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Signaling by Epidermal Growth Factor (EGF) Family of Growth Factors during Implantation

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We previously described spatiotemporal expression of various epidermal growth factor (EGF)-like ligands and receptor subtypes, ErbB1 and ErbB2, during the peri-implantation period. To better understand the roles of these ligands and their possible signaling schemes in implantation, it is important to define the status of all the ligands and receptor subtypes in the uterus/embryo. No information is available about uterine and embryonic status of ErbB3 or ErbB4 during implantation. We cloned mouse *erbB3* and *erbB4* cDNAs and examined their expression and bioactivity in the peri-implantation uterus (days 1–8). Two *erbB3* (cytoplasmic and extracellular) and three *erbB4* (two cytoplasmic and one extracellular) clones were generated. Both forms of the *erbB3* clone showed similar transcript profiles, while different transcript profiles were obtained with *erbB4* clones. The steady-state levels of *erbB3* and *erbB4* mRNAs in whole uterine poly(A)$^+$ RNA samples showed little changes during the peri-implantation period, while their unique cell-specific accumulation was noted. *erbB3* is predominantly expressed in the epithelial cells, although decidual and embryonic cells also accumulate this mRNA. In contrast, the *erbB4* mRNA is primarily expressed in the submyometrial stroma and myometrial connective tissues during this period. Additionally, the extracellular form of the *erbB4* clone detected signals in a subpopulation of stromal cells. Autophosphorylation and immunoprecipitation studies provided evidence that uterine ErbB3 and ErbB4 are biologically active. This study provides a comprehensive analysis of possible ligand–receptor signaling schemes for EGF-like ligands in implantation.

Key Words: **ErbBs; uterus; implantation; mouse.**

INTRODUCTION

Synchronized development of the embryo to the blastocyst stage and preparation of the uterus to the receptive state are essential for successful implantation (Psychoyos, 1973; Paria *et al.*, 1993b). The establishment of the receptive uterus is achieved by the coordinated effects of progesterone (P_4) and estrogen in a temporal and cell type-specific manner (Huet-Hudson *et al.*, 1989). In the mouse, the uterine epithelium on days 1 and 2 of pregnancy undergoes proliferation under the influence of preovulatory estrogen. In contrast, increasing levels of P_4 from the newly formed corpora lutea on day 3 result in the switching of proliferation from the epithelium to the stroma which is further potentiated by preimplantation ovarian estrogen secretion on day 4. The uterine receptivity is achieved on day 4 when epithelial cells cease to proliferate and become differentiated (Huet-Hudson *et al.*, 1989). The attachment reaction occurs at 2200–2300 h on this day and is followed by penetration by trophoblast cells through the underlying basement membrane. This is accompanied by transformation of stromal cells into decidual cells (decidualization) (Dey, 1996). Although the molecular mechanism(s) that directs steroid hormone-mediated proliferation/differentiation of uterine cells for implantation and decidualization is poorly defined, the regulated expression of polypeptide growth factors and their receptors in the uterus and embryo during the peri-implantation period or under steroid hormonal stimulation suggests that these factors serve as local mediators of steroid hormone actions (Das *et al.*, 1992, 1994a,b, 1995, 1997; Huet-Hudson *et al.*, 1990; Kapur *et al.*, 1992; Lim *et al.*, 1997; Murphy *et al.*, 1987; Paria *et al.*, 1992, 1993a, 1994; Paria and Dey, 1990; Rappollee *et al.*, 1988; Tamada *et al.*, 1991).

Our laboratory has been engaged in defining the roles of

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the EGF family of growth factors in uterine biology and implantation. The members of the EGF family are EGF itself, transforming growth factor-α (TGF-α), amphiregulin (Ar), heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epiregulin, and neuregulins 1–3 (NRGs) (Cohen, 1962; Derynck *et al.*, 1984; Shoyab *et al.*, 1988; Higashiyama *et al.*, 1991; Holmes *et al.*, 1992; Shing *et al.*, 1993; Toyoda *et al.*, 1995; Chang *et al.*, 1997; Carraway *et al.*, 1997; Zhang *et al.*, 1997). These ligands can interact with the receptor subtypes of the *erbB* gene family which is composed of four receptor tyrosine kinases: ErbB1 (EGF-receptor), ErbB2, ErbB3, and ErbB4. They share a common structural feature, but differ in their ligand specificity and kinase activity (Carraway and Cantley, 1994; Heldin, 1995; Lemke, 1996). While EGF, TGF-α, HB-EGF, Ar, betacellulin, and epiregulin all can directly bind to ErbB1, all NRGs require either ErbB3 and/or ErbB4 for direct binding (Tzahar *et al.*, 1994; Riese *et al.*, 1996; Komurasaki *et al.*, 1997; Carraway *et al.*, 1997; Chang *et al.*, 1997; Zhang *et al.*, 1997). Additionally, betacellulin, HB-EGF, and epiregulin can serve as direct ligands for ErbB4 (Riese *et al.*, 1996, 1998; Elenius *et al.*, 1997a; Komurasaki *et al.*, 1997; Shelly *et al.*, 1998). Epiregulin is unique since it can interact with many different combinations of heterodimers with low affinity (Shelly *et al.*, 1998). Although there is no ligand known to interact directly with ErbB2, this receptor possesses the strongest kinase activity and is considered as the preferred partner in forming dimers with other members of the family (Karunagaran *et al.*, 1996; Graus-porta *et al.*, 1997). In contrast, ErbB3 exhibits impaired kinase activity and requires heterodimerization with other receptor subtypes for intracellular signaling (Guy *et al.*, 1994; Chen *et al.*, 1996). The initial dimerization between the coexpressed receptors upon specific ligand binding constitutes the classical mechanism of actions of EGF-like ligands. However, the incidence of the "secondary dimerization" after receptor dissociation suggests that the receptor activation is not necessarily correlated with direct ligand binding (Gamett *et al.*, 1997). Furthermore, coexpression of ErbB2 and ErbB3 in an engineered cell line creates previously undescribed binding sites for EGF and betacellulin (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998). These observations suggest extensive "cross-talk" among the receptor subtypes involving various mechanisms. Therefore, depicting the expression patterns of the EGF and ErbB family members in the peri-implantation uterus with changing cellular phenotypes is important in defining their possible ligand–receptor signaling network during this period.

We have previously examined the expression and regulation of EGF, TGF-α, Ar, HB-EGF, betacellulin, epiregulin, and NRG1 in the mouse uterus during the peri-implantation period (Huet-Hudson *et al.*, 1990; Tamada *et al.*, 1991; Das *et al.*, 1994b, 1995, 1997; Reese *et al.*, 1998). Collectively, the results suggest that Ar may be involved in uterine preparation for implantation, while HB-EGF could interact with blastocyst ErbB1/EGF-R for the attachment reaction (Das *et al.*, 1994b; Raab *et al.*, 1996). Further, overlapping uterine cell-specific expression of Ar, HB-EGF, betacellulin, epiregulin, and NRG1 surrounding the implanting blastocyst suggests that these growth factors are important for the attachment reaction (reviewed in Das *et al.*, 1997). However, distinct spatiotemporal expression of these growth factors in the pre- and postimplantation uterus also suggests that they serve specific functions associated with uterine preparation for implantation. These studies also suggested that steroid hormonal regulation of uterine cell proliferation and differentiation is mediated locally by these growth factors. With respect to ErbB receptors, ErbB1 and ErbB2 are expressed in a spatiotemporal manner in the peri-implantation uterus and show steroid hormone responsiveness (Das *et al.*, 1994a; Lim *et al.*, 1997). In the present investigation, we examined whether ErbB3 and ErbB4 can also serve as potential mediators for EGF-like growth factors in implantation. We cloned the mouse *erbB3* and *erbB4* cDNAs to examine their spatiotemporal expression and their bioactivity in response to various ligands in the peri-implantation uterus. We identified two species of the *erbB4* mRNA spliced transcripts in the cytoplasmic region which exhibited differential expression in the uterus. These observations coupled with our previous results (Das *et al.*, 1994a; Lim *et al.*, 1997) depict for the first time the potential compartmentalized signaling by EGF-like growth factors in a complex *in vivo* system.

MATERIALS AND METHODS

Growth Factors and Antibodies

Receptor-grade mouse EGF was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), while recombinant human betacellulin and amphiregulin were procured from R & D Systems (Minneapolis, MN). Human recombinant HB-EGF and NRG1β were kindly provided by Dr. Judith Abraham (Scios Nova Inc., Mountain View, CA) and Dr. K. Carraway, III (Harvard Medical School, Boston, MA), respectively. Mouse epiregulin was provided by Dr. T. Komurasaki (Taisho Pharmaceutical Co., Japan). The rabbit polyclonal antibodies to mouse ErbB3 and ErbB4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and these antibodies do not show cross-reactivity with other ErbB members.

Animals and Tissue Preparation

CD-1 mice (Charles River Laboratory, Raleigh, NC) were housed in the animal care facility at the University of Kansas Medical Center according to NIH and institutional guidelines for the care of laboratory animals. Adult female mice (20–25 g, 48–60 days old) were mated with fertile males of the same strain. The morning of finding a vaginal plug was designated day 1 of pregnancy. Mice were killed between 0830 and 0900 h on days 1–8 of pregnancy. Embryos were recovered from the reproductive tract on days 1–4 to confirm pregnancy. Implantation sites on days 5 and 6 were visualized by intravenous injection (0.1 ml/mouse) of Chicago blue dye solution (0.1% in saline). Implantation sites were demarcated by discrete blue bands along the uterus (Paria *et al.*, 1993b). On days 7–8, implantation sites are distinct and blue dye injection is not

required. Uteri were collected for Northern blot hybridization, *in situ* hybridization, or autophosphorylation studies.

Cloning and Sequencing of the Mouse erbB3 and erbB4 Partial cDNAs

Reverse-transcription polymerase chain reaction (RT-PCR) was employed to generate the mouse-specific *erbB3* and *erbB4* cDNA clones. RT-PCR conditions were essentially the same as described previously (Paria *et al.*, 1993a). Two sets of primers (sense and antisense), based on the human cDNA sequence, were used to generate the mouse *erbB3* cDNAs. The first set of primers, 5′-GATGCTGAGAACCAATACCAGACAC-3′ (sense) and 5′-CAATATAAACACCCCCTGACAGAATC-3′ (antisense), were used to generate partial cDNA clones of the extracellular domain in the mouse *erbB3* gene (*erbB3E*). The sense strand primer corresponds to 220–244 nt, whereas the antisense strand primer encompasses 522–547 nt in the human *erbB3* gene. The second set of primers, 5′-TCTGGATACATGCCCATGAACCAGGGTAATCT-TGG-3′ (sense) and 5′-CCTTTGAGGTGTGTATCTGGCAT-GACATAACC-3′ (antisense), were used to generate partial cDNA clones of the cytoplasmic domain in the mouse *erbB3* gene (*erbB3C*) (Moscoso *et al.*, 1995). These primers encompass 3253–3287 and 3571–3602 nt of the human *erbB3* gene, respectively. Partial cDNA clones of the extracellular domain in the mouse *erbB4* gene (*erbB4E*) were generated by using 5′-CAGAAA-ATCATTGCCAGACTTTGAC-3′ (sense) and 5′-CACGCACACA-AGAACTGGAATCTAC-3′ (antisense) primers, which encompass 635–659 and 928–952 nt in the human sequence (Plowman *et al.*, 1993). Mouse-specific primers 5′-GCTGAGGAATATTTGGT-CCC-3′ (sense) and 5′-CTACTGGAGCCTCTGGTATG-3′ (antisense) were designed to clone the cytoplasmic domain of the *erbB4* (*erbB4C*) (Moscoso *et al.*, 1995). Day 4 pregnant mouse uterine total RNA (1 μg) was reverse transcribed using each antisense primer as indicated above. RT products (3 μl) were amplified by PCR for 45 cycles using the following cycle parameters: 94°C, 1 min 30 s; 55°C, 2 min; 72°C, 2 min 30 s. The RT-PCR products were analyzed by Southern hybridization using a ^{32}P end-labeled internal primer designed from the human *erbB3* or *erbB4* sequence. RT-PCR products were cloned into pCR-Script SK(+) cloning vector (Stratagene, La Jolla, CA) and nucleotide sequences of the clones were determined on both strands by dideoxy nucleotide chain termination method (Sanger *et al.*, 1977) and the Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH).

Hybridization Probes

Antisense ^{32}P-labeled cRNA probes for Northern hybridization and ^{35}S-labeled cRNA probes (sense and antisense) for *in situ* hybridization were generated from cDNAs of the mouse *erbB1*, *erbB2*, *erbB3E*, *erbB3C*, *erbB4E*, *erbB4C/L*, *erbB4C/S*, and *rpL7* using appropriate polymerases (Das *et al.*, 1994a; Lim *et al.*, 1997; Lim and Dey, 1997). For Southern hybridization, specific internal primers were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Probes had specific activities of about 2×10^9 dpm/μg.

Northern Blot Hybridization

Total RNAs of whole uteri pooled from 10–15 mice on indicated days of pregnancy were extracted by a modified guanidine thiocyanate procedure (Das *et al.*, 1994a,b). Poly(A)$^+$ RNAs were isolated from total RNAs by oligo(dT)–cellulose column chromatography (Sambrook *et al.*, 1989). Poly(A)$^+$ RNA (2 μg) was denatured, separated by formaldehyde–agarose gel electrophoresis, transferred to nylon membranes, and cross-linked by UV irradiation (Spectrolinker XL-1500, Spectronics Corp., Westbury, NY). The blots were prehybridized, hybridized, and washed as described previously (Das *et al.*, 1994a). After hybridization, the blots were washed under stringent conditions and the hybrids were detected by autoradiography. The blots were stripped and rehybridized with ribosomal protein L7 (rpL7) probe as described previously (Lim and Dey, 1997).

In Situ Hybridization

In situ hybridization was performed as described previously (Das *et al.*, 1994b). Uteri were cut into 4- to 6-mm pieces and flash frozen in Histo-Freeze (Fisher Scientific, St. Louis, MO). Frozen sections (11 μm) from days 1, 4, 5, and 8 or days 2, 3, 6, and 7 of pregnancy were mounted onto poly-L-lysine-coated slides and fixed in cold 4% paraformaldehyde in PBS. The sections were acetylated and hybridized at 45°C for 4 h in 50% formamide hybridization buffer containing the ^{35}S-labeled antisense cRNA probe. After hybridization and washing, the sections were incubated with RNase A (20 μg/ml) at 37°C for 20 min. RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY). Sections hybridized with the corresponding sense probe served as negative controls. Slides were poststained with hematoxylin and eosin.

Autophosphorylation of Uterine ErbB3 and ErbB4

The method essentially followed the protocol described by us previously (Das *et al.*, 1994a). In brief, day 4 pregnant uteri were homogenized in buffer A [10 mM Tris–HCl (pH 7.5), 250 mM sucrose, 2 mM EGTA, 10 μg/ml leupeptin, 20μg/ml PMSF, 10 μg/ml aprotinin] and centrifuged at 900*g* for 10 min at 4°C. The supernatant was centrifuged at 144,000*g* for 1 h at 4°C. The pellet was resuspended in the same buffer and subjected to similar centrifugation for another hour. The pellet was resuspended in buffer B [10 mM Tris–HCl (pH 7.4), 0.15 mM NaCl, 1 mM EGTA, 10 μg/ml leupeptin, 20 μg/ml PMSF, 10 μg/ml aprotinin]. Membranes (150 μg protein) were suspended in 50 μl phosphorylation reaction buffer [50 mM Pipes (pH 7.0), 1 mM $MnCl_2$, 0.1 mM Na–vanadate] and preincubated with or without a specific ligand (100 ng/ml) for 10 min at 4°C. The labeling reaction was performed for 2 min at 4°C after addition of 5 μCi [γ-^{32}P]ATP (1 μM) in the presence of 0.1% Triton X-100. The reaction was terminated by 15 μl of an ice-cold mixture of 1 mM ATP and 0.1% BSA followed by an equal volume (w/v) of 10% trichloroacetic acid. After incubation on ice for 1 h, the mixture was centrifuged. The precipitates were washed three times with a mixture of diethylether and ethanol (1:1) and suspended in 50 μl of 50 mM Tris buffer (pH 7.5). An equal volume of protein A/Sepharose–antibody conjugate (3 mg:0.5 μg) was added to this mixture and incubated for 90 min at 4°C with constant shaking. The protein A/Sepharose–antibody conjugates were washed sequentially with buffer A (50 mM Hepes, 0.1% Triton X-100, 0.1% SDS, 5 mM EGTA, pH 8.0), buffer B (50 mM Hepes, 0.1% Triton X-100, 0.1% SDS, 150 mM NaCl, pH 8.0), and buffer C (10 mM Tris–HCl, pH 8.0). The pellets were boiled in 1× SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) for 3 min and centrifuged. The supernatants were subjected to 7.5% SDS–PAGE in parallel with molecular weight markers. The gel was transferred to nitrocellulose membrane and the products were visualized by autoradiography.

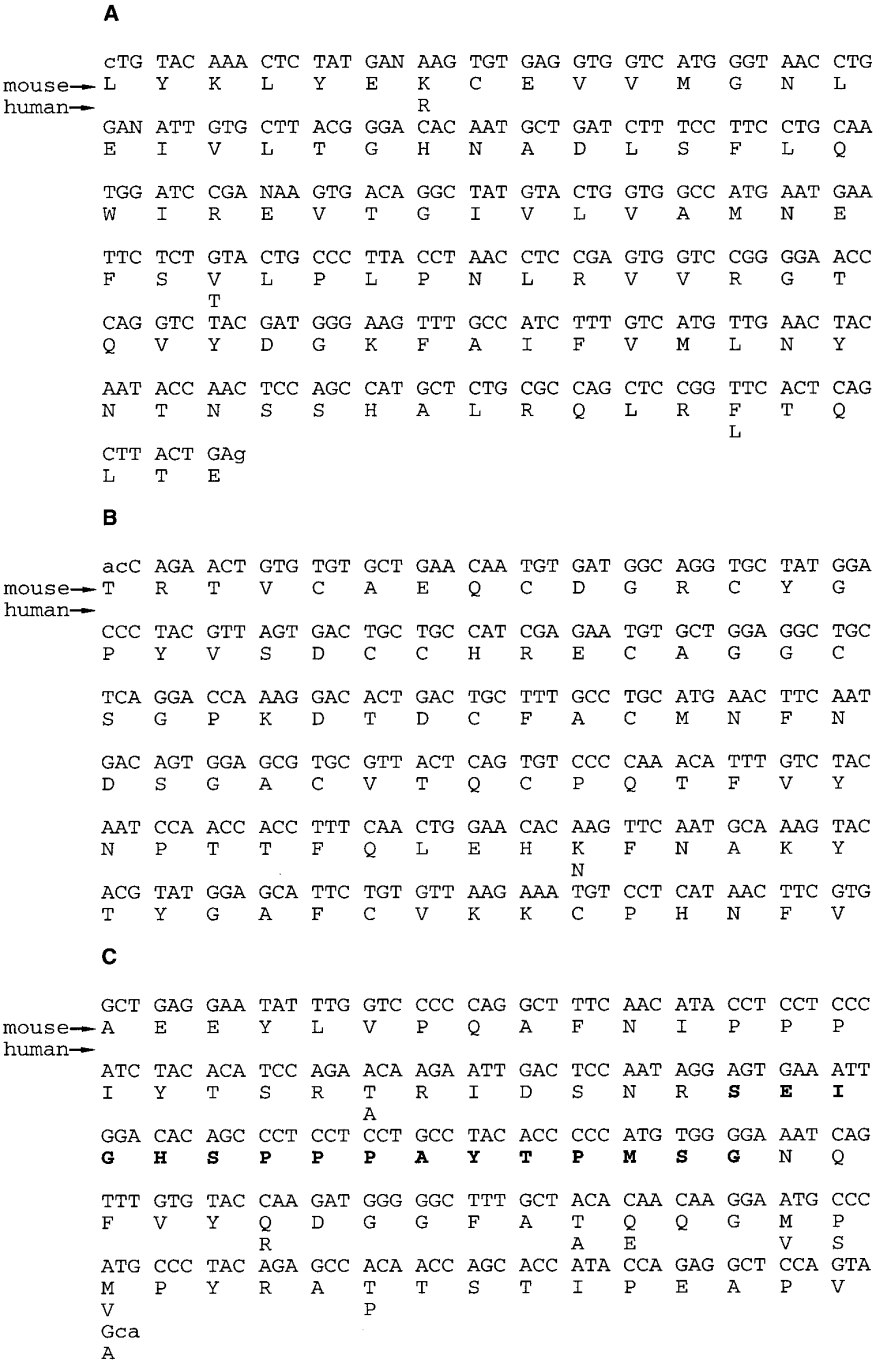

FIG. 1. Partial nucleotide and amino acid sequences of the mouse *erbB3* and *erbB4* cDNAs. Nucleotide sequences of the mouse *erbB3* and *erbB4* partial cDNA clones isolated by RT-PCR are shown. The deduced amino acid sequence is shown by one-letter amino acid code. Differences in amino acid residues in the mouse sequences with those in the human are indicated. (A) *erbB3E;* (B) *erbB4E;* (C) *erbBC/L.* Amino acid residues that are present only in the *erbB4C/L* are shown in bold characters. The sequence data for *erbB3E*, *erbB4E*, and *erbB4C/L* are available from GenBank/EMBL/DDBJ under Accession Nos. AF059175, AF059176, and AF059177, respectively.

RESULTS

Mouse erbB3 and erbB4 cDNA Sequences

The partial clone of the extracellular domain in the mouse *erbB3* cDNA (*erbB3E*) is 277 bp encompassing 245–521 nt (codons 49–141) of the human *erbB3* cDNA (Kraus *et al.*, 1989) (Fig. 1). This region shows 87 and 92% nucleic acid sequence homologies to the human and rat *erbB3*, while the deduced amino acid sequence is 97 and 98% homologous to human and rat ErbB3, respectively (Kraus *et al.*, 1989; Hellyer *et al.*, 1995). As reported previously (Moscoso *et al.*, 1995), the partial clone of the cytoplasmic domain in the mouse *erbB3* cDNA (*erbB3C*) is composed of 283 bp encompassing 3288–3570 nt (codons 1064–1157) of the human sequence. The cloned *erbB4E* (extracellular domain) is 268 bp encompassing 660–927 nt (codons 209–298) of the human *erbB4*. This region has 92% nucleic acid sequence homology to the human *erbB4* cDNA with 99% amino acid sequence homology (Plowman *et al.*, 1993). The primers used to generate a partial clone of the cytoplasmic domain in the *erbB4* (*erbB4C*) produced two different products. As reported previously (Moscoso *et al.*, 1995), a short form (*erbB4C/S*) of 178 bp was generated. In addition, a 226-bp fragment (*erbB4C/L*) with in-frame insertion of 48 nt was identified. This cDNA encompasses 3088–3313 nt in the human *erbB4* sequence with 85% nucleic acid and 88% amino acid sequence homologies (Plowman *et al.*, 1993). Although the significance of these two *erbB4C* products is not clear, *erbB4C/L* and *erbB4C/S* clearly exhibited differential expression patterns in several mouse tissues examined by RT-PCR. For example, the *erbB4C/S* was predominantly expressed over the *erbB4C/L* in the brain, kidney, and heart (data not shown). In contrast, primarily the *erbB4C/L* was detectable in pregnant mouse uterine tissues, albeit at much lower levels than those in the brain, kidney, or heart (data not shown).

Northern Blot Analysis of erbB3 and erbB4 mRNAs in the Peri-implantation Uterus

Each of the *erbB3* and *erbB4* clones was used to analyze the steady-state levels of their mRNAs in the peri-implantation mouse uterus (days 1–8). Both of the *erbB3* clones (*erbB3E* and *erbB3C*) detected a single ≈5.5-kb transcript. The *erbB3* mRNA is expressed on all days of pregnancy examined; data of the *erbB3E* are shown (Fig. 2A). Each of the three *erbB4* clones showed distinct transcript profiles (Fig. 2B). The common transcripts detected by these clones are ≈5.5, ≈4.0, and ≈2.5 kb (marked with an asterisk). However, *erbB4E* also detected additional transcripts of ≈10 kb (a) and ≈1.6 kb (b). Further, the *erbB4C/L* clone detected ≈10-kb (a) and ≈1.4-kb (c) transcripts, while the *erbB4C/S* clone detected ≈1.4-kb (c) and ≈1.8-kb (d) transcripts. In general, these transcripts were expressed in the mouse uterus without showing much variation during the peri-implantation period. The significance of multiple transcripts is not yet clearly understood.

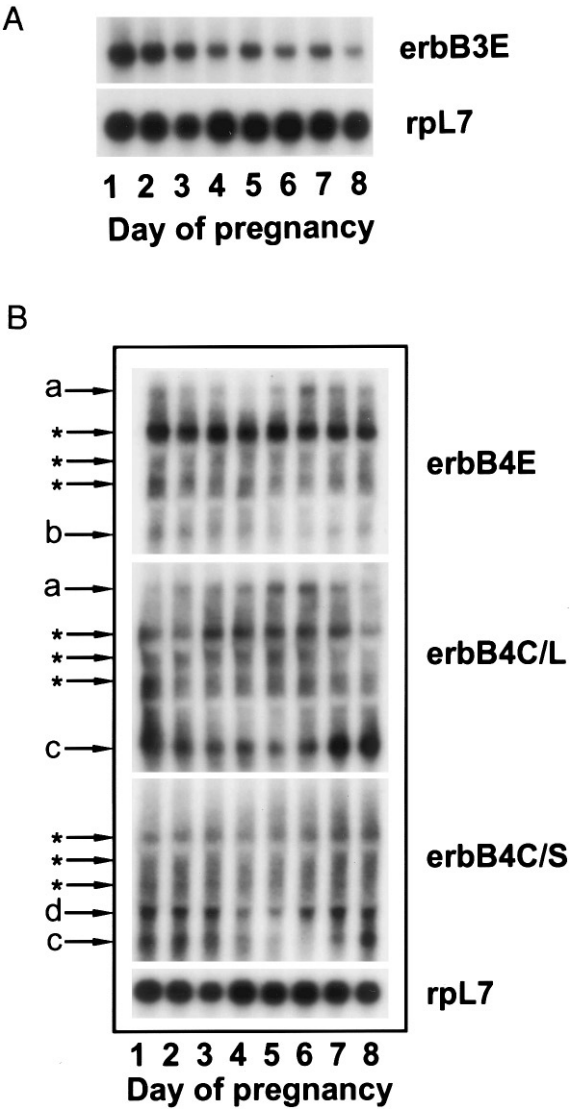

FIG. 2. Northern blot analysis of *erbB3* and *erbB4* mRNAs in the peri-implantation mouse uterus. The mRNA levels were detected in poly(A)$^+$ samples obtained from the whole uterus on days 1–8 of pregnancy. Autoradiographic exposures were as follows: (A) *erbB3E,* 7.5 h; (B) *erbB4E,* 16 h; *erbB4C/L,* 16 h; *erbB4C/S,* 2 days; *rpL7,* 1 h. Three common transcripts (5.5, 4.0, and 2.5 kb) detected by all three *erbB4* clones are marked with an asterisk (*). Additional transcripts are *erbB4E:* (a) ≈10 kb, (b) ≈1.6 kb; *erbB4C/L:* (a) ≈10 kb, (c) ≈1.4 kb; and *erbB4C/S:* (c) ≈1.4 kb, (d) ≈1.8 kb. These experiments were repeated twice using independent RNA samples with similar results.

In Situ Hybridization Analysis of erbB3 and erbB4 mRNAs in the Peri-implantation Uterus

The steady-state levels of the *erbB3* and *erbB4* mRNAs in whole uterine RNA samples showed little changes. However, *in situ* hybridization exhibited unique cell-specific

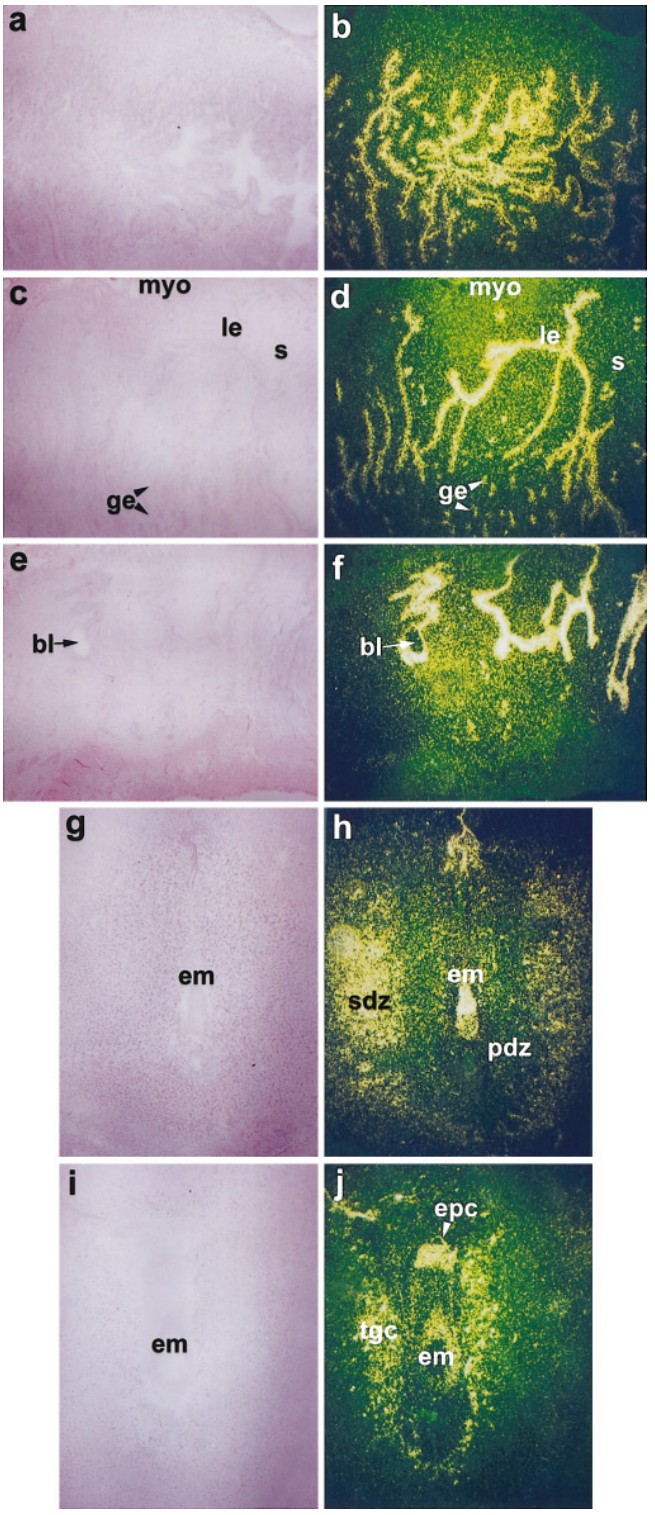

FIG. 3. *In situ* hybridization of the *erbB3* mRNA in the peri-implantation mouse uterus. Uterine sections on days 1, 4, 5, and 8 or days 2, 3, 6, and 7 of pregnancy were mounted onto the same slide. Sections were hybridized with ^{35}S-labeled antisense cRNA probes. RNase-A resistant hybrids were detected by autoradiography after 7 days of exposure. Uterine *erbB3* mRNA distribution on days 1 (a, b), 4 (c, d), 5 (e, f), 7 (g, h), and 8 (i, j) of pregnancy is shown in brightfield (left column) and darkfield (right column) photomicrographs at 40×. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; bl, blastocyst; pdz, primary decidual zone; sdz, secondary decidual zone; em, embryo; epc, ectoplacental cone; tgc, trophoblastic giant cells. These experiments were repeated three times using independent samples with similar results.

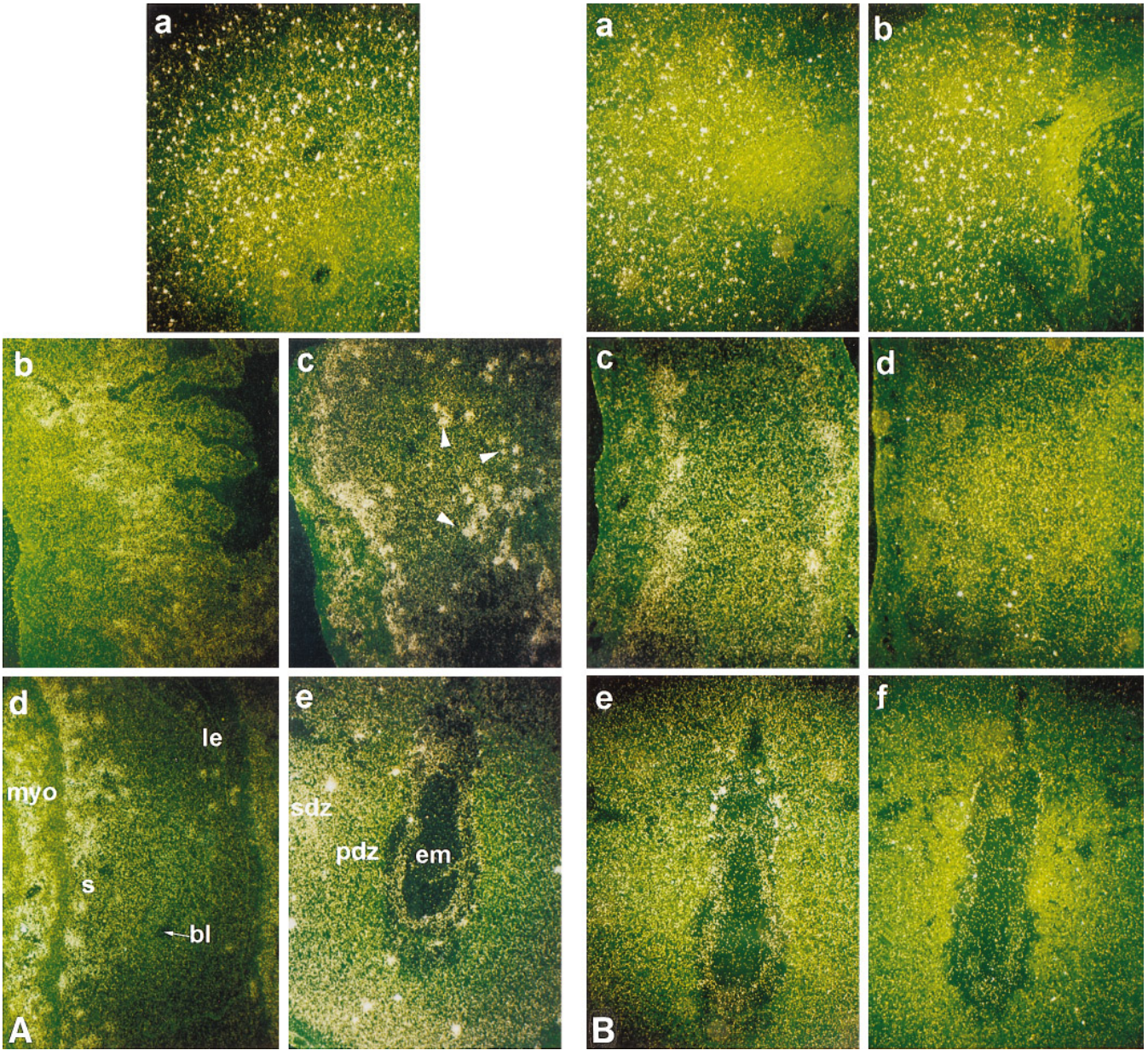

FIG. 4. *In situ* hybridization of the *erbB4* mRNAs in the peri-implantation mouse uterus. (A) distribution of *erbB4E* mRNA. a, brain; b–e, uterine sections from days 1, 4, 5, and 8 of pregnancy. (B) distribution of *erbB4C/L* (left column) and *erbB4C/S* (right column) mRNAs. a and b, brain; c and d, day 4 uterus, e and f, day 8 uterus. Exposure times for A and B are 8 and 12 days, respectively. All photomicrographs are shown in darkfield at 40×. le, luminal epithelium; s, stroma; myo, myometrium; bl, blastocyst; pdz, primary decidual zone; sdz, secondary decidual zone; em, embryo. These experiments were repeated three times using independent samples with similar results.

accumulation of these mRNAs in the peri-implantation uterus. In the preimplantation and early postimplantation uterus (days 1–5), the *erbB3* mRNA was predominantly localized in the luminal and glandular epithelial cells (Figs. 3a–3f). Epithelial cell accumulation of *erbB3* mRNA was retained on days 6 and 7. In addition, signals were present in the secondary decidual zone (Figs. 3g and 3h). On day 8, trophoblastic giant cells surrounding the embryo, ectoplacental cone, and the embryo exhibited strong signals for *erbB3* mRNA (Figs. 3i and 3j).

In contrast to the predominant epithelial accumulation of the *erbB3* mRNA, the *erbB4* mRNA was primarily expressed in the submyometrial stroma and myometrial connective tissues. The accumulation was low in other areas of

the stroma and in epithelial cells. The *erbB4E* clone detected abundant signals in the stroma, primarily in the submyometrial region of the day 1 uterus (Fig. 4A, b). On days 4 and 5, the distinct accumulation was again evident in the myometrial connective tissues and submyometrial stromal cells (Fig. 4A, c and d). In addition, a subpopulation of cells dispersed in the stromal bed exhibited strong signals (see arrowheads in Fig. 4A, c); the identities of these cells are not clear. In contrast, stromal and myometrial accumulation was downregulated on day 8, except with modest expression in the secondary decidual zone and a subpopulation of cells in the decidual bed. Accumulation of the *erbB4E* mRNA was also distinct in the day 8 embryo (Fig. 4A, e). Interestingly, a clear distinction was noted when two clones of cytoplasmic regions were used for *in situ* hybridization (Fig. 4B). The uterine expression pattern with the *erbB4C/L* clone closely followed that of the *erbB4E* clone, although the levels of signals in the subpopulation of stromal cells were lower (Fig. 4B, c and e). The signals detected by the *erbB4C/S* clone were very low in periimplantation uterine cells (Fig. 4B, d and f). Mouse brain sections served as positive controls. As reported (Pinkas-Kramarski *et al.*, 1997), a subpopulation of neuronal cells throughout the cortex exhibited accumulation of the *erbB4* mRNA. All three clones showed similar expression patterns in these cells (Fig. 4A, a; 4B, a and b). Sections hybridized with sense probes did not exhibit any positive signals (data not shown).

Phosphorylation of ErbB3 and ErbB4 in the Uterus

Employing immunoprecipitation with specific antibodies, we examined bioactivities of ErbB3 and ErbB4 in uterine membrane preparations in response to various EGF-like ligands. Because various EGF-like ligands are expressed from the time of implantation on day 4 night (Das *et al.*, 1997), day 4 morning uterine samples were used to avoid possible desensitization of these receptors by *in vivo* exposure to these ligands (Countaway *et al.*, 1992). EGF, TGFα, HB-EGF, epiregulin, NRG1β, and betacellulin were used in our experiments. As shown in Fig. 5, phosphorylation of ErbB3 was stimulated by EGF, TGFα, HB-EGF, and betacellulin, while epiregulin and NRG1β were ineffective. Likewise, phosphorylation of ErbB4 occurred when membranes were challenged with EGF, TGFα, HB-EGF, and betacellulin. In addition, epiregulin induced phosphorylation of ErbB4 as reported previously (Komurasaki *et al.*, 1997; Riese *et al.*, 1998; Shelly *et al.*, 1998), but to a lesser extent. Ar was ineffective in phosphorylating uterine ErbB3 and ErbB4, although this ligand induced phosphorylation of uterine (Das *et al.*, 1995) and liver ErbB1 in our control experiments (data not shown). Although NRG1β is known as a ligand for both ErbB3 and ErbB4, it showed very little phosphorylation of these receptors under our experimental conditions.

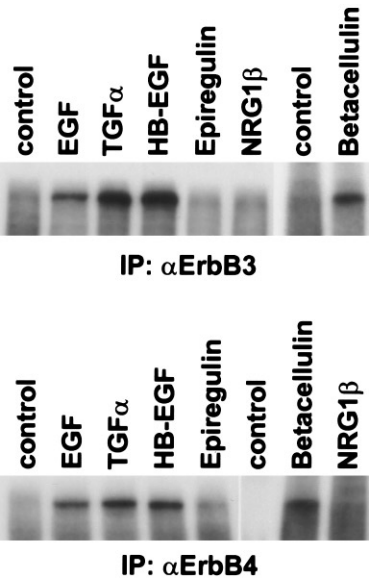

FIG. 5. Phosphorylation of uterine ErbB3 and ErbB4 by EGF-like ligands. Phosphorylation of ErbB3 or ErbB4 was determined in day 4 uterine membranes after preincubation with or without (control) EGF, TGF-α, HB-EGF, epiregulin, NRG1β, or betacellulin (100 ng/ml). The labeling reaction was initiated by the addition of [γ-^{32}P]ATP. After 2 min labeling, immunoprecipitations were performed with antibodies that are specific to ErbB3 or ErbB4. Precipitated proteins were separated by 7.5% SDS–PAGE, transferred to nitrocellulose membrane, and detected by autoradiography for 5 and 10 h for ErbB3 and ErbB4, respectively. IP, immunoprecipitation. These experiments were repeated three times using independent membrane preparations with similar results.

Composite Analysis of in Situ Hybridization of erbBs in Day 5 Implantation Sites

An analysis of *in situ* localization of *erbB1*, *erbB2*, *erbB3*, and *erbB4* mRNAs at the implantation sites on day 5 of pregnancy is shown (Fig. 6A). As reported previously (Das *et al.*, 1994; Tong *et al.*, 1996), the full-length *erbB1* mRNA is expressed in the stroma surrounding the blastocyst, while *erbB2* is predominantly expressed in the epithelium irrespective of the location of blastocysts and to a lesser extent in the stroma (Lim *et al.*, 1997). The localization of *erbB3* and *erbB4E* has already been described (Figs. 3e and 3f and Fig. 4A, d). The proposed ligand–receptor signaling network in the uterus during implantation is primarily based on this analysis (Fig. 6B).

DISCUSSION

The EGF family of growth factors and their receptor subtypes are being studied more extensively in cancer cells because their overexpression or constitutive expression is correlated with tumorigenesis (Slamon *et al.*, 1989; Juhl *et*

al., 1998). However, little is known regarding the roles of these growth factors/receptors in normal cellular processes due to lack of information about their spatiotemporal expression and complex signaling pathways. We have previously observed that EGF-like ligands and their receptor subtypes, ErbB1 and ErbB2, are expressed in the peri-implantation uterus and embryo in a spatiotemporal manner (Das *et al.,* 1994, 1997; Lim *et al.,* 1997). These observations coupled with our present results of ErbB3 and ErbB4 have enabled us to describe a comprehensive analysis and a scheme for cellular sites of ligand–receptor availability and signaling network during early pregnancy (Fig. 6B).

In this study, we have identified previously undescribed isoforms of the mouse *erbB4* mRNA spliced variants in the cytoplasmic region (*erbB4C/L* and *erbB4C/S*). The *erbB4C/S* is 48 nt shorter than the *erbB4C/L* which is homologous to the human *erbB4* (Plowman *et al.,* 1993). However, the significance of these forms in the mouse is not yet clear. It is interesting to note that all three *erbB4* clones exhibited distinct transcript profiles, localization, and abundance in the uterus (Fig. 2B). This implies multiple truncation of mouse ErbB4 involving both cytoplasmic and extracellular regions. A recent work described two alternatively spliced isoforms of the human *erbB4* (HER4) gene that differ at the juxtamembrane region with distinct cellular functions (Elenius *et al.,* 1997b). Likewise, an intracellular truncated product of the human ErbB2 and various alternate transcripts of the human *erbB3* were isolated from carcinoma cell lines, suggesting their implications in both pathogenic and normal cellular processes (Scott *et al.,* 1993; Lee and Mailhe, 1998). The mechanisms by which these uterine isoforms of the mouse *erbB4* are generated and whether they possess distinct and/or overlapping functions require further investigation.

Our observation of predominant epithelial expression of the *erbB3* gene in the peri-implantation mouse uterus is consistent with its expression in many human cell types of epithelial origin (Kraus *et al.,* 1989), suggesting its direct involvement in epithelial cell proliferation and/or differentiation (Fig. 3). However, the decidual expression of the *erbB3* mRNA on days 6 and 7 of pregnancy suggests its role in stromal cell transformation into decidual cells. The expression of *erbB3* in the ectoplacental cone and subsets of trophoblastic giant cells is similar to that of the *erbB2* gene (Lim *et al.,* 1997). This coexpression suggests that signaling through these receptors is involved in decidualization, regulation of trophoblast invasion, and perhaps subsequent placentation. Among the three *erbB4* clones, *erbB4E* showed the most abundant expression in the uterus. Further, this clone detected intense signals in a subpopulation of uterine cells throughout the stroma (Fig. 4A), suggesting that truncation at the extracellular region is involved in this expression. In contrast, all three *erbB4* clones exhibited similar cell-specific expression in the brain cortex, albeit with modest differences in intensity. Collectively, the uterine expression pattern of *erbB4* is unique among the *erbB* gene family members in that it is expressed in cells distant from the implanting embryo. Uterine ErbB3 and ErbB4 responded to different EGF-like ligands and exhibited autophosphorylation, suggesting that they are biologically active in the uterus (Fig. 5). Both *erbB3* and *erbB4* genes are also expressed in day 8 embryos. This early embryonic expression suggests their involvement in cardiac and nervous system development, because midgestational abnormalities of these organs are manifested in mice mutated for these genes (Gassman *et al.,* 1995; Riethmacher *et al.,* 1997; Erickson *et al.,* 1997).

The process of implantation involves dynamic cellular changes. The epithelial cell differentiation and stromal cell proliferation on day 4 of pregnancy (day of implantation) prepare the uterus to the receptive state for implantation (Dey, 1996). The earliest known molecular interaction between the trophectoderm and luminal epithelium is the induction of HB-EGF in the luminal epithelium exclusively at the sites of blastocyst apposition prior to the attachment reaction (1600 h) on day 4 (Das *et al.,* 1994b). This epithelial HB-EGF appears to interact with blastocyst ErbB1 in a paracrine/juxtacrine manner (Das *et al.,* 1994b; Raab *et al.,* 1996) and is followed by similar inductions of Ar, betacellulin, epiregulin, and NRG1 (reviewed in Das *et al.,* 1997) during the attachment reaction (2300 h). The expression of these ligands persists through early stages of implantation on day 5. Induction of these ligands requires the presence of active blastocysts, though the mechanism of induction is unclear (reviewed in Das *et al.,* 1997). The spatiotemporal analysis of the EGF family and their receptor subtypes has allowed us to describe potential ligand–receptor networking during implantation. As reported previously (Das *et al.,* 1994a; Tong *et al.,* 1996) and as shown in Fig. 6A, the full-length ErbB1 is expressed in the stroma surrounding the implanting blastocyst on day 5. In contrast, ErbB2 and ErbB3 are predominantly expressed in the epithelium throughout the uterus, albeit ErbB2 is expressed at basal levels throughout the stroma. ErbB4 is expressed primarily at the submyometrial stroma and myometrial connective tissues with basal levels of expression throughout the stroma. Distinct expression patterns of these receptor subtypes suggest possible dimerization schemes (Fig. 6B). Because ErbB2 is the most favored partner for other ErbBs (Karunagaran *et al.,* 1996; Graus-Porta *et al.,* 1997), the ErbB2/ErbB3 heterodimer could be functional in the epithelium upon ligand activation, while the ErbB1/ErbB2 and ErbB2/ErbB4 heterodimer could be operative in the subepithelial stroma and submyometrial stroma, respectively. However, the formation of the ErbB1/ErbB1 or ErbB4/ErbB4 homodimer is also possible in these regions. Figure 6B also depicts possible cellular sources of EGF-like ligands and their sites of action. As mentioned above, Ar, HB-EGF, epiregulin, betacellulin, and NRG1 are expressed in the luminal epithelium and/or stroma at the sites of blastocyst attachment. In contrast, while EGF is not expressed in the peri-implantation uterus, TGF-α is expressed in major uterine cell types regardless of the blastocyst location (reviewed in Das *et al.,* 1997). Thus, for the formation of the ErbB1/

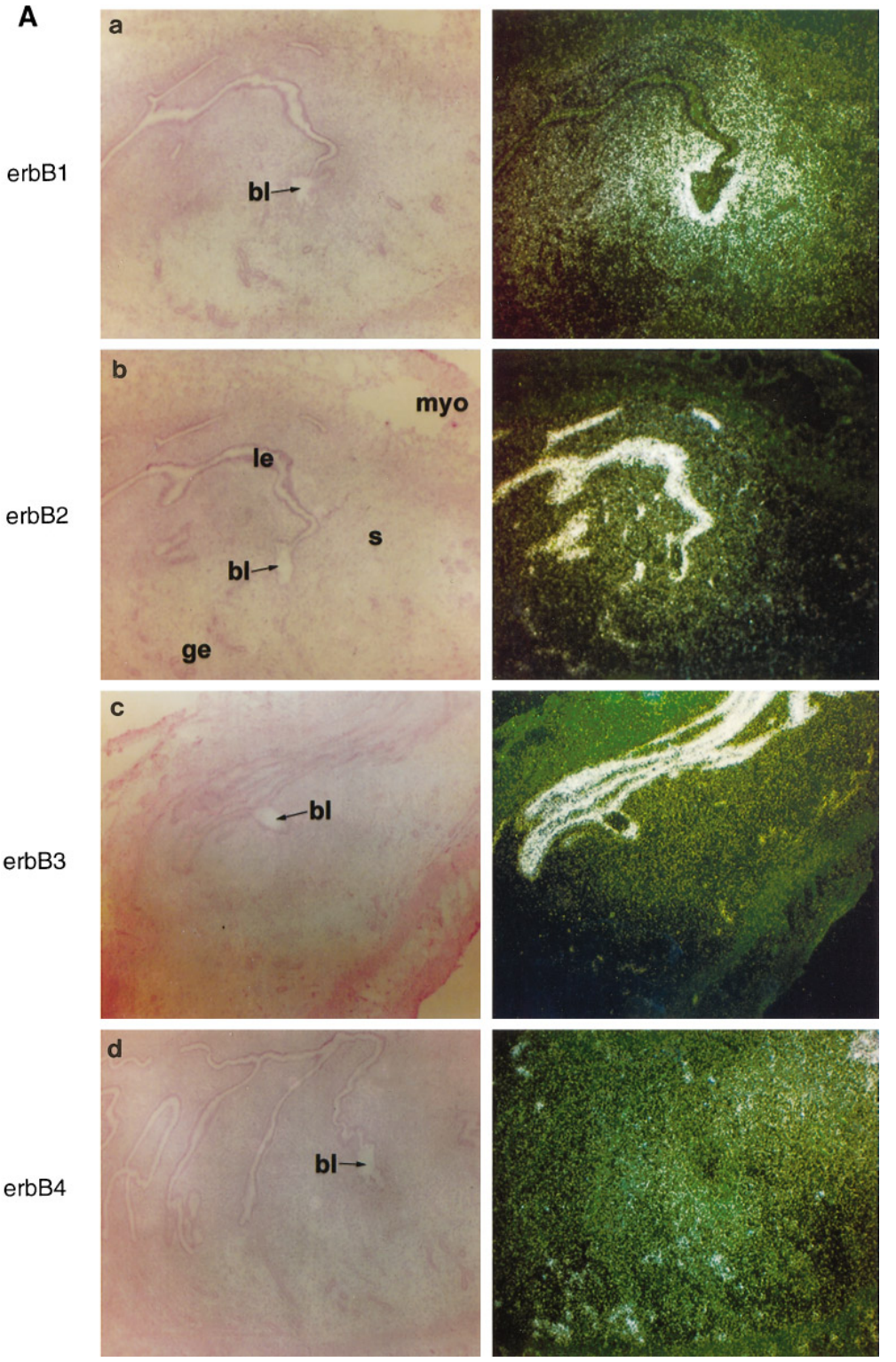

FIG. 6. A model for the ligand–receptor signaling network with the EGF family of growth factors in the uterus during implantation. (A) Expression of the erbB gene family at the sites of implantation on day 5 is shown in darkfield photomicrographs at 40×. a, *erbB1* (3-day exposure); b, *erbB2* (3-day exposure); c, *erbB3E* (7-day exposure); d, *erbB4E* (12-day exposure). le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; bl, blastocyst. (B) A schematic diagram depicting the potential ligand–receptor signaling network with the EGF family of growth factors in the uterus during implantation is shown. This scheme is based on the mapping of the ligands and receptors in the uterus at the sites of blastocyst implantation on day 5 of pregnancy. The scheme primarily focuses on the uterine aspects of the signaling network that is considered important for uterine preparation for implantation. However, our preliminary results suggest that the signaling between the blastocyst ErbBs and uterine ligands is also important for implantation, but is not within the scope of the study.

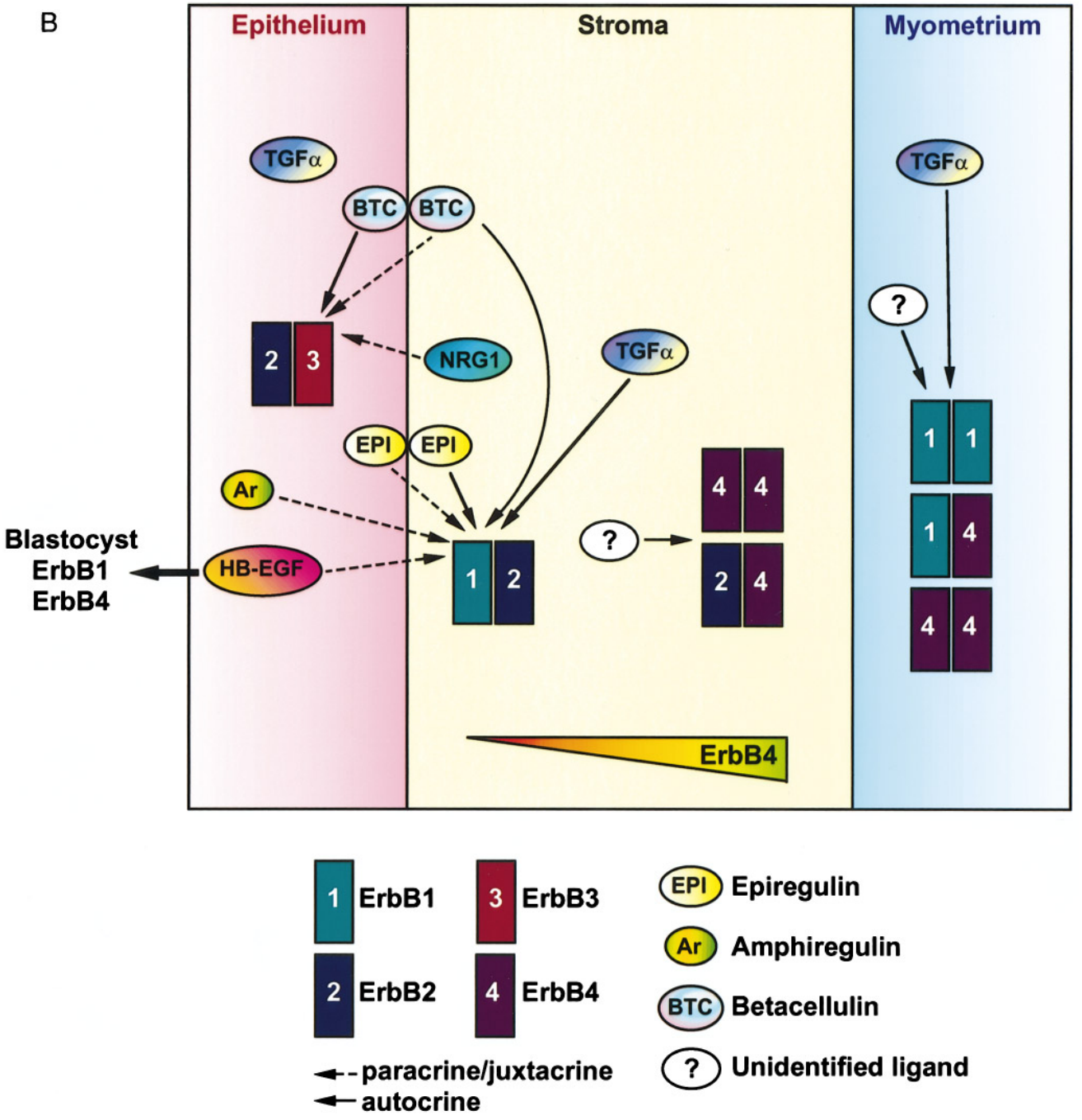

FIG. 6—*Continued*

ErbB2 heterodimer, Ar, HB-EGF, and epiregulin are available to function as autocrine/paracrine/juxtacrine factors for stromal cell proliferation and/or differentiation. Although ErbB4 is primarily localized at the submyometrial region of the stroma, no known ErbB4 ligands such as HB-EGF, betacellulin, and epiregulin are expressed at its close proximity during implantation. Nonetheless, it is possible that these ligands are secreted in the stroma and affect ErbB4 as paracrine factors. Alternatively, novel ligands may exist for this receptor in the uterus.

As described above, preferential ErbB2/ErbB3 dimer formation is likely to occur in the epithelium because of their

predominant coexpression in these cells. This dimerization serves as a high-affinity receptor for NRG1 (Sliwkowski *et al.*, 1994) and also creates recently identified potential binding sites for EGF and betacellulin (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998). An intriguing possibility is that betacellulin utilizes the ErbB2/ErbB3 dimer in mediating its signaling in the epithelium for implantation. Therefore, colocalization of ErbB2 and ErbB3 suggests that dimerization of these subtypes can directly transduce signals for epithelial cell proliferation and/or differentiation in the peri-implantation uterus in the absence of ErbB1 (the classic receptor for EGF and betacellulin). To test further this possibility, coimmunoprecipitation of ErbB2 and ErbB3 upon betacellulin challenge could be studied using uterine membrane preparations. However, difficulties are anticipated because these uterine receptors are expressed at relatively low levels compared with those of overexpressor cell lines. As mentioned above, another possible ligand for this dimer is NRG1 which is expressed only in the stroma at the sites of blastocyst attachment (Reese *et al.*, 1998). Thus, stromal NRG1 is likely to act on epithelial ErbB2/ErbB3 in a paracrine manner. This notion is consistent with the previous observation that NRG1 is a mesenchymal factor which interacts with the receptors of neighboring epithelial cells in a paracrine manner during mouse development (Meyer and Birchmeier, 1994; Lemke, 1996).

Our mapping of the EGF-like growth factors and their receptor subtypes in the peri-implantation mouse uterus is the first to provide the potential site-specific networking of these growth factors in the uterus during implantation which could also be important and applicable to other organ systems.

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