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Dual source and target of heparin-binding EGF-like growth factor during the onset of implantation in the hamster

Xiaohong Wang[1], Haibin Wang[2], Hiromichi Matsumoto[2], Shyamal K. Roy[4], Sanjoy K. Das[2,3] and Bibhash C. Paria[2,*]

[1]Departments of Pediatrics, [2]Molecular and Integrative Physiology and [3]Obstetrics and Gynecology, Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66160-7338, USA
[4]Department of Obstetrics and Gynecology and Physiology, University of Nebraska, Medical Center, Omaha, NE 68198-4515, USA
*Author for correspondence (e-mail: bparia@kumc.edu)

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SUMMARY

Heparin binding EGF-like growth factor (HB-EGF), encoded by the *Hegfl* gene, is considered as an important mediator of embryo-uterine interactions during implantation in mice. However, it is unknown whether HB-EGF is important for implantation in species with different steroid hormonal requirements. In mice and rats, maternal ovarian estrogen and progesterone (P_4) are essential to implantation. In contrast, blastocyst implantation can occur in hamsters in the presence of P_4 alone. To ascertain whether HB-EGF plays any role in implantation in hamsters, we examined the expression, regulation and signaling of HB-EGF in the hamster embryo and uterus during the periimplantation period. We demonstrate that both the blastocyst and uterus express HB-EGF during implantation. *Hegfl* is expressed solely in the uterine luminal epithelium surrounding the blastocyst prior to and during the initiation of implantation. Hypophysectomized P_4-treated pregnant hamsters also showed a similar pattern of implantation-specific *Hegfl* expression. These results suggest that uterine *Hegfl* expression at the implantation site is driven by either signals emanating from the blastocyst or maternal P_4, but not by maternal estrogen. However, in ovariectomized hamsters, uterine induction of *Hegfl* requires the presence of estrogen and activation of its nuclear receptor (ER), but not P_4. This observation suggests an intriguing possibility that an estrogenic or unidentified signal from the blastocyst is the trigger for uterine HB-EGF expression. An auto-induction of *Hegfl* in the uterus by blastocyst-derived HB-EGF is also a possibility. We further observed that HB-EGF induces autophosphorylation of ErbB1 and ErbB4 in the uterus and blastocyst. Taken together, we propose that HB-EGF production and signaling by the blastocyst and uterus orchestrate the 'two-way' molecular signaling to initiate the process of implantation in hamsters.

Key words: Uterus, Blastocyst, HB-EGF, ErbB receptor, Implantation, Hamster

INTRODUCTION

The process of implantation involves complex cell-to-cell communications between the blastocyst trophectoderm and luminal epithelium of the receptive uterus. Steroid hormonal requirements for this process are well documented in several species. A brief preimplantation ovarian estrogen secretion is required for the initiation of implantation in the progesterone (P_4)-primed uterus in mice and rats in which blastocysts do not have the capacity to produce estrogen (Psychoyos, 1973; Paria et al., 1993a; Dey, 1996; Stromstedt et al., 1996). In contrast, conceptus-derived estrogen is considered important for implantation in pigs and rabbits (George and Wilson, 1978; Heap et al., 1981; Hoversland et al., 1982; Geisert et al., 1990). In hamsters, maternal estrogen is not an absolute requirement for implantation. Implantation can occur in this species in the absence of any ovarian and/or adrenal estrogen provided P_4 is given exogenously (Prasad et al., 1960; Orsini and Meyer, 1962; Harper et al., 1969). There is also no molecular evidence that hamster preimplantation embryos produce estrogen that could be a source of estrogen for implantation. It is also not clear whether species-specific differential steroid hormonal requirements are reflected in differential regulation of local implantation-specific factors.

Molecular and cellular evidence suggests that HB-EGF, a member of the EGF family of growth factors (Abraham et al., 1993), plays important roles in implantation in several species including humans (reviewed by Paria et al., 2000; Paria et al., 2001a). HB-EGF is produced as a transmembrane form, which is proteolytically cleaved to produce the soluble mature form. Both forms of HB-EGF can interact with cell surface proteins of the EGF receptor family (ErbBs) and cell surface heparan sulphate proteoglycan (HSPG) molecules (reviewed by Das et al., 1994; Elenius et al., 1997). The *Erbb* gene family comprises four receptor tyrosine kinase genes: *Erbb1*, *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 all of the members of the EGF family can directly bind to ErbB1, HB-EGF, betacellulin and epiregulin act as distinct ligands for ErbB4. ErbB2 and ErbB3 require heterodimerization with either ErbB1 or ErbB4 for intracellular signaling (reviewed by Lim et al., 1998). Most of the recent work to elucidate the mechanism by which HB-EGF exerts its biological effects has focused on activation of tyrosine phosphorylation of ErbB1. The binding of HB-EGF to the extracellular domain of ErbB1 activates its cytoplasmic tyrosine kinase that phosphorylates the receptor itself (Hunter and Cooper, 1980). The direct interaction of HB-EGF with ErbB1 plays a crucial role in cell-cell adhesion and signal transmission between neighboring cells. HB-EGF stimulates blastocyst growth and zona hatching in vitro (Das et al., 1994; Martin et al., 1998; Mishra and Seshagiri, 2000; Wang et al., 2000; Seshagiri et al., 2002). Furthermore, cells expressing the transmembrane form of HB-EGF adhere to the trophectoderm surface of implantation-competent mouse blastocysts by interacting with ErbB1 or ErbB4 and HSPG molecules displayed on the blastocyst cell surface (Raab et al., 1996; Paria et al., 1999). Several studies have shown that uterine expression of HB-EGF is highly relevant to implantation in several species including humans (Das et al., 1994; Yoo et al., 1997; Leach et al., 1999; Yue et al., 2000). We previously demonstrated that HB-EGF is first induced solely at the site of blastocyst implantation several hours before the onset of the attachment reaction and persists through the early stages of implantation in mice (Das et al., 1994). This unique uterine HB-EGF expression requires the presence of a blastocyst that gains implantation-competence in the presence of estrogen in the P_4-primed uterus (Das et al., 1994). Although, the presence of immunoreactive HB-EGF has been demonstrated in hamster uterus and blastocyst (Mishra and Seshagiri, 2000), the expression of *Hegfl* and its hormonal regulations during implantation are not known in hamsters in which P_4 alone, but not estrogen, is sufficient to induce this process. Here we demonstrate that *Hegfl* is preferentially expressed in the uterine luminal epithelial cells surrounding the blastocysts prior to, during and after the initiation of implantation process. HB-EGF is also expressed in blastocysts and it is a potent inducer of ErbB1 and ErbB4 phosphorylation both in the blastocyst and uterus.

MATERIALS AND METHODS

Animals and tissue preparation

Adult virgin male and female golden hamsters (*Mesocricetus auratus*) of 8- to 12-week old were purchased from Sasco, Nebraska. They were maintained in a light 14 hours light:10 hours dark cycle in the Laboratory Animal Facility of the University of Kansas Medical Center with ad libitum supply of water and food. Hamsters with three consecutive 4-day estrous cycles were used in this study. One female was housed with two fertile males overnight on the evening of proestrus. Finding of sperm in the vaginal discharge the next morning (estrus) was designated day 1 of pregnancy. Hamsters on days 1-3 of pregnancy were killed at 0900 hours and whole uteri were flash frozen. Pregnancy was confirmed by recovering embryos from oviducts and/or uterine horns. While whole uteri were collected on the morning of day 4 (0900 hours), implantation and interimplantation sites were collected in the afternoon (1600 hours) of day 4 and morning (0900 hours) of days 5 and 6 after an intravenous injection of Chicago Blue B dye solution (0.25 ml of 1% in saline). Implantation sites on these days were visualized as discrete blue bands along the horns. On days 6-8, implantation sites are distinct and their identification does not require blue dye injection (Sengupta et al., 1981). Uterine tissues were flash frozen in cold Super Friendly Freeze'it (Curtin Matheson Scientific, Houston, TX) for extraction of RNA and in situ hybridization.

Implantation occurs without delay in hamsters ovariectomized or hypophysectomized on day 2 of pregnancy and given P_4 daily (Prasad et al., 1960; Orsini and Meyer, 1962; Harper et al., 1969). This suggests that implantation in hamsters is P_4 dependent. However, it is possible that implantation occurred with correct expression of implantation-specific genes because basal levels of estrogen still persisted. To address this issue, a group of pregnant hamsters were hypophysectomized on day 2 (0900 hours) and given P_4 (1 mg/hamster) on either day 3 or days 3 and 4. Control hamsters underwent sham operations and were injected with oil. Whole uteri were collected on the morning of day 4 (0900 hours). Implantation sites, determined by blue dye injection, were collected on the evening of day 4 (1600 hours) and the morning of day 5 (0900 hours) for in situ hybridization. Blood samples were also collected to obtain sera for the measurement of circulating estradiol-17β (E_2) levels. Serum E_2 levels were measured as described previously using a standard curve ranging from 2-1000 pg/tube (Roy and Greenwald, 1987).

To determine the effects of steroid hormones on uterine *Hegfl* expression, hamsters were ovariectomized without regard to their stages of the estrous cycle and rested for 12 days. One group of these hamsters was treated with a subcutaneous injection of P_4 (500 μg/hamster), E_2 (1.0 μg/hamster) or E_2 plus P_4. All steroids were dissolved in sesame oil. Hamsters were killed at 2, 6, 12 and 24 hours after injection of hormones and uteri were collected for RNA extraction and in situ hybridization. In the second group, each hamster received an injection of 0.01, 0.10 or 1.0 μg E_2 to determine the minimum amount of E_2 required for the induction of *Hegfl*. The third group of hamsters each received an injection of ICI-182,780 (1 mg/hamster) 30 minutes before an injection of 1.0 μg E_2 to determine whether nuclear ER mediated the estrogen effects. Animals from the second and third groups were killed 2 or 6 hours after the injection of E_2. Uteri were processed for in situ hybridization.

For collection of preimplantation embryos, females having a regular 4-day estrous cycle were superovulated by intraperitonial injection of 20 IU of pregnant mare serum gonadotrophin (PMSG) before 0900 hours on the day of the post-estrous discharge and were mated 3 days later (Kane and Bavister, 1988). Oviducts were flushed at 0900 hours on days 1 and 2 and at 0200 hours on day 3 to collect one-cell, two-cell and four-cell embryos, respectively. Eight-cell/morula stage embryos were collected at 1700 hours on day 3 and blastocysts at 0800 hours on day 4 by flushing uteri. Embryos were washed several times to avoid any contamination of maternal cells in Hamster embryo culture medium-2 (HECM-2) (Ain and Seshagiri, 1997). An average of 40 to 70 embryos were recovered from each superovulated female. One hundred embryos at each developmental stage were quickly frozen in a small volume of medium in a sterile 1.5 ml microcentrifuge tube for total RNA isolation. Blastocysts were also collected for immunofluorescence, and HB-EGF-stimulated EGFR autophosphorylation studies.

Total uterine RNA preparation

Uterine RNA was extracted using TRIZOL reagent (Gibco Life technologies, USA) according to the manufacturer's instruction. In brief, tissues were homogenized in TRIZOL reagent (1 ml/50 mg tissue). Homogenates were mixed with 0.2 ml of chloroform/ml of TRIZOL, shaken vigorously for 15 seconds and centrifuged at 12,000 ***g*** for 15 minutes at 4°C. The aqueous phase was collected and added with isopropyl alcohol (0.5 ml/ml TRIZOL reagent) for precipitation of RNA. After 10 minutes at room temperature, samples

Table 1. Sequences of primers used in RT-PCR analysis

Genes		Oligomers	Product size (bp)
Hegfl	Sense	5′-ACG CCA AGC AAA GAA AAG AA-3′	247
	Sense-internal	5′-GCA AGG GAT TGG GAA AGA AG-3′	
	Antisense	5′-CCA CCA CAG CCA AGA TTG TA-3′	
Erbb1	Sense	5′-GAC TGT CTG GTC TGC CAC AG-3′	180
	Sense-internal	5′-GAA GCC ACA TGC AAA GAC AC-3′	
	Antisense	5′-TGA GCC ATG ATC TGT CAC CA-3′	
Erbb4	Sense	5′-CTA TGG ACC CTA CGT TAG TGA CTG C-3′	207
	Sense-internal	5′-TAC AAT CCA ACC ACC TTT CAA CTA G-3′	
	Antisense	5′-ACA CAG AAT GCT CCG TAT GTG TAC TT-3′	
Rpl7	Sense	5′-TGA ATG GAG TAA TCC CAA AG-3′	246
	Sense-internal	5′-ATT GCA TGG GGG TAC CCC AAC C-3′	
	Antisense	5′-CAA GAG ACC GAG CAA TCA AG-3′	

were again centrifuged to re-precipitate RNA. The precipitated RNA was mixed with 75% ethanol in TRIZOL for washing and centrifuged at 7,500 ***g*** to obtain final RNA pellet.

Total embryo RNA preparation

Total RNA from preimplantation embryos was extracted as previously described (Andrews et al., 1991; Paria et al., 1993b). After addition of *E. coli* rRNA (20 μg) as a carrier in each tube, total RNA was extracted using sodium dodecyl sulphate/phenol/chloroform buffers. Percentage recovery of total RNA using this method is approximately 59-61% as determined by recovery of a labeled tracer RNA.

RNA probes

Linearized plasmids bearing hamster cDNAs were transcribed using appropriate RNA polymerases to generate sense and antisense probes and labeled with either ^{32}P or ^{35}S for Northern or in situ hybridizations, respectively (Das et al., 1994). A partial clone of rpL7 cDNA was also used as a template for the synthesis of ^{32}P-labeled antisense cRNA probe. All labeled sense and antisense cRNA probes had specific activities of approximately 2×10^9 dpm/μg.

Northern blot hybridization

Total RNA (6 μg) was denatured and separated by formaldehyde/agarose gel electrophoresis, transferred to nylon membranes and UV cross-linked. Northern blots were prehybridized, hybridized, and washed at 68°C as described previously (Das et al., 1994). Quantitation of hybridized bands was analyzed by densitometric scanning.

In situ hybridization

The protocol followed was as described by Das et al. (Das et al.1994). Briefly, uterine cryosections were mounted onto poly-L-lysine-coated slides and fixed in cold 4% paraformaldehyde solution in phosphate-buffered saline (PBS) for 15 minutes. After prehybridization, sections were hybridized with ^{35}S-labeled sense or antisense probes at 45°C for 4 hours in 50% formamide hybridization buffer. After hybridization and washing, sections were treated with RNase A (20 μg/ml) at 37°C for 20 minutes. RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak Company, Rochester, NY). The slides were poststained with Hematoxylin and Eosin. Sections hybridized with the sense probes served as negative controls.

Analysis of *Hegfl*, *Erbb1* and *Erbb4* mRNAs in preimplantation embryos and day 4 uteri

To examine the expression of *Hegfl*, *Erbb1* and *Erbb4* mRNAs in preimplantation embryos and day 4 uteri, RT-PCR was employed using isolated total RNAs. Uterine total RNA (1 μg) and 25% of the embryonic total RNA were used for RT reaction using random oligonucleotide primers according to the Manufacturer's instruction (Invitrogen, Carlsbad, CA). One-third of the RT product was PCR amplified using sense and antisense primers that were designed from the cloned sequences of the hamster genes, except for ribosomal protein L7 (*Rpl7*), a house keeping gene. Sense and antisense primers for *Rpl7* were designed from mouse *Rpl7* sequence (GenBank accession no. M29016), while sense-internal primers used for Southern hybridization was designed from cloned sequence of hamster *Rpl7* (GenBank accession no. AF394540). The GenBank accession number for the hamster *Hegfl* sequence is AF327896 (Table 1).

PCR was performed under the following conditions: 94°C for 5 minutes denaturing, 45 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 10 minutes. The reactions were carried out in a final volume of 25 μl. One-fifth of the amplified PCR product was electrophoresed on agarose gels (1.5%) and stained with ethidium bromide. Experimental and appropriate negative controls were run simultaneously. The product size was determined by concurrently separating a 1000-bp DNA ladder on the gel. The DNA gel was blotted and analyzed by Southern blot hybridization at 45°C using end-labeled (γ-^{32}P)ATP internal oligonucleotides. The hybrids were detected by autoradiography and the intensity of the bands was quantified by densitometry. Cloning and sequencing also confirmed the identity of each PCR product.

Immunodetection of HB-EGF, ErbB1 and ErbB4 in blastocysts

Blastocysts were fixed in 3.7% formaldehyde in PBS at room temperature for 30 minutes, permeabilized in 2.5% Tween 20 in PBS for 5 minutes and then incubated overnight at 4°C with goat polyclonal antibodies to HB-EGF (2 μg/ml) or rabbit polyclonal antibodies to ErbB1 and ErbB4 at a dilution of 1:1000 in PBS. Goat anti-human HB-EGF antibody and rhHB-EGF were obtained from R&D systems, Minneapolis, MN. Rabbit polyclonal antibodies to mouse liver ErbB1 was kindly provided by Eileen Adamson, La Jolla Cancer research Foundation (La Jolla, CA). A mouse monoclonal anti-human ErbB1 and its blocking peptide were purchased from ICN (Costa Mesa, CA). The rabbit polyclonal antibody to mouse ErbB4 and its blocking peptide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Blastocysts incubated with either non-immune sera or antibodies preneutralized with 200-fold molar excess of the antigenic proteins or peptides served as negative controls. After several washes with PBS containing 0.5% Triton X-100 and 0.5% bovine serum albumin (BSA), blastocysts were incubated with TRITC-labeled rabbit anti-goat antibody or TRITC-labeled goat anti-rabbit antibody (Zymed Laboratories, San Francisco,

CA) for 1 hour at room temperature. Nuclei were labeled with Hoechst 33,342 (1 μg/ml; Molecular Probes, Eugene, OR) for 30 minutes at room temperature. After several washes with PBS containing 0.1% BSA, blastocysts were mounted, antigen labeled with TRITC (red) and nuclei stained with Hoechst (blue). They were then viewed in a Zeiss LSM 510 confocal scanning laser microscope (Axioplan 2 Imaging) using excitation wavelengths of 543 nm and 364 nm, respectively. Images shown in the Results section are representative of at least 8 blastocysts from different animals that produced similar patterns.

Autophosphorylation and immunoprecipitation of ErbBs in the uterus and blastocyst

Day 4 blastocysts (0800 hours) collected in groups of 100 were placed in microfuge tubes containing 10 μl of buffer A [10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 μg/ml leupeptin, 10 μg/ml aprotinin and 20 μg/ml phenylmethanesulfonyl fluoride (PMSF)] and sonicated. Membranes from day-4 uteri were prepared as previously described (Das et al., 1994). In brief, day-4 pregnant uteri were homogenized in buffer A and centrifuged at 900 ***g*** for 10 minutes at 4°C. The supernatant was centrifuged at 144,000 ***g*** for 1 hour at 4°C. The pellet was resuspended again in buffer A and subjected to recentrifugation for 1 hour. The resultant pellet was resuspended in buffer B (10 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 20 μg/ml PMSF).

Blastocyst homogenates and uterine membranes were suspended in 50 μl of phosphorylation buffer (50 mM Pipes (pH 7.0), 1 mM $MnCl_2$, 0.1 mM sodium vanadate) and preincubated with or without HB-EGF (100 ng/ml) for 10 minutes at 4°C. The labeling reactions were performed for 2 minutes 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 the addition of equal volume of 10% trichloroacetic acid (TCA). To further confirm that HB-EGF induces autophosphorylation of ErbB1 and ErbB4, radiolabeled phosphorylated products were subjected to immunoprecipitation using antibodies to ErbB1 or ErbB4. Trichloroacetic acid (10%)-precipitated phosphorylated products, after washing in 50 mM Tris-HCl (pH 8.0), were resuspended in 100 μl of 50 mM Tris buffer (pH. 8.0). An equal volume of protein A sepharose-antibody conjugates (3 mg:0.8 μg) was added to the mixture. A mouse monoclonal anti-human ErbB1 and a rabbit polyclonal antibody to mouse ErbB4 were purchased from ICN (Costa Mesa, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. In a separate set of experiments, specificity of these antibodies was confirmed by western blotting using day-4 hamster uterine membranes and preneutralized antibodies with 500-fold excess of the antigenic peptides (data not shown). These reaction mixtures were incubated for 90 minutes at 4°C with constant shaking. The protein A sepharose antibody and antigen complexes were subjected to repeated washes in 10 mM Tris-HCl (pH 8.0). The resultant pellets were boiled in 1× SDS sample buffer for 5 minutes and centrifuged. The supernatants were subjected to 6% SDS-PAGE in parallel with molecular mass markers. The gel was transferred to Immuno-Blot™ PVDF membrane (BIO-RAD, Hercules, CA) and radioactive products were visualized by autoradiography.

RESULTS

Hegfl is expressed in an implantation-specific manner in the hamster uterus

To assess the expression levels of *Hegfl* mRNA during the periimplantation period, uterine total RNA samples from day-1 to -8 of pregnancy were used for northern blot hybridization. As previously reported in mice (Das et al., 1994; Wang et al., 1994), northern blotting detected an approx 2.5 kb transcript

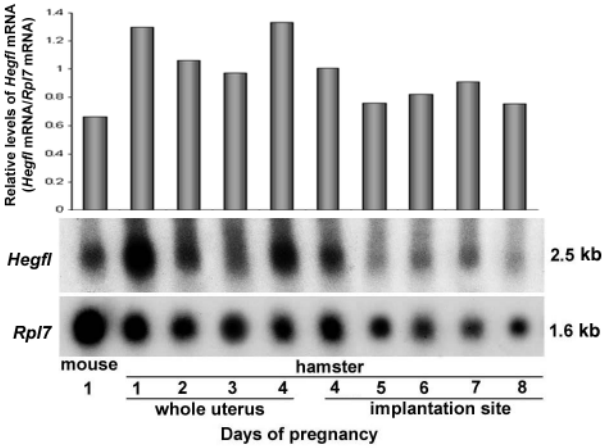

Fig. 1. Northern blot hybridization of *Hegfl* mRNA in the periimplantation (days 1-8) hamster uterus. The expression of *Hegf1* mRNA was detected in total RNA samples obtained from whole hamster uteri on days 1-4, and implant sites on day 4 afternoon and days 5-8 of pregnancy. Total RNA from day-1 mouse uterus was used as a control. An approx. 2.5 kb transcript for *Hegfl* was detected in the uterus. The blot was hybridized with *Rpl7* to confirm integrity, equal loading and blotting of RNA samples. These experiments were repeated twice with similar results. Bar diagram above the autoradiogram shows the relative levels of *Hegfl* mRNA (*Hegfl* mRNA/*Rpl7* mRNA) after densitometric scanning of the autoradiogram.

of *Hegfl* mRNA in the hamster uterus (Fig. 1). The hamster uterine expression of *Hegfl* was high on day 1, but was lower on days 2 and 3. The level of expression was again high on day 4, but declined thereafter.

Since *Hegfl* is expressed in a spatiotemporal manner in the mouse uterus (Das et al., 1994), we speculated that its expression in the hamster uterus would be similar to that in mice if this growth factor were important for implantation. Indeed, this study shows a distinct spatiotemporal localization of *Hegfl* mRNA during the periimplantation period. *Hegfl* expression was restricted to the luminal epithelium on day 1 of pregnancy, but its expression was undetectable on days 2 and 3 (Fig. 2). However, the expression was again visible in the luminal epithelial lining throughout the uterus, but more intensely at the location of the blastocyst on the morning of day 4 (0900 hours). Interestingly, *Hegfl* was exclusively expressed in the luminal epithelium surrounding the implanting blastocyst on the afternoon of day 4 (1600 hours) and on the morning of day 5. Signals were undetectable in decidual cells at the site of implantation on days 6-8, except at low levels in the mesometrial luminal epithelium or in developing embryos.

Since implantation in hamsters occurs presumably in the absence of circulating estrogen (Prasad et al., 1960; Orsini and Meyer, 1962; Harper et al., 1969), we sought to examine whether uterine *Hegfl* expression in hypophysectomized hamster induced to implant by exogenous administration P_4 alone is similar to normal pregnancy. Luminal epithelial *Hegfl* expression surrounding the blastocyst, as observed during normal pregnancy (Fig. 2), was also noted on days 4 and 5 under this experimental condition (Fig. 3). These results suggest that implantation in P_4-treated hypophysectomized

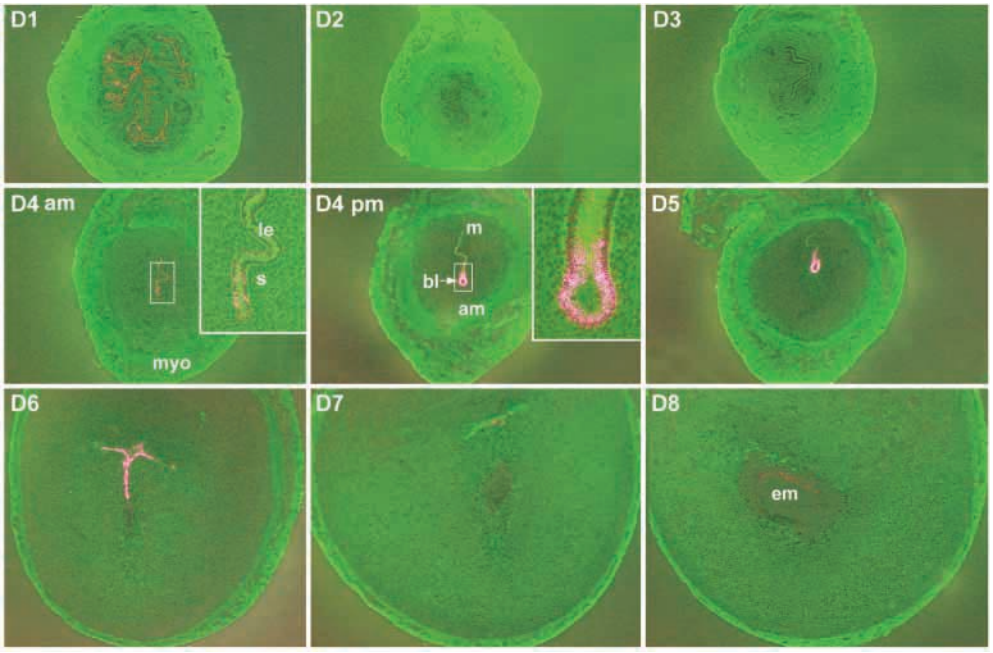

Fig. 2. In situ hybridization of *Hegfl* mRNA in the periimplantation (days 1-8) hamster uterus. Dark-field photomicrographs of representative uterine sections on days 1-8 of pregnancy are shown. Inserts show higher magnification of *Hegfl* mRNA accumulation in the presumptive implantation site (D4 am; 0900 hours) and implantation site containing a blastocyst (D4 pm; 1600 hours). am, antimesometrial side; bl, blastocyst; em, embryo; le, luminal epithelium; m, mesometrial side; myo, myometrium; s, stroma.

hamsters could becaused by *Hegfl* expression in the uterus. However, the mechanism of uterine *Hegfl* expression after hypophysectomy with P_4-treatment is not clearly understood. Two possibilities are envisioned. Uterine *Hegfl* is regulated either by P_4 as observed in rats (Zhang et al., 1994), or by estrogen still present in the circulation after hypophysectomy. The latter is not likely the cause since serum E_2 levels in hypophysectomized pregnant hamsters were near or below the detection limit. These results led us to examine the steroid hormonal regulation of uterine *Hegfl* in a more defined system.

Hegfl is regulated by E_2 in the ovariectomized hamster uteri

To examine whether *Hegfl* expression is induced by E_2, northern blot hybridization experiments were performed in the uterine samples obtained from ovariectomized hamsters treated with E_2, P_4, or P_4 plus E_2. *Hegfl* expression was very low in ovariectomized uterine RNA samples. However, a single injection of E_2 up-regulated the levels by 2 hours that reached a peak at 6 hours followed by a gradual decrease by 24 hours (Fig. 4). An injection of P_4 alone had no effect on uterine *Hegfl* expression. P_4 injected together with E_2 also upregulated *Hegfl* expression, suggesting that E_2 is the primary trigger for uterine *Hegfl* expression and P_4 fails to antagonize E_2 effects.

To examine whether E_2-induced expression of *Hegfl* was limited to the epithelial cells, in situ hybridization was performed in uterine sections of ovariectomized hamsters treated similarly with E_2 or P_4 or P_4 plus E_2. No hybridization signals were observed in uterine sections of either oil- (Fig. 5) or P_4-injected ovariectomized hamsters (data not shown). However, uterine epithelial cells showed distinct expression of *Hegfl* by 6 hours of E_2 treatment with reduced expression by 24 hours. An injection of P_4 together with E_2 did not antagonize E_2-induced epithelial *Hegfl* expression at 6 hours.

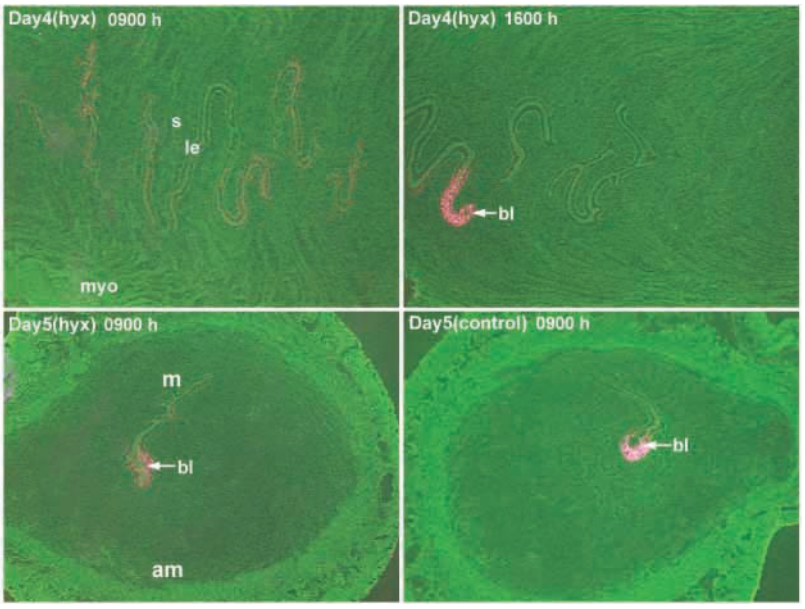

Fig. 3. In situ hybridization of *Hegfl* mRNA in hypophysectomized (hyx) progesterone (P_4)-treated pregnant (days 4 and 5) hamster uterus. Dark-field photomicrographs of representative uterine sections are shown. Pregnant hamsters were hypophysectomized on day 2 (0900 hours) and treated with P_4 (1 mg/hamster) daily from day 2. Whole uterine tissues were collected on the morning of day 4 (0900 hours). Implantation sites were collected on the afternoon of day 4 (1600 hours) and morning of day 5 (0900 hours) after an intravenous injection of blue dye as described in the Materials and Methods. Day 5 implantation sites obtained from sham operated pregnant hamsters treated with oil, were used as control. am, antimesometrial side; bl, blastocyst; le, luminal epithelium; m, mesometrial side; myo, myometrium; s, stroma.

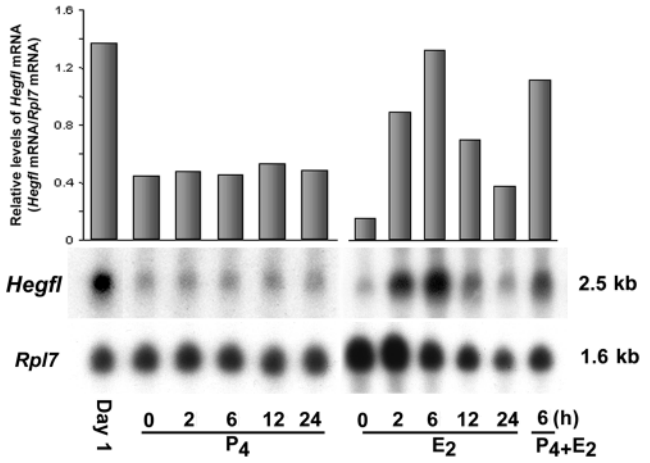

Fig. 4. Northern blot hybridization of uterine *Hegfl* mRNA in steroid-treated adult ovariectomized hamsters. The expression of *Hegfl* mRNA was detected in total RNA samples obtained from whole ovariectomized hamster uteri after various steroid treatments. Ovariectomized hamsters were given a single injection of progesterone (P_4), estradiol-17β (E_2) or P_4 plus E_2. Control ovariectomized hamsters received an injection of oil (0 hour). Uteri were collected at the indicated hour (h) after steroid treatments. As mentioned in the Fig. 1, an approx. 2.5 kb transcript for *Hegfl* was detected in the uterus. The blot was hybridized with *Rpl7* to confirm integrity, equal loading, and blotting of RNA samples. These experiments were repeated twice with similar results. Bar diagram above the autoradiogram shows the relative levels of *Hegfl* mRNA (*Hegfl* mRNA/*Rpl7* mRNA) after densitometric scanning of this autoradiogram.

There is evidence that ovariectomized hamsters require more estrogen to elicit an uterotrophic response than ovariectomized mice (Giannina et al., 1971). Thus to determine the minimum dose of E_2 required to stimulate uterine epithelial *Hegfl* expression, we treated ovariectomized hamsters with three doses of E_2 (0.01, 0.10 and 1.00 μg/hamster). A dose-dependent epithelial expression of *Hegfl* was noted. While the lowest dose of E_2 (0.01 μg/hamster) was unable to induce uterine *Hegfl* expression (Fig. 6), a dose of 0.10 or 1.00 μg stimulated epithelial *Hegfl* expression by 6 hours. The lowest dose of E_2 required to stimulate uterine *Hegfl* should not be considered high, considering the difference in body weights between mice and hamsters (25 g versus 100 g). We also examined whether E_2-stimulated uterine *Hegfl* expression is mediated via the nuclear estrogen receptor (ER). ICI 182,780 (Faslodex^{TM}) is a selective antagonist of both ERα and ERβ (Howell et al., 2000). An injection of ICI 182,780 (1 mg/hamster) alone had no effect on uterine epithelial *Hegfl* expression (data not shown), while a combined treatment of ICI 182,780 and E_2 completely inhibited the uterine *Hegfl* expression inducible by E_2. These results suggest that E_2 effects on uterine *Hegfl* expression are mediated by classical nuclear estrogen receptors.

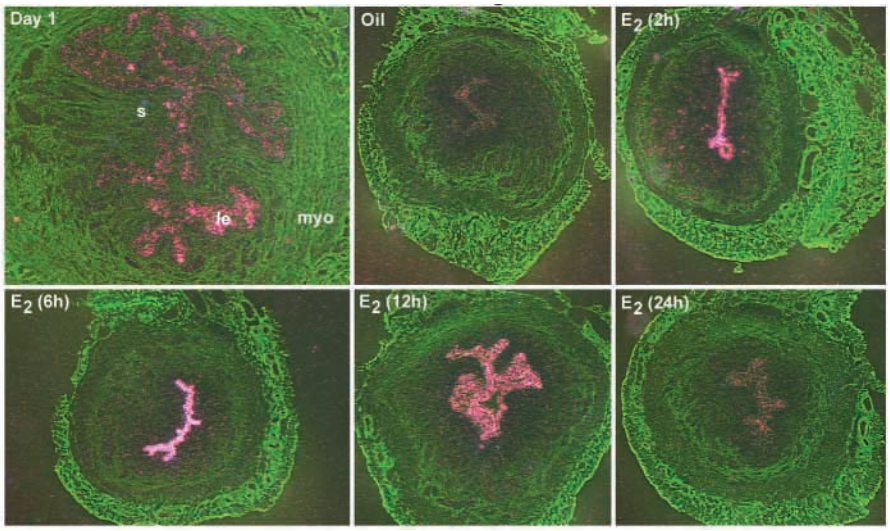

Fig. 5. *In situ* hybridization *Hegfl* mRNA in uteri of ovariectomized hamster after estradiol-17β (E_2) treatment. Photomicrographs of representative uterine sections are shown at 40× magnification. Ovariectomized hamsters were given an injection of E_2 (1.0 μg/hamster) and killed 2, 6, 12 and 24 hours after the treatment. (Day 1) Control unoperated uterus; (Oil) control ovariectomized hamsters received an injection of oil. le, luminal epithelium; myo: myometrium; s, stroma.

HB-EGF and ErbBs are expressed in preimplantation embryos and uterus.

Since HB-EGF can accelerate development, zona-hatching and trophoblast outgrowth of blastocysts in vitro in mice and humans (Das et al., 1994; Martin et al., 1998; Wang et al., 2000), the expression of HB-EGF by hamster preimplantation embryos was analyzed. RT-PCR detected the expression of *Hegfl* mRNA from the 4-cell stage onwards (Fig. 7). Immunostaining of day-4 blastocysts by confocal microscopy confirmed that HB-EGF is present in both the trophectoderm and the inner cell mass (ICM). The staining was mostly cytoplasmic, although some localization was also evident on the blastocyst cell surface (Fig. 8A). These results demonstrate that hamster preimplantation embryos express HB-EGF for potential interaction with ErbBs. RT-PCR analysis showed that both *Erbb1* and *Erbb4* mRNAs are present in two-cell embryos, but absent in one-cell, four-cell and eight-cell/morulae (Fig. 7). The presence of *Erbb1* mRNA, but not of *Erbb4*, was again noted at the blastocyst stage. While immunostaining with anti-ErbB1 and anti-ErbB4 antibodies confirmed the absence of both ErbB1 and ErbB4 in fertilized one-cell embryos (data not shown), these proteins were detected primarily in the embryonic cell surface from the two-cell stage onwards (Fig. 8B). Although there are no obvious changes in the basic immunostaining patterns using these two antibodies, at the blastocyst stage the staining for ErbB1 showed surface localization, while ErbB4 appeared to be localized both at the cell surface and in the cytoplasm. Preneutralized antibodies with an excess of the antigenic peptide failed to show any positive immunostaining in hamster blastocysts (data not shown). Cytoplasmic localization of ErbB4 has previously been reported in the mouse blastocyst (Wang et al., 2000). Since the formation of blastocysts from

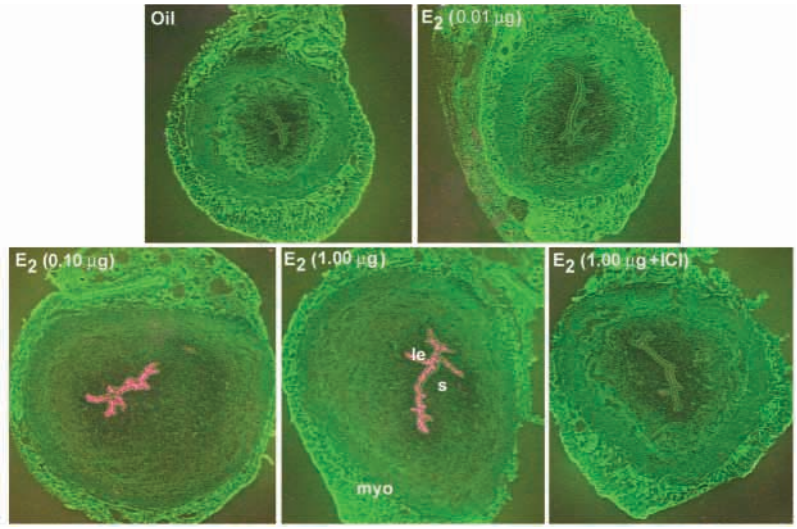

Fig. 6. In situ hybridization of *Hegfl* mRNA in uteri of ovariectomized hamster after various doses of estradiol-17β (E_2) or E_2 plus ICI 18,2780 treatments. Photomicrographs of representative uterine sections are shown. Ovariectomized hamsters were killed 6 hours after an injection of either of the three doses of E_2 (0.01, 0.1 and 1.0 μg/hamster) or E_2 (1 mg/hamster) plus ICI 18,2780 (1 mg/hamster). Control ovariectomized hamsters received an injection of oil. le, luminal epithelium; myo, myometrium; s, stroma.

two-cell embryos occurs rapidly (24-30 hours) in hamsters, the presence of ErbB1 and ErbB4 proteins in embryos after the two-cell stage in the absence of its mRNA expression is not surprising. It appears that these proteins were stable and carried over to the later stages of embryonic development. There is evidence that maternal oogenetic products, for example E-cadherin, can influence embryo development, at least through the blastocyst stage (reviewed by Watson and Barcroft, 2001). RT-PCR analysis also showed the expression of both *Erbb1* and *Erbb4* mRNAs in day-4 pregnant uteri (Fig. 7). The expression of *Erbb1* and *Erbb4* in two-cell stage embryos is correlated with the first genomic activation of embryos (Seshagiri et al., 1992), while blastocyst ErbB1 and ErbB4 could interact with blastocyst or uterine HB-EGF for implantation-related signaling. Taken together, these results show that HB-EGF signaling via ErbB1 and ErbB4 are potential signaling pathways for reciprocal interactions between the uterus and blastocyst during implantation.

HB-EGF induces phosphorylation of uterine and blastocyst ErbB1 and ErbB4

The expression of HB-EGF and ErbBs in the blastocyst and uterus at the time of implantation led us to speculate that HB-EGF should interact with ErbBs. Thus, we examined HB-EGF-induced phosphorylation of blastocyst and uterine ErbB1 and ErbB4 in vitro, employing immunoprecipitation using specific antibodies.

The results demonstrate that HB-EGF indeed induces autophosphorylation of ErbB1 and ErbB4 in the day-4 uterus (ErbB1: Fig. 9A, lane 2; ErbB4: Fig. 9B, lane 2) and blastocyst (ErbB1: Fig. 9A, lane 6; ErbB4: Fig. 9B, lane 6). Mouse day-4 uterine membranes were used as controls (ErbB1: Fig. 9A, lane 4; ErbB4: Fig. 9B, lane 4). The total protein content of a mouse blastocyst is about 20 ng (Brinster, 1973), but it is unknown for hamster blastocysts. We assume that the total protein content of hamster blastocysts would be similar to mouse blastocysts or even lower, since cell numbers in hamster blastocysts are lower than those in mice blastocysts (Seshagiri et al., 2002). Thus, efficient phosphorylation of ErbB1 and ErbB4 by HB-EGF in extracts of 100 hamster blastocysts compared with the use of 45 μg of isolated uterine membrane protein suggests that blastocyst ErbBs are remarkably active in response to HB-EGF signaling.

DISCUSSION

Initiation of implantation is the result of reciprocal signaling between a receptive uterus and an implantation-competent blastocyst. The uterine receptivity, blastocyst activation and the coordinated cross-talk between them are driven by signaling from a specific sets of gene products expressed in the blastocyst and uterus. Numerous signaling molecules have been identified that are relevant to implantation (reviewed by Carson et al., 2000; Paria et al., 2000; Paria et al., 2001a). Among them, HB-EGF, a member of the EGF family, has an expression pattern highly pertinent to the implantation process. In mice, *Hegfl* is expressed exclusively in the uterine luminal epithelium closely apposed to the blastocyst prior to and during implantation. Furthermore, HB-EGF promotes blastocyst growth and function in a number species including mice, humans and hamsters (Das et al., 1994; Martin et al., 1998; Mishra and

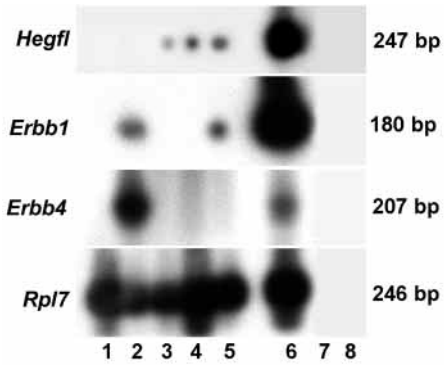

Fig. 7. Southern blot analysis of RT-PCR amplified products of *Hegfl, Erbb1 and Erbb4* in preimplantation embryos and day-4 uteri of hamsters. Lanes 1-5, one-cell, two-cell, four-cell, eight-cell/morula and blastocyst stages, respectively; lane 6, day 4 uterus; lane 7, day 4 uterine RNA without RT; lane 8, primer control. These experiments were performed twice with similar results.

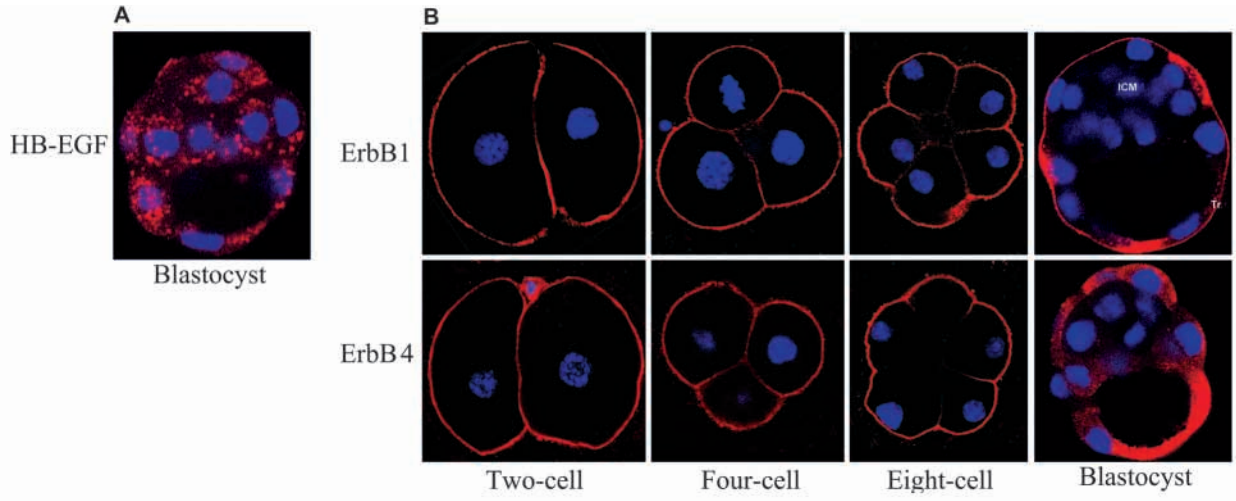

Fig. 8. Cellular localization of HB-EGF (A) and ErbB1 and ErbB4 (B) in hamster preimplantation embryos. Various stages of hamster preimplantation embryos were fixed, permeabilized and incubated with polyclonal HB-EGF, ErbB1 or ErbB4 antibodies. Localization of antigens (red) and nuclei (blue) were visualized using TRITC-conjugated secondary antibodies and Hoechst 33,342, respectively. Indirect immunofluorescence staining of HB-EGF, ErbB1 and ErbB4 was visualized by scanning laser confocal microscopy with 6 μm optical sections. HB-EGF was mainly in the cell cytoplasm of both ICM (inner cell mass) and trophectoderm (Tr) cells of the blastocyst (A). Both ErbB1 and ErbB4 were mainly in the cell surface of two-cell, four-cell and eight-cell embryos (B). At the blastocyst stage ErbB1 was present in the cell surface of Tr cells only. However, ErbB4 was present in both the surface and the cytoplasm of blastocyst Tr cells (B).

Seshagiri, 2000; Wang et al., 2000; Seshagiri et al., 2002). Here we provide a comprehensive account of the site of HB-EGF synthesis and its targets for signaling in implantation in hamsters, a species in which implantation can occur in the presence P_4 alone and unlike mice does not require estrogen. Our present observation of expression of *Hegfl* in the luminal epithelium surrounding the blastocyst prior to and during implantation in the presence of P_4 alone suggests that although maternal estrogen is not a prerequisite for implantation, HB-EGF is important for this process in hamsters. This finding appears paradoxical since estrogen is critical for the induction of uterine *Hegfl* in ovariectomized hamsters. Two intriguing possibilities are raised. First HB-EGF produced by the hamster blastocyst serves as an autoinductive stimulus for the expression of uterine HB-EGF locally during implantation. Indeed, there is evidence in other systems that EGF-like growth factors can induce their own genes (Barnard et al., 1994). Second, embryonic estrogen could function locally to influence uterine gene expression during implantation, although maternal estrogen is not an absolute requirement for implantation in hamsters. We have preliminary evidence that hamster preimplantation embryos express the gene encoding aromatase (unpublished observation). The local induction of *Hegfl* in the luminal epithelium of the mouse uterus by activated, but not dormant, blastocysts lends support to the speculation that an embryonic signal is critical to the induction of this gene at the time of implantation (Das et al., 1994). The identification of the signal remains a seminal question.

The expression of *Hegfl* and its cognate receptors ErbB1 and ErbB4 in both the luminal epithelium and blastocyst suggests a dual site of action of this growth factor. The autophosphorylation of these uterine and blastocyst receptors by HB-EGF provides evidence that HB-EGF signaling via these receptors is operative during implantation. It should be noted that the signaling of the EGF family growth factors requires heterodimerization among ErbB isoforms (reviewed by Lim et al., 1998). Thus, it is possible that in addition to ErbB1 and ErbB4, other isoforms also participate in signaling by HB-EGF. However, the greater magnitude of phosphorylation of blastocyst receptors advocates a

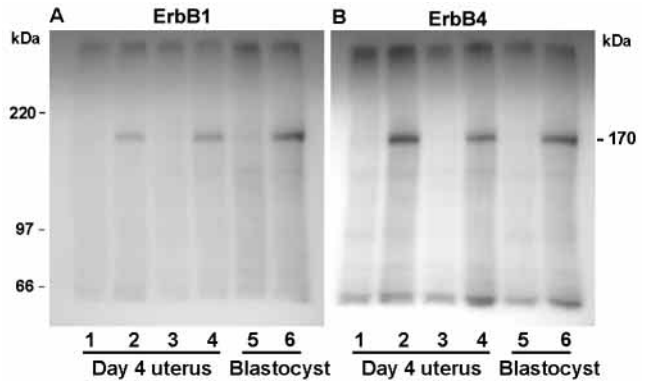

Fig. 9. HB-EGF-induced autophosphorylation of ErbB1 (A) and ErbB4 (B) in day 4 uteri and blastocysts of hamsters. Autophosphorylation of ErbBs was determined in uterine membrane and blastocyst homogenate after preincubation (10 minutes) with or without HB-EGF (4 ng/ml, human recombinant HB-EGF; a gift from Dr M. Klagsbrun, Harvard medical School, Boston, MA). Day 4 mouse uterine membranes were used as controls. The labeling reaction was initiated by the addition of [γ-^{32}P]ATP for 2 minutes. After labeling, proteins were precipitated with 10% trichloroacetic acid. The precipitates were then used for immunoprecipatation with antibodies to ErbB1 and ErbB4. The immunoprecipates were separated by SDS-PAGE (6%), and phosphorylated proteins were detected by autoradiography. No growth factor was added to the samples in lanes 1, 3 and 5. HB-EGF was added to those in lanes 2, 4 and 6. Lanes 1 and 2 show hamster uterine membranes; lanes 3 and 4 show day 4 mouse uterine membranes; lanes 5 and 6 show hamster blastocyst homogenates. These experiments were performed twice.

preferential role of HB-EGF toward embryonic functions. The potentiation of embryonic growth and functions by HB-EGF is also evident in other species including mice, hamsters and humans (Das et al., 1994; Martin et al., 1998; Mishra and Seshagiri, 2000; Wang et al., 2000; Seshagiri et al., 2002). However, the source of HB-EGF that influences embryonic function is not clearly understood. It is possible that HB-EGF produced by the blastocyst influences its own functions in an autocrine or juxtacrine manner, since ErbB1 and ErbB4 are displayed on blastocyst cell surface. Alternatively, uterine HB-EGF influences blastocyst functions in a paracrine manner or directs blastocyst homing into the uterus by a juxtacrine manner. The role of soluble and transmembrane HB-EGF in these processes has been documented in mice (Raab et al., 1996; Paria et al., 1999). HB-EGF may also be involved in uterine functions that are important for embryo-uterine signaling during implantation. Our findings of dual sites of HB-EGF synthesis and action show diversification of specific gene functions in a physiologically relevant system. We have recently shown that transfer of blastocyst-size affigel blue beads pre-soaked in HB-EGF into uterine lumens of pseudopregnant mice elicit implantation-like responses with appropriate gene expression in the uterus similar to those observed in the presence of living embryos (Paria et al., 2001b). This observation shows that HB-EGF is locally active to influence uterine and embryonic functions relevant to implantation. In this respect, deletion of the *Hegfl* gene is warranted to establish the importance of this molecule in embryo development and implantation.

In conclusion, the expression of HB-EGF in the uterus and embryos of various species with different hormonal requirements for implantation underscores the importance of HB-EGF in this process. Particularly, the hamster serves as an intriguing model to study gene regulation and functions during implantation because of its exclusive dependence on P_4 during implantation.

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