Endometrial signals improve embryo outcome: functional role of vascular endothelial growth factor isoforms on embryo development and implantation in mice

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STUDY QUESTION: Does vascular endothelial growth factor (VEGF) have important roles during early embryo development and implantation?

SUMMARY ANSWER: VEGF plays key roles during mouse preimplantation embryo development, with beneficial effects on time to cavitation, blastocyst cell number and outgrowth, as well as implantation rate and fetal limb development.

WHAT IS KNOWN ALREADY: Embryo implantation requires synchronized dialog between maternal cells and those of the conceptus. Following ovulation, secretions from endometrial glands increase and accumulate in the uterine lumen. These secretions contain important mediators that support the conceptus during the peri-implantation phase. Previously, we demonstrated a significant reduction of VEGF in the uterine cavity of women with unexplained infertility. Functional studies demonstrated that VEGF significantly enhanced endometrial epithelial cell adhesive properties and embryo outgrowth.

STUDY DESIGN, SIZE, DURATION: Human endometrial lavages (n = 6) were obtained from women of proven fertility. Four-week old Swiss mice were superovulated and mated with Swiss males to obtain embryos for treatment with VEGF in vitro. Preimplantation embryo development was assessed prior to embryo transfer (n = 19–30/treatment group/output). Recipient F1 female mice (8–12 weeks of age) were mated with vasectomized males to induce pseudopregnancy and embryos were transferred. On Day 14.5 of pregnancy, uterine horns were collected for analysis of implantation rates as well as placental and fetal development (n = 14–19/treatment).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Lavage fluid was assessed by western immunoblot analysis to determine the VEGF isoforms present. Mouse embryos were treated with either recombinant human (rh)VEGF, or VEGF isoforms 121 and 165. Preimplantation embryo development was quantified using time-lapse microscopy. Blastocysts were (i) stained for cell number, (ii) transferred to wells coated with fibronectin to examine trophoblast outgrowth or (iii) transferred to pseudo pregnant recipients to analyze implantation rates, placental and fetal development.

MAIN RESULTS AND THE ROLE OF CHANCE: Western blot analysis revealed the presence of VEGF121 and 165 isoforms in human uterine fluid. Time-lapse microscopy analysis revealed that VEGF (n = 22) and VEGF121 (n = 23) treatment significantly reduced the preimplantation mouse embryo time to cavitation (P < 0.05). VEGF and VEGF165 increased both blastocyst cell number (VEGF n = 27; VEGF165 n = 24: P < 0.001) and outgrowth (n = 15/treatment: 66 h, P < 0.001; 74, 90, 98 and 114 h, P < 0.01) on fibronectin compared with control. Furthermore, rhVEGF improved implantation rates and enhanced fetal limb development (P < 0.05).

LIMITATIONS, REASONS FOR CAUTION: Due to the nature of this work, embryo development and implantation was only examined in the mouse.
Introduction

Infertility affects ~48.5 million people globally (Mascarenhas et al., 2012); while many causes of infertility can be treated with assisted reproduction therapies, such as IVF, ~70–75% of IVF cycle attempts will fail. Those that succeed to a viable pregnancy are at an increased risk of pregnancy complications and demonstrate a correlation with behavioral and developmental problems (Ludwig et al., 2006; Ceelen et al., 2008; Rinaudo and Lamb, 2008; Scherrer et al., 2012; Dar et al., 2013; Makinen et al., 2013; Pinborg et al., 2013; Talaulikar and Arulkumaran, 2013). Therefore, improving current IVF procedures, including embryo culture conditions and optimizing endometrial receptivity, will likely improve both pregnancy rates and the long-term health and developmental outcome for children conceived through IVF. Over the past 30 years there have been considerable advances in clinical embryo culture techniques, including culturing embryos in low oxygen (Gardner, 2007), addition of important metabolites to the medium (Gardner, 2008; Gardner, 2014) and the development of sequential culture media and stage of embryo/blastocyst transfer (Gardner and Lane, 1997; Papanikolaou et al., 2006a,b), which resulted directly in an increase in implantation rates and a decrease in pregnancy loss (Papanikolaou et al., 2006a). However, even with these advancements in embryo culture, there is still a 70–75% IVF failure rate. An improved understanding of the ‘physiological’ microenvironment of preimplantation embryo development in vivo will likely lead to a more optimal pregnancy outcome.

The proteins present in the uterine secretions may be important for endometrial-embryonic communication and therefore improved preimplantation development. Throughout most of the human menstrual cycle the endometrium is hostile or non-receptive to embryo implantation. For a short window of time, ~5–9 days following ovulation, the endometrium becomes receptive to allow blastocyst implantation (Navot and Bergh, 1991). At this time the glandular and luminal epithelium become highly secretory, producing and secreting a wide array of factors into the uterine cavity (Gray et al., 2001; Burton et al., 2002; Hemptstock et al., 2004; Hannan et al., 2010). Thus, the blastocyst is bathed in maternal uterine secretions from the time it first enters the uterine cavity and throughout the peri-implantation period.

Disrupted secretion of individual soluble factors including cytokines, growth factors and proteases from the endometrium into the uterine lumen has been correlated with infertility (Dimitriadis et al., 2006; Mikolajczyk et al., 2006; Boomsma et al., 2009). Studies identifying the products of glandular secretions, bioactivity and function are therefore a necessary prerequisite to further understand endometrial receptivity and maternal-embryonic communication and to increase the efficacy of in vitro culture conditions. In addition, there are currently no definitive markers of endometrial receptivity which can reliably identify receptive endometrium during IVF procedures.

Previously, we assessed the cytokine and growth factor profile of human uterine fluid using multiplex assays (Hannan et al., 2011) in both fertile and infertile women during the mid-secretory phase. Women with unexplained infertility had overall reduced levels of vascular endothelial growth factor A (VEGFA). Importantly, we further demonstrated that VEGFA has substantial effects on both endometrial epithelial receptivity (adhesive capacity) and on blastocyst outgrowth in vitro (Hannan et al., 2011).

VEGF is most well known for its roles in angiogenesis, driving both endothelial cell proliferation and migration. Both VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) are expressed by mouse blastocysts (Baston-Buest et al., 2011). There are three secreted isoforms of VEGFA, namely VEGF121, VEGF145 and VEGF165, the two most abundant being VEGF121 and VEGF165, both signal through VEGFR-2. We therefore examined the role of both VEGF121 and VEGF165 on the pre-implantation embryo.

Our previous study identified, for the first time, a non-vascular role of endometrial-derived VEGFA with functional actions on the maternal surface and on the peri-implantation blastocyst (Hannan et al., 2011). The current study aimed to further characterize the role of VEGF on the peri-implantation embryo. We identified the predominant VEGF isoforms secreted into the human uterine cavity by western blot, revealing that both VEGF121 and 165 isoforms are present. These isoforms and total VEGF were added to mouse embryo cultures and embryos were monitored using high temporal time-lapse microscopy, examining blastocoeel formation and hatching. Embryos were then analyzed for (i) cell number, (ii) blastocyst outgrowth or (iii) transferred to pseudo pregnant recipient females to analyze implantation rates, and placental and embryo development. Supplementing culture media with VEGF, VEGF121 and VEGF165 had beneficial effects on post-compaction mouse embryo development, outgrowth, implantation and fetal development.

Materials and Methods

Ethical approval

Ethical approval was obtained from Monash Surgical Private Hospital; Human Research Ethics Committee (Project No. 04056) and Southern Health Human Research Ethics Committee (Project No. 030668) for all human sample collections. Written informed consent was obtained from all subjects prior to sample collection. Ethical approval was obtained from the University of Melbourne Animal Ethics Committee (Project ID: 0811074.2) prior to experimentation. All animal experimentation was conducted in accordance...
with accepted standards of humane animal care, as outlined in the Ethical Guidelines of the National Health and Medical Research Council.

Sample collection and patient details
Human endometrial lavage fluid (n = 6/group) was obtained from women with proven fertility (undergoing tubal ligation) and women of unknown fertility status and with unexplained infertility during the late proliferative—early secretory phase (Days 14—19) of the menstrual cycle who were undergoing hysteroscopy, dilatation and curettage. Cycle stage was confirmed by histological dating, according to the criteria of Noyes et al., (1975). The infertile women had been screened for non-endometrial causes of their infertility, tubal patency and their partner did not have male factor infertility. Patients with uterine abnormalities, such as endometrial polyps, fibroids, endometriosis and endometritis, or who had received steroid hormone therapy in the last 6 months were excluded from the study. As previously described (Hannan et al., 2009, 2010, 2011, 2012), prior to hysteroscopy 3 ml of sterile saline was gently infused into the uterine cavity through a fine flexible catheter for a few seconds; the saline solution was then aspirated and centrifuged to remove contaminating cellular debris (including leukocytes, red blood cells and mucus) and stored at −80 °C as 0.5 ml aliquots.

Heparin capture of uterine lavage fluid
Heparin agarose suspension (100 μl) (heparin agarose, H6508, Sigma-Aldrich) was loaded on to Pierce spin columns (69705, Pierce). Storage buffer was removed by centrifugation at 1000g for 5 min, and agarose washed three times with 0.01 M Tris–HCl. Uterine lavage fluid (100 μl) (equal amounts pooled from n = 6 women) was mixed with 100 μl of binding buffer (0.01M Tris–HCl), added to washed heparin agarose and incubated at room temperature for 90 min with end over end mixing. Unbound proteins were removed by centrifugation at 1000g for 5 min and weakly bound proteins were removed with 0.01 M Tris–HCl/150 mm NaCl. Heparin-bound proteins were removed by three sequential elutions with 200 μl 0.01 M Tris–HCl/2M NaCl. Eluted proteins were dialyzed and concentrated to 50 μl with phosphate-buffered saline (PBS) using 3 kDa cut-off spin filters.

Western immunoblot analysis
Fifty nanograms of recombinant VEGF121, VEGF165 or 50 μl of heparin-bound proteins was mixed with 5X non-reducing loading buffer, heated to 95°C for 5 min and loaded on to a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis gel. Protein was blotted onto 0.45 μm PVDF transfer membrane (GE Healthcare) using a TransBlot turbo transfer system (BioRad). Immunoblots were washed in 0.1% Tris-buffered saline (TBS)– Tween 20 before incubation in 5% skim milk/0.1% TBS–TWEEN 20 to block non-specific binding. Immuno-blot was then washed in 0.1% TBS–TWEEN 20 before incubation with goat anti-VEGF antibody (R&D Systems) at a 1:500 dilution overnight at 4°C. Immunoblots were washed thoroughly in 0.1% TBS–TWEEN 20 before incubation with rabbit anti-goat horse-radish peroxidase antibody (Dako) at 1:2000 dilution at room temperature for 1 h. After washing in 0.1% TBS–TWEEN 20, enhanced chemiluminescence (ECL, BioRad) was applied and immunoreactive bands visualized using a Chemidoc XRS+ system (BioRad). Recombinant human (rh) VEGF was used as a positive control to confirm antibody specificity.

Animals and hormonal stimulation
Four-week-old Swiss female mice (n = 50) were superovulated with intra-peritoneal injections of 5 IU pregnant mare’s serum gonadotrophin (Folligon; Intervet, UK) followed 48 h later by 5 IU hCG (Chorulon; Intervet). Females were mated with Swiss males overnight. The presence of a vaginal plug the following morning was used as an indicator of successful mating.

Embryo collection and culture
Pronucleate oocytes (n = 400) were collected ~21–22 h post-hCG in G-MOPS embryo handling medium (Lane, 2004) supplemented with 5 mg/ml human serum albumin (HSA) (Vitrolife, Sweden), followed by cumulus removal in G-MOPS (Vitrolife) containing 300 IU/ml hyaluronidase (bovine testes, type IV; Sigma-Aldrich). Pronucleate oocytes were removed from the hyaluronidase immediately once the cumulus cells had detached, washed twice in G-MOPS and then once in G1 medium (Vitrolife) (Gardner, 2007) before culture. Pronucleate oocytes were then combined and randomly assigned to different treatment groups, either cultured in 20 μl (Group: 10 embryos/drop) or 2 μl (individual; single embryo/drop) drops of G1 medium supplemented with 5 mg/ml HSA under paraffin oil (Ovol; Vitrolife) at 37°C in 6% CO2, 5% O2 and 89% N2. After 48 h, embryos were randomly transferred into their respective treatment media either in groups (20 μl) or as individuals (2 μl): (i) control; (ii) G2 media (Vitrolife) (Gardner, 2007) with 5 mg/ml HSA; (iii) total VEGF containing rhVEGF (R&D systems, Minneapolis, MN, USA) at a dose of 50 ng/ml in G2 control media, as previously established (Hannan et al., 2011); (iv) VEGF121 containing 50 ng/ml rhVEGF isoform 121 (Isokine™ ORF Genetics, Keldnaholt, Iceland) in G2 media and (v) VEGF165 containing 50 ng/ml rhVEGF isoform 165 (Isokine™ ORF Genetics) in G2 media. Embryos were cultured under paraffin oil (Ovol) at 37°C under the same gas phase conditions to the blastocyst stage.

Embryo development
Embryos were analyzed for development and cell cycle kinetics were cultured individually in 2 μl drops of G1 medium supplemented with 5 mg/ml HSA under paraffin oil (Ovol, Vitrolife) in the same gas phase conditions at 37°C in a humidified multi-gas imaging incubator (Sanyo MCOK-SM[RJC]) for 48 h and then transferred to treatment groups (as detailed above). Time-lapse images of individual embryos were generated every 15 min across culture using the imaging incubator. From these images, the timing of the start of cavitation and hatching were calculated as hours post-hCG.

Blastocyst staining
Blastocyst cell number was determined following final morphological assessment at 114 h post-hCG (Day 5) as described previously (Hannan et al., 2011; Binder et al., 2012). Blastocysts (n = 24–30 per treatment group) were stained in a solution of 0.1 mg/ml Bisbenzimide (Hoescht 33342; Sigma Chemical Co.) in 10% ethanol, for 1 h at 37°C, and rinsed in G-MOPS with 5 mg/ml HSA. Blastocysts were mounted in glycerol under cover slips on glass slides before being observed under fluorescent light (Nikon TS100-F), and cell numbers counted manually.

Embryo outgrowth
Blastocyst outgrowth was assessed as described previously (Hannan et al., 2011). In brief, flat bottomed 96-well tissue culture dishes (BD Biosciences, USA) were coated with fibronectin (Fn) (10 μg/ml) (BD Biosciences), rinsed twice with sterile PBS and incubated with 4 mg/ml bovine serum albumin (Sigma Diagnostics, St. Louis, USA). Wells were rinsed and subsequently filled with 150 μl of appropriate experimental medium and equilibrated at 37°C under paraffin oil (Ovol) for 3 h prior to the addition of blastocysts. Hatched blastocysts (on Day 5 of development) that had been pre-cultured in appropriate medium were placed into the coated wells (1 embryo per well) and incubated for 114 h. Outgrowth was examined and images were taken at a matching magnification (×10) at sequential times (66, 74, 90, 98 and 114 h following transfer to outgrowth plate) during the culture period with an inverted microscope (Eclipse TS100-F; Nikon, Coherent Scientific Pty. Ltd., SA, Australia) equipped with heated stage at 37°C. The extent of outgrowth for each treatment was obtained by measuring the area of outgrowth in

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VEGF improves embryo development and implantation

Embryo transfer, implantation and fetal development

F1 female mice between 8 and 12 weeks of age were mated with vasectomy-mated males to induce pseudopregnancy. Mating was confirmed by the presence of a vaginal plug. Embryos were transferred on Day 4 of development (asynchronous to female reproductive tract which was staged as Day 3.5 of pregnancy). Recipient female mice were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg Ketalar, Pfizer, Australia) and medetomidate (1 mg/kg Domitor, Pfizer, Australia). Five embryos were transferred through a small dorsal incision with a glass pipette into the lumen of each uterine horn. Recipient female mice received embryos cultured in media treatment groups as described above: (i) control; (ii) total VEGF, 50 ng/ml of rhVEGF; (iii) VEGF121, 50 ng/ml of rhVEGF isoform 121 and (iv) VEGF165, 50 ng/ml of rhVEGF isoform 165. Alternate groups were transferred to both the right and left horn per recipient to avoid any preferential implantation bias of the left or right horn. Following embryo transfer, the skin wound was sealed with sterile surgical clips, and the recipient female underwent post-operative recovery with an intraperitoneal injection of atipamezole (1 mg/kg Antisedan, Pfizer, Australia) to reverse the effects of medetomidate. Pregnant females were sacrificed 10 days later (Day 14.5 of fetal development). The number of fetuses and implantation/absorption sites were recorded to determine the rates of implantation and fetal development. Fetal and placental weight was recorded and crown-rump length measured. Fetal ear, eye and limb development was assessed as reported previously (Wahlsten and Wainwright, 1977; Lane and Gardner, 1994; Binder, Hannan and Gardner, 2012).

Statistical analysis

After testing for normal distribution, statistical analysis was performed on raw data.

Time-lapse (embryo developmental kinetics): the time, in hours (h) from hCG, to cavitation and to hatching was not normally distributed and statistically analysed with a non-parametric Mann–Whitney test. Blastocyst cell counts were normally distributed and statistically analysed by analysis of variance followed by Tukey’s multiple comparison test. Embryo outgrowth data were not normally distributed and were tested non-parametrically using the Kruskal–Wallis test. Embryo implantation following transfer—the proportion of embryos implanted per transfer was assessed; data were not normally distributed and were analysed using a Kruskal–Wallis test. Fetal and placental development: weight (g), crown-rump length (mm) and fetal morphological grade (not normally distributed) were analysed with a Kruskal–Wallis test (non-parametric). P values < 0.05 were taken as significant. All statistical analysis was carried out using PRISM version 6.00 for Mac (GraphPad, SanDiego, CA, USA).

Results

VEGF isoforms are present in receptive phase human uterine fluid

Western immunoblot analysis following heparin capture of human uterine lavage fluid revealed two bands corresponding with the VEGF121 (~28 kDa) and VEGF165 (~38 kDa) molecular weight (Fig. 1). VEGF121 appears to predominate in heparin-captured uterine lavage fluid.

VEGF enhanced embryo development

Mouse embryos treated with either VEGF (n = 22) or the VEGF121 isoform (n = 23) cavitated significantly earlier compared with control embryos (n = 19) (Fig. 2A). VEGF165 (n = 24) treatment had no significant effect on time to cavitation (Fig. 2A). Blastocyst hatching rates were variable but no significant difference in hatching rates were observed with treatment (data not shown).

VEGF enhanced blastocyst cell number

Mouse blastocyst cell number was significantly increased when either VEGF (n = 27) or VEGF165 (n = 24) was added to the culture media (Fig. 2B) compared with control (n = 28) (control 88 ± 26 cells versus VEGF 101 ± 12 cells; P < 0.001; control 88 ± 26 cells versus VEGF165 114 ± 27 cells; P < 0.001). Addition of VEGF121 (n = 29) to the culture had no effect on blastocyst cell number (Fig. 2B).

VEGF enhanced blastocyst outgrowth in vitro

Mouse embryos were used to assess the functional effects of VEGF, VEGF121 and VEGF165 on blastocyst outgrowth in vitro. A significant increase in the area of blastocyst outgrowth was observed with rhVEGF treatment (Fig. 2C; n = 15). VEGF121 treatment (n = 15) increased blastocyst outgrowth area (P < 0.05; 66 h post-hatching) compared with control cultured embryos (Fig. 2D; n = 15). VEGF165 (n = 15) caused a highly significant increase in outgrowth (at all time points examined; 66 h (P < 0.001), 74, 90, 98 and 114 h (P < 0.01)) compared with control (Fig. 2D), while VEGF121 was without effect after 66 h.

Figure I Vascular endothelial growth factor in human uterine lavage fluid. Heparin capture and western blot analysis revealed the presence of two VEGF isoforms in human uterine lavage fluid. Both VEGF isoforms 121 (~28 kDa) and 165 (~38 kDa) are abundant. Lane 1: molecular mass (Mr) markers are included to indicate Mr; Lane 2: lavage fluid from ‘Fertile’ women (n = 6 pooled); Lane 3: lavage fluid from ‘women of unknown fertility’ (n = 6 pooled); Lane 4: lavage fluid from ‘Infertile’ women (n = 6 pooled). The dotted lines demarcate the borders between two juxtaposed images due to the removal of two lanes with over-developed VEGF signal.
Figure 2  Effects of rhVEGF and VEGF isoforms, VEGF121 and VEGF165, on mouse embryo development in culture. (A) Total VEGF and VEGF121 improved embryo development in culture, significantly reducing the time taken to reach cavitation (early blastocyst stage). VEGF165 had no effect. Data are expressed as the proportion of embryos at the designated developmental stage by the arbitrarily set time for each cleavage event (n = 19–24 embryos /treatment group). Data were statistically assessed by a Mann–Whitney test. (B) Blastocysts cultured in the presence of total rhVEGF or VEGF165, but not VEGF121, had significantly more cells on Day 5 of culture than controls. Data are expressed as mean ± SEM (n = 24–30/treatment group). Data were statistically analyzed by analysis of variance followed by Tukey’s multiple comparison test. (C) Blastocyst outgrowth (on fibronectin 10 μg/ml) was significantly enhanced by 66 h when embryos were cultured in the presence of rhVEGF compared with control. (D) VEGF121 and VEGF165 (50 ng/ml) both significantly enhanced outgrowth. VEGF165 was most potent throughout the 114 h of outgrowth culture. Data expressed as mean ± SEM (n = 15 blastocysts/treatment). *P < 0.05, **P < 0.01, ***P < 0.001 versus control. Data were tested non-parametrically using Kruskal–Wallis.
Table 1 Implantation and ongoing pregnancy rates on embryonic Day 14.5 in mice following embryo transfer of embryos cultured in standard IVF media (Control) and with added rhVEGF and two VEGF isoforms.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>rhVEGF</th>
<th>VEGF121</th>
<th>VEGF165</th>
</tr>
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<tr>
<td>Implantation/transfer</td>
<td>0.76 ± 0.06</td>
<td>0.90 ± 0.04*</td>
<td>0.80 ± 0.06</td>
<td>0.63 ± 0.09</td>
</tr>
<tr>
<td>Ongoing pregnancy/transfer</td>
<td>0.41 ± 0.07</td>
<td>0.48 ± 0.05</td>
<td>0.43 ± 0.06</td>
<td>0.40 ± 0.09</td>
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<tr>
<td>Ongoing pregnancy/implantation</td>
<td>0.57 ± 0.10</td>
<td>0.53 ± 0.05</td>
<td>0.56 ± 0.08</td>
<td>0.61 ± 0.11</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.
*P < 0.05 versus control. Embryo implantation following transfer (proportion of embryos implanted per transfer) was assessed statistically using a Kruskal–Wallis test (n = 28–41/treatment).

VEGF improves embryo implantation rates and limb development

Mouse embryos cultured with rhVEGF had significantly higher implantation rates following blastocyst transfer compared with control (P < 0.05) (Table I). VEGF121 showed an overall improved implantation rate compared with control, but this was not significant. Whilst not significant, there was a trend for embryos cultured in rhVEGF to have higher rates of ongoing pregnancy per transfer and embryos cultured in rhVEGF165 also showed a trend to higher rates of ongoing pregnancy per implantation (Table I).

Implanted embryos were further characterized at embryonic Day 14.5 to examine development. There was a significant advance in fetal limb development when VEGF165 was present during embryo culture (Fig. 3A). In particular, preimplantation embryo culture in VEGF165 resulted in embryos with clearly defined, webbed digits at this time (Fig. 3B) compared with those derived from control embryos (Fig. 3C).

Neither fetal nor placental weights were significantly altered when rhVEGF or the two isoforms were added to the embryo culture medium: neither were there significant differences in crown-rump length, eye or ear morphology (Table II).

Discussion

Previously, we identified that VEGFA was significantly reduced in uterine lavage fluid in women with unexplained infertility (Han et al., 2011) and that functionally, VEGF acted on both human uterine epithelium and on mouse embryo attachment and outgrowth in vitro (Han et al., 2011). The current study identifies that the predominant VEGF isoforms secreted into the human uterine cavity are VEGF121 and 165. Addition of VEGF, VEGF121 and VEGF165 to embryo culture post-compaction enhanced preimplantation mouse embryo development (reduced time to cavitation and increased blastocyst cell number). Consistent with our previous findings addition of total rhVEGF enhanced blastocyst outgrowth in vitro. In the present study, the addition of the VEGF121 and VEGF165 isoforms caused a significant increase in blastocyst outgrowth compared with control. Furthermore, this study demonstrates for the first time that VEGF and its secreted isoforms VEGF121 and VEGF165 enhanced implantation rates and fetal limb development in mice.

VEGF is most well known for its roles in angiogenesis. The VEGF family (consisting of five members: VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor) and their receptors are key mediators of vascular growth and remodeling in a variety of tissues, including the human endometrium (Hornung et al., 1998; Girling and Rogers 2009). VEGFA binds to and signals via two tyrosine kinase receptors, VEGFR1 and VEGFR2, both of which are immunolocalized apically in endometrial glandular epithelium (Moller et al., 2001). VEGFA enhances the mitogenic activity of endothelial cells via the adhesion molecule integrin αvβ3 (Soldi et al., 1999), an important adhesion molecule during embryo implantation and a potential marker of endometrial receptivity (Lessey, 2002; Illera et al., 2003). VEGF is expressed by granulated metrial
glands in the murine uteri (Wang et al., 2000). Both VEGFR1 and 2 are expressed by mouse blastocysts (Baston-Buest et al., 2011). There are three secreted isoforms of VEGFA, namely VEGF121, VEGF145 and VEGF165, the two most abundant being VEGF121 and VEGF165 (Poltorak et al., 1997; Nakatsu et al., 2003). While VEGF121 and VEGF165 have similar functions, it has been suggested that VEGF165 may be more potent than 121 (Ke et al., 2002) and while VEGF165 and VEGF121 bind to their receptors with equal affinity, their ability to activate VEGFR-2 is not equivalent (Keyt et al., 1996; Soker et al., 1997; Ogawa et al., 1998). Therefore, we examined the role of both VEGF121 and VEGF165 in the preimplantation embryo.

VEGFA is expressed in both human endometrial endothelial and epithelial cells (Moller et al., 2001) and is readily detected in uterine fluid, supporting its secretion by endometrial epithelium (Hannan et al., 2011) into the microenvironment where the blastocyst undergoes its final development for implantation. Moreover, its secretion is increased in the presence of the blastocyst-derived factor, hCG (Paiva et al., 2011) highlighting a mechanism by which the presence of a human blastocyst could enhance receptivity (Licht et al., 2001). Furthermore, VEGFA is significantly reduced in the mid-secretory phase in uterine fluid from infertile women compared with women of proven fertility (Hannan et al., 2011). Given that VEGF can exist in a number of isoforms that can have different actions and potency, identification of the specific isoforms secreted into the uterine cavity was important. Using western blot analysis we showed that heparin-binding forms of VEGF121 and 165 were readily detectable in human uterine fluid. These likely represent the active forms present in vivo as it is known that they bind to heparin and this binding enhances their potency (Ashikari-Hada et al., 2005).

While embryo culture has improved over the past 30 years, in vitro cultured embryos have been observed to typically develop more slowly and have reduced blastocyst cell numbers than embryos developed in utero (Bowman and McLaren, 1970; Paria and Dey, 1990). Analysis of media used to support human embryos in clinical IVF shows that until the mid-1990s the media were based on simple salt solutions supplemented with glucose, pyruvate, lactate and HSA (Quinn et al., 1985). Such culture media have been shown to impart significant stress to the developing mammalian embryo from the zygote to blastocysts stage (Gardner and Lane, 2005; Lane and Gardner, 2005). Human embryo culture media have become more complex and effective over the past 15 years, and now include amino acids and vitamins (Gardner, 2008). However media used in human IVF typically lack key regulators of development present in many other culture media, particularly growth factors and cytokines. Recently, the addition of granulocyte–macrophage colony-stimulating factor (GM-CSF) has been evaluated in human IVF, where a significant increase in survival of transferred embryos was observed but only when the embryos were cultured at reduced levels of HSA (2 mg/ml) (Ziebe et al., 2013). Furthermore, in our previous work analyzing the abundance of cytokines and growth factors in human uterine fluid, we observed higher levels of VEGF compared with GM-CSF in the uterine cavity (Hannan et al., 2011).

Using sophisticated time-lapse analysis we demonstrated here that rhVEGF and the specific VEGF121 isoform significantly improved on-time blastocoel cavity formation. Furthermore, embryos cultured in the presence of either VEGF or VEGF165 had significantly increased cell numbers compared with embryos cultured in standard IVF media.

The potential effect of VEGF and its isoforms on embryos during culture post-compaction was also assessed by blastocyst outgrowth (a functional in vitro analysis of embryo implantation potential). As previously identified (Hannan et al., 2011), addition of VEGF to standard IVF culture media significantly increased outgrowth area. The present study extended these findings demonstrating that both VEGF121 and VEGF165 significantly increase outgrowth area by >5-fold.

Any benefit of adding VEGF to embryo culture in terms of pregnancy rates and embryo development was assessed in vivo following embryo transfer to pseudo pregnant recipients. Addition of rhVEGF to embryo culture media caused a significantly increased implantation rate compared with control standard IVF media. Whilst not significant but perhaps more importantly when considering the quality and health of the pregnancy, VEGF165-treated embryos showed a trend towards increased number of viable pregnancies following transfer.

Interestingly, of a number of other parameters examined (placental and fetal weights, embryo crown-to-rump length, eye or ear development), the only change observed with VEGF was that of limb development. In fetuses developing in vivo from embryos cultured in standard IVF medium there was a lack of defined digitation at Day 14.5 of pregnancy compared with those embryos that were cultured in media supplemented with VEGF165.

Taken together, the data in mice clearly demonstrate that the addition of both VEGF isoforms VEGF121 and VEGF165 to embryo culture medium has benefits compared with standard IVF media. VEGF121 and VEGF165 have unique actions at different stages of preimplantation embryo development and both are abundant in the human uterine microenvironment during the peri-implantation phase. Therefore, addition of VEGF121 and VEGF165 should be considered for use in

Table II Fetal development following transfer of mouse embryos cultured in standard IVF media (control) and with VEGFs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>rhVEGF</th>
<th>VEGF121</th>
<th>VEGF165</th>
<th>P value</th>
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<tbody>
<tr>
<td>Fetal weight (mg)</td>
<td>121.8 ± 4.2</td>
<td>120.4 ± 4.6</td>
<td>117.7 ± 6.6</td>
<td>119.8 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>Placental weight (mg)</td>
<td>115.6 ± 4.5</td>
<td>114.5 ± 4.1</td>
<td>118.4 ± 5.2</td>
<td>114.8 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>Fetal/placental ratio</td>
<td>1.05</td>
<td>1.05</td>
<td>0.99</td>
<td>1.04</td>
<td>NS</td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td>10.2 ± 0.1</td>
<td>10.4 ± 0.3</td>
<td>10.2 ± 0.2</td>
<td>10.4 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Limb morphological grade</td>
<td>13.4 ± 0.05</td>
<td>13.4 ± 0.2</td>
<td>13.5 ± 0.09</td>
<td>13.6 ± 0.1</td>
<td>0.023</td>
</tr>
<tr>
<td>Eye morphological grade</td>
<td>13 ± 0.03</td>
<td>13 ± 0.04</td>
<td>13 ± 0.04</td>
<td>13 ± 0.0</td>
<td>NS</td>
</tr>
<tr>
<td>Ear morphological grade</td>
<td>13.1 ± 0.08</td>
<td>13.2 ± 0.08</td>
<td>13.1 ± 0.08</td>
<td>13.2 ± 0.07</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. Fetal and placental development was statistically analysed using a Kruskal–Wallis test (n = 14–19/treatment). NS, not significant.
human embryo culture medium. A randomized controlled prospective clinical trial should be considered to test the efficacy of VEGF121 and VEGF165 in humans.

Conclusions
We previously identified high levels of VEGFA in human uterine fluid in women during the window of implantation and that this was significantly reduced in women with unexplained infertility. Here we demonstrate that VEGF121 and VEGF165 are the major isoforms in the human uterine cavity and that addition of rhVEGF to mouse embryo culture significantly improves preimplantation embryo development and embryo outgrowth in vitro. Furthermore, improvements in mouse implantation rates and aspects of fetal development in vivo were identified. These findings further support the concept that there is a precise paracrine and autocrine dialog between the blastocyst and endometrial epithelium during embryo implantation, and clearly highlight the importance of the changing microenvironment as embryos develop. Furthermore, the potential for improvement of clinical IVF outcomes by the addition of VEGF to human embryo culture media needs further investigation.

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Authors’ roles
All authors contributed to the conception and design of experiments, or the acquisition of data, or analysis and interpretation of data, manuscript preparation and revision, as well as final approval for publication.

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Conflict of interest
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