

Expression of Messenger Ribonucleic Acids for Fibroblast Growth Factors 7 and 10, Hepatocyte Growth Factor, and Insulin-Like Growth Factors and Their Receptors in the Neonatal Ovine Uterus¹

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ABSTRACT

In sheep, uterine development begins during fetal life but is only completed postnatally with proliferation and branching morphogenetic differentiation of the endometrial glandular epithelium (G) from the luminal epithelium (L) between birth or Postnatal Day (PND) 0 and PND 56. In other epithelial-mesenchymal organs, fibroblast growth factor (FGF)-7 and FGF-10, hepatocyte growth factor (HGF), and insulin-like growth factor (IGF)-I and IGF-II play essential roles in ductal branching morphogenesis. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization analyses were used to study temporal and spatial alterations in expression of mRNAs for growth factors (FGF-7, FGF-10, HGF, IGF-I, IGF-II) and their respective receptors (FGF receptor or FGFR2IIIb, *c-met*, and IGF-IR) in the developing neonatal ovine uterus. The RT-PCR analyses indicated that expression of FGF-10, HGF, IGF-I, and IGF-II mRNAs increased in the neonatal uterus between PND 1 and 56. In situ hybridization analyses indicated that FGFR2IIIb and *c-met* mRNAs were expressed solely in uterine L and developing G, whereas IGF-IR was expressed in all uterine cell types, with highest levels in L and developing G. Both IGF-I and IGF-II mRNAs were expressed in the endometrial stroma and myometrium, with IGF-I predominantly in the intercaruncular endometrial stroma. The highest levels of IGF-I and IGF-II mRNA expression were detected in the intercaruncular endometrial stroma surrounding the nascent and proliferating glands. Immunohistochemistry revealed that phosphorylated extracellular regulated kinases-1 and -2 were most abundantly expressed in the nascent and proliferating glands of the developing neonatal uterine wall. These results implicate FGF-7, FGF-10, HGF, IGF-I, IGF-II, and their epithelial receptors in epithelial-mesenchymal interactions regulating endometrial gland morphogenesis in the neonatal sheep uterus.

development, developmental biology, female reproductive tract, growth factors, signal transduction, uterus

INTRODUCTION

The dichotomous nature of the adult ovine endometrium, consisting of both aglandular caruncular areas and glandular intercaruncular areas, provides an excellent model for the study of mechanisms underlying establishment of di-

vergent structural and functional areas within a single, mesodermally derived organ [1]. As in other mammals, the ovine uterus develops as a specialization of the paramesonephric (i.e., müllerian) ducts, which give rise to the infundibula, oviducts, uterus, cervix, and anterior vagina [2]. Paramesonephric duct fusion occurs between Gestational Day 34 and 55 in sheep, is partial, and produces a bicornuate uterus [1, 3, 4]. Postnatal ovine uterine morphogenesis primarily involves the emergence, proliferation, and branching morphogenesis of endometrial glands; development of endometrial folds; and to a lesser extent, growth of endometrial caruncular areas and myometrium (M) between birth or Postnatal Day (PND) 0 and PND 56 [5–8]. Development of endometrial glands in the neonatal ovine uterus is coincident with postnatal increases in serum prolactin and estradiol-17 β concentrations [8]. These hormones are hypothesized to regulate endometrial gland morphogenesis or adenogenesis, because the nascent and proliferating glandular epithelium (G) expresses receptors for both hormones [7, 8].

In addition to the potential hormonal regulatory mechanisms associated with prolactin and estradiol-17 β , epithelial-mesenchymal interactions involve additional systems for local control and coordination of morphogenetically important cell behaviors, including motility, adhesion, differentiation, and proliferation [9, 10]. Tissue recombination studies in rodents clearly indicate that uterine mesenchyme directs and specifies patterns of epithelial development, whereas epithelium is required to support organization of endometrial stroma (S) and myometrial differentiation [11–13]. Complex communication between the epithelium and mesenchyme is mediated by paracrine and autocrine pathways within uterine tissues [14].

Stromal-derived growth factors, such as fibroblast growth factor (FGF)-7, FGF-10, hepatocyte growth factor (HGF), and insulin-like growth factor (IGF)-I and IGF-II, play vital roles in gland proliferation, differentiation, and branching morphogenesis in other developing epithelial-mesenchymal organs. An established paracrine growth factor, FGF-7 stimulates epithelial cell proliferation and differentiation [15, 16]. Originally, FGF-10 was isolated from rat lung mesenchyme and identified as essential for patterning early branching and morphogenic events [17–19]. The actions of FGF-7 and FGF-10 are mediated by an epithelial-specific splice variant of the FGF receptor 2 gene, FGFR2IIIb [16, 20]. Scatter factor, or HGF, functions as a paracrine mediator of mesenchymal-epithelial cell interactions that govern development of the lung and mammary gland [21, 22]. The mitogenic, motogenic, and morphogenic actions of HGF on epithelia are mediated by the *c-met* protooncogene product [23]. Recently, FGF-7, FGF-10, HGF, and their receptors were identified as paracrine growth factor systems in both the developing neonatal uter-

¹Supported in part by NIH HD 38274 to T.E.S., NRI Competitive Grants Program/USDA grants 98-35203-6322 to T.E.S., and NIH grant P30 ES09106.

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Received: 20 September 2000.

First decision: 18 October 2000.

Accepted: 28 November 2000.

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ISSN: 0006-3363. <http://www.biolreprod.org>

TABLE 1. Summary of PCR primer sequences and expected product size.

Primer	Sequence of forward and reverse primers (5'→3')	GenBank accession	Annealing temperature (°C)	Product size (bp)
FGF-7	GCTTGAATGACATGACTCC TGCCATAGGAAGAAAGTGGG	AF217463	55	486
FGF-10	CTTCTTGGTGTCTCCGTCC CTCCTTTTCCATTCAATGCC	AF213396	55	491
FGFR2IIIb	TCTGTTCAATGTGACCGAGG TCGGTCACATTGAACAGAGC	AF213380	55	298
HGF	TGCACAATTCCTGAAAAGACC CGGACAAAAATACCAGGACG	AF213397	55	314
<i>c-met</i>	CGGTCTTCAAGTAGCCAAGG ACCACTTCAGAAAACGGATGG	AF213398	55	450
IGF-I	GACAGGAATCGTGGATGAGTG AACAGGTAACCTGTGACGAGC	M30653	59	270
IGF-II	CGTGGCATCGTGAAGAGTGT GGTGACTCTTGGCCTCTCTGA	X55638	61	277
IGF-IR	GAGATTGCAGATGGCATGG GCTGATGATCTCCAGGAACG	X54980	56	441
β-actin	CATCCTGACCCTCAAGTACCC GTGGTGGTGAAGCTGTAGCC	U39357	55	420

us [7] and the adult ovine uterus [24, 25]. However, the ontogeny of these factors and their receptors in ovine uterine development has not been investigated.

Insulin-like growth factors are multifunctional regulators of cell function and promote mitosis and differentiation of a number of cell types in virtually every organ [26, 27]. The IGF system in many tissues mediates responses to steroid hormones, including the effects of estrogen on uterine growth and development in the rodent [28, 29]. Similar to that in the neonatal ewe [8], uterine development in the neonatal rodent is coincident with an increase in serum estradiol-17 β levels from the ovary [30]. In the perinatal rodent uterus, the IGF system is involved in postnatal uterine morphogenesis and growth [31]. The biological effects of IGFs (IGF-I and IGF-II) are mediated by two types of cell membrane receptors, but most effects of IGF-I and IGF-II are mediated by the type I IGF receptor (IGF-IR) in most tissue and cell systems [27]. In the adult ovine uterus, IGF-I and IGF-II are expressed in the S, and IGF-IR is expressed in all uterine cell types, with highest levels in G [32]. As in the ovine mammary gland [33], IGF-I and IGF-II represent paracrine growth factors for regulation of uterine epithelial morphogenesis. However, the ontogeny of the IGF system in the neonatal ovine uterus has not been reported. Given the importance of stromal-derived growth factors in epithelial morphogenesis, this study tested the hypotheses that neonatal ovine uterine development involves temporal and spatial alterations in the expression of mRNAs for FGF-7, FGF-10, HGF, IGF-I, IGF-II, and their receptors (FGFR2IIIb, *c-met*, and IGF-IR). Because each of these growth factors activate the mitogen-activated protein (MAP) kinase pathway, temporal and spatial alterations in tyrosine-phosphorylated extracellular regulated kinases-1 and -2 (p-ERK1/2), often referred to as p44 and p42 MAP kinases, were also assessed during neonatal ovine uterine development.

MATERIALS AND METHODS

Animals

All experiments and procedures were in accordance with the Guide for the Care and Use of Agriculture Animals and approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University (Animal Use Protocol 8-182AG).

Experimental Design, Tissue Collection, and Histology

Thirty-five cross-bred Rambouillet ewe lambs were assigned randomly on PND 0 or birth to be necropsied on PND 1 (n = 6), 7 (n = 5), 14 (n = 5), 21 (n = 4), 28 (n = 5), 42 (n = 5), or 56 (n = 5). The entire reproductive tract was excised, and the uterus was trimmed free of the broad ligament, oviduct, and cervix. Cross-sections from the midportion of each uterine horn were fixed in 4% v/v paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast Plus (Oxford Labware, St. Louis, MO). The remainder of the uterine tissue was frozen in liquid nitrogen and stored at -80°C for RNA extraction and analysis.

RNA Isolation and Analyses

RNA extraction and isolation. Total cellular RNA was isolated from frozen uteri using Trizol (Gibco BRL, Grand Island, NY) according to the manufacturer's recommendations.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Expression of mRNAs for FGF-7, FGF-10, FGFR2IIIb, HGF, *c-met*, IGF-I, IGF-II, and IGF-IR was assessed in uterine total RNA using semiquantitative RT-PCR as described elsewhere [7, 8, 34]. Briefly, cDNA was synthesized from total cellular RNA (5 μg) isolated from neonatal uteri using random (Life Technologies, Gaithersburg, MD) and oligo(dT) primers and SuperScript II Reverse Transcriptase (Life Technologies). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 μl of water, and stored at -20°C . The cDNAs were diluted (1:10 or 1:100) with water before use in PCR. Primers were designed to amplify partial cDNAs as summarized in Table 1. The PCR reactions were performed using AmpliTaq DNA polymerase (Perkin-Elmer Corp.-PE Applied Biosystems Div., Foster City, CA) and Optimized Buffer D (Invitrogen, Carlsbad, CA) for β -actin, IGF-II, and IGF-IR or Optimized Buffer F (Invitrogen) for FGF-7, FGF-10, FGFR2IIIb, HGF, *c-met*, and IGF-I according to the manufacturer's recommendations. The amount of cDNA template, annealing temperature, and number of cycles used for PCR were initially optimized using cDNA derived from PND 1, 28, and 56 uteri to ensure that PCR conditions were within the linear range of amplification for each primer pair.

TABLE 2. RT-PCR analysis of growth factor and receptor mRNAs in uterine total RNA from neonatal ewe lambs.

mRNA	Relative light units ^a							SEM	P value	Effect of PND ^b
	PND 1	PND 7	PND 14	PND 21	PND 28	PND 42	PND 56			
FGF-7	4696	6128	6235	4997	8428	9964	6721	673	—	NS
FGF-10	2033	1570	1978	1512	8286	10 942	12 537	680	0.01	Quadratic
FGFR2IIIb	5271	5093	6109	7411	9076	9890	9589	456	0.05	Quadratic
HGF	2503	3470	2350	2332	8413	8420	7851	496	0.01	Quadratic
c-met	4153	6170	3376	5403	6985	11 609	9118	456	—	NS
IGF-I	2208	1545	2454	4216	7238	6077	4977	450	0.01	Quadratic
IGF-II	3657	3682	3505	5741	6381	6639	6416	107	0.01	Quadratic
IGF-IR	2129	1425	1136	1415	1691	2617	2115	171	0.05	Cubic

^a Measurements of cDNA synthesized in PCR reactions were made from ethidium-bromide-stained agarose gels by using an AlphaInnotech Imaging System. Data are shown as least squares mean with overall SEM.
^b NS, No significant effect of PND.

as described by Gray et al. [7]. β-Actin PCR reactions contained 5 μl of cDNA (1:100); FGF-7 reactions 5 μl of cDNA (1:10); FGF-10 and HGF reactions 3 μl of cDNA (1:10); FGFR2IIIb reactions 3 μl of cDNA (1:100); c-met reactions 5 μl of cDNA (1:100); and IGF-I, IGF-II, and IGF-IR reactions 10 μl of cDNA (1:100). All PCR reactions were performed at 95°C for 30 sec, 55–61°C for 1 min, and 72°C for 1 min. Exact annealing temperatures used for each primer pair are provided in Table 2. Cycle number was 30 for FGF-7, FGF-10, FGFR2IIIb, HGF, c-met, IGF-I, and IGF-IR and 25 for β-actin and IGF-II. After PCR, equal amounts of reaction product were analyzed using a 2% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of

DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using an AlphaImager (Alpha Innotech Corporation, San Leandro, CA). The β-actin values were used as a covariate in statistical analyses to correct for differences in the amounts of cDNA used for each endometrial sample. All RT-PCR products were cloned into pCR II (Invitrogen) and fully sequenced in both directions to confirm identity.

In situ hybridization analysis. The FGFR2IIIb, c-met, IGF-I, IGF-II, and IGF-IR mRNAs were localized in uterine tissue sections by in situ hybridization analysis as described elsewhere [35]. Briefly, deparaffinized, rehydrated, and deproteinized tissues sections (6 μm) were hybridized with radiolabeled sense or antisense ovine cRNA probes generated from linearized plasmid templates using in vitro transcription with α-³⁵S-uridine triphosphate. Plasmid templates were partial cDNAs for ovine FGFR2IIIb (AF213380) [24] and ovine c-met (AF213398) [25]. For the IGF system, plasmid templates were derived by cloning partial cDNAs generated by RT-PCR into pCRII using a T/A Cloning Kit (Invitrogen). Slides were then dipped in Kodak NTB-2 liquid photographic emulsion (Kodak, Rochester, NY) and exposed at 4°C for 2 wk. Slides were developed in Kodak D-19 developer, counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), and dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher).

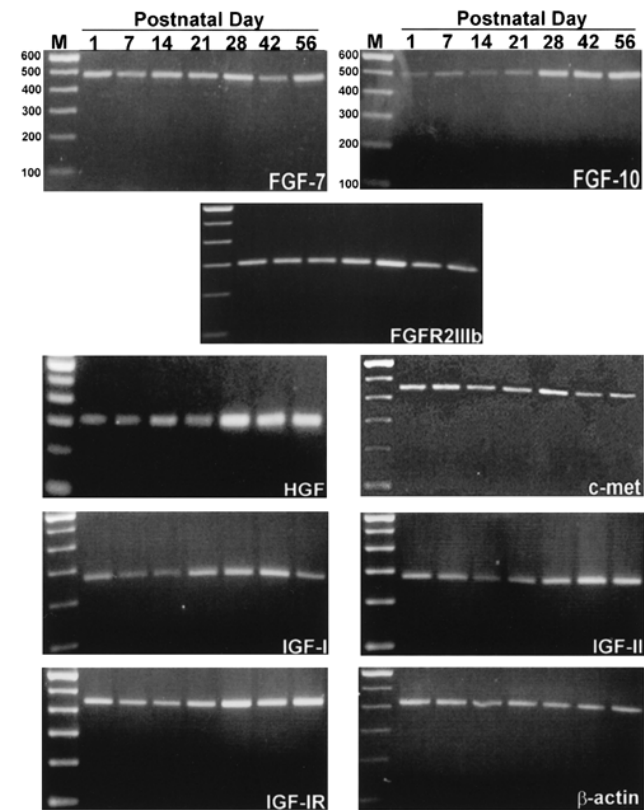


FIG. 1. Semiquantitative RT-PCR analysis of growth factors and their receptor mRNAs in total RNA isolated from uteri of neonatal ewes. All PCR products were separated in a 2% agarose gel and stained with ethidium bromide. Positions of the 100-base pair (bp) DNA marker (M) ladder are shown. Results of the analyses of changes in band intensity due to postnatal day are summarized in Table 2.

Immunohistochemistry

Expression of immunoreactive p-ERK1/2 was detected in uterine tissue cross-sections (5–7 μm) using mouse anti-phospho-ERK1/2 monoclonal IgG_{2a} antibody (p-ERK E-4; catalog no. sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA) and a Super ABC Mouse/Rat IgG Kit (Biomeda, Foster City, CA) according to methods described elsewhere by Spencer et al. [36]. The final working antibody concentration was 2 μg/ml. Negative controls were performed in which the primary antibody was substituted with the same concentration of purified normal mouse IgG from Sigma Chemical Co. (St. Louis, MO). Antigen retrieval utilizing pronase was performed as described elsewhere [36]. The chromagen used for peroxidase localization was 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Tissue sections from both uterine horns of each ewe were processed as sets within an experiment.

Relative staining intensity for immunoreactive p-ERK1/2 protein expression was visually assessed in multiple tissue sections from each uterine horn of each ewe by two independent observers and scored as follows: absent (–; i.e.,

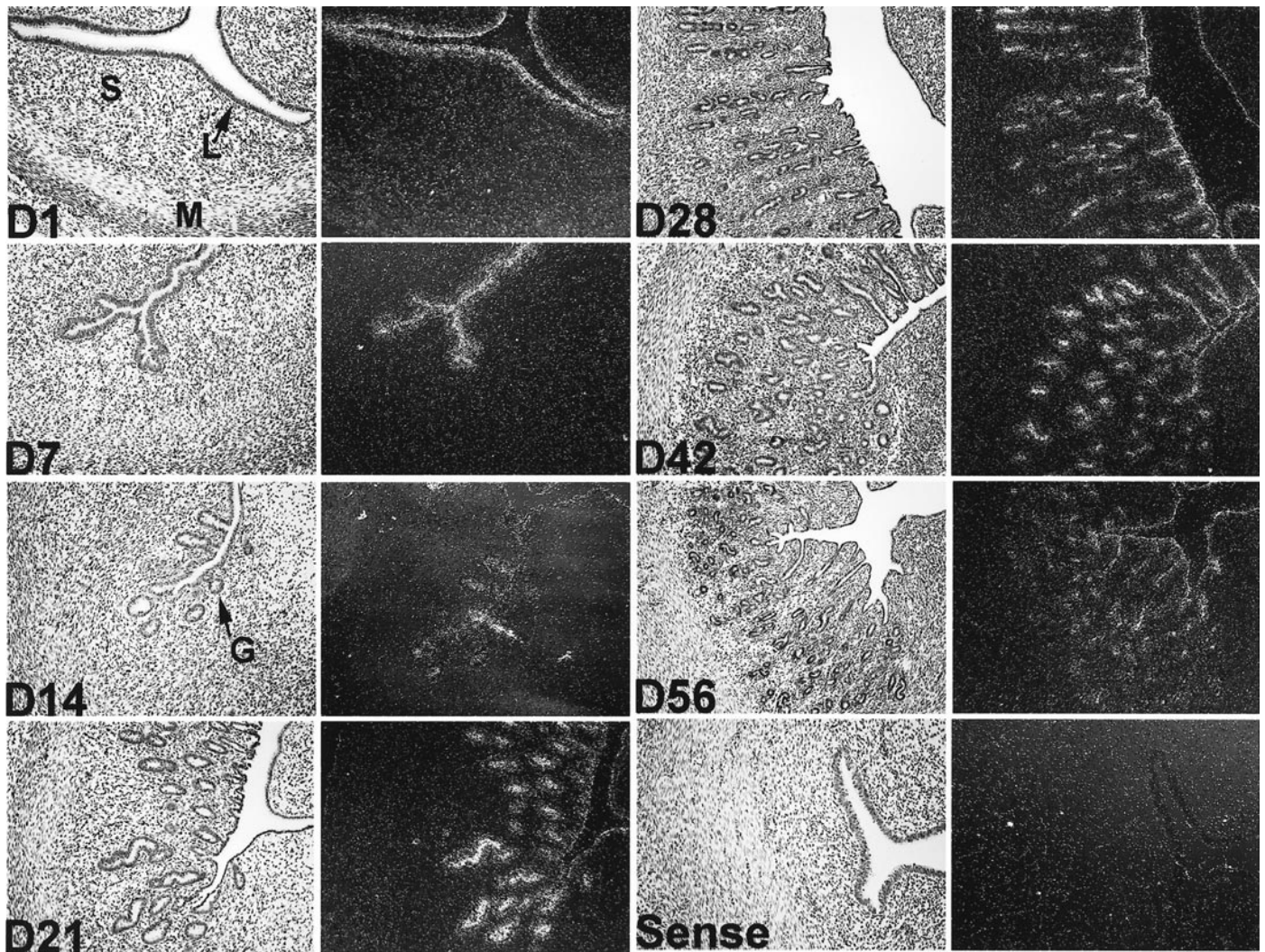


FIG. 2. In situ localization of FGFR2IIIb mRNA in the developing neonatal ovine uterus. Cross-sections of the uterine wall from neonatal ewes were hybridized with α - 35 S-labeled antisense or sense ovine FGFR2IIIb cRNA probe. Hybridized sections were digested with ribonuclease A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under bright-field or dark-field illumination. $\times 260$.

no staining above IgG control), weak (+), moderate (++), or strong (+++). If histologically discernable, intercaruncular endometrial tissues, including L, S, and G; caruncular endometrial tissues, including L and S; and myometrial tissues were scored.

Photomicroscopy

Photomicrographs were taken using a Zeiss Axioplan2 photomicroscope (Carl Zeiss, Inc., Thornwood, NY) fitted with a Hamamatsu chilled 3CCD color camera (Hamamatsu Corporation, Bridgewater, NJ). Digital images were captured and assembled using Adobe Photoshop 4.0 (Adobe Systems, Seattle, WA) and a MacIntosh PowerMac G3 computer (Apple Computer, Cupertino, CA). Black-and-white prints were electronically printed using a Kodak DS8650 color printer.

Statistical Analyses

The RT-PCR data were subjected to least-squares regression analyses using the Statistical Analysis System [37]. The β -actin values were used as a covariate in the model to correct for differences in the amounts of RT

cDNA analyzed for each uterus. All data are presented as least-square means relative light units with overall experimental SEMs.

RESULTS

Quantitation of mRNAs for FGF-7, FGF-10, FGFR2IIIb, HGF and c-met in Neonatal Uteri

The RT-PCR analyses indicated that mRNAs for FGF-7, FGF-10, FGFR2IIIb, HGF, and *c-met* were expressed in the developing neonatal ovine uterus (Fig. 1). Each RT-PCR product was of the expected size (Table 1) and sequenced to confirm identity (data not shown). As summarized in Table 2, regression analyses indicated that FGF-7 mRNA was constitutively expressed between PND 1 and 56. The FGF-10 mRNA levels were low on PND 1 to 21, increased substantially between PND 21 and 28, and remained abundant thereafter ($P < 0.01$, quadratic). The FGFR2IIIb mRNA was constant between PND 1 and 21, increased between PND 21 and 28, and remained high thereafter ($P < 0.05$, quadratic). Similar to FGF-10 mRNA expression, steady-state levels of HGF mRNA were low on PND 1 to 21, increased markedly between PND 21 and 28, and re-

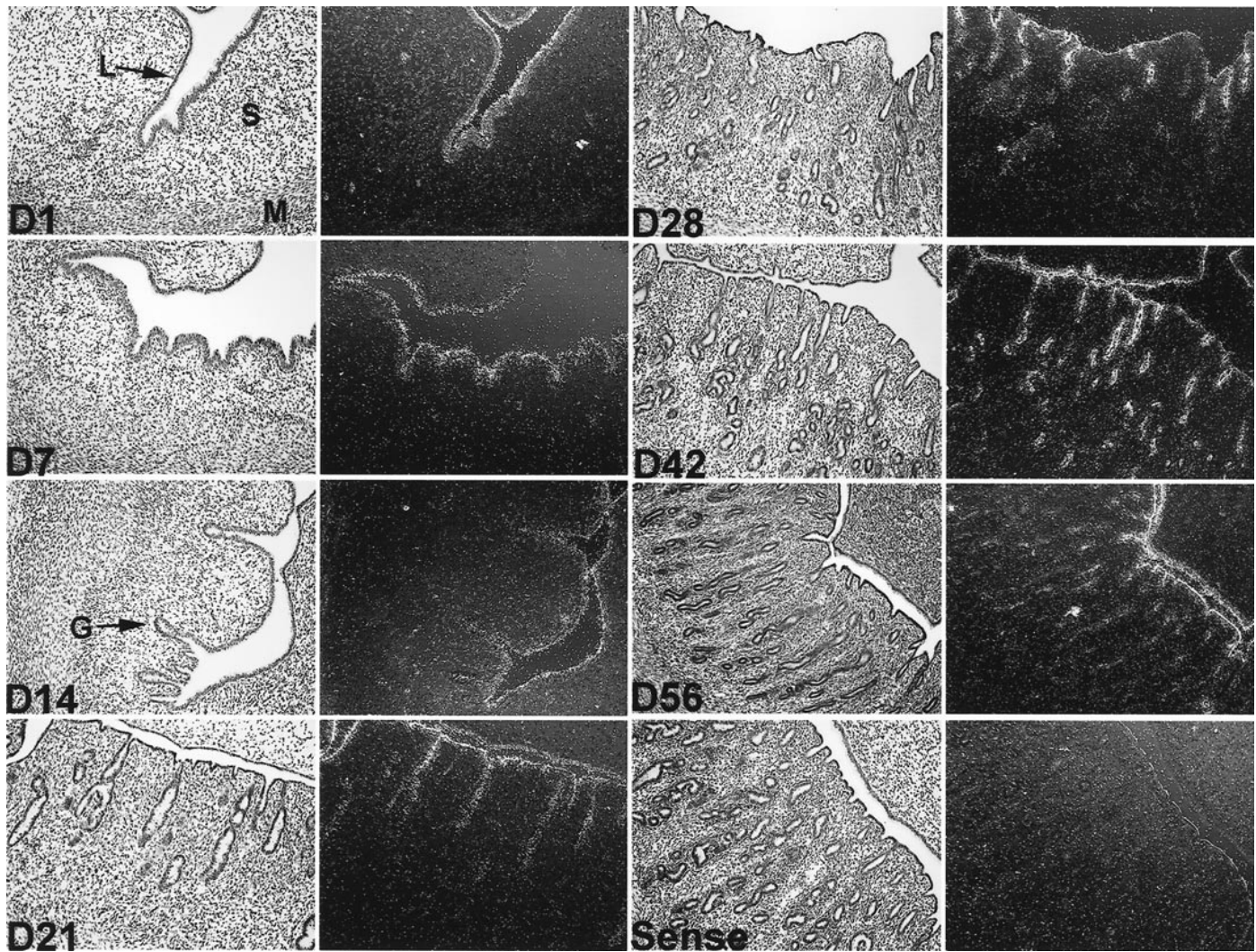


FIG. 3. In situ localization of *c-met* mRNA in the developing neonatal uterus. Cross-sections of the uterine wall from neonatal ewes were hybridized with α - 35 S-labeled antisense or sense ovine *c-met* cRNA probe. Hybridized sections were digested with ribonuclease A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under bright-field or dark-field illumination. $\times 260$.

maintained high thereafter ($P < 0.01$, quadratic). Steady-state levels of mRNA for *c-met* were not different between PND 1 to 56 ($P > 0.10$). No differences in β -actin mRNA expression were detected (data not shown).

In Situ Hybridization Analysis of FGFR2IIIb and c-met mRNA

In situ hybridization revealed cell type-specific expression of FGFR2IIIb mRNA in developing neonatal uterine tissues (Fig. 2). On PND 1, FGFR2IIIb mRNA was detected only in the luminal epithelium (L), with no specific hybridization detected in any other uterine cell type compared to sections probed with sense cRNA. Between PND 7 and 56, FGFR2IIIb mRNA was expressed abundantly in the L and the nascent and proliferating G. The mRNA hybridization signal intensity did not differ between L, shallow G, and deep G. Several of the uterine sections contained black melanocytes, which appeared intensely white under dark-field illumination.

On PND 1, *c-met* mRNA was detected in the L (Fig. 3). On PND 7 and 14, *c-met* mRNA was detected in both the L and nascent G; however, higher levels of *c-met* mRNA

expression were detected in the L compared to G. Between PND 21 and 56, *c-met* mRNA levels were greater in the G of the upper-stratum compactum compared to the G in the lower-stratum spongiosum near the M. No specific hybridization signal was detected in any other uterine cell type compared to sections hybridized with sense cRNA. Although low levels of FGF-7, FGF-10, and HGF mRNA were observed in endometrial S of adult ovine uteri [24, 25], a hybridization signal for these mRNAs in neonatal uteri was not detectable compared to sections hybridized with the appropriate sense cRNA (data not shown).

Quantitation of mRNAs for IGF-I, IGF-II, and IGF-IR in Neonatal Uteri

The RT-PCR analyses indicated that mRNAs for IGF-I, IGF-II, and IGF-IR were expressed in the developing neonatal ovine uterus (Fig. 1). Each RT-PCR product was of the expected size (Table 1) and sequenced to confirm identity (data not shown). As summarized in Table 2, regression analyses of RT-PCR data indicated that expression of uterine IGF-I and IGF-II mRNAs increased between PND 1 and 56 ($P < 0.01$, quadratic). Uterine IGF-IR mRNA ex-

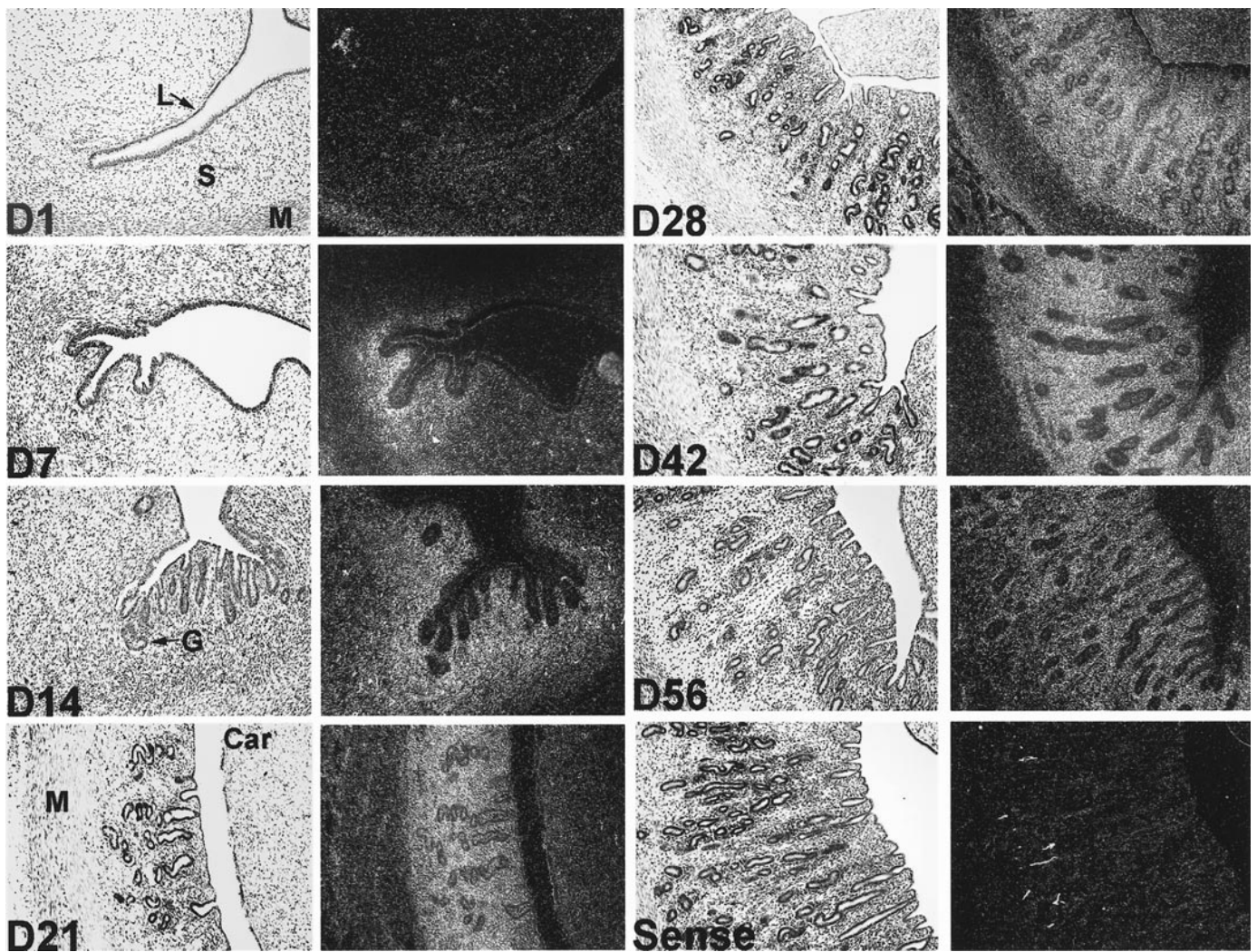


FIG. 4. In situ localization of IGF-I mRNA in the developing neonatal ovine uterus. Cross-sections of the uterine wall from neonatal ewes were hybridized with α - 35 S-labeled antisense or sense ovine IGF-I cRNA probe. Hybridized sections were digested with ribonuclease A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under bright-field or dark-field illumination. Car, Caruncle. $\times 260$.

pression was high on PND 1, decreased to PND 14, then increased to PND 56 ($P < 0.05$, cubic).

In Situ Hybridization Analysis of IGF-I, IGF-II, and IGF-IR mRNA

In situ hybridization analyses revealed distinct temporal and spatial alterations in the expression of IGF-I, IGF-II, and IGF-IR mRNAs in the neonatal ovine uterus. The IGF-I mRNA expression was specifically located in the developing intercaruncular endometrium and was very low or undetectable in the aglandular caruncular areas (Fig. 4). On PND 1, IGF-I mRNA was very low or undetectable in the endometrium and abundant in the M. The IGF-I mRNA expression declined thereafter in the M. In contrast, IGF-I mRNA was strikingly abundant in the intercaruncular S underlying differentiating, developing, and proliferating endometrial glands on PND 7 to 56.

The IGF-II mRNA was expressed in both areas (caruncular and intercaruncular) of the endometrium and M (Fig. 5). Expression of IGF-II mRNA in caruncular areas and M was not affected by neonatal age. The IGF-II mRNA was most abundant in the S surrounding the differentiating and developing endometrial glands on PND 21 to 42.

The IGF-IR mRNA was located in all uterine cell types. It was predominantly expressed in the developing G and L but was also detected at lower levels in the S and M (Fig. 6). The IGF-IR mRNA was particularly abundant in the L on PND 1 and in the morphogenetically active nascent and proliferating glands on PND 21 to 56.

p-ERK-1/2 Expression

The mouse monoclonal antibody utilized in this study specifically detects tyrosine-phosphorylated forms of both ERK1 and ERK2. The complex temporal and spatial alterations in immunoreactive p-ERK1/2 staining intensity of individual uterine tissues and cell types is presented in Figure 7 and summarized in Table 3.

On PND 1, p-ERK1/2 expression was moderate to strong in the L and moderate in the S and M. On PND 7, p-ERK1/2 protein was strong in the L and nascent G but moderate to weak in the S and M. On PND 14 and 21, p-ERK1/2 was abundant in the developing glands but weak in the S and M. Between PND 21 and 56, immunostaining for p-ERK1/2 was low to moderate in the neck regions of glands in the upper S; however, staining intensity in the middle to the tip regions of the developing glands was very

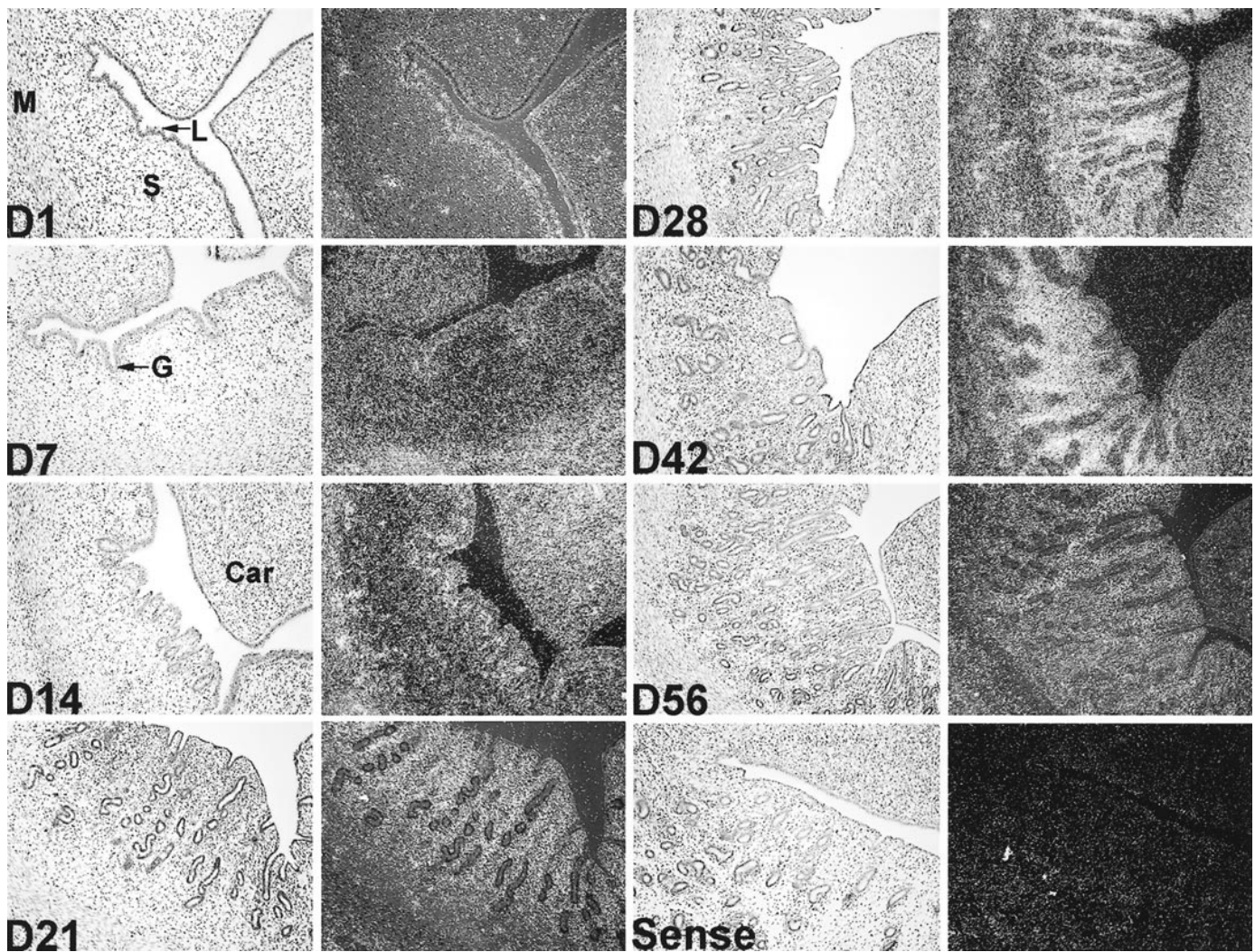


FIG. 5. In situ localization of IGF-II mRNA in the developing neonatal ovine uterus. Cross-sections of the uterine wall from neonatal ewes were hybridized with α - 35 S-labeled antisense or sense ovine IGF-II cRNA probe. Hybridized sections were digested with ribonuclease A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under bright-field or dark-field illumination. Car, Caruncle. $\times 260$.

strong. In these G cells, immunoreactive p-ERK1/2 protein was abundant, punctate, and concentrated beneath the apical surface. Consistently, moderate levels of p-ERK1/2 protein were detected in the endothelial cells of blood vessels (V).

DISCUSSION

Collectively, results from the present study support the hypothesis that FGF-7, FGF-10, HGF, IGF-I, IGF-II, and their epithelial receptors play roles in postnatal ovine uterine morphogenesis. Neonatal uterine development in the sheep is dramatic and includes the appearance, proliferation, and branching morphogenesis of uterine glands in the intercaruncular areas; development of endometrial folds; and to a lesser extent, differentiation of intercaruncular stromal layers and growth of the caruncular areas and M [5, 8]. Administration of a 19-norprogesterin to neonatal ewes from birth permanently ablates uterine gland differentiation, resulting in a uterine gland knockout (UGKO) phenotype in adults [35, 38, 39]. The endometrium of UGKO uteri is thin and lacks delineation of stratum compactum and stratum spongiosum S, suggesting that epithelial-stromal interactions occur between the nascent G and underlying S in

the neonatal uterus [7, 35]. Mature UGKO ewes do not exhibit normal estrous cycles, because they cannot generate sufficient oxytocin-stimulated pulses of prostaglandin $F_{2\alpha}$ from the endometrium [39]. However, UGKO ewes respond to exogenous prostaglandin $F_{2\alpha}$ with luteolysis and mating behavior but fail to establish or maintain pregnancy [39, 40]. Recent results using the UGKO model demonstrate unequivocally that endometrial glands are required for peri-implantation conceptus survival and growth between Days 9 and 15 of early pregnancy [40]. The highly branched and coiled endometrial glands synthesize and secrete or transport substances into the uterine lumen that are collectively termed histotroph [41]. During pregnancy, endometrial glands are required for peri-implantation conceptus survival and growth [39, 40] and undergo hypertrophy and hyperplasia, presumably to provide increasing amounts of histotroph for the developing fetus and placenta [34]. Given the importance of endometrial glands in uterine function, the discovery of hormonal, cellular, and molecular mechanisms regulating endometrial adenogenesis is important, because the success of these mechanisms determines, in part, the embryotrophic potential and functional capacity of the uterus.

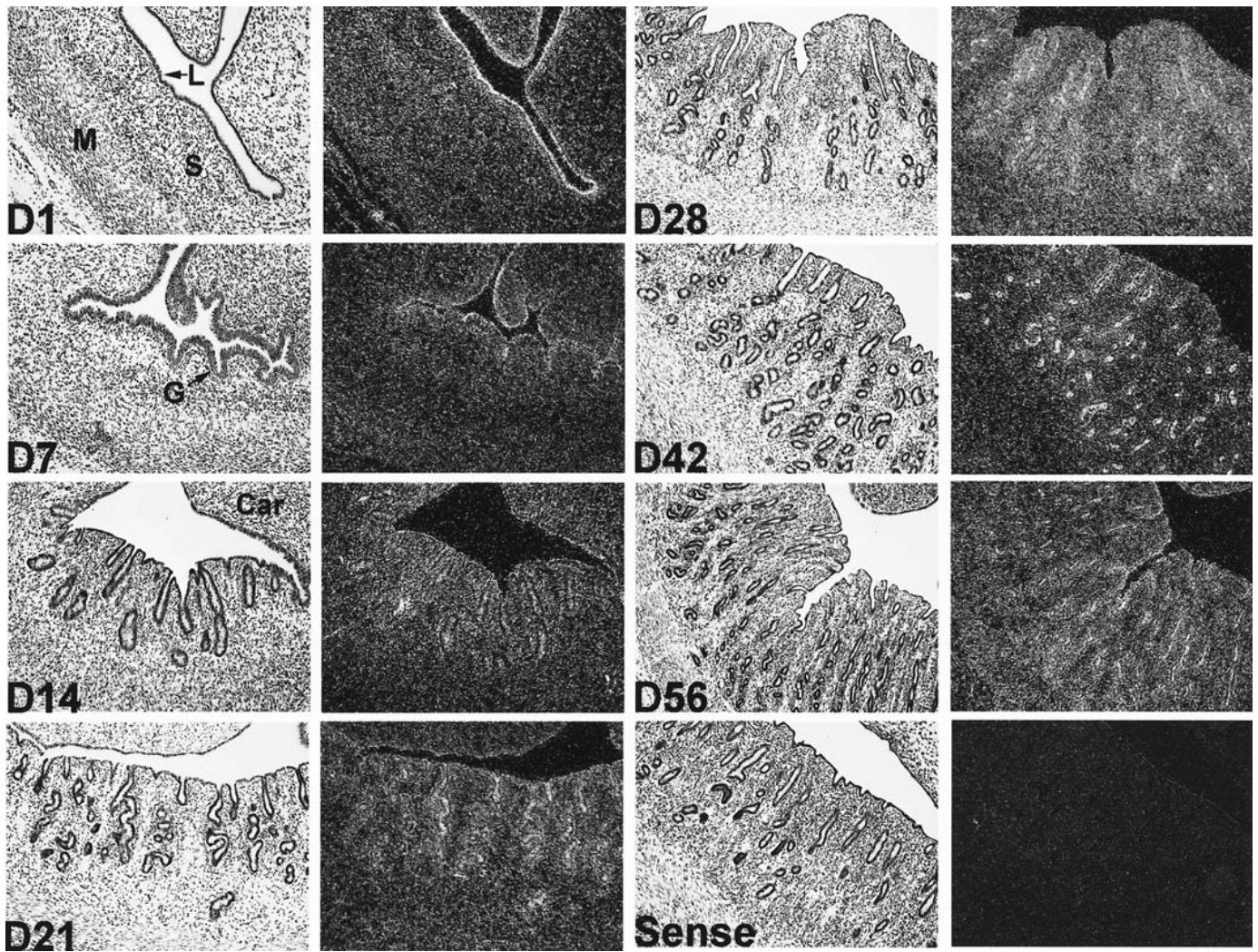


FIG. 6. In situ localization of IGF-IR mRNA in the developing neonatal ovine uterus. Cross-sections of the uterine wall from neonatal ewes were hybridized with α - 35 S-labeled antisense or sense ovine IGF-IR cRNA probe. Hybridized sections were digested with ribonuclease A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under bright-field or dark-field illumination. Car, Caruncle. $\times 260$.

In the present study, semiquantitative RT-PCR indicated that FGF-7 mRNA was constitutively expressed in uteri from PND 1 to 56. Although uterine FGF-10 and HGF mRNA levels did not change from PND 1 to 21, their expression increased markedly after PND 21. The period between PND 14 and 56 is characterized by the coiling and branching morphogenetic development of the endometrial glands in the neonatal ovine uterus [8]. Results from in situ hybridization indicate that FGFR2IIIb and *c-met* mRNA are expressed exclusively in L and nascent and proliferating G of neonatal ovine uteri. In the adult ovine uterus, FGF-7 is expressed by the smooth muscle of the vasculature or the tunica muscularis [24]. In contrast, FGF-10 and HGF are expressed in the endometrial S [25]. The actions of stromal-derived FGF-7, FGF-10, and HGF on their epithelial receptors may be substantial in neonatal uterine adenogenesis. This hypothesis is supported by results of a recent study by Gray et al. [7] that compared the expression of FGF-7, FGF-10, HGF, and their receptors in uteri of normal and progestin-exposed PND 28 ewes. The HGF and FGFR2IIIb mRNA expression was lower in progestin-treated ewes, but the expression of FGF-7, FGF-10, and *c-met* mRNAs was not affected. These results indicate that progestin-induced

ablation of endometrial adenogenesis involves disruptions in expression of paracrine growth factors and/or receptors involved in epithelial-mesenchymal interactions.

The roles of FGF-10 and HGF have been established in lung morphogenesis, which is similar to that of uterine gland morphogenesis. Fibroblast growth factor-10 is a short-range signal for guidance of lung epithelial bud development [42, 43]. In vitro experiments have demonstrated that lung epithelial buds can grow toward FGF-10-coated beads and form concentric layers of epithelial cells around the beads [42]. Fibroblast growth factor-7 is a potent mitogen for lung epithelial proliferation that leads to formation of cyst-like structures [43]. Compared to FGF-7, FGF-10 exerts a modest effect on lung epithelial cell proliferation [42]. Therefore, one can speculate that during ovine uterine branching morphogenesis, FGF-10 may serve as a chemotactic molecule, stimulating gland budding and branching, whereas constitutive expression of FGF-7 may stimulate epithelial cell proliferation. Hepatocyte growth factor also plays a crucial role in the morphogenesis of alveolar and bronchial epithelia [44] and is regulated by estrogen in the primate endometrium [45]. The coincidental increase in HGF and FGF-10 between PND 21 and 56 sug-

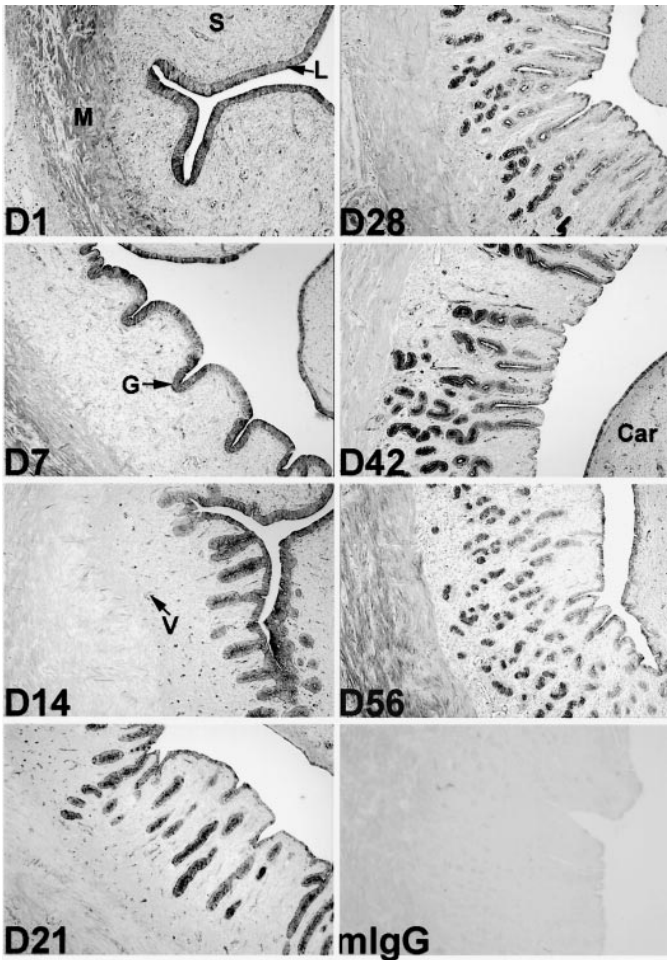


FIG. 7. The p-ERK1/2 protein in the developing neonatal ovine uterus. Immunoreactive protein was detected using mouse anti-phospho-ERK1/2 monoclonal IgG and a BioStain Super ABC kit. The negative IgG control was performed by substituting irrelevant mouse IgG for primary antibodies (mlgG). Car, Caruncle; V, blood vessel. $\times 260$.

gests that these growth factors may act synergistically to regulate uterine gland branching morphogenesis. In situ hybridization analyses indicate a similar but differential expression pattern for their receptors, with FGFR2IIIb expressed by all epithelia and *c-met* expressed predominantly by L and upper G in the stratum compactum. This slightly different expression pattern in the G suggests a functional distinction between FGF-10 and HGF on gland proliferation and morphogenesis. The potential specific roles for FGF-7, FGF-10, and HGF during postnatal uterine endo-

metrial development will be a subject of further investigation. The receptors for these three growth factors utilize the MAP kinase pathway to regulate cell proliferation and differentiation [18, 23]. In the present study, abundant levels of p-ERK1/2 protein were detected in the developing endometrial glands, which express FGFR2IIIb and *c-met* mRNAs and are rapidly proliferating [8].

Results from the present study support the idea that IGF-I and IGF-II expressed in the S act via epithelial IGF-IR to stimulate and maintain gland development and proliferation in the neonatal ovine uterus. The localization of IGF-I and IGF-II and their receptors is similar to that reported in adult uteri from cyclic and pregnant ewes [32]. The IGFs are synthesized de novo and are multifunctional regulators of proliferation and differentiation within the local tissue environment through autocrine and/or paracrine mechanisms in almost every organ system [26], including the fetal human uterus [46], adult human uterus [47], and adult ovine uterus [32]. In addition to their roles as mitogens, IGF-I and IGF-II induce cellular differentiation and promote the expression of differentiated functions in a variety of cells [27]. Functionally, most actions of both IGF-I and IGF-II are mediated by IGF-IR, which is a member of the receptor tyrosine kinase family and activates the MAP kinase pathway [48, 49].

In the uterus of a number of species, IGFs and their receptors regulate endometrial development, differentiation, and function. The IGFs in many tissues regulate responses to steroid hormones, including complex responses of the immature rodent uterus to estrogens [28, 29, 31] and human endometrial growth in the proliferative phase to ovarian estradiol [47]. Null mutation of the *IGF-I* gene in mice has demonstrated its critical role in normal development of the female reproductive tract [50] and the requirement for estrogen-induced uterine growth in cycling female rodents [51]. Gu et al. [31] observed that IGF-I mRNA expression in the neonatal rat uterus was only in S and M and increased during uterine gland genesis. Expression of IGF-II was not detected in the postnatal rat uterus, although it is present in nonpregnant and pregnant human endometrium [47].

In the present study, IGF-I mRNA was particularly abundant in the intercaruncular endometrial S surrounding the nascent and proliferating G. In the developing intercaruncular endometrium of the neonatal ovine uterus, Taylor et al. [8] observed that both the developing G and underlying adjacent S expressed high levels of estrogen receptor- α (ER- α) mRNA and protein. Studies in both the rodent and ovine uterus indicate that estrogen can up-regulate IGF-I mRNA expression in uterine S [28, 29, 34]. Given that circulating levels of estradiol-17 β increase in the neonatal

TABLE 3. Distribution and relative abundance of immunoreactive phosphorylated ERK-1/2 protein in the neonatal ovine uterus.^a

PND	Intercaruncular endometrium			Caruncular endometrium		Myometrium
	L	G	S	L	S	
1	+++	NP	+/++	+++	+	++
7	++	+++	+	+++	+	+
14	++	+++	+	+++	+	+
21	+/+++	+++	+	+	+	+
28	+	+++ ^b	+	+/-	+	+
42	+	+++ ^b	+	+/-	+	+
56	+	+++ ^b	+	+/-	+	++

^a Protein staining intensity was evaluated visually as absent (-), weak (+), moderate (++), or strong (+++) for endometrial and myometrial tissues obtained from neonatal ewes (n = 5–6 per PND). L, Luminal epithelium; G, glandular epithelium; S, stroma; NP, not present.

^b Staining was greater and more uniform in the tips of the glands as compared with the necks.

ewe between PND 1 and 28, estrogen likely acts via stromal ER- α to increase local *IGF-I* gene expression. In addition to local IGF-I production, serum IGF-I concentrations in neonatal ewes are high at birth and are maintained at high levels until 4 wk after birth [52]. In contrast, serum IGF-II concentrations decline precipitously after birth and remain low thereafter. Results from the present study support the hypothesis that stromal IGF-I and IGF-II act via epithelial IGF-IR to promote epithelial development in the neonatal uterus.

The developing G expresses high levels of ER- α in neonatal pig and sheep uteri [8, 53, 54]. In the neonatal pig uterus, ligand-independent activation of ER- α appears to be required for endometrial adenogenesis and uterine growth [54]. The precise role of ligand-dependent and ligand-independent activation of ER- α in development of the neonatal ovine uterus is not known. Cross-talk between ER- α and IGF-IR signaling pathways results in synergistic growth stimulation in a number of model systems [55, 56]. Activation of ER- α by growth factors such as IGF-I involves direct serine phosphorylation by p-ERK1/2. In addition, estrogen increases IGF-IR protein in the immature rat uterus and modulates IGF-IR function by inducing tyrosine phosphorylation of IGF-IR and insulin receptor substrate-1, which is followed by enhanced MAP kinase activation [48, 49]. Results of the present study support the hypothesis that ER- α activation in a ligand-independent manner by stromal growth factors identified in this study plays a crucial role in endometrial adenogenesis of the neonatal ovine uterus.

In summary, results from the present study and others [7, 8, 38] indicate that development of the endometrial glands in the neonatal ovine uterus is complex and involves a number of endocrine and paracrine growth factors, including prolactin, IGF-I, IGF-II, FGF-7, FGF-10, and HGF. Activation of the respective receptors in the developing glands by these growth factors most likely results in stimulation of the MAP kinase pathway and, thus, cell proliferation and differentiation as in other model systems [57]. Future research will determine which of these growth factors, if any, activate ER- α in a ligand-independent manner via the MAP kinase pathway. Another intriguing question is to determine the precise role of each growth factor that activates the MAP kinase pathway and regulates endometrial gland proliferation and differentiation.

ACKNOWLEDGMENTS

The authors thank Dr. Shawn Ramsey and Mr. Todd Taylor (Texas A&M University Sheep and Goat Center) for assistance with animal husbandry and Drs. Quint Winger and Mark Westhusin (Department of Veterinary Physiology and Pharmacology, Texas A&M University) for assistance with RT-PCR analyses.

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