

**EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR
IN PREIMPLANTATION MOUSE EMBRYOS**

by

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I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

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Expression of Vascular Endothelial Growth Factor in PreImplantation Mouse Embryos

Abstract

Establishment of an adequate endometrial vascular supply is crucial for successful implantation and development of mammalian embryos. Uterine endometrial receptivity determines the success of implantation, as human embryos and gametes are routinely harvested, maintained and fertilized in vitro in assisted reproduction. Once a viable embryo is transferred to a uterus, however, its chance of establishing a pregnancy in the recipient female is only approximately 20%. Embryos placed in the distal portion of the fallopian tube typically show a higher rate of pregnancy success, thus suggesting that embryo-derived factors may contribute to uterine receptivity.

Angiogenesis, the extensive development and growth of capillaries, is essential to the establishment of the vascular network needed for maternal-fetal exchange through the placenta. Prior to implantation, the endometrial vascular network is random and contains no specific orientation. Factors released at the site of implantation cause the vessels to orient towards the developing embryo and provide it with an ample blood supply. The factor(s) that directs blood vessel formation during implantation is not known.

Vascular endothelial growth factor (VEGF) is specific to vascular endothelial cells and has significant angiogenic and permeability effects. There are four main variants of VEGF that are due to alternative exon splicing of a single mRNA strand. Two variants are cell-associated (206 and 189), while two are soluble (165 and 121). There are no known functional differences between the variants. Receptors for VEGF are *fms*-like tyrosine kinase (flt) and kinase domain-containing receptor (KDR). They are predominantly expressed on endothelial cells, although the embryo may also contain receptors on its trophoblast cells.

The purpose of this study was to determine if VEGF is expressed in the early preimplantation mouse embryo. The uterus is known to make VEGF but it was not known if the preimplantation embryo itself made VEGF or if it contained VEGF receptors. Mouse eggs and embryos were used because their embryonic implantation is similar to human implantation. The availability of samples and ethics concerns also made mouse specimens the viable choice.

RNA was extracted from the eggs/embryos then made into a complementary strand of DNA (cDNA) by the process of reverse transcriptase. An oligo (dT) primer was used to differentiate mRNA being actively translated, which contained poly A⁺ tails, from other mRNA in the sample. Once the cDNA was obtained, it was amplified by polymerase chain reaction (PCR). Mouse kidney cDNA was used for the positive control tissue as it was known to contain both VEGF and actin and was the same tissue type as the samples. Actin was used as the control gene within the sample because it is highly expressed in all cells. The primers used were expected to produce a 548 base pair fragment for actin, and a 349 base pair fragment for VEGF that is common to all VEGF variants. To test for contamination, a negative control containing only the stock reagents and ddH₂O was also prepared. The PCR products were loaded into non-denaturing polyacrylamide gels for electrophoresis analysis.

Both mouse eggs and blastocysts appear to express VEGF. It is difficult to envision a role for VEGF in the egg stage, but it may be involved in early folliculogenesis. VEGF, if secreted by the embryo, may flow down the fallopian tube and prime the uterus for implantation. When the blastocyst begins to implant, VEGF may promote blood vessel redirection and growth at the implantation site. At the blastocyst stage, VEGF may act as both a permeability and an angiogenic factor. A third function for VEGF may also exist at this stage. The blastocyst produces VEGF and the trophoblast cells are believed to contain VEGF receptors, therefore the blastocyst may induce its own growth.

Introduction

Establishment of an adequate endometrial vascular supply is crucial for successful implantation and development of mammalian embryos. Uterine endometrial receptivity determines the success of implantation, as human embryos and gametes are routinely harvested, maintained and fertilized in vitro in assisted reproduction. Once a viable embryo is transferred to a uterus, however, its chance of establishing a pregnancy in the recipient female is only approximately 20%. Embryos placed in the distal portion of the fallopian tube typically show a higher rate of pregnancy success, thus suggesting that embryo-derived factors may contribute to uterine receptivity.

Angiogenesis, the extensive development and growth of capillaries, is essential to the establishment of the vascular network needed for maternal-fetal exchange through the placenta. It differs from vasculogenesis in that angiogenic growth occurs from pre-existing cells and vessels rather than creating totally new ones. Prior to implantation, the endometrial vascular network is random and contains no specific orientation. Factors released at the site of implantation cause the vessels to orient towards the developing embryo and provide it with an ample blood supply (Torry R and Rongish B, 1992). The factor(s) that directs blood vessel formation during implantation is not known.

Vascular endothelial growth factor (VEGF) is specific to vascular endothelial cells and has significant angiogenic and mitogenic effects (Ferrara N *et al.*, 1992). It has also been shown to increase microvascular permeability and was originally named vascular permeability factor (Brown L *et al.*, 1992). Preventing tumor angiogenesis has been the main focus of much recent research involving VEGF, as tumors cannot survive if their blood supply is ceased. The role of

VEGF in reproduction is now being explored and its full involvement in that field is not yet known.

There are four isoforms of VEGF that originate from alternative exon splicing of a single mRNA strand (Ferrara *et al.*, 1992). There are thought to be no functional differences between the four variants, but they do have significant biological differences. VEGF₂₀₆ and VEGF₁₈₉ are cell-associated, meaning they only affect cells adjacent to cells that produce these variants. The other two isoforms, VEGF₁₆₅ and VEGF₁₂₁, are diffusible into the extracellular matrix. Thus, the variant determines what cells VEGF acts on in relation to the cells that express it. If secreted by the embryo, VEGF could contribute to local permeability and angiogenesis and/or prime the uterus for implantation, depending on the variant(s) expressed.

Receptors for VEGF are the *fms*-like tyrosine kinase (flt) and kinase domain-containing receptor (KDR). They are predominantly expressed on endothelial cells, although the embryo may also contain receptors on its trophoblast cells (Charnock-Jones D *et al.*, 1994, Torry D *et al.*, unpublished). Since VEGF is known to have mitogenic effects, if embryos secrete VEGF and also contain receptors for it, they may use VEGF as a means to promote their own growth.

The purpose of this study was to determine if VEGF is expressed in the early preimplantation mouse embryo. The uterus is known to make VEGF (Torry D *et al.*, 1995) but it was not known if the preimplantation embryo itself makes VEGF or if it contains VEGF receptors. Mouse eggs and embryos were used because their embryonic implantation is similar to human implantation. The ready availability of samples and ethical concerns also made mouse specimens the viable choice.

Materials and Methods

Mouse Kidney Samples

A whole mouse kidney was surgically removed from a sacrificed mouse and snap-frozen in liquid nitrogen immediately upon removal. The kidney was stored at -80°C until used. GITC solution was added and the kidney was ground with a tissue homogenizer. RNA was extracted via the GITC method (see below).

MCF-7 Breast Cancer Cells

A T₂₅ flask was grown to confluency and RNA was extracted via the GITC method (see below).

Mouse Embryo Samples

The embryos used in these studies were collected at different stages of preimplantation development (Hogan, *et al.*, 1986). Egg samples were collected by first priming the mice with 5IU pregnant mare serum gonadotropin (PMSG). After 48 hours they were given human chorionic gonadotropin (hCG) to induce superovulation. The oviducts were flushed with Hams F10 culture media containing 1% Human Serum Albumin (HSA) 12 hours later to collect the free eggs. Cumulus cells were removed by hyuronidase (1mg/10mL). Typically a final volume of 2 microliters (μL) of media contained 10 eggs.

More advanced stages of embryonic development were collected after in vitro culturing of fertilized eggs. The mice were induced to superovulate with 5IU of PMSG followed 48 hours later with 5IU hCG. They were then placed in cages with proven fertile mates. The females

were sacrificed at day 1.5 post coitus (d1.5 p.c.) and their oviducts were flushed to collect the embryos at the 2 cell stage. A portion of the embryos were cultured under standard conditions and samples were collected at the zona pellucida-free late blastocyst stage (d4.5 p.c.). The late blastocyst stage corresponds to the time of implantation in vivo in mice. The GITC method was predominantly used to extract RNA (see below), however the crude lysate method (see below) was used when GITC failed to obtain enough RNA for amplification.

Extraction of Ribonucleic Acid (RNA) by the Guanidinium Isothiocyanate (GITC) Method

To extract RNA from the samples, the cells were first lysed (Chomczynski and Sacchi, 1987). A lysing stock solution was made of 4M GITC (83.3g), 25 mM Na Citrate (pH=7, 5.87mL of .75M), 0.5% N-lauroylsarcosine (8.8mL of 10%), and 97.67 mL diethylpyrocarbonate treated water (DEPC H₂O). This stock solution was combined with 2- β -mercaptoethanol (2-ME) in the following ratio to create a working solution: 14 μ L 2-ME to 2mL of stock solution. 500 μ L of the working solution was added to the cell sample and the cells were lysed on ice for 15 minutes.

After denaturization occurred, RNA was extracted from the eggs/embryos. The following reagents were added in order on ice for 500 μ L of denaturing solution: 50 μ L 2M Na-acetate (pH=4.0), 500 μ L phenol (H₂O saturated, not buffered), and 100 μ L chloroform/isoamylalcohol (24:1). The solution was mixed and kept on ice for 15 minutes, then centrifuged at 10,000 x g for 20 minutes. The aqueous phase of the solution was removed and 1mL of isopropanol was added. The RNA was placed at -20°C to -80°C and allowed to precipitate overnight. The precipitated solution was centrifuged at 10,000 x g for 20 minutes and the supernatants were removed. The pellet was resuspended in 300 μ L GITC/2-ME (working solution) and 300 μ L

isopropanol was added. The solution was allowed to re-precipitate at -20°C to 80°C for at least 30 minutes. The precipitated solution was centrifuged at 10,000 x g for 15 minutes and the supernatants were removed. The pellet was washed with 500µL 75% ethanol/DEPC H₂O solution and centrifuged. The supernatants were again removed. The pellet was dried under vacuum and then resuspended in DEPC H₂O and 10U of RNasin. The optical density at 260 nanometers was taken to determine the RNA concentration of the solution according to the formula: absorbance of 1.0 = 30µg/mL RNA.

Preparation of RNA Using the Crude Lysate Method

In this method, all reactions took place in the crude lysates to avoid loss of RNA during the purification process (Pal *et al.*, 1994). Snap-frozen eggs/embryos were lysed by adding an equal volume of 0.5% Nonidet-P 40 (NP-40), 10mM dithiothreitol (DTT), and 10U Rnasin. The solution was reverse transcribed (see below) and run through a spin column to concentrate the RNA and DNA. It was then dried under a vacuum, resuspended in 20µL double distilled water (ddH₂O), and heated at 50°C to 60°C for 10 minutes.

Synthesis of Complementary Deoxyribonucleic Acid (cDNA) by Reverse Transcriptase (RT) Reaction

10µg of RNA (usually in <5µL) were mixed with 10 pmol of oligo (dT)₁₈ in a 0.5µL RNA-safe Eppendorf tube. The primer oligo (dT)₁₈ was used to differentiate messenger RNA (mRNA) being actively translated, which contain poly A⁺ tails, from non-translated mRNA. The mixture was placed at 70°C for 2 minutes in a water bath and then slow cooled to 35°C. A reverse transcriptase reagent mix consisting of 20U avian myeloblastosis virus reverse

transcriptase (AMV-RT), 4 μ L 5x AMV-RT buffer, 1 μ L of each 10mM deoxyribonucleotide triphosphate (A,C,G,T), 0.2 μ L 1M DTT, and 14U RNasin, was added to the RNA and oligo(dT)₁₈. DEPC H₂O was added to the mixture to make the final volume of the RT reaction equal 20 μ L. The entire mixture was incubated at 42°C for two hours and then was placed for 2 minutes at 95°C to inactivate the RT. Afterwards it was chilled on ice for 1 minute and centrifuged at maximum for a few seconds. The newly prepared cDNA was kept frozen and stored at -20°C.

Amplification of Genes Present in cDNA Sample by Polymerase Chain Reaction (PCR)

Under an isolation hood, a working stock solution of PCR reagents was prepared proportionally to the number of samples tested. For each sample tube, 5 μ L 10x PCR Buffer 1, 1 μ L of each 10mM deoxyribonucleotide triphosphate (A,C,G,T), 1 μ L sense primer (100 pmol), 1 μ L anti-sense primer (100 pmol), and 2.5U AmpliTaq® DNA Polymerase were added. The primers for each gene were:

Actin sense 5'-CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC-3'

Actin anti-sense 5'-GTG-GGG-CGC-CCC-AGG-CAC-CA-3'

VEGF sense 5'-ATG-AAC-TTT-CTG-CTG-TCT-TGG-T-3'

VEGF anti-sense 5'-CTC-ATC-TCT-CCT-ATG-TGC-3'

These primers were expected to produce a 548 base pair fragment for actin and a 349 base pair fragment for VEGF that is common to all VEGF isoforms.

Aliquots of 11.5 μ L of the stock solution were put into each PCR tube. 5 μ L of cDNA and 33.5 μ L ddH₂O were added to the solution to make the final volume equal 50 μ L. In some cases the amount of cDNA assayed was increased to raise sensitivity. Mouse kidney cDNA was used

for the positive control as it is known to contain both VEGF and actin (Brown *et al.*, 1992). To test for contamination, a negative control containing only the stock solution and 38.5 μ L ddH₂O was also prepared. 55 μ L of mineral oil was added to each sample tube and one drop of mineral oil was placed into the wells of a Perkin-Elmer Model PCR thermal cycler. The tubes were seated into the wells and the machine was set for 35 cycles for 1 minute each at 94°C, 61°C, and 72°C. At the end of the cycles, the samples stayed at 72°C for 5 minutes and then went to a 4°C hold until their removal. They were stored at -20°C until analyzed by gel electrophoresis. In some cases heightened sensitivity was needed so a "nested PCR" was performed. Instead of cDNA, 1 μ L of PCR product was added to 11.5 μ L of stock solution and 37.5 μ L ddH₂O. The tube was then placed in the thermal cycler and the machine was set for 25 cycles of the same temperatures and durations as above.

Analyses of PCR Products by Non-Denaturing Polyacrylamide Gel Electrophoresis

A 5% non-denaturing acrylamide gel solution was prepared using 2.0 mL 40% Acryl-Bisacryl, 1.6 mL 10x Tris (Hydroxymethyl) Aminomethane-Borate-Ethylenediaminetetraacetic acid buffer (TBE), 12.4 mL ddH₂O, 20 μ L Tetramethyl-ethylenediamine (TEMED), and 100 μ L 10% NH₄SO₂. The solution was gently mixed so as not to introduce air bubbles and was poured between two glass plates enclosed in a gel caster. A comb was placed at the top of the plates to form wells for the samples. When solidified, the gel and the glass plates were removed from the caster and clamped onto an electrophoresis unit. 1x TBE buffer was added to the top chamber until it spilled over into the sample wells (~ 150 mL). 1x TBE buffer was also added to the bottom tank chamber until it was approximately half full (~ 250 mL).

5 μ L of DNA sample loading buffer was added to 20 μ L aliquots of the PCR products and

to a 0.7 μL aliquot of 123 base pair ladder molecular weight marker. The samples were loaded into the sample wells using a long-nosed pipette tip.

The electrodes were attached to the electrophoresis unit and 100 volts of electricity was applied until the bromophenol blue in the loading buffer ran off the bottom of the gel. The glass plates containing the gel were unclamped from the unit and carefully pried apart. The gel and the remaining glass plate were placed in a gel staining tray containing ddH_2O . The water released the gel from the plate and the plate was removed. 3 μL ethidium bromide (EtBr) was added to the water. The gel was allowed to soak in the EtBr solution for 10 minutes with slow agitation to stain the DNA bands on the gel for interpretation under UV light. For band clarity, the gel was destained by draining the EtBr solution from the tray and adding fresh ddH_2O . The gel was then photographed under UV light with a Polaroid MP-4 photography set using type 57 film.

Results

Expression of VEGF in MCF-7 Cells and Mouse Kidney

Preliminary tests were conducted on non-precious samples to test procedures and primers. MCF-7 breast cancer cells show an overexpression of VEGF, as cancerous cells typically do (Boocock C *et al.*, 1995). Since determining VEGF expression in a normal tissue was ultimately the goal of these tests, mouse kidney was used as a comparison to the MCF-7 cells.

RNA was extracted via the GITC method and stored at -80°C until analyzed by RT-PCR. The RNA concentration was 1.6 µg/µL for MCF-7 and 4.2 µg/µL for mouse kidney. 10µg of RNA from each sample were reverse transcribed to produce 20µL of cDNA. For PCR, 2.5µL of cDNA were taken from the MCF-7 sample to test for actin, and 5µL of cDNA were taken to test for VEGF. The amounts of cDNA taken to test for actin and VEGF were the same for the mouse kidney.

Through the use of non-denaturing polyacrylamide gel electrophoresis, the number of base pairs in a sample were gauged against a 123 bp ladder molecular weight marker. In Fig. 1, actin in MCF-7 cells produced a strong band at the predicted 548 bp level (lane 2). Mouse kidney also expressed actin, as its band can clearly be seen at that level in lane 3. The expression of actin in these two samples shows that the RNA extraction, RT, and PCR procedures were successful and thus gives credence to the VEGF lanes. A 349 bp fragment, the size predicted for VEGF, was detected for MCF-7 in lane 6 and for mouse kidney in lane 7. Both negative control lanes were blank (lanes 4 and 8), indicating no contamination was present.

Expression of VEGF in Mouse Eggs, Cumulus Cells, Blastocysts, and Mouse Kidney

After several unsuccessful attempts to isolate an adequate amount of RNA from eggs using the GITC method, it was determined that another method might yield more RNA. The crude lysate method was then used to prepare RNA from 19 mouse eggs. 10U RNasin and 10 μ L of the NP-40/DTT mix were added to the eggs to lyse them. After reverse transcription, 80 μ L of cDNA were obtained from the eggs. 50 μ L were removed and run through a spin column, dried under a vacuum, and resuspended in 20 μ L ddH₂O. For PCR, 10 μ L of the concentrated cDNA were used to test for actin, while the other 10 μ L were used to test for the presence of VEGF. In order to increase sensitivity, a nested PCR was also performed for both actin and VEGF using 1 μ L of their respective PCR products to serve as a substitute for cDNA.

The RNA from cumulus cells, blastocysts, and kidney tissue were extracted using the GITC method. The RNA concentration was 11.1 μ g/ μ L for cumulus cells, 2.3 μ g/ μ L for blastocysts, and 4.2 μ g/ μ L for the mouse kidney. 10 μ g of RNA from each sample were reverse transcribed, producing 20 μ L of cDNA from each of them. 5 μ L of both blastocyst cDNA and cumulus cell cDNA were removed to test for actin. The same volume of each was also removed to test for the presence of VEGF. From the kidney cDNA, 2 μ L were removed to test for actin, while 4 μ L were removed to test for VEGF. Negative controls lacking cDNA were also prepared. All of these samples produced a final PCR product volume of 50 μ L after stock solutions and primers were added.

The PCR products were analyzed by gel electrophoresis. The RT-PCR process succeeded, as fig. 2 shows a 548 bp fragment of actin in the egg (lane 1), nested egg (lane 2), blastocyst (lane 4), and mouse kidney (lane 7). However, it was not seen in cumulus cells (lane 3). VEGF appears to be expressed throughout embryonic development, as Fig. 3 shows the

predicted 349 bp fragment of VEGF in the egg (lane 2), nested egg (lane 3), cumulus cells (lane 4), blastocysts (lane 5), and mouse kidney (lane 7). The negative controls (lane 6 in figs. 2 and 3) were both blank, indicating no contamination was present.

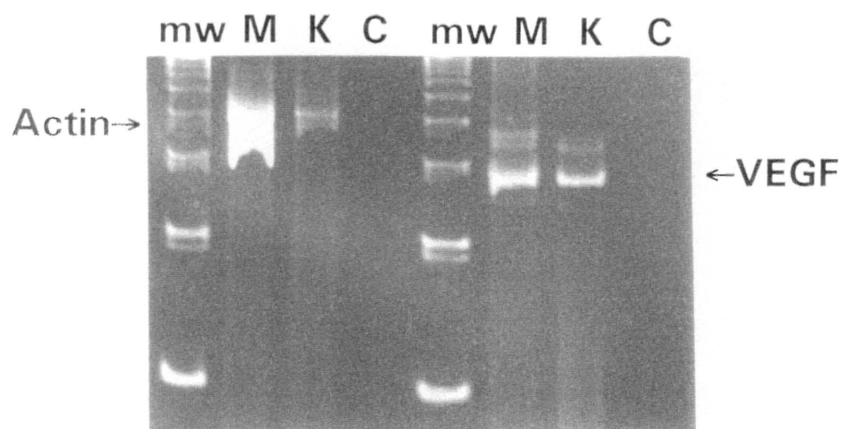


Fig. 1. Expression of actin and VEGF analyzed by RT-PCR. Ethidium bromide stained polyacrylamide gel showing actin in MCF-7 cells (lane 2), actin in mouse kidney (lane 3), VEGF in MCF-7 cells (lane 6), VEGF in mouse kidney (lane 7), and controls (no cDNA added, lanes 4 and 8). Molecular weight markers (MW) are a 123 bp ladder. Sizes predicted for actin and VEGF were 548 and 349 bp, respectively.

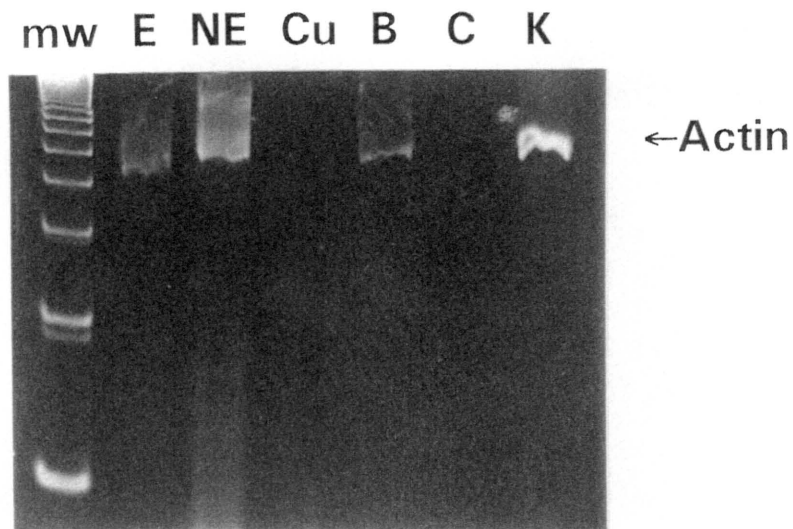


Fig. 2. Expression of actin analyzed by RT-PCR. Ethidium bromide stained polyacrylamide gel showing actin in eggs (lane 2), nested eggs (lane 3), blastocysts (lane 5), and mouse kidney (lane 7). No actin is evident in cumulus cells (lane 4). The control (no cDNA added, lane 6) is negative for contamination. The molecular weight marker (MW) is a 123 bp ladder. The predicted actin size was 548 bp.

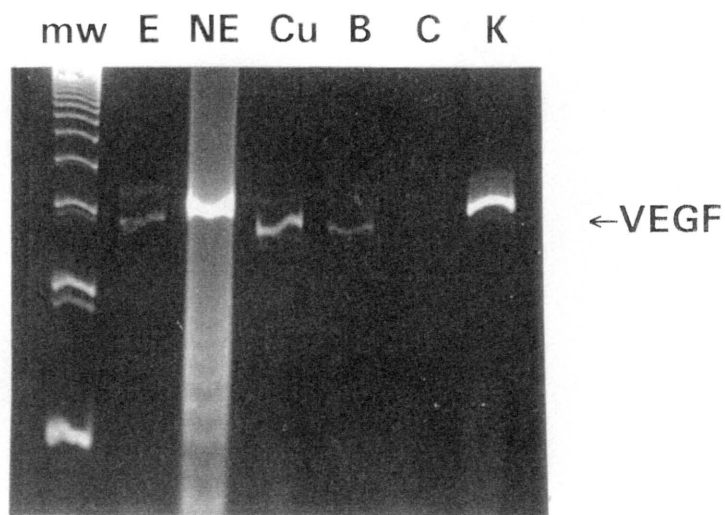


Fig. 3. Expression of VEGF analyzed by RT-PCR. Ethidium bromide stained polyacrylamide gel showing VEGF in eggs (lane 2), nested eggs (lane 3), cumulus cells (lane 4), blastocysts (lane 5), and mouse kidney (lane 7). The control (no cDNA added, lane 6) is negative for contamination. The molecular weight marker (MW) is a 123 bp ladder. The predicted VEGF size was 349 bp.

Discussion

By RT-PCR analysis, both mouse eggs and blastocysts appear to express VEGF. Since the VEGF fragment produced by PCR is common to all four variants, it is impossible to differentiate which variant(s) is being produced. Since cumulus cells are known to express VEGF (fig. 1B, Kamat B *et al.*, 1995), egg samples that are not totally cumulus-free may appear to express VEGF when none is actually present. The egg samples tested in this study, however, were visibly free of any cumulus cells. The crude lysate method of preparing the egg RNA may also have contributed to the appearance of a VEGF band. The egg cDNA that was used was heavily concentrated and PCR may have amplified a miniscule amount of VEGF message that is not biologically relevant. It is believed, based on this study, that VEGF is expressed in the egg although its role at that stage is not yet understood. One possibility for VEGF involvement at the egg stage is in folliculogenesis. Future studies could compare equal volumes of cumulus-free egg samples with cumulus-attached egg samples to determine the validity of VEGF expression.

At the blastocyst stage, VEGF may be important in implantation by initiating reorientation of blood vessels and increasing vascular permeability. VEGF variants 121 and 165 are soluble and, if present in the embryo, could precede it and prime the uterus for implantation. VEGF variants 189 and 206, if present, may influence local permeability and angiogenic events at the implantation site. Therefore, VEGF may be necessary for successful implantation of the embryo and for placental growth. Future studies could test for the individual variants of VEGF in the blastocyst to determine which one(s) is expressed.

A possible additional function of VEGF besides angiogenesis and permeability may be trophoblast growth promotion. Since trophoblast cells are believed to contain VEGF receptors

(Charnock-Jones D *et al.*, 1994, Torry D *et al.*, unpublished) and the blastocyst was shown to produce VEGF, the blastocyst may induce its own trophoblast growth.

The major source of VEGF within the blastocyst is not known, but it is likely to be the trophoblast. Therefore, trophoblast production of VEGF could induce trophoblast receptors and possibly promote both placental formation and trophoblast growth. In future studies, trophoblast cells could be separated from the inner cell mass and tested individually for VEGF expression. This would determine the exact location of VEGF secretion from the blastocyst.

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Dr. Pradeep Warikoo generously provided the mouse egg and blastocyst samples for the experiments. Despite his busy patient schedule, he felt that the research was important enough to spend several hours harvesting and culturing mouse embryos. I appreciate his commitment to this study, as it would have been impossible to conduct it without his expertise in embryology.

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