

Growth factor expression by human oviduct and buffalo rat liver coculture cells*†

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Objective: To characterize growth factor gene expression by passaged coculture cell lines demonstrated to enhance in vitro pre-embryo growth.

Design: Ribonucleic acids isolated from the isthmus, ampullary, and fimbriae portions of the human oviduct, and from buffalo rat liver cell monolayers were subjected to Northern analysis using probes for growth factors.

Setting: Academic tertiary care hospital.

Patient(s): Two reproductive age women undergoing a hysterectomy and bilateral salpingectomy for benign gynecologic conditions consented to experimental use of their oviducts.

Intervention(s): Cell cultures were established from fresh human oviduct segments and commercially purchased buffalo rat liver cells. After two passages, total RNA was isolated from these confluent monolayers, fractionated on denaturing agarose gels, transferred to nylon membranes, and analyzed by Northern hybridization using complementary DNAs from epidermal growth factor (EGF), stem cell factor, also known as Kit-ligand, colony-stimulating factor-1 (CSF), leukemia inhibitory factor, and interleukin-6 (IL-6). Radioactively labeled probes were prepared by in vitro transcription or by 5' end labeling. After hybridization, blots were washed at increasing stringencies to remove nonspecifically bound radioactivity and subjected to autoradiography.

Result(s): Human oviduct coculture cells express EGF (kit-ligand), CSF, leukemia inhibitory factor, and IL-6. Buffalo rat liver cells contain the messenger RNA transcripts for kit-ligand and CSF.

Conclusion(s): Human oviduct and buffalo rat liver coculture cells express specific growth factors. These results support the theory that coculture systems may enhance pre-embryo growth via the production of embryotrophic factors. The identification of these ligands may provide the rationale for selecting specific growth factors for media supplementation as well as contribute to our understanding of the general mechanisms involved in regulating early embryonic growth and development. (Fertil Steril® 1997;67:775-9. © 1997 by American Society for Reproductive Medicine.)

Key Words: In vitro fertilization, coculture, growth factors

The demonstration more than 25 years ago that mouse embryos are capable of autonomous preimplantation development from the zygote to the blas-

tocyst stage in chemically defined media in vitro (1) provided embryologists with the means for experimentally manipulating embryos to investigate mechanisms of early mammalian development. Remarkable progress has been made since toward understanding mammalian embryogenesis and the ap-

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plication of this information for the treatment of human infertility. Unfortunately, despite numerous technological advances, only 16.8% of babies are born per retrieval in human IVF-ET (2). One factor that may contribute to such poor success rates are the in vitro culture conditions currently in use, which sustain only approximately 20% to 50% of pre-embryos to the blastocyst stage (3–6). In many IVF-ET programs, efforts to minimize in vitro fragmentation and degeneration and to maximize the opportunity for embryo replacement include premature transfer of zygote to eight-cell stage embryos into the uterus. Many mammalian embryos, including human, also display specific blocks to development in vitro that are related to activation of the embryonic genome, transport of the embryo from oviduct to uterus, and asynchronous in vitro development with respect to uterine receptivity (7). These developmental delays can be alleviated partially by culturing embryos in the presence of somatic cells or with conditioned and/or growth factor-supplemented media (8). In fact, growth rates and morphology have been improved significantly for pre-embryos maintained in coculture systems (8). Presumably, one mechanism for the enhancement of embryonic development in coculture systems is via the expression of growth factors. Indeed, recent reports on the expression of several ligands and receptors by preimplantation embryos and reproductive tract epithelium indicate that the embryo is capable of responding to both autocrine and paracrine factors (9–14). The primary aim of this research was to characterize gene expression at the transcriptional level of specific growth factors by human oviduct and buffalo rat liver coculture cell lines.

MATERIALS AND METHODS

Cell Lines

Human oviduct tissue was obtained from two healthy premenopausal women who underwent hysterectomy for benign conditions and tested negative for human immunodeficiency virus and hepatitis. The patients were in the proliferative phase of their menstrual cycle based on history. The research protocol for use of human tissue was approved by the hospital's institutional review board and written consent was received from both patients. After surgery, the specimens were transported to the lab in a centrifuge tube with $1\times$ Dulbecco's phosphate-buffered saline (GIBCO, Grand Island, NY) solution on ice. Fimbriae, ampullary, and isthmic portions of the oviduct were isolated by dissecting the appropriate segments based on gross morphology. Epithelial cells were dislodged chemically by incubation of ovi-

duct segments with 0.05% trypsin-ethylenediamine-tetraacetic acid (GIBCO). After a 40-minute incubation period, the segments were flushed with M199-Hank's media (GIBCO) supplemented with 5% penicillin-streptomycin (GIBCO). Dispersed cells were harvested, centrifuged, washed, and plated with M199-Earle's media (GIBCO) supplemented with 1% penicillin-streptomycin and 10% heat-inactivated fetal calf serum (GIBCO) into 25 cm² tissue flasks. The cells were incubated in an atmosphere of 5% CO₂ in air at 37°C. Primary monolayers were established in 6 to 7 days and subcultured twice before analysis. The growth of epitheloid cells versus the formation of fibroblast-like monolayers was confirmed by gross morphology. Buffalo rat liver cells were obtained commercially from American Type Culture Collection (Rockville, MD).

Molecular Techniques

RNA Isolation and Northern Blotting

Total RNA was isolated from cell lysates, fractionated on denaturing agarose gels, transferred to nylon membranes, and analyzed by Northern blot hybridization according to standard protocols (15). Briefly, monolayers were trypsinized and the cells were harvested by centrifugation. Cell lysates were subjected to phenol-chloroform extraction followed by ethanol precipitation. RNA recovery was assessed spectrophotometrically. The RNA samples were fractionated on 1.2% agarose denaturing gels, transferred to nylon filters, and immobilized by ultraviolet crosslinking.

Probe Preparation and Hybridization

The complementary DNAs (cDNAs) used for radioactive probe preparation included: epidermal growth factor (EGF), a 1.9-kb human cDNA; stem cell factor, also known as kit-ligand, a 1-kb mouse cDNA; colony-stimulating factor-1 (CSF), a 0.15-kb mouse cDNA purchased from ATCC; interleukin-6 (IL-6), a 30-base rat oligomer; and human leukemia inhibitory factor, an equimolar mixture of five 28 to 29 base pair human leukemia inhibitory factor oligomers purchased from R and D Systems (Minneapolis, MN).

The cDNA sequences in plasmid vectors were propagated and amplified in *Escherichia coli* bacteria. Riboprobes labeled with α -phosphorus-cytidine 5' triphosphate were generated by in vitro transcription of appropriately linearized plasmids using RNA polymerases. Oligomers for leukemia inhibitory factor and IL-6 were 5' end labeled by a polynucleotide kinase in the presence of γ -phosphorus-adenosine 5' triphosphate. Radiolabeled probes were hybridized

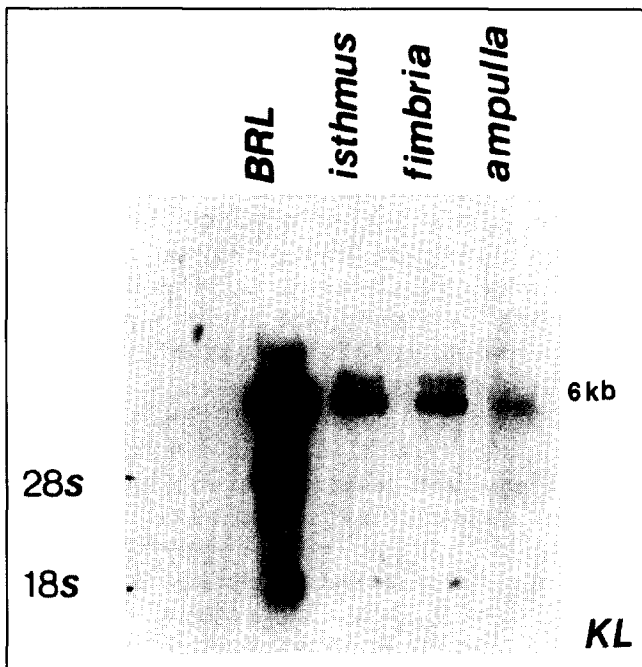


Figure 1 Northern blot analysis of kit-ligand transcripts in buffalo rat liver, human isthmus, ampulla, and fimbria coculture cells. Each lane contains 2 μ g of total RNA. Positions of 28S and 18S ribosomal RNA are indicated.

to Northern blots according to established protocols (15). Blots were subjected to autoradiography after posthybridization washes at increasing stringencies to remove nonspecifically bound radioactivity.

RESULTS

Northern blot hybridization to transcripts complementary to the human EGF sequence demonstrate the presence of a band at approximately 5 kb in RNA from human isthmus and fimbria portions of the oviduct, confirming the expression of this growth factor by the oviduct cell lines (not shown). Because a human EGF probe was used, we cannot say with certainty whether the two hybridizing species in the rat cell line represent authentic EGF or unrelated messages with sequence homology to human EGF. The ligand for the c-kit receptor (at approximately 5.5 kb) is expressed by each of the oviduct cell lines, as well as buffalo rat liver cells (Fig. 1). Hybridization of CSF to Northern blots containing RNA species from buffalo rat liver cells and human fimbriae demonstrate the presence of bands of appropriate molecular weight, suggesting that this factor is expressed by both cell lines (Fig. 2). The differences in band intensities may be attributed to a higher degree of expression of CSF by oviduct versus rat liver cells or may be due to differences in sequence homology as a mouse-derived probe was used. Interleukin-6 (at approximately 1.3 kb) and leukemia inhibitory

factor (at approximately 3 kb) appear to be expressed constitutively in all cell lines derived from the human oviduct (not shown). The second, higher molecular weight species in fimbriae cells hybridized to leukemia inhibitory factor may represent a transcript selectively expressed by fimbriae.

DISCUSSION

This study represents the first analysis at the transcriptional level of growth factor gene expres-

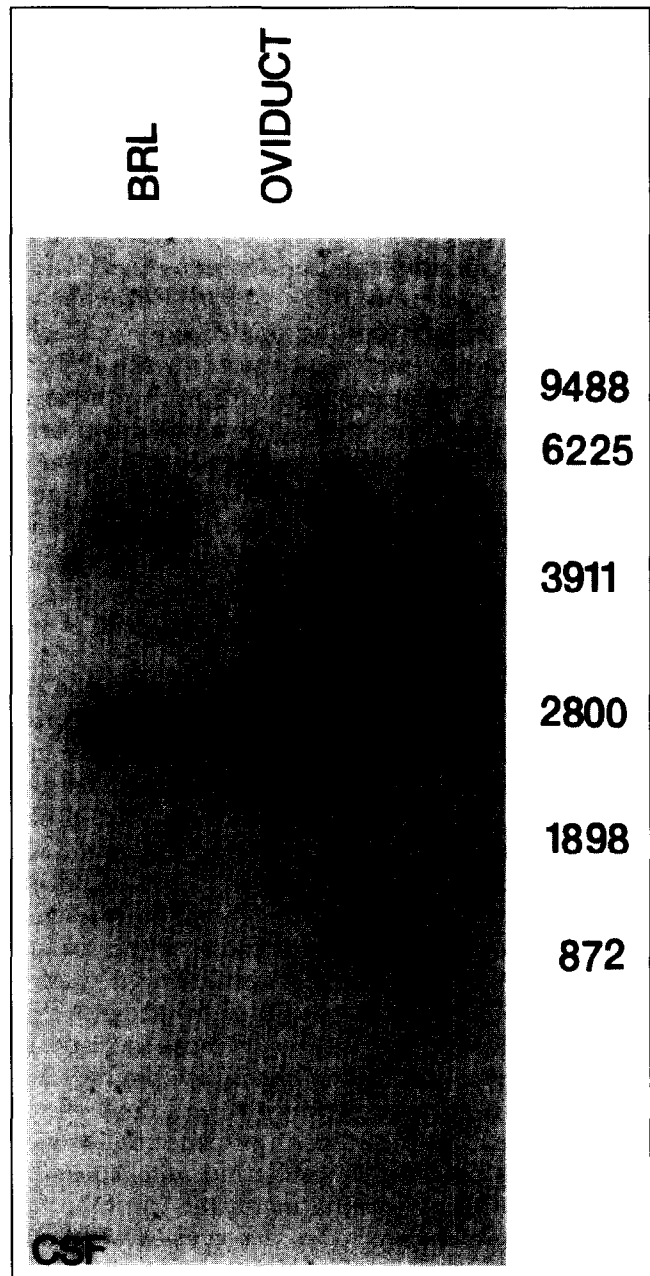


Figure 2 Northern blot analysis of CSF transcripts in buffalo rat liver and human fimbria coculture cells. Each lane contains 2 μ g of total RNA. The numbers to the right represent λ HIND-III markers.

sion by cells with demonstrated ability to influence positively *in vitro* pre-embryo development and possibly the outcome of human IVF-ET (16) (Colonna Worrirow K, Goldsmith GE, Hu YX, Zacher M, Munabi AK, abstract). We have identified transcripts encoding EGF, kit-ligand, CSF, leukemia inhibitory factor, and IL-6 in RNA derived from human oviduct cell lines. Furthermore, kit-ligand and CSF also appear to be expressed by buffalo rat liver cells. These data demonstrate that somatic cells in pre-embryo coculture systems express growth promoting factors.

The biologic importance of autocrine-paracrine growth factors in regulating embryonic development has been supported by recent studies in both mouse and humans in which improved developmental rates and pregnancy rates were noted when pre-embryos were cultured in groups (9, 17, 18). Apparently, autocrine growth factors are responsible for the cooperative interactions among embryos developing *in vitro* because the addition of growth factors known to be produced by preimplantation embryos (i.e., transforming growth factors [TGF- α and β] [11]) enhances growth of inferiorly growing single embryo cultures (9). The autocrine factors produced by the embryo may not be of biologic significance *in vivo* because singleton pregnancies are the norm in humans and, in lower mammalian species, embryo distribution in the reproductive tract may not allow for these interactions. However, autocrine regulation of embryonic development *in vitro* probably plays a significant role and may compensate partially for the lack of paracrine factors normally secreted by cells of the reproductive tract *in vivo*.

Several growth factors have been shown to be expressed by secretory epithelial cells lining the uterine lumen and by cells of lymphohematopoietic origin localized to uterine tissues after the onset of pregnancy (10). These include EGF, insulin-like growth factors (IGF-I and IGF-II), CSF, stem cell factor, granulocyte-macrophage colony stimulating factor (GM-CSF), and leukemia inhibitory factor. The hypothesis that autocrine and/or paracrine factors may be relevant to estrogen-induced uterine proliferative response is supported by evidence that levels of EGF and its receptor increase in response to estrogen and that EGF enhances cell proliferation in uterine cell cultures. Similarly, IGF-I expression by the uterus also increases in response to estrogen and may be involved in mediating mitogenesis in the uterus. Colony stimulating factor-1 mRNA and protein have been detected in the mouse oviduct and in luminal and glandular epithelium of the pregnant uterus and appear to be regulated by E₂-17 β and P. Stem cell factor, GM-CSF, TGF- β , and leukemia inhibitory factor also have been shown to be expressed by the mouse oviduct and/or uterus from

the onset of pregnancy. Thus, the epithelium of the oviduct and uterus as well as lymphocytes and macrophages that are localized to the reproductive tract from the onset of pregnancy appear to be the primary sources of paracrine growth factors, which have the potential to influence early embryonic development. The effects of growth factors present in the reproductive tract during the preimplantation period are likely to complement those elicited by growth factors produced by the embryo, and the full repertoire of autocrine and paracrine factors is probably essential for embryonic differentiation.

There are both randomized (5, 19, 20) and nonrandomized studies (16, 21–23) that demonstrate the beneficial effects of helper cell lines on *in vitro* pre-embryo development as well as clinical outcome. Furthermore, a recent study suggests that cocultured pre-embryos are more likely than their noncocultured counterparts to implant after cryopreservation (24). However, other investigators have shown that, although pre-embryo growth is improved on helper cell lines, there are no significant effects of coculture on pregnancy rates (4, 25). Therefore, the role of feeder cell lines in human IVF-ET remains controversial. These studies illustrate the potential benefit of pre-embryo exposure to helper cell lines on *in vitro* development and possibly pregnancy rates.

Because the natural site of gamete transport, fertilization, and preimplantation development is the oviduct, it is likely that cells of the oviduct produce growth factors that promote these activities. Only recently have EGF, TGF- α , and their common receptor (EGF-TGF- α -R) been identified in tissue specimens of human oviducts (12, 13). The expression of these regulatory molecules depends on anatomical region and reproductive stage: transcripts are more abundant in ampullary than isthmic regions and less abundant in the secretory than proliferative stages. The specificity of the effects of these growth factors with regard to preimplantation embryonic development has been verified by the observation that enhancement of embryonic growth on oviductal monolayers was diminished by the addition of monoclonal antibodies to EGF and TGF- α (13). This was the first demonstration that embryotrophic factors of oviduct cell origin do support growth of the preimplantation embryo *in vivo* and *in vitro*.

The results of this study demonstrate that human oviduct and buffalo rat liver coculture cells express growth factors. Our data suggest that coculture cells may mimic more closely the *in vivo* environment than conventional media because of the secretion of regulatory substances. These results will be important from the clinical perspective in providing the rationale for selecting specific growth factors for media supplementation with the eventual goal of re-

placing labor intensive coculture systems. Furthermore, the identification of embryotrophic factors produced by oviduct and/or buffalo rat liver cells may contribute to our understanding of the general mechanisms involved in controlling human embryonic growth and differentiation.

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