

Angiopoietin-1 and Angiopoietin-2 Activate Trophoblast Tie-2 to Promote Growth and Migration during Placental Development

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Human placental development involves coordinated angiogenesis and trophoblast outgrowth that are compromised in intrauterine growth restriction (IUGR). As Tie-2^(-/-) mice exhibit growth retardation and vascular network malformation, the expression of Tie-2 and its ligands, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), were investigated in human placenta from normal pregnancies and those complicated by severe IUGR. Ribonucleotide protection assays showed no significant change in the expression of Ang-2 mRNA between gestationally matched normal and IUGR placentas; however, immunoblots revealed that Ang-2 protein was significantly decreased in IUGR, suggesting that this may contribute to the abnormal development of the villous vasculature. *In situ* hybridization studies showed that Ang-1 and Tie-2 were detected in the cyto/syncytiotrophoblast bilayer in first-trimester placenta, whereas Ang-2 mRNA was restricted to the cytotrophoblast, suggesting their role in trophoblast function. At term, Ang-1 mRNA and immunoreactive protein were restricted to the paravascular tissues of the primary stem villi, supporting its role in vessel maturation. In contrast, Ang-2 was expressed throughout the term villous core, perhaps to permit the developing placental vascular network to remain in a state of fluidity. As these studies also revealed that trophoblast, in addition to endothelial cells, expressed Tie-2 receptors, we investigated the potential role of Ang-1/Ang-2 on trophoblast proliferation, migration, and the release of NO. Using spontaneously transformed first-trimester trophoblast cell lines that exhibit cytotrophoblast-like (ED₂₇) and extravillous trophoblast-like (ED₇₇) properties, we show that the addition of Ang-2 (250 ng/ml) stimulated DNA synthesis in ED₂₇ trophoblast cells and triggered the release of NO. Ang-1 stimulated trophoblast (ED₇₇) migration in a dose-dependent manner that was inhibited by recombinant Tie-2-FC. These

data thus imply, for the first time, a specific role for angiopoietins as regulators of trophoblast behavior in the development of the utero/fetoplacental circulation, an action independent of their well-established roles in vascular endothelium. (Am J Pathol 2000, 156:2185–2199)

Successful placentation requires the development of a low-impedance uteroplacental circulation after transformation of the maternal intramyometrial portion of the spiral arterioles by trophoblast invasion.¹ Hemochorial placentation is also dependent on the establishment and maintenance of a competent fetoplacental vascular network formed by the processes of vasculogenesis and branching (first and second trimesters) and nonbranching (third trimester) angiogenesis.¹ Consequently, a careful coordination of trophoblast and endothelial cell development, proliferation, invasion, and differentiation must occur during the early stages of placental development. This is considered to be mediated by locally acting angiogenic growth factors.²

Vascular endothelial growth factor (VEGF) and its two high-affinity receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR), are expressed in human placenta.^{3,4} Although VEGF is generally known as an endothelial cell-specific mitogen,⁵ it was also demonstrated to act as a mitogen for trophoblast^{3,6} and to stimulate NO release from first-trimester trophoblast.⁷ Recently described angiopoietin-1 (Ang-1) is a secreted angiogenic factor that binds and induces the tyrosine phosphorylation of *Tunica interna* endothelial cell kinase-2 (Tie-2) receptor on endothelial cells.⁸ Ang-1-deficient mice, in a manner similar to that of Tie-2^{-/-} mice, display a lethal phenotype caused by a severe defect in the embryonic vasculature.⁹ This has led to the proposal that Ang-1 mediates stabilization of developing blood vessels by recruiting and interacting

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with periendothelial cells.^{9,10} Ang-1 *per se* does not stimulate *in vitro* proliferation or tube formation of endothelial cells, although Ang-1 has been described as stimulating endothelial cell migration.¹¹ In the presence of VEGF, Ang-1 potentiates and sustains capillary growth in an *in vitro* system.^{8,12} In contrast, angiopoietin-2 (Ang-2) is the natural antagonist of Ang-1 on endothelium, and transgenic overexpression of Ang-2 caused disruption of blood vessel formation.¹³

In intrauterine growth-restricted (IUGR) pregnancies with reduced or absent end-diastolic flow velocity, the appearance of increased syncytial nuclei, grouped into syncytial knots, suggests an aged syncytium.¹⁴ In addition, the morphology of straight, unbranched capillaries and erythrocyte congestion of the terminal villi¹⁴ suggests poor placental blood vessel development due to disturbances in angiogenic growth factor expression/function.¹⁵ Interestingly, Tie-2^(-/-) mice exhibit growth retardation and malformation in the vascular network, mainly consisting of a dilated vasculature with limited branching and capillary sprouting.^{16,17} It thus seemed reasonable to define the role of Tie-2/angiopoietin system in placental development. We hypothesized that IUGR may be associated with overexpression of Ang-1 or underexpression of Ang-2 in these placentas and trophoblast expressing functional Tie-2 receptors. The aim of the study was first to examine the expression of the Tie-2/angiopoietin system in normal placenta and those complicated with IUGR. As these studies revealed that trophoblast in addition to endothelial cells also expressed Tie receptors, we investigated the potential role of Ang-1/Ang-2 in trophoblast proliferation, migration, and the release of NO.

Materials and Methods

Reagents

Recombinant Ang-1, Ang-2, and Tie-2-Fc proteins; monoclonal anti-Ang-1, anti-Ang-2 antibodies; and plasmids containing cDNAs for Ang-1, Ang-2, and Tie-2 were all generously provided by Regeneron Pharmaceuticals (Tarrytown, NY). A polyclonal rabbit anti-Tie-2 antibody (sc-324) was purchased from Autogen Bioclear (Stevenage, UK). VEGF₁₆₅ and VEGF₁₂₁ were purchased from Strahmann Biotech (Hannover, Germany). All cell culture reagents were purchased from Sigma (Dorset, UK).

Tissue Collection

First-trimester placenta (FT) (7–12 weeks' gestation; *n* = 11) and second-trimester placenta (ST) (14–17 weeks' gestation; *n* = 5) were collected from surgical termination of pregnancies. Third trimester placenta (TT) (27–36 weeks' gestation; *n* = 5) and uncomplicated term placenta (Term) (38–42 weeks' gestation; *n* = 14) delivered by elective cesarean section for breech presentation or a recurring indication in otherwise uncomplicated pregnancies were collected and stored as previously described.⁴ In addition to normal pregnancies, similar gestationally

matched tissues were collected by elective cesarean section from pregnancies complicated by IUGR (IUGR) (28–36 weeks' gestation; *n* = 6) or preeclampsia (PE) (28–37 weeks' gestation; *n* = 5). Placental tissues for IUGR were obtained from women with absent end diastolic flow velocity¹⁸ and babies who were small for date, with a fetal weight less than the fifth centile for gestational age. Full-thickness sections of placental tissues were dissected from a central location lying between the basal and chorionic plates. After dissection, the tissue was surrounded in embedding medium (OCT compound; Miles Scientific, Elkhart, IN) and rapidly frozen over dry ice and then stored at -80°C . Ten-micron sections were cut and thaw-mounted on superfrost glass slides (Surgipath, Peterborough, UK). These sections were stored (for less than 2 weeks) at -80°C until they were used for *in situ* hybridization. Alternatively, tissues were immersed in 10% formaldehyde, embedded in paraffin wax, and processed for immunocytochemistry (3- μm sections) or snap-frozen in liquid nitrogen immediately after collection before homogenization. Ethical committee approval for tissue collection was obtained from the South Birmingham Ethical Committee.

In Situ Hybridization

Human Ang-1 (570-bp) *SpeI-EcoRI* fragment was subcloned into *XbaI/EcoRI* of pKS⁺ transcription vector. *In vitro* transcription was carried out using T₃ for the generation of antisense cRNA and using T₇ for the generation of sense cRNA from plasmid linearized with *NotI* and with *EcoRI*, respectively. Human Ang-2 (640-bp) *EcoRI-HindIII* fragment was subcloned into *EcoRI/HindIII* of pKS⁺ transcription vector. *In vitro* transcription was carried out as described for Ang-1. Human Tie-2 (640-bp) *EcoRI-HindIII* cDNA fragment was subcloned into *EcoRI/HindIII* of pKS⁺ transcription vector. *In vitro* transcription was carried out using T₃ for the generation of antisense cRNA and using T₇ for the generation of sense cRNA from plasmid linearized with *EcoRI* and *HindIII*, respectively. The probes were diluted to a specific activity of 1×10^6 dpm/ml of plasmid template. *In situ* hybridization was carried out as previously described.¹⁹ Pretreated placental sections from first trimester (*n* = 7) and term (*n* = 9) were hybridized in hybridization buffer containing either [³⁵S]dUTP labeled sense or antisense probe. Hybridization was carried out in a humidified oven at 55°C overnight. After sequential washing in a high-stringency solution of 20 \times standard saline citrate, the slides were dried and dipped in photographic emulsion. The sections were developed after 6 weeks of storage at 4°C .

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from cultured cells and endometrial and placental tissues according to the method of

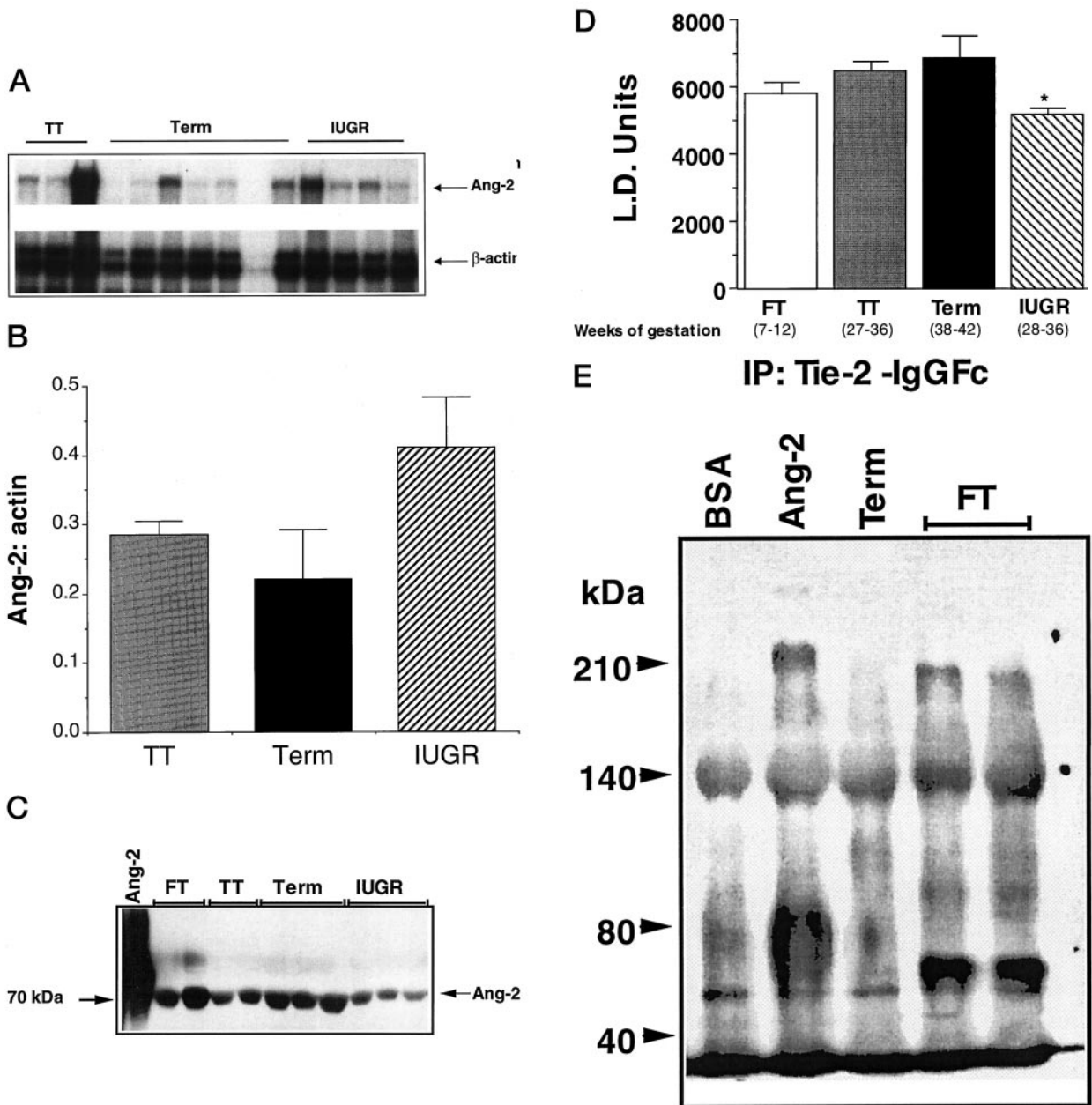


Figure 1. Quantification of Ang-2 mRNA and protein levels in IUGR and gestationally matched normal third trimester placenta. **A:** Identification of a 388-bp protected mRNA fragment for Ang-2 by RNase protection assay, using total placental RNAs from third trimester (TT) ($n = 3$), term ($n = 7$), and IUGR ($n = 4$). The abundance of mRNA for actin is also shown for each sample for comparison of RNA amounts. **B:** Graphical representation of Ang-2 mRNA levels. The ratio of Ang-2 to actin was calculated from the protected band intensities as assessed by laser densitometric analysis. Levels of Ang-2 were not significantly increased in severe IUGR as compared to gestationally matched third-trimester placenta. **C:** A representative Western blot analysis of Ang-2 protein in human placenta. A single major band of 70 kDa, corresponding to recombinant Ang-2 protein, was detected by the monoclonal anti-Ang-2 antibody. (1:2500). **D:** Laser densitometric analysis of Ang-2 protein expression in the human placenta. Data are presented as laser densitometric (LD) units of band intensity from Western blots of placental protein from first trimester (FT) ($n = 4$), third trimester (TT) ($n = 4$), term ($n = 5$), and IUGR ($n = 5$). Ang-2 protein levels demonstrate a significant decrease in placenta complicated by IUGR as compared to gestationally matched TT placenta. Statistical analysis of the intensity of bands was performed with Student's unpaired *t*-test. * $P < 0.05$ versus TT. **E:** Identification of Ang-2 in placental lysates. Total placental protein lysates (250 μ g/250 μ l) (FT and term) were immunoprecipitated overnight at 4°C with rTie-2-IgGFC-linked protein as described in Materials and Methods. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Ang-2 monoclonal antibody (1:2500). A specific 70-kDa band corresponding to recombinant Ang-2 protein was detected in first trimester (FT) placenta.

Chomczynski and Sacchi,²⁰ using TRIzol Reagent (Gibco BRL, Paisley, UK). RNA was reverse transcribed as previously described.²⁰ The erythroblastic leukemia tumor cell line (K562) was used as the positive control for Ang-1 and Ang-2.²¹ In addition, secretory endometrial tissues

collected from patients with a history of regular menstrual cycles undergoing hysterectomy for nonmalignant conditions and primary cultures of endometrial stromal cells generated from these tissues were also tested for Ang-1 and Ang-2. Ang-1 primers, reported previously,²² re-

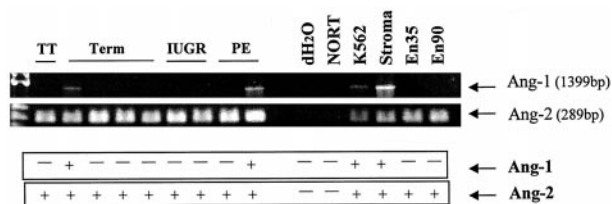


Figure 2. RT-PCR for Ang-1 and Ang-2 mRNA in placenta. Total RNA was extracted from third trimester (TT) ($n = 1$), term ($n = 4$), IUGR ($n = 2$), and preeclamptic (PE) ($n = 2$) placenta. Ang-1 primers detected a specific 1399-bp band in two of nine placental samples tested. The band detected corresponded to the erythroblastic leukemia tumor cell line (K562) used as the positive control. Primary cultures of endometrial stromal cells (stroma) and endometrial tissues (En35, En90) were also tested. Ang-2 primers detected a specific 289-bp band in all of the samples from placenta, endometrium, and cultured stromal cells corresponding to that detected in the positive control cell line K562.

sulted in the amplification of a 1399-bp product. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Ang-2 was performed using the upstream primer 5' GTCCACCTGAGGAAGTCT 3' and downstream primer 5' TTGTGACAGCAGCGTCTGTA 3', resulting in the amplification of a specific 289-bp product. The PCR reaction consisted of 35 cycles of denaturing temperature 94°C for 1 minute, annealing temperature 65°C for 2 minutes, extension temperature at 72°C for 3 minutes, and a further 4 minutes at 72°C.

Ribonuclease Protection Assay

Total RNA was isolated from snap-frozen placental tissue by homogenization in TRIzol reagent.²⁰ The human Ang-1 probe template consisted of a cDNA fragment in pBluescript KS⁺ (Stratagene, Amsterdam, The Netherlands), corresponding to nucleotides 240–804 of the sequence described by Davis et al.⁸ Ang-1 antisense RNA was transcribed by T₃ RNA polymerase after linearization with *Not*I, in the presence of [α -³²P]UTP (Amersham, Little Chalfont, UK), using standard methods, and with hybridization it protected a 570-bp fragment. The Ang-2 probe template consisted of nucleotides 1–635 of the sequence described by Maisonpierre et al,¹³ also in pBluescript KS⁺. Antisense RNA was transcribed by T₃ RNA polymerase after linearization with *Nco*I, and with hybridization it protected a 388-nucleotide fragment. The Tie-2 receptor probe protected 265 nucleotides after hybridization and was transcribed from the same construct used for *in situ* hybridization, after linearization with *Sty*I. Probes for human β -actin and 28S ribosomal RNA (28S rRNA) were used as internal standards. These were transcribed from the pTRI- β -Actin-125-human and pTRI-RNA-28S antisense control templates (Ambion, Witney, Oxon), respectively, with T3 RNA polymerase. When hybridized these probes protected 127 and 115 nucleotides, respectively. Probes for Ang-1, Ang-2, and Tie-2 (1.0 – 5.0×10^5 cpm), and β -actin or 28S rRNA (1.0 – 5.0×10^4 cpm) were combined with 10 μ g of total placental RNA and coprecipitated. Ribonucleotide (RNase) protection assays were then performed with the RNase protection assays II kit (Ambion) according to the manufacturer's instructions. Protected fragments were resolved on

6% denaturing polyacrylamide gel electrophoresis (PAGE) gels, transferred to Whatmann 3MM paper, and dried under vacuum in a gel drier. Autoradiography was performed using X-ray film (Kodak Biomax MR) with intensifying screens at -70°C for 2–3 days. After autoradiography, the intensities of protected species and their respective β -actin signals were quantitated using UVP Gelbase densitometry software.

Immunohistochemistry

Immunocytochemistry was performed as described previously.⁴ Briefly, serial 3- μm formalin-fixed, wax-embedded sections were deparaffinized by two incubations in xylene for 10 minutes, and endogenous peroxidase activity quenched by incubation in 0.3% hydrogen peroxide in methanol for 15 minutes. All subsequent incubations were carried out in the presence of excess serum (10% goat serum) to reduce nonspecific binding. Sections were incubated with either a monoclonal anti-Ang-1 or anti-Ang-2 antibody or a polyclonal anti-Tie-2 antibody (1:100 dilution in 10% goat serum) for 1 hour at room temperature. Amplification of primary antibody reaction was achieved by incubation of sections with goat anti-rabbit secondary antibody (Dako, High Wycombe, UK) for 30 minutes at room temperature, followed by a complex of streptavidin/biotinylated peroxidase (Dako) for a further 30 minutes at room temperature. Binding was visualized by incubation of sections in 0.5 mg/ml diaminobenzidine (Sigma), 0.1% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 minutes. Sections were counterstained with Mayer's Hemalum (Richard A. Lamb, London), dehydrated, and mounted. Control sections were performed in duplicate, where the primary antibody was replaced with nonimmune rabbit IgG or was omitted.

Immunoprecipitation and Western Blotting

Proteins were extracted from first-trimester ($n = 4$), third-trimester ($n = 3$), term ($n = 5$), and IUGR ($n = 5$) placental tissues and subjected to either immunoprecipitation and/or Western blot analysis as previously described.²³ Immunoprecipitation was carried out on placental tissue lysates and a first-trimester trophoblast ED₂₇ cell line, using either recombinant Tie-2-FC (rTie-2-FC) or polyclonal anti-Tie-2 antibody, respectively. For Western blotting, immunoprecipitates or 100 μ g of extracted protein were resuspended in 2 \times sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 minutes. Equal amounts of total protein (100 μ g) was separated on a 10% SDS-PAGE gel by electrophoresis and transferred to nitrocellulose membranes (Amersham) at room temperature overnight. Membranes were blocked with 1% milk fat (Premier Beverages, Staffordshire, UK) in Tween Tris-buffered saline (TTBS) (10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 0.1% Tween 20) for 6 hours at room temperature and washed in TTBS at room temperature for 15 minutes, and the wash was repeated twice for 5 minutes. Membranes were incubated with anti-Ang-2 antibody (1:

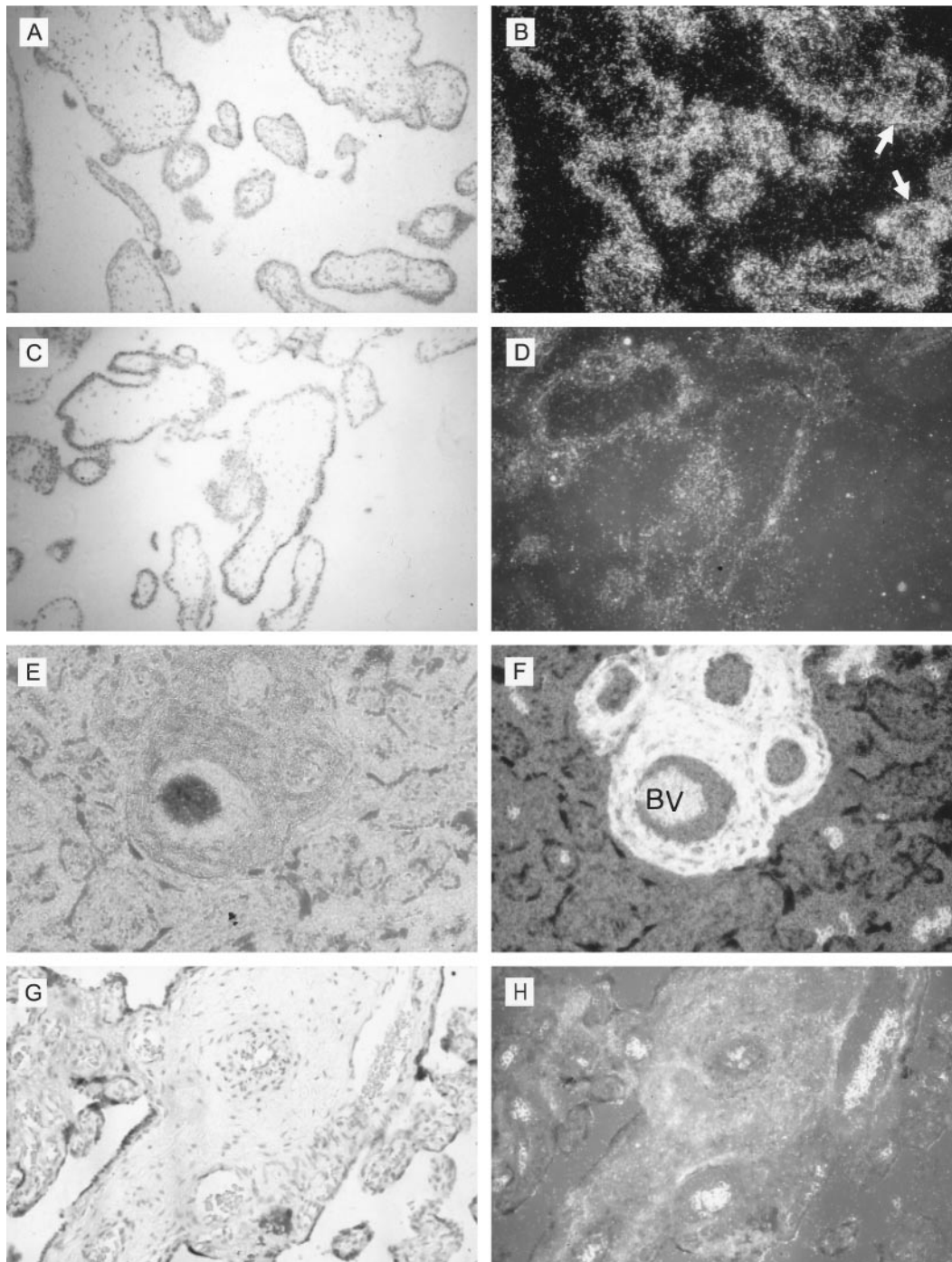


Figure 3. Localization of Ang-1 mRNA in early and late gestational placental villi. **A–D:** Photomicrographs showing the hybridization signal detected in first trimester placental villi (9 weeks) with an antisense ³⁵S-Ang-1 riboprobe. **A and B:** Intense hybridization signal localized to the syncytiotrophoblast and cytotrophoblast of the villi (**arrowhead**) in bright-field (**A**) and dark-field optics (**B**). **C and D:** The specificity of the signal was confirmed by incubating a serial section with ³⁵S-labeled sense strand. **E–H:** Localization of Ang-1 mRNA in term placenta. An intense hybridization signal was detected in the media of the large placental blood vessels (bv) (**E** and **F**). **G** and **H:** An adjacent section hybridized with the control sense cRNA probe, by bright-field (**G**) and dark-field (**H**) microscopy. Original magnification: ×200.

2500) at 4°C overnight. Membranes were washed and antibody reactions were detected using the ECL detection kit (Amersham), followed by detection of chemiluminescence on X-ray film. The intensity of detected bands was quantified by laser densitometry and presented as laser densitometric units (LD units).

Preparation and Stimulation of Cells

Spontaneously transformed first-trimester human cytotrophoblast-like (ED₂₇) and extravillous-like (ED₇₇) trophoblasts were generated by repeated passaging of trophoblasts obtained from chorionic villous sampling.²⁴ The

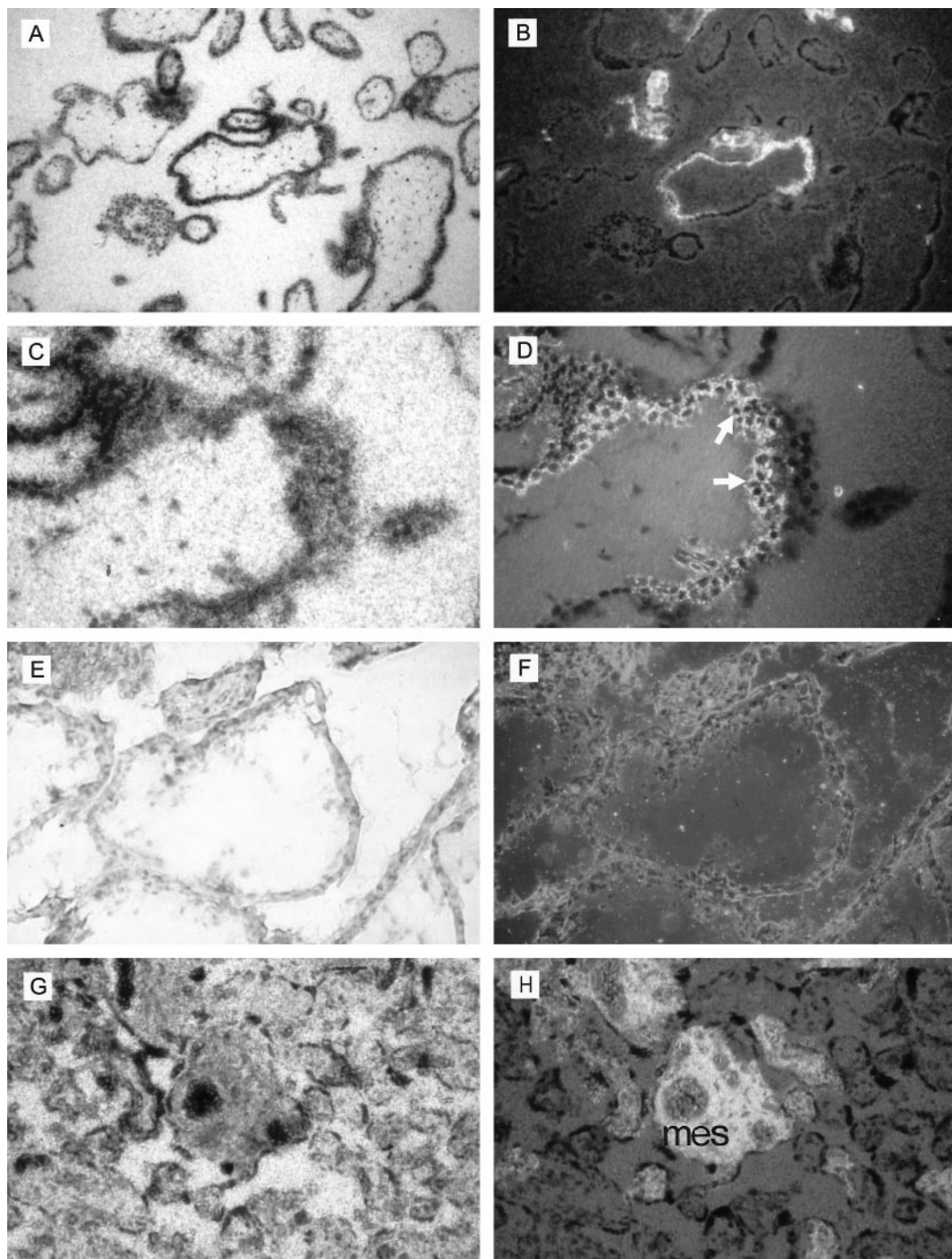


Figure 4. Localization of angiopoietin-2 mRNA in early and late gestational human placental villi. **A–F:** Photomicrographs showing the hybridization signal with antisense ^{35}S -Ang-2 riboprobe. An intense hybridization signal was seen only in the cytotrophoblast layer lining the villi in first trimester (7 weeks' gestation, **bold arrows**) in bright-field (**A**) and dark-field (**B**) optics. Higher magnification of this section is seen in **C** and **D** ($\times 400$). The specificity of the signal was confirmed by incubating a serial section with ^{35}S -labeled sense strand (**E** and **F**, $\times 400$). **G–H:** Localization of Ang-1 mRNA in term placenta. The Ang-1 hybridization signal was localized to the mesenchymal stromal core (MSC) of the placental stem villi with bright-field (**G**) and dark-field (**H**) optics. Original magnification: $\times 200$.

ED₂₇ cell line has been characterized and demonstrated to express the α and β subunits of human chorionic gonadotrophin, placental alkaline phosphatase, and cytokeratin peptide-8 but not vimentin and factor VIII.²⁵ The ED₇₇ cells exhibit an extravillous trophoblast-like characteristic of cellular migration and matrix metalloproteinase production.²⁶ Cells were maintained in 75-cm² flasks in a

1:1 mixture of Dulbecco's minimum essential medium (DMEM):Hams F12 (ICN, Oxford, UK), 15% fetal bovine serum, containing 1% L-glutamine, 5000 U penicillin, and 5 mg/ml streptomycin. Both cell lines were grown at 37°C, 5% CO₂ in a humidified incubator and routinely passaged when 90% confluent. For the *in vitro* assays cells were seeded in 24-well plates, at the densities indicated,

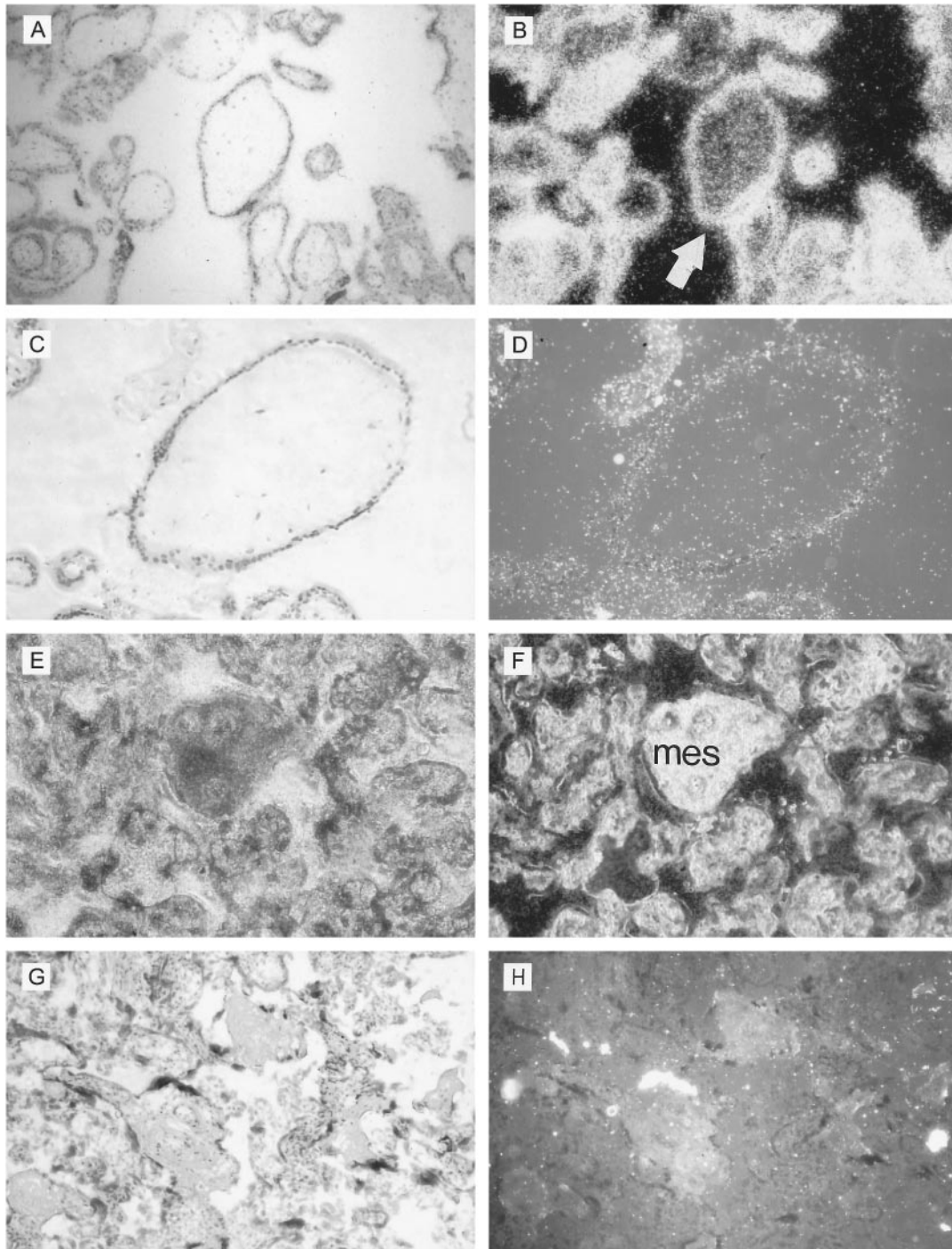


Figure 5. Localization of Tie-2 receptor mRNA in early and late gestational human placental villi. **A–D:** Photomicrographs showing hybridization signal detected by antisense ³⁵S-Tie-2 riboprobe probes in the first trimester (10 weeks' gestation) placenta. Intense Tie-2 signal was detected in the syncytiotrophoblast-cytotrophoblast bilayer of first-trimester villi in bright-field (**A**) and dark-field optics (**B**). Higher magnification of this section is seen in **C** and **D** (×400). **E–H:** Photomicrographs of Tie-2 mRNA in sections from uncomplicated term placentae. Positive hybridization signal was seen most intensely in cells within the mesenchymal stromal core (MSC) of the villous tissue (**E** and **F**). The fused syncytiotrophoblast/cytotrophoblast lining of the villi demonstrated weak Tie-2 hybridization. The specificity of the signal was confirmed by incubating a serial section with ³⁵S-labeled sense strand (**G** and **H**). Original magnification: ×200

in 15% fetal calf serum (FCS) DMEM/F-12, were allowed to attach overnight, and then quiesced for 24 hours in serum-free 0.2% bovine serum albumin (BSA) DMEM/F-12 before stimulation with agonist.

[³H]Thymidine Incorporation

First-trimester trophoblast ED₂₇ were seeded in 24-well plates in 15% FCS DMEM/F-12 at a density of 30,000

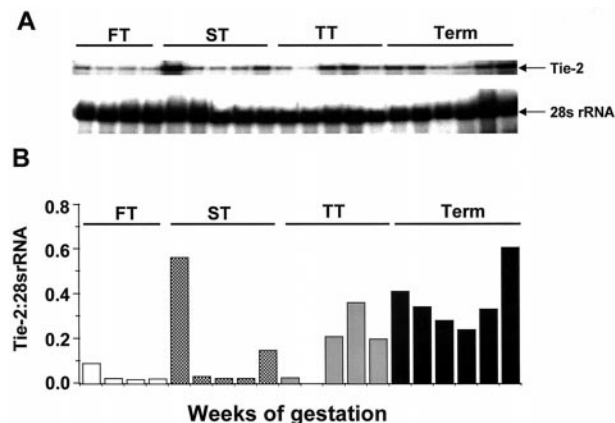


Figure 6. Quantification of Tie-2 mRNA gestational human placenta. **A:** Identification of a 265-bp protected mRNA fragment for Tie-2 by RNase protection assay, using total placental RNAs from first (FT) ($n = 4$), second (ST) ($n = 5$), and third (TT) ($n = 5$) trimester, as well as term ($n = 6$). The abundance of mRNA for 28S rRNA is also shown for each sample for comparison of RNA amounts. **B:** Graphical representation of Tie-2 mRNA levels. The ratio of Tie-2 to 28S rRNA was calculated from the protected band intensities as assessed by laser densitometric analysis. Levels of Tie-2 increased with increasing gestation.

cells/well. Cells were grown to 70–80% confluence and rendered quiescent by incubation for 24 hours in 0.2% BSA media. Stimulations were initiated by the addition of recombinant Ang-1 or Ang-2 in increasing concentrations (250–500 ng/ml) and incubated for 30 hours. During the last 6 hours of incubation with angiopoietins, cells were labeled with [methyl- ^3H]thymidine at 0.2 $\mu\text{Ci/ml}$ (Amersham). Cells were stimulated with 10 ng/ml VEGF₁₂₁ as a positive control because VEGF₁₂₁ binds exclusively to VEGFR-2 receptor and stimulates trophoblast proliferation.⁶ After completion of the incubation cells were washed with PBS, fixed in 5% ice-cold trichloroacetic acid, and washed with 100% ethanol. Cells were lysed in PBS, 0.2% BSA, 1% Triton X-100, and 1 mmol/L NH₄OH, and incorporated [^3H]thymidine was measured with a beta scintillation counter (Packard, Pangbourne, UK). Results are expressed as a mean of three independent experiments. Data were analyzed by unpaired Student's *t*-test.

Measurement of Nitric Oxide

First-trimester trophoblast ED₂₇ were seeded in 24-well plates in 15% DMEM/F-12 at a density of 250,000 cells/well. Cells were grown to 70–80% confluence and rendered quiescent by incubation for 24 hours in 0.2% BSA media. After a 24-hour serum starvation, the ED₂₇ cells were exposed to increasing concentrations (250–500 ng/ml) of Ang-1 or Ang-2 in serum-free culture medium for 30 minutes. VEGF₁₆₅ (10 ng/ml) was used as a positive control, as we have previously shown that VEGF₁₆₅ stimulates NO release from ED₂₇ cells.⁷ Reactions were terminated by removal of the supernatant that was subsequently centrifuged and stored at -80°C for NO analysis. Levels of NO were measured in the gas phase, using a Sievers NOA 280B chemiluminescence analyzer (Sievers, Boulder, CO), as previously described.⁷ Samples of

cell culture medium (100 μl) were injected into a nitrogen purge vessel containing a 1% solution of sodium iodide in glacial acetic acid to liberate gaseous NO from dissolved NO and nitrite. The sample gas was then exposed to ozone in the reaction vessel to form activated nitrogen dioxide (NO*), which was detected by a red-sensitive photomultiplier tube, and the output was recorded with an integrating pen recorder. For each sample, the area under the curve was converted to pmoles NO, using a calibration curve constructed from the analysis of a series of sodium nitrite standards. Results are expressed as the mean \pm SEM of three independent experiments in triplicate determination.

Chemotaxis Assay

Chemotaxis of trophoblast cells was performed in modified Boyden's chambers. Polycarbonate filters (8- μm pore size) were coated with 0.1% gelatin (Sigma) for 8 hours at room temperature. DMEM/F-12 medium containing 0.5% FCS and 100–200 ng/ml Ang-1 or Ang-2 or the vehicle alone was placed in the lower compartment of the chamber, and 2×10^5 cells suspended in the same medium were then seeded into the upper compartment of the Boyden's chamber. Incubation was carried out at 37°C for 6 hours. For inhibition studies, Ang-1 or Ang-2 was preincubated with rTie-2-FC for 30 minutes. At the end of the incubation period the upper surface of the filter was scraped with a rubber policeman. The filters were then fixed and stained with Diff-Quick (Harleco, Gibbstown, NJ), and 10 fields at $\times 200$ magnification were counted.

Results

Analysis of Ang-1/-2 mRNA and Protein Expression in Normal and IUGR Placenta

RNase protection assays using [^{32}P]UTP-labeled specific riboprobes were used to test the hypothesis that expression of Ang-1 or Ang-2 was altered in IUGR compared to gestationally matched normal placenta. Autoradiographic analysis demonstrated in all placental samples the presence of a 388-bp specific protected fragment corresponding to the size of the Ang-2 probe loaded as a control (Figure 1A). We were unable to detect any protected species corresponding to Ang-1 with this technique. No band was detected in the experimental yeast tRNA control (data not shown). Laser densitometric analysis of the Ang-2 and control β -actin bands demonstrated no significant change in the ratio of Ang-2 to β -actin in severe IUGR placenta as compared to gestationally matched third-trimester placenta (Figure 1B).

Semiquantitative immunoblot analysis of Ang-2 placental protein levels in gestational and IUGR placenta demonstrated a 70-kd band in all samples (Figure 1C). Laser densitometric analysis showed a small increase in Ang-2 protein levels throughout gestation from first trimester to term. Interestingly, in contrast to mRNA data, Ang-2 immunoprotein was significantly decreased in se-

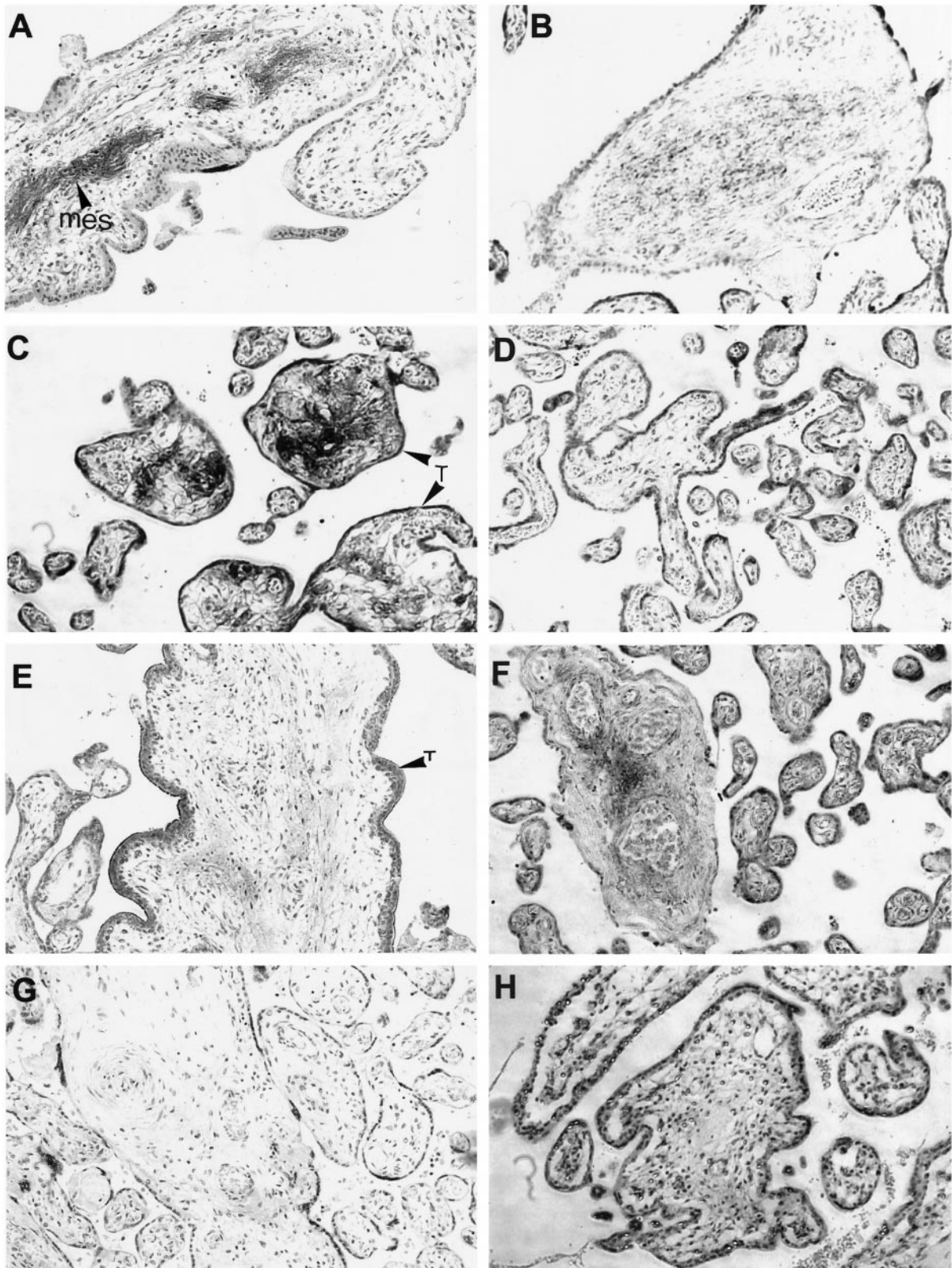


Figure 7. Immunohistochemical localization of Ang-1 and Ang-2 protein in the human placenta. Serial placental sections were incubated with monoclonal rabbit antibodies raised against Ang-1 and Ang-2 (1:100). **A** and **B**: Intense staining for Ang-1 protein is demonstrated by mesenchyme surrounding placental blood vessels in first trimester placenta. **C**: In the term placenta strong staining for Ang-1 is observed in the perivascular tissues of stem villi. **D**: No immunostaining for Ang-1 was observed in the terminal villi of human term placenta. **E**: Weak Ang-2 immunostaining was detected in the cytotrophoblast-syncytiotrophoblast bilayer of first-trimester placental villi. **F**: In the term placenta moderate immunostaining for Ang-2 was detected in the villous core and was associated with vascular tissues in the stem villi. Staining was also observed surrounding the capillaries of terminal villi. **G**: Omission of primary antibody. **H**: Negative control with nonimmune antibody. Original magnification: **A**, **B**, and **E**, $\times 400$; **C**, **D**, and **F**, $\times 100$; **G** and **H**, $\times 200$.

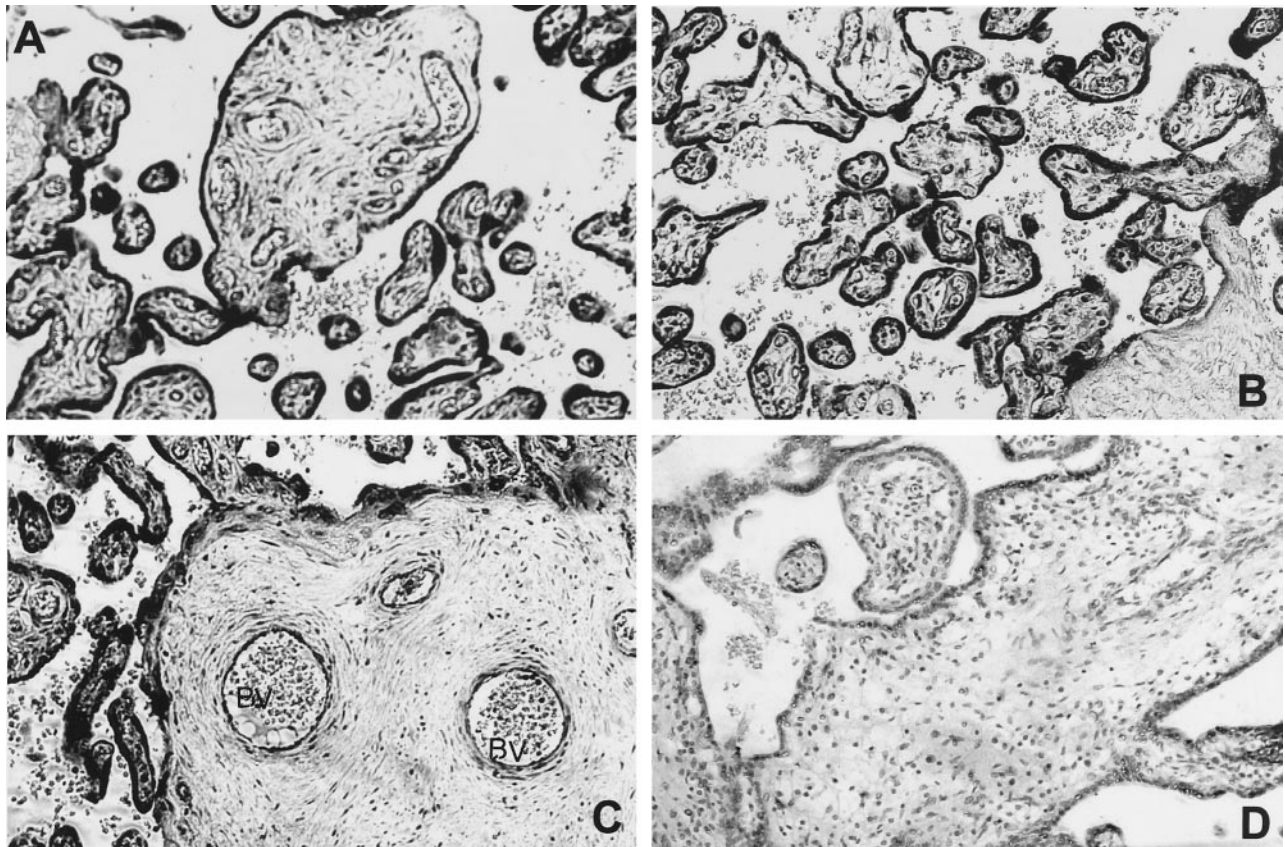


Figure 8. Immunohistochemical localization of Tie-2 receptor in the human placenta. Serial sections were incubated with a rabbit polyclonal anti-Tie-2 antibody (1:100). **A and B:** Intense immunostaining staining for Tie-2 receptor is demonstrated in the cytotrophoblast-syncytiotrophoblast bilayer of placental villi of term placenta. **C:** Intense immunostaining for Tie-2 is demonstrated in the cytotrophoblast and syncytiotrophoblast of first-trimester placenta. **D:** The endothelial cells of the placental blood vessels also display strong tie-2 immunostaining. The control is negative, with nonimmune antibody. Original magnification: **A and B,** $\times 100$; **C and D,** $\times 400$.

were IUGR as compared to gestationally matched third-trimester placenta (Figure 1D; $P < 0.03$, $n = 5$). To confirm the specificity of the Ang-2 antibody used in this study, a recombinant Tie-2 protein conjugated to the IgG-Fc region was used to immunoprecipitate Ang-2 from first-trimester and term placental protein lysates. The anti-Ang-2 antibody recognized a single specific major band of 70 kd corresponding to the positive control recombinant Ang-2 protein (Figure 1E).

RT-PCR Analysis of Ang-1 and Ang-2 mRNA in Human Placenta

RT-PCR analysis using Ang-1/-2-specific primers demonstrated a 1399-bp band corresponding to Ang-1 in two of nine third-trimester placental samples (Figure 2). Ang-1 was also detected in total RNA from primary cultures of endometrial stromal cell, and the erythroblastic leukemia tumor cell line K562 was used as the positive control (Figure 2). In contrast, Ang-2-specific primers detected a 289-bp band in all placental samples tested as well as in the tumor cell line, stromal cells, and uterine endometrial samples (Figure 2). No bands were detected in the negative controls.

In Situ Localization of Ang-1 and Ang-2 in Gestational Human Placenta

In first-trimester placenta ($n = 7$), the Ang-1 mRNA hybridization signal localized to both the cytotrophoblast and syncytiotrophoblast of the primary chorionic villi (Figure 3, A and B, arrows). At term ($n = 9$), Ang-1 mRNA hybridization was seen only in the surrounding perivascular tissues of the mature stem villi (Figure 3, E and F). The specificity of the signal was confirmed by incubating the adjacent section with ^{35}S -labeled sense Ang-1 (first-trimester, Figure 3, C and D; term, Figure 3 G and H).

In contrast to Ang-1, the Ang-2 mRNA hybridization signal localized only to the innermost cytotrophoblast layer in first-trimester tissues (Figure 4, A-D). The specificity of the signal was confirmed by minimal hybridization with sense probe (Figure 4, E and F). Like Ang-1, the highest levels of Ang-2 mRNA hybridization were detected in the perivascular and stromal tissues of the mature stem villi at term. However, some of the small intermediate and terminal villi also displayed Ang-2 mRNA hybridization in the mesenchymal core (Figure 4, G and H).

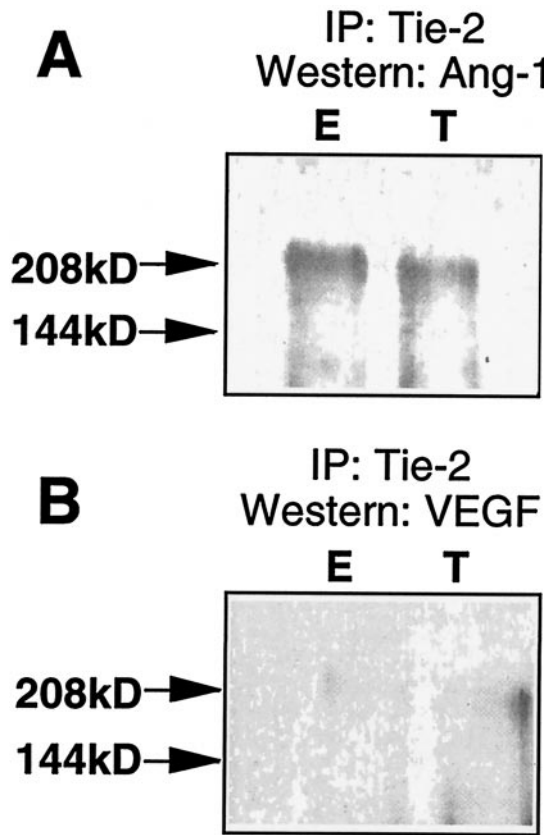


Figure 9. Identification of Tie-2 in a human first-trimester trophoblast cell line. **A:** Proteins were extracted from serum-starved HUVECs and trophoblast cells (ED₂₇) and immunoprecipitated with anti-Tie-2 antibody (1 μg/100 μl) overnight at 4°C before Western blotting with anti-angiopoietin 1 (1:2000). Both endothelial (E) and trophoblast (T) extracts show a band of 208 kd corresponding to the Tie-2 receptor plus Angiopoietin 1. **B:** Cells were prepared and immunoprecipitated as in **A** before Western blotting with an anti-VEGF antibody (1:1000). No specific bands were observed in either endothelial or trophoblast cell extracts. The data shown are representative of three independent experiments.

Localization and Quantification of Tie-2 in Gestational Human Placenta

A very strong hybridization signal for Tie-2 mRNA was seen in the cytotrophoblast-syncytiotrophoblast bilayer of first-trimester placenta (Figure 5, A–D). Weaker Tie-2 mRNA hybridization was displayed by the endothelium of the placental blood vessels (Figure 5C). At term, a strong hybridization for the Tie-2 mRNA signal was observed throughout the mesenchymal stromal core of the villous tissue (Figure 5, E and F) and in the outer vasculosyncytiotrophoblast bilayer. The sense strand showed minimal hybridization (Figure 5, G and H). An RNase protection assay revealed that the Tie-2 receptor probe protected a 265-bp fragment in all placental samples that corresponded to the undigested Tie-2 probe (Figure 6A). Laser densitometric analysis of Tie-2 and control 28S rRNA bands demonstrated an increase in Tie-2 expression levels as gestation increased to term (Figure 6B).

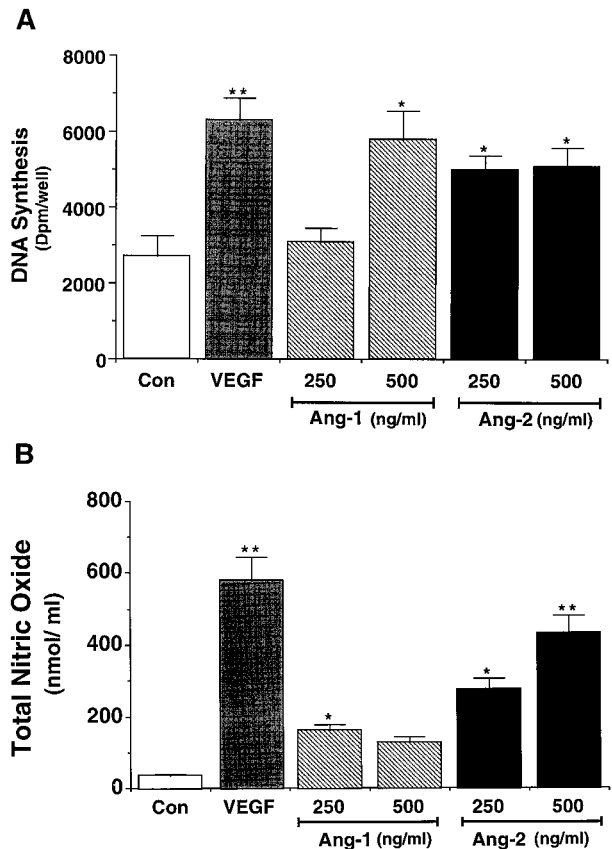


Figure 10. Effect of angiopoietins on DNA synthesis and NO release in a first-trimester trophoblast cell line. **A:** Addition of Ang-1 (hatched columns) or Ang-2 (black columns) at 250–500 ng/ml to quiescent ED₂₇ cells resulted in a significant increase in DNA synthesis, as assessed by incorporation of [³H]thymidine. VEGF₁₂₁ (10 ng/ml, gray column) was used as a positive control. **B:** Addition of Ang-1 (hatched columns) or Ang-2 (solid columns) to confluent ED27 cells for 30 minutes resulted in the release of NO into the culture media. VEGF₁₆₅ (10 ng/ml) was used as a release control. Conditioned medium was removed and assayed for NO release with a Sievers NOA 270B chemiluminescence analyzer. Data are represented as a mean ± SEM of three independent experiments and statistically tested using Student's unpaired *t*-test. **P* < 0.05; ***P* < 0.01 versus control; *n* = 3.

Immunolocalization of Ang-1, Ang-2, and Tie-2 in Gestational Human Placenta

Intense immunostaining for Ang-1 protein was detected in isolated cells within the villous mesenchyme and in the perivascular stroma around the blood vessels in first-trimester placental villi (Figure 7, A and B). In the term placenta, intense Ang-1 immunostaining was detected in the fibrosed stromal core and the media of mature blood vessels in primary placental stem villi (Figure 7C). In contrast, no Ang-1 immunostaining was detected in the terminal villi of term placenta (Figure 7D). Weak immunostaining for Ang-2 protein was detected in the cytotrophoblast-syncytiotrophoblast bilayer of first-trimester placental villi (Figure 7E). In the term placenta the immunostaining for Ang-2 localized to the villous mesenchyme and the media surrounding blood vessels in stem placental villi and the perivascular tissues of the smaller intermediate and terminal villi (Figure 7F). No immunostaining was detected in negative control, with either

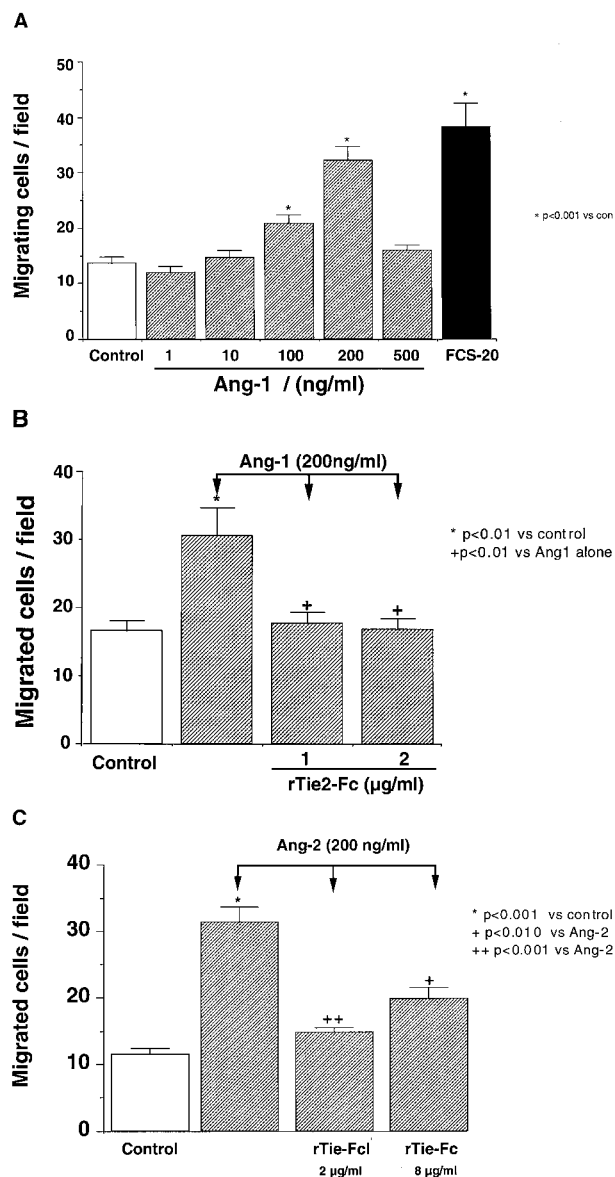


Figure 11. Effect of Ang-1 on trophoblast migration. Migration of ED₇₇ was observed with a modified Boyden chamber. Cells (2×10^5) were seeded in the upper compartment in 0.5% FCS. **A:** Increasing concentrations of Ang-1 were placed in the lower compartment. **B:** Ang-1 was premixed with recombinant Tie-2-FC for 30 minutes before its addition to the lower compartment. **C:** The effect of Ang-2 on trophoblast migration was assessed in a fashion similar to that of the analysis of Ang-1 in **B**. Cells that migrated across the polycarbonate filters in response to the vehicle alone or to Ang-1/Ang-2 were counted after a 6-hour incubation. Chemotaxis in response to FCS (20%) (black column) was used as a positive control. Results are expressed as the mean (\pm SEM) ED₇₇ counted per 10 fields ($\times 200$) in representative experiments performed in duplicate and quadruplicate, respectively. Five similar experiments were performed on different cell passages with similar results.

omission of primary antibody or use of nonimmune antibody (Figure 7, G and H).

Intense immunostaining for the Tie-2 receptor protein was detected in the syncytiotrophoblast of both primary and terminal placental villi in the term placenta (Figure 8, A and B). As expected, strong immunostaining for Tie-2 receptor was observed in the endothelial cells of the placental blood vessels (Figure 8C). No immunostaining

was observed on incubation of the sections with a non-immune rabbit IgG (Figure 8D).

Identification of Tie-2 in Cultures of First-Trimester Trophoblast

The localization studies demonstrated that in human placenta, trophoblasts, in addition to endothelial cells, express Tie-2 receptor. Western blot analysis of Tie-2 immunoprecipitates, using a monoclonal anti-Ang-1 antibody, demonstrated a band of approximately 208 kd in both trophoblast and endothelial cell lysates, corresponding to the reported molecular mass of Tie-2 (140 kd)^{16,17} plus Ang-1 (70 kd)⁹ (Figure 9A). Blots probed with a polyclonal anti-VEGF antibody showed no band, thus confirming the specificity of the Tie-2 antibody used for immunoprecipitation (Figure 9B).

Effect of Ang-1 and Ang-2 on Trophoblast Proliferation

To investigate the potential role of Ang-1/2 on trophoblast function, the effect of these factors on cell proliferation was assessed using [³H]thymidine incorporation as a measure of DNA synthesis. Stimulation of quiescent ED₂₇ trophoblast with Ang-2 (black bars) caused a significant increase in trophoblast [³H]thymidine incorporation at both 250 ng/ml and 500 ng/ml (Figure 10A). However, addition of Ang-1 (hatched bars) stimulated trophoblast DNA synthesis only at the higher concentration (Figure 10A). Addition of 10 ng/ml VEGF₁₂₁, used as a positive control, caused a doubling in DNA synthesis.

Effect of Ang-1 and Ang-2 on Trophoblast NO Release

We have previously reported that VEGF₁₆₅ stimulates trophoblast NO release.⁷ To determine whether Ang-1 or Ang-2 also stimulated the release of NO from trophoblast, we measured total NO as described in Materials and Methods. The addition of Ang-2 (250–500 ng/ml) caused a significant increase in NO levels in the ED₂₇ trophoblast cell line ($P < 0.05$, $n = 3$; Figure 10B). The addition of Ang-1 also stimulated an increase in NO release but to a lesser extent than did Ang-2 (Figure 9B). As expected, VEGF₁₆₅ (10 ng/ml) induced a marked increase in NO release that was greater than that induced by either Ang-1 or Ang-2 (Figure 10B).

Effect of Ang-1 and Ang-2 on Trophoblast Migration

Ang-1 has chemotactic properties for endothelial cells.¹¹ To determine whether Ang-1 or Ang-2 acts as a migratory stimulus for trophoblast, the first-trimester extravillous-like ED₇₇ trophoblast cell line was exposed to Ang-1 or Ang-2, and migration was assessed. Ang-1 induced a dose-dependent migration of ED₇₇ trophoblast (Figure

11A). The specificity of the chemotactic effect of Ang-1 was demonstrated by the inhibitory effect of rTie-2-FC on Ang-1-induced migration (Figure 11B). Ang-2 (200 ng/ml) also stimulated trophoblast migration in a manner that was attenuated by preincubation of Ang-2 with rTie-2-FC (Figure 11C). ED₂₇ trophoblasts showed little migration in response to Ang-1 or Ang-2 (data not shown).

Discussion

The present study identifies the sites of expression for Ang-1, Ang-2, and Tie-2 receptor in the human placenta throughout gestation. In first-trimester placentas Ang-1, Ang-2, and Tie-2 colocalized to the trophoblasts, suggesting that the angiopoietins may play an autocrine role in trophoblast function. Indeed, Ang-2 stimulates an increase in trophoblast DNA synthesis and the release of NO, whereas Ang-1 acted as a potent chemotactic factor for trophoblasts. At term, Ang-1 expression was restricted to the perivascular stroma of stem villi surrounding the large blood vessels, supporting the hypothesis that Ang-1 plays a role in maturation and maintenance of the placental vessels in late gestation. In contrast, Ang-2 was expressed by the perivascular stroma of all placental villi. Furthermore, this study shows that levels of Ang-2 protein were significantly reduced in IUGR placentas and suggests that this decreased expression may therefore contribute to the reported poor angiogenesis within the intermediate and terminal villi associated with severe IUGR.^{14,27}

Fetal growth restriction is characterized by a small placenta demonstrating decreased cytotrophoblast proliferation and increased syncytial knot formation, along with poorly developed vasculature in the terminal villi, leading to erythrocyte congestion and fetal hypoxia.^{14,27} We have previously hypothesized that altered levels of locally acting angiogenic growth factors may contribute to the morphological features of severe IUGR.²⁸ The present study shows that Ang-2 protein levels were markedly decreased in severe IUGR as compared to gestationally matched third-trimester normal placentas. As Ang-2 is known to act as an antagonist for Ang-1 in endothelial cells,¹³ the present study suggests that the decreased levels of Ang-2 in IUGR may permit premature maturation of the vessels, thus contributing to the abnormal development of the terminal villous vasculature. In addition to the poor angiogenesis, IUGR terminal villi demonstrate a thickened basal lamina and increased deposition of collagens and laminin.¹⁴ Interestingly, Ang-2 inhibits trophoblast-derived laminin production (unpublished data), supporting our hypothesis that decreased Ang-2 may contribute to the failure of adequate terminal villous formation. Although Ang-2 protein was decreased in IUGR, mRNA levels as detected by RNase protection assay remained unchanged compared to gestationally matched third-trimester normal placenta, suggesting a potential failure of translational regulatory mechanism in IUGR.

First-trimester placental development is dominated by vasculogenesis and branching angiogenesis.¹ Expres-

sion of mRNA encoding for Ang-1 and Tie-2 receptor in the first trimester over the syncytiotrophoblast bilayer suggests an autocrine role for the Ang-1 in trophoblast function. In contrast, immunoreactive Ang-1 localized to the core of primary chorionic villi, suggesting that trophoblast-derived Ang-1 was secreted to this site for paracrine interaction with the developing blood vessels of first-trimester placenta. Interestingly, Ang-2 mRNA transcript and immunoprotein were restricted to the cytotrophoblast layer of the first-trimester placental villi. The pattern of Ang-2 expression colocalizing with that of VEGF⁴ and fibroblast growth factor (FGF)¹⁹ during the period of vascularization of the early placental villi supports the hypothesis that Ang-2 may interact with VEGF by blocking a constitutive stabilizing or maturing function for Ang-1 on placental blood vessels.⁹ This would thus permit the placental vessels to remain in a plastic state, so that they can respond to the sprouting signal of VEGF and FGF.

The villous vascular growth undergoes a change from branching to nonbranching angiogenesis from 26 weeks' gestation until term.²⁹ This is due to the formation of the mature intermediate and terminal villi that specialize in gas exchange. In term placenta, where angiogenesis is complete, expression of Ang-1 and Ang-2 mRNA expression was very low in the trophoblastic villous membrane, but they were highly expressed in the perivascular and stromal tissue surrounding the large blood vessels of the stem villi. These findings contrasted with the RNase protection assay analysis that detected mRNA encoding for Ang-2 in third-trimester placenta but did not detect Ang-1. RT-PCR analysis using specific Ang-1 primers detected a band in only two of nine placental samples obtained from term deliveries. Immunolocalization studies, however, confirmed the *in situ* hybridization data showing that Ang-1 protein was restricted to the larger stem villi and was associated with the core surrounding the mature placental vessels. Clearly, the lack of Ang-1 mRNA detection with the RNase protection assay and RT-PCR is likely to be due to variation in sampling and extraction procedures.

In the present study, the relatively high level of expression of Ang-1 in the media of stem villous vessels at term is consistent with its reported role in vessel maturation and stabilization.⁹ Formation of a mature stem villous (trunk of the placental tree) involves concentration of contractile cells around the central lumina of the immature stem villi, acquiring the full spectrum of cytoskeletal antigens, including smooth muscle actin.³⁰⁻³² As Ang-1 is reported to be a chemoattractant,^{10,11} it further supports our contention that its specific expression around the central lumina of the immature stem villi contributes to the maturation of the placental vascular tree.

The current study shows that in contrast to Ang-1, Ang-2 was detected in the endothelium and mesenchyme of the smaller capillaries of the intermediate and terminal villi. The capillaries within these villi grow in excess of the trophoblastic sheath a result of a net decreased trophoblast and increased endothelial proliferation,²⁹ leading to coiling and bulging of the capillaries through the trophoblastic surface, forming the balloon-

like structures of the terminal villi.¹ The association of Ang-2 with these capillary structures, together with the observation that it acts as an antagonist of the Ang-1 effect on endothelium,¹³ suggests that it may play a role in the formation and maintenance of these specialized nonmuscularized structures by preventing vessel maturation.

This study showed that Tie-2 receptor mRNA and protein localized to the endothelium of the placental blood vessels and vasculosyncytial trophoblast membrane of both first-trimester and term placental villi. The perceived gestational increase in the expression of placental Tie-2 mRNA observed by *in situ* hybridization studies was quantitatively confirmed by RNase protection assay. In addition, the hybridization signal for the Tie-2 receptor mRNA was detected within the mesenchyme of first-trimester chorionic villi. The Tie-2 synthesis by the vascular and mesenchymal tissues in first-trimester placenta may reflect the competence of the developing endothelial cells to respond to an angiogenic stimulus. This is consistent with earlier ultrastructural studies identifying the precursors of fetal endothelium in early fetal villi as mesenchymally derived hemangioblastic cell cords.³³ Moreover, the persistent expression of Tie-2 in term placenta, where endothelial cell turnover is low, favors a role for Ang-1 in the maintenance of an endothelium-specific phenotype.

In contrast to endothelial cells, our study for the first time demonstrates that the angiopoietins and Tie-2 are coexpressed on trophoblasts, suggesting an autocrine function for angiopoietins in these cells. These findings were confirmed by the identification of immunoreactive Tie-2 receptor in immunoprecipitates from both cultured endothelial and trophoblast cells. This is supported by our findings that Ang-2 and, to a lesser extent, Ang-1 stimulate DNA synthesis and NO release from cytotrophoblast-like cells (ED₂₇), suggesting that Ang-2 in particular, like VEGF,⁷ contributes to the maintenance of vascular homeostasis and the regulation of trophoblast growth. Conversely, Ang-1 and, to a lesser extent, Ang-2 promoted migration of the ED₇₇ extravillous trophoblast-like cells, as assessed with the Boyden chamber assay. The demonstration that cultured endometrial stromal cells expressed Ang-1 mRNA suggests that Ang-1 produced by the decidua may act as a chemoattractant to promote extravillous trophoblast migration and invasion during the remodeling of the spiral arteries to establish uteroplacental circulation in pregnancy. Although Ang-2 has no effect on endothelial Tie-2 receptor, clearly trophoblasts are able to respond to both Ang-1 and Ang-2. Similarly, fibroblasts stably transfected with Tie-2 receptor exhibit chemotactic responses to both Ang-1 and Ang-2.¹¹

In conclusion, the findings presented here that Ang-1 and Ang-2 display differential temporal and spatial expression implies an important role for this growth factor system in the development and differentiation of the placental villous tree. Moreover, as Ang-2 was demonstrated to act as an endothelial antagonist for Ang-1,¹³ the present study suggests that the decreased levels of Ang-2 in IUGR placenta may permit premature maturation of the vessels, thus contributing to the abnormal

development of the terminal villous vasculature. Finally, the presence of functional Tie-2 receptor on trophoblast demonstrates a specific role for angiopoietins as regulators of trophoblast behavior in the development of the uteroplacental circulation, an action independent of their well-established roles in vascular endothelium.

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