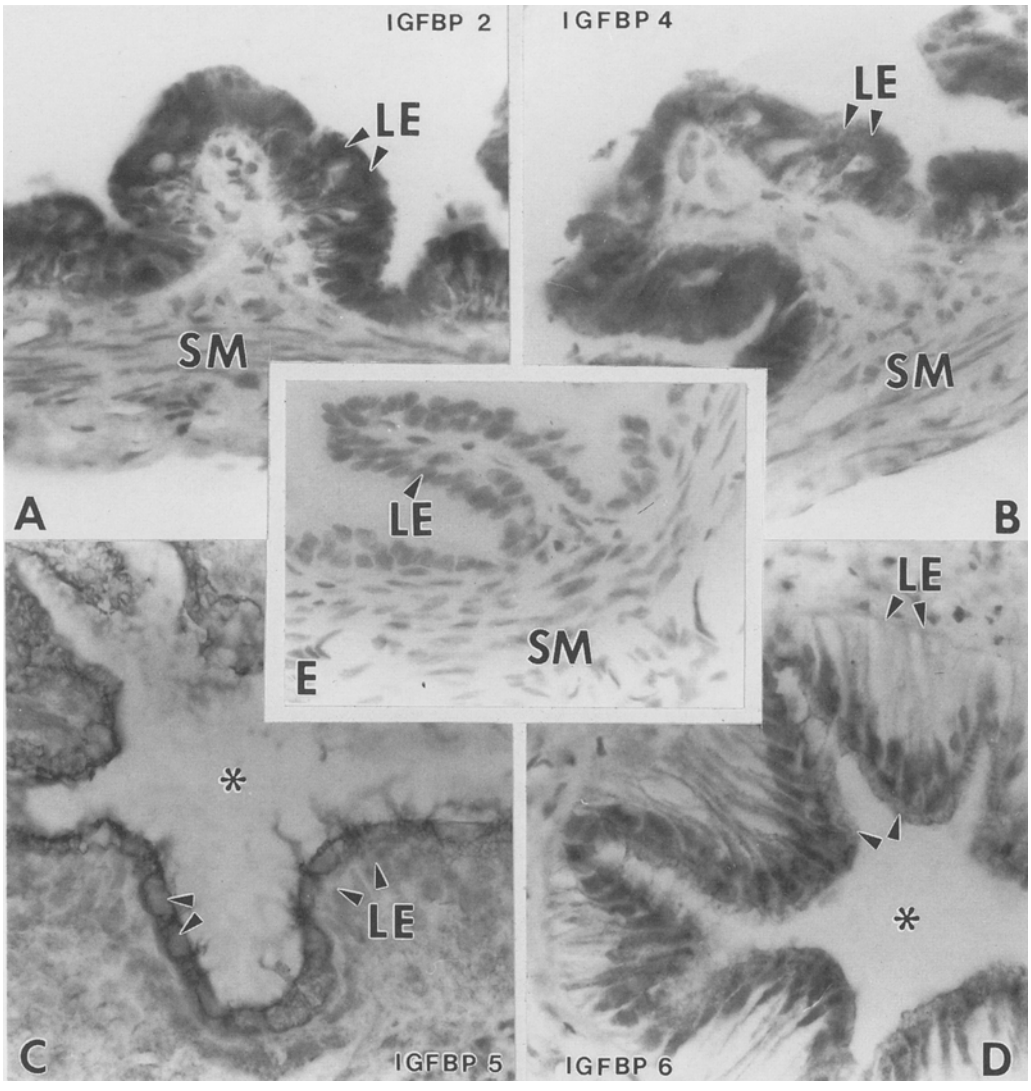

Figure 6 Cellular localization of immunoreactive IGFBP-2, -4, -5 and -6 in the oviduct of adult cycling rats. LE, luminal epithelium; SM, smooth muscle; arrowheads, ciliated border; *, luminal fluid. (**A**) IGFBP-2; (**B**) IGFBP-4; (**C**) IGFBP-5; (**D**) IGFBP-6; (**E**) Representative control section stained in the absence of primary IGFBP-2, -4, -5 or 6 antisera. **A,B,E** are at DII1100 h. **C** and **D** are at P1000 h

observation that requires further investigation.

It is apparent from this physiological study that there is tissue specific and cycle dependent expression of the IGFBP family in the adult rat oviduct. The capacity for IGFBP production argues that intrinsic IGFBPs may be able to locally modulate, either increase or decrease, IGF-I and II signaling within the oviduct. A similar case can be made for the oocyte-cumulus complex and perhaps for the preimplantation embryo as well. These results raise a number of intriguing and potentially important hypothesis; however, the physiological significance of these intrinsic IGFBPs must await the elucidation of the physiological roles of IGF-I and II in the oviduct and oocyte-cumulus complexes.

Materials and methods

Animals

The animal studies were conducted in accord with the recommendations outlined in 'Guidelines for Care and Use of Experimental Animals' and the protocols were approved by the Animal Care and Use Committees. Female Sprague-Dawley rats (60–90 days old) were used in these experiments. The stages of the estrous cycle were determined by vaginal smears. Animals were sacrificed at proestrus 1000 h and 2000 h (P1000 h, P2000 h), estrus 0200 h and 1000 h (E0200, E1000 h, and at 1100 h on diestrus I (DI) and diestrus II (DII).

Tissue fixation

Animals were anesthetized and perfused through the femoral artery with cold physiological saline (0.9% NaCl), followed by perfusion with fixative according to the pH shift method (Simmons *et al.*, 1989).

In situ *hybridization*

The IGFBPs mRNAs were localized by *in situ* hybridization (Nakatani *et al.*, 1991). Frozen sections (10 μm) were mounted onto poly-L-lysine coated glass slides, digested with proteinase-K (5 μg/mL); 37°C; 10 min), acetylated and dehydrated. Sections were hybridized with ^{35}S-labeled RNA probe ($4-6 \times 10^6$ c.p.m./mL) in a solution containing 50% (vol/vol) deionized formamide, 0.3 M NaCl, 10 mM. Tris (pH 8.2), 1 mM EDTA 0.05% yeast tRNA, 10 mM dithiothreitol, 1 × Denhardt's solution and 10% dextran sulfate. Hybridization solution (60 μL) was placed onto acid-washed siliconized 60 × 22 mm coverslips and applied to each slide.

Coverslips were then sealed with liquid DPX. Sections were hybridized for 16 h at 58–60°C in a humidified chamber. After hybridization, the sections were treated with ribonuclease-A (20 μg/mL); 37°C; 30 min and washed in 15 mM NaCl/1.5 mM sodium citrate at 60–62°C for 30 min and dehydrated. Dehydrated slides were defatted in xylene, rinsed in 100% ethanol, air dried and coated with Kodak NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY). Slides were exposed for 4 days at 4°C in a desiccated dark box. After exposure, the slides were developed (Kodak D19; 14°C; 3.5 min), rinsed briefly in distilled water and fixed (Kodak Rapid Fix; 14°C; 5 min). After washing in distilled water for 1 h, slides were counterstained with hematoxylin.

Before preparation of the cRNA probes for IGFBP-1 and -2, part of the coding regions of the cDNAs [nucleotides 486–892 of the rat IGFBP-1 cDNA (Murphy *et al.*, 1990) and 643–1037 of the rat IGFBP-1 cDNA (Brown *et al.*, 1989)] were prepared by polymerase chain reaction (PCR). PCR was performed by a TwinBlock system (Ericcomp, San Diego, CA) with a GeneAmp DNA amplification Reagent Kit (Perkin-Elmer Cetus, Norwald, CT), using a rat liver cDNA library as a template. The primers were synthesized by a Cyclone Plus DNA synthesizer (Milligen/Biosearch, Novato, CA). Annealing reactions were performed at 60°C for 30 s followed by a 30 s extension at 72°C and 15 s denaturation at 94°C. After 35 cycles of amplification, PCR-derived fragments were purified, kinased by ATP and cloned into the EcoRV site of pBluescript SK$^+$ (Stratagene, San Deigo, CA). The DNA sequence of the amplified fragment was determined by the double strand dideoxy chain termination method, using Sequenase (U.S. Biochemical Corp., Cleveland, OH). In the cases of IGFBP-3, -4, -5 and -6 the ApaI–RsaI 699-basepair (bp) restriction fragment derived from the rat IGFBP-3 cDNA clone pRF1507 (Shimasaki *et al.*, 1989), and the SmaI-HindIII 444 bp restriction fragment derived from the rat IGFBP-4 cDNA clone pRBP4-501 (Shimasaki *et al.*, 1990), the SacII–HindIII 300 bp restriction fragment derived from the rat IGFBP-5 cDNA (Shimasaki *et al.*, 1991a), and the PstI–PstI 246 bp restriction fragment derived from the rat IGFBP-6 cDNA (Shimasaki *et al.*, 1991b), were subcloned into the ApaI–SmaI, SmaI–HindIII, SacII–HindIII and PstI–PstI sites of pBluescript SK$^+$, respectively. Antisense strand cRNAs were prepared using these plasmids, which contain a partial coding region of IGFBP-1, -2, -3, -4, -5 and -6 by *in vitro* transcription with the T3 or T7 RNa polymerase and [^{35}S]UTP and used as probes for the *in situ* hybridization. In each experiment, the cRNA probe corresponding to the matched sense strand was used on control tissue sections. All hybridizations for each quantitative comparison were done at the same time and with the same amount of radiolabeled cRNA probe.

Immunocytochemistry

Specific rat polyclonal antibodies were used for immunohistochemical localization of the IGFBPs (Liu *et al.*, 1993). Paraffin sections (8 μm) were mounted onto geletain-coated slides. The sections were incubated for 30 min with 0.3% H_2O_2 in methanol, washed three times for 5 min in 0.01 M PBS, and incubated for 30 min in 0.01 M PBS containing 0.25% Triton X-100 and 3% normal goat serum. After removing the medium, the sections were incubated overnight with primary antiserum diluted 1:1000 or 1:2000 in 0.01 M PBS containing 3% normal goat serum and then washed three times for 30 min in PBS. IGFBP antibody complexes were demonstrated using an avidin-biotin immunocomplex method (Vector Labs, Burlingame, CA). Reaction products were developed during 3–5 min incubation in a mixture of 0.03% (wt/vol) 3,3'-diamino-benzidine tetrahydrochloride, 0.05% (wt/vol) $NiCl_2$ and 0.015% H_2O_2 dissolved in 0.05 M TriosHCl (pH 7.4). Controls were incubated without the primary antibodies or with preimmune rabbit serum instead of primary antibodies.

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