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Severe Intrauterine Growth Restriction Pregnancies Have Increased Placental Endoglin Levels

Hypoxic Regulation via Transforming Growth Factor-β3

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Endoglin, a co-receptor for transforming growth factor (TGF)-β_1 and -β_3 is expressed in the human placenta and plays an important role in the pathogenesis of preeclampsia. Because preeclampsia is associated with hypoxia, and because TGF-β3 is overexpressed in preeclamptic pregnancies, we examined the effect of oxygen and TGF-β3 on placental endoglin expression and investigated its expression in pathological models of placental hypoxia such as intrauterine growth restriction (IUGR) pregnancies. Endoglin expression was high at 4 to 9 weeks of gestation, when oxygen tension is low, and decreased after 10 weeks, when oxygen tension increases. Exposure of villous explants to low oxygen (3% O_2) resulted in elevated expression of both membrane and soluble endoglin compared to standard conditions (20% O_2). Moreover, addition of TGF-β_3 to villous explants under low oxygen conditions increased the expression of endoglin compared to nontreated explants whereas addition of TGF-β_3-neutralizing antibodies inhibited the low oxygen stimulatory effect on endoglin expression. Endoglin and soluble endoglin expression were significantly increased in placentas of IUGR singletons compared to controls and in the IUGR twin placentas relative to both the control co-twin and the normal twins. These data demonstrate that oxygen regulates the placental expression of endoglin via TGF-β_3. Reduced placental perfusion leading to placental hypoxia might contribute to the increased expression of endoglin in IUGR pregnancies. ***(Am J Pathol 2008, 172:77–85; DOI: 10.2353/ajpath.2008.070640)***

Intrauterine growth restriction (IUGR), defined as failure of the fetus to achieve its genetically determined growth potential, complicates 4 to 7% of births and is linked to a 6- to 10-fold increased risk of perinatal mortality.[1,2] Fetal growth restriction may be caused by a variety of conditions, such as infections, maternal diseases, and chromosomal disorders, but most often is related to a defect in proper placental development that originates early in pregnancy.[3] Low placental oxygenation is believed to play a pivotal role in the development of IUGR based on observations indicating increased expression of genes regulated by hypoxia in placentas of IUGR pregnancies.[4,5] Fetal hypoxia, which can either lead to impaired growth or just be associated with it,[6] may result from shallow trophoblast invasion in the decidua and myometrium and failure to invade the spiral arteries, a key requirement to establish an efficient utero-placental circulation.[7] Low placental oxygenation is also associated with other placental pathologies such as preeclampsia.[8] However, the pathways linking hypoxia to these placental pathologies remain to be elucidated. We have reported that low oxygen increases transforming growth factor (TGF)-β_3 expression in villous explants[9] and that TGF-β_3 is overexpressed in preeclamptic placentas.[10] Moreover, antisense inhibition of TGF-β_3 in villous explant cultures restores the invasive capabilities of trophoblast cells.[10] Endoglin (Eng), a co-receptor for TGF-β_1 and -β_3, is highly expressed on cell membranes of vascular endothelium and syncytiotrophoblasts.[11,12] The function of Eng in vascular morphogenesis has been demonstrated

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in knockout mice whose embryos die at midgestation because of vascular and cardiac abnormalities.[13,14] In addition, mutations in the gene encoding Eng are the underlying cause of hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant disorder characterized by arteriovenous malformations and focal loss of capillaries.[15] We have previously reported that Eng is a negative regulator of trophoblast differentiation.[16] These findings raised the possibility that inappropriate expression of Eng might contribute to preeclampsia, which is associated with abnormal trophoblast invasion. Recent studies have indicated that Eng expression is elevated in preeclamptic placentas, which is accompanied by high circulatory levels of its soluble form, an N-terminal cleavage product of the full-length protein.[17,18] The administration of soluble endoglin (sEng) with soluble VEGF receptor 1 (sFlt1), which has also been found to be elevated in preeclamptic pregnancies,[19] to pregnant rats leads to preeclampsia-like disease, suggesting that sEng may act in concert with sFlt1 to induce this syndrome.[17] Although Eng might play a key role in the pathogenesis of preeclampsia, its regulation in the human placenta and expression in other hypoxic placental pathologies such as IUGR is unknown. Herein, we examined the effect of low oxygen and TGF-β_3 on placental Eng expression and investigated Eng expression in physiological (early gestation) and pathological (IUGR singletons and discordant twins) models of placental hypoxia.

Materials and Methods

Tissue Collection

Local ethics committee approvals were obtained for the study, and all women gave written informed consent. The tissues were collected at Mount Sinai Hospital, Toronto, Canada, and at Ospedale Infantile Regina Margherita S.Anna Hospital, Turin, Italy. All women were healthy nonsmokers, with no signs of preeclampsia, infections, or other known causes of IUGR. The clinical characteristics of the singleton pregnancies are shown in Table 1. Gestational age was determined by the date of the last menstrual period and first trimester ultrasound measurement of crown-rump length. IUGR was defined as birth weight below the fifth percentile accompanied by abnormal umbilical artery Doppler defined as absence or reverse of end diastolic velocity and in most of cases with increased resistance to flow in uterine arteries defined as early diastolic notch or pulsatility index >1.45. None of the neonates had evidence of infection, anomalies, or abnormal chromosomes. Umbilical blood gas analysis at delivery of the IUGR cases demonstrated low oxygen tension indicating impaired oxygenation of these IUGR fetuses. The mean arterial and venous pO_2 was 13 ± 4.64 mmHg and 24.2 ± 4.55 mmHg, respectively. Controls were selected as age-matched and term healthy pregnancies with normal grown fetuses that did not have signs of placental dysfunction. Data regarding maternal characteristics, mode of delivery, birth weight, gestational age, laboratory values, and ultrasound findings were collected from the clinical records. Patients suffering from diabetes, essential hypertension, or renal disease were excluded. The preterm control group included only spontaneous preterm deliveries without an identifiable cause. All preterm and term control groups did not show clinical or pathological signs of preeclampsia, infections, or other maternal or placental disease. Specimens were randomly collected immediately after delivery from normal-looking cotyledons. Areas with calcified, necrotic, or visually ischemic tissue were omitted from sampling. The samples were stored at −80°C. All placentas from preterm deliveries were examined by a perinatal pathologist, and cases that had histological evidence of chorioamnionitis were excluded.

Discordant twins were selected as a separate group in which a normal twin and a growth-restricted twin developed in the same maternal and uterine environment. Discordant growth was defined as discordancy of more than 25% in birth weight in which the small twin's birth weight was below the fifth percentile and had evidence of absence or reverse of end diastolic velocity in the umbilical artery, and the normal co-twin was appropriate for gestational age with normal Doppler studies. The control group included normal twins without discordancy and without signs of growth restriction in either of the twins. None of the twin pregnancies in all groups had evidence of twin-to-twin transfusion syndrome and none of the twins had evidence of infection, anomalies, or abnormal chromosomes. Chorionicity was determined by first tri-

Table 1. Clinical Characteristics of Participating Singleton Pregnancies

Maternal/fetal characteristics	TC (n = 12)	PTC (n = 18)	Severe IUGR (n = 18)
Maternal age (yr)	30.8 ± 5.6	31.5 ± 6.3	29.7 ± 5.8
Gestational age (wk)	39 ± 1.1	30.1 ± 2.7	30.2 ± 2.4
Gravidity/parity (mean)	2/1	2/0	2/1
Birth weight (gm)	3325 ± 430	1361 ± 470	920 ± 276
Birth weight centile	41.6 ± 18	33.1 ± 24.6	3.2 ± 1.8
A/REDV (% of women)	0	0	100
Bilateral abnormal uterine artery Doppler (% of women)	0	0	70
Mode of delivery (no.)			
CS	8 (67%)	8 (44%)	18 (100%)
VD	4	10	0

Values are mean ± SD.
A/REDV, absence/reverse diastolic velocity in the umbilical artery; CS, cesarean section; VD, vaginal delivery.

Table 2. Clinical Characteristics of Participating Twin Pregnancies

	Control twins (n = 8)		Discordant DCDA (n = 4)		Discordant MCDA (n = 5)	
Maternal/fetal characteristics	Twin A	Twin B	IUGR	Cotwin	IUGR	Cotwin
Maternal age (yr)	31 ± 6		35.7 ± 4.1		38.0 ± 6.2	
Gestational age (wk)	34.7 ± 2.3		32.3 ± 1.9		34.6 ± 1.5	
Gravidity/parity	2/1		2/1		3/2	
Birth weight (gm)	2325 ± 215	2410 ± 200	1005 ± 358	1631 ± 374	1484 ± 427	2282 ± 487
Birth weight centile	31.2 ± 16.5	33.7 ± 18.9	1.2 ± 0.5	25.0 ± 10.8	2.8 ± 2.05	31.0 ± 13.9
Discordancy (%)	5.7 ± 1.5		39.0 ± 15.4		36 ± 8	
A/REDV (%)	0	0	100	0	100	0
Mode of delivery						
CS	3		4		4	
VD	2		0		1	

Values are mean ± SD.
A/REDV, absence/reverse diastolic velocity in the umbilical artery; CS, cesarean section; DCDA, dichorionic/diamniotic twins; MCDA, monochorionic/diamniotic twins; VD, vaginal delivery.

mester ultrasound scan and confirmed after delivery by histopathological examination of the placenta and membranes. The placentas of the monochorionic twins were sampled from the area of the cord insertion of each twin and lateral to it, avoiding the territory between the cord insertions to reduce the chances of sampling from shared cotyledons. The clinical characteristics of the twin pregnancies are shown in Table 2.

First Trimester Villous Explant Culture

Villous explant cultures were established from first trimester human placentas (5 to 8 weeks of gestation) obtained from elective terminations of pregnancies by dilatation and curettage. Villous explant cultures were established as described previously.[16] To explore O_2 effects, explants were maintained in either standard condition of 20% O_2 (5% CO_2 in 95% air) or in an atmosphere of 3% or 8% O_2 (5% CO_2 in 92% or 87% N_2, respectively) for 48 hours at 37°C. Six sets of explants from six different placentas were used. To validate the effect of low oxygen on villous explants, we performed Western blot analysis using rabbit polyclonal antibody to HIF-1α (no. 3716; Cell Signaling Technology, Danvers, MA). In separate experiments, villous explants, prepared from placentas of 5 to 8 weeks of gestation, were incubated under different oxygen tensions (3% O_2, 8% O_2, and 20% O_2) in the presence or absence of TGF-β_3 or TGF-β_1 (5 ng/ml). Five sets of explants from five different placentas were used for these experiments. In addition, villous explants were incubated at 3% and 20% O_2 with or without neutralizing antibodies to TGF-β_3 (10 μg/ml). For each experimental condition a minimum of three explants harvested from the same placenta were used.

RNA Isolation and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (qPCR)

Total RNA, extracted from placental tissues, was treated with DNase I to remove genomic DNA contamination After column purification (Qiagen, Mississauga, Canada), 1 μg of total RNA was reverse transcribed using random hexamers (Applied Biosystems, Inc., Foster City, CA). The resulting templates (50 ng of cDNA for Eng and 5 ng for 18S) were quantified by real-time PCR (MJ Research Inc., Waltham, MA) as previously described.[20] TaqMan Universal MasterMix and specific TaqMan primers and probe for Eng and 18S were purchased from Applied Biosystems, Inc. (Assay ID HS00164438_m1). Relative quantification of data were performed using logarithmic curves. Expression level of Eng was normalized based on 18S expression using the 2$\Delta\Delta$Ct formula as previously described.[20]

Western Blot Analysis

Western blot analysis for Eng was performed using 50 μg of total placental protein lysates that were subjected to 8% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Nonspecific binding was blocked by incubation in 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 60 minutes. Membranes were then incubated with 1:1000 diluted rabbit polyclonal antibody to human Eng (Santa Cruz Biotechnology, Santa Cruz, CA) in 5% milk in TBST at 4°C. After overnight incubation, membranes were washed with TBST and incubated for 60 minutes at room temperature with 1:5000 diluted horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) in 5% milk in TBST. After washing with TBST, blots were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Oakville, Canada).

Immunohistochemistry

Paraffin sections were mounted on glass slides, dewaxed in xylene, and rehydrated in descending ethanol gradient. Antigen retrieval was performed by heating in 10 mmol of sodium citrate solution. Endogenous peroxidase was quenched with 3% (v/v) hydrogen peroxide in phosphate-buffered saline (PBS) for 30 minutes. After block-

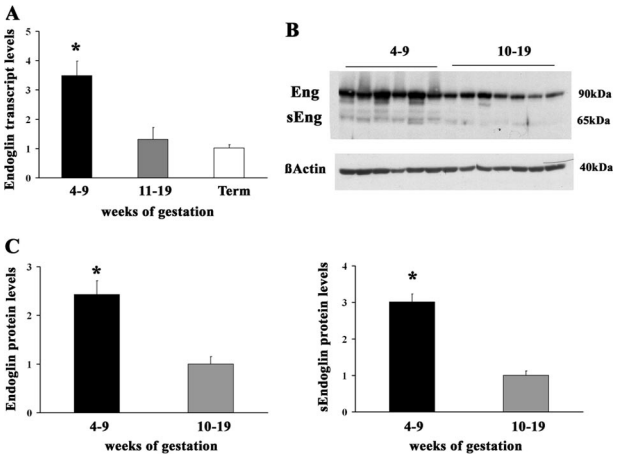

Figure 1. Eng expression during placental development. **A:** Expression of Eng mRNA in early first trimester placental samples versus late first and second trimester and term placental samples as assessed by qRT-PCR (4 to 9 weeks, $n = 7$; 11 to 19 weeks, $n = 6$; term, $n = 4$); $^*P < 0.01$, 4 to 9 weeks versus 11 to 19 weeks and 4 to 9 weeks versus term. **B:** Representative Western blot analysis for Eng and sEng in first and second trimester placental samples. **C:** Eng (**left**) and sEng (**right**) protein densitometric analysis in placental tissues from early first trimester (4 to 9 weeks, $n = 7$) compared to late first and second trimester (11 to 19 weeks, $n = 7$), $^*P < 0.01$.

ing (5% normal goat serum for 1 hour), the slides were incubated overnight with 1:600 diluted primary antibody (H-300, SC-20632; Santa Cruz Biotechnology). Slides were washed in PBS and exposed to biotinylated secondary antibody (1:300, goat anti-rabbit; Vector Laboratories, Burlingame, CA) for 45 minutes at room temperature. Finally, avidin biotin complex (Vector Laboratories) was applied for 1 hour, and positive staining was detected with the diaminobenzidine chromogen. Slides were counterstained with hematoxylin. In negative controls, primary antibody was omitted and replaced by blocking solution. Six samples of IUGR, four samples of preterm and term control, and six pairs of discordant twins were used for immunohistochemistry.

Statistics

Statistical analyses were performed using GraphPad Prism software (San Diego, CA). For comparison of data between multiple groups one-way analysis of variance with posthoc Dunnett's test was used. For comparison between two groups Wilcoxon matched paired test and unpaired *t*-test were performed when applicable. Significance was defined as $P < 0.05$. Results are expressed as the mean ± SEM (SE).

Results

Placental Eng Expression Is Increased at the Early Stages of Pregnancy

Early placental development is marked by a switch in oxygen tension, which is low at 5 to 9 weeks and increases at 10 to 12 weeks.[21,22] To establish whether the expression of Eng is affected by this physiological change in placental oxygenation, we examined its expression in placentas from different stages of pregnancy. Eng mRNA expression was significantly higher in early first trimester placental samples (4 to 9 weeks of gestation) compared to samples from 10 to 19 weeks of gestation (3.48 ± 0.5 versus 1.3 ± 0.4, $P < 0.05$) and term placentas (3.48 ± 0.5 versus 1.02 ± 0.1, $P < 0.05$) (Figure 1A). Eng expression was not different between late first/second trimester and term placental samples. Quantitative PCR analysis of GAPDH, which is a housekeeping gene, showed no difference in its placental mRNA expression levels between early first trimester, late first/second trimester, and term gestation (1.084 ± 0.3, 0.8 ± 0.02, and 1.17 ± 0.6, respectively), excluding the possibility that early placentas are more transcriptionally active than the later first trimester and term placenta. Western blot analysis of placental lysates demonstrated increased expression of both Eng and its soluble form (sEng) in placental samples from 4 to 9 weeks of gestation compared to samples from 10 to 19 weeks of gestation (Figure 1B). Densitometric analysis revealed that both Eng and sEng protein levels were significantly increased in early first trimester samples compared to late first/second trimester samples (Eng: 2.43 ± 0.28-fold increase, $P < 0.01$; sEng: 3.01 ± 0.22-fold increase, $P < 0.01$) (Figure 1C).

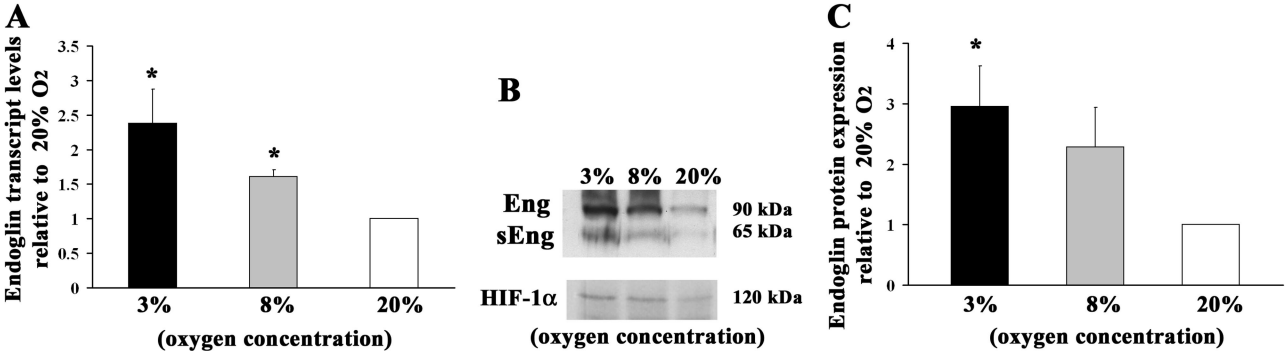

Figure 2. Effect of low oxygen on Eng expression in first trimester villous explants. **A:** Expression of Eng mRNA in explants cultured at 3%, 8%, and 20% O_2 measured by qRT-PCR ($n = 6$; $^*P < 0.05$, 3% versus 20%). **B: Top:** Representative Western blot of Eng and sEng shows full-length Eng (90 kDa) and sEng (65 kDa), which are highly expressed in first trimester villous explants exposed to 3% O_2 compared to 8% O_2 and 20% O_2; **bottom:** representative Western blot of HIF-1α. **C:** Eng protein densitometric analysis in first trimester villous explants exposed to different oxygen conditions, $n = 6$. $^*P < 0.05$, 3% versus 20%.

Low Oxygen Increases Placental Eng Expression

We next examined the effect of different oxygen conditions on Eng expression. Exposure of villous explants to 3% O_2 resulted in a significant increase in Eng message levels compared to explants kept at 20% O_2 (2.37 ± 0.5-fold, $P < 0.05$; Figure 2A). Exposure to 8% O_2 resulted in a trend toward increased Eng mRNA expression, although not statistically significant, compared to 20% O_2 (Figure 2A). Both Eng and sEng protein levels were greater in explants kept at 3% O_2 compared to those maintained at 20% O_2 (Figure 2, B and C). As anticipated, exposure of explants to low oxygen levels also increased HIF-1α protein levels (Figure 2B, bottom).

TGF-β_3 Increases Placental Eng Expression under Hypoxic Conditions

To study the effect of TGF-β_3 on Eng expression we performed a series of experiments in which explants were maintained overnight at 3% and 20% O_2 and then cultured in the presence or absence of TGF-β_3 for 24 hours. Addition of TGF-β_3 to explants exposed to 3% O_2 significantly increased Eng mRNA expression compared to explants maintained at 3% O_2 alone (4.1 ± 0.3-fold increase, $P < 0.05$) (Figure 3A). Addition of TGF-β_3 to explants cultured at 20% O_2 also increased Eng mRNA levels, but the effect was not significant (2.1 ± 0.5-fold increase, $P = 0.07$) (Figure 3B). Similar to the mRNA expression, addition of TGF-β_3 resulted in elevated Eng protein levels in explants cultured in 3%, but not 20%, O_2 when compared to explants maintained in 3% and 20% O_2 alone, respectively (Figure 3, C–E). In contrast to TGF-β_3, we found no significant difference in Eng mRNA and protein expression after TGF-β_1 treatment (data not shown). To examine whether the effect of low oxygen on the expression of Eng is mediated via TGF-β_3, we cultured explants maintained at 3% O_2 with neutralizing antibodies to TGF-β_3 for 24 hours. Addition of TGF-β_3-neutralizing antibodies inhibited the 3% O_2-induced expression of Eng (Figure 3, F and G). No changes were noted after addition of TGF-β_3-neutralizing antibodies to explants maintained at 20% O_2 (Figure 3, F and G).

Eng Expression Is Increased in Placentas of IUGR Pregnancies

We next examined the expression of Eng and sEng in placentas of IUGR singleton pregnancies with documented absence or reverse of end diastolic velocity in the umbilical artery and without signs of preeclampsia. Placentas from age-matched preterm deliveries (preterm control, PTC) and term deliveries (term control, TC) were used as controls. Quantitative PCR analysis revealed a significant increase in the number of Eng transcripts in IUGR placentas compared to PTC (3.15 ± 1.0 versus 1.05 ± 0.1, $P < 0.05$) (Figure 4A). In accordance with the qPCR results, Western blot analysis demonstrated increased levels of both Eng and its soluble form (sEng) in placental tissue of IUGR pregnancies compared to preterm and term controls (Eng: IUGR, 3.4 ± 0.4 versus PTC, 1.6 ± 0.2 versus TC, 1 ± 0.6, $P < 0.05$; sENG: IUGR, 2.29 ± 0.27 versus PTC, 0.93 ± 0.08 versus TC, 1 ± 0.14, $P < 0.05$) (Figure 4, B–D). No difference in Eng levels was noted between the IUGR cases with normal uterine Doppler and the ones with abnormal uterine Doppler (data not shown). Immunohistochemical analysis showed that Eng expression was primarily restricted to the trophoblast layer (see Figure 6A). In line with the protein data, strong positive immunoreactivity for Eng was observed in the IUGR placentas compared to the absent/low staining found in sections from placentas of preterm and term control. We also tested the expression of Eng in placentas of discordant twin pregnancies, in which one twin exhibited IUGR with birth weight below the fifth percentile for gestational age and abnormal umbilical artery flow, whereas the other twin was appropriate for gestational age. Normal twin pregnancies without discordancy

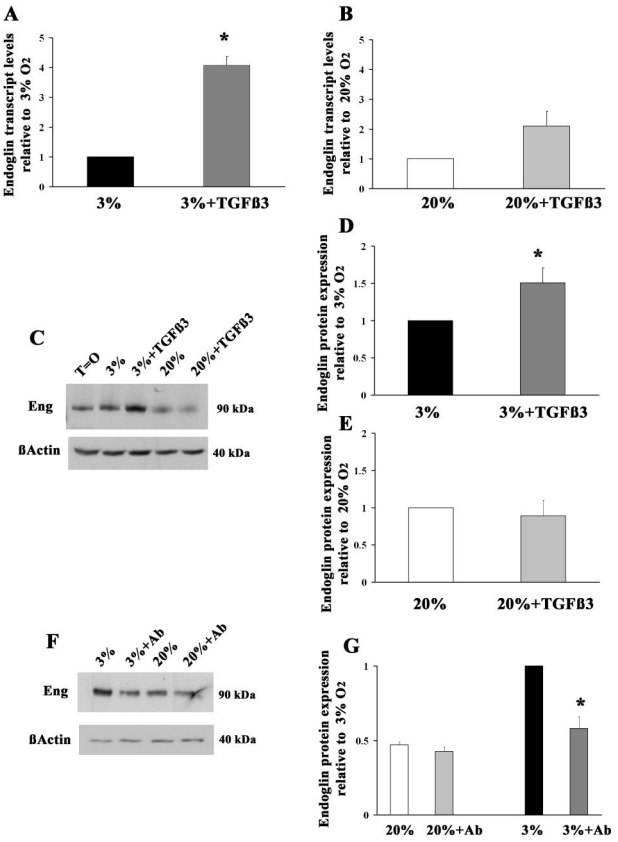

Figure 3. Effect of TGF-β_3 on Eng expression in first trimester villous explants exposed to different oxygen conditions. **A:** Eng mRNA levels in explants cultured at 3% oxygen with and without TGF-β_3 as measured by qRT-PCR ($n = 3$, $^*P < 0.05$). **B:** Eng mRNA levels in explants cultured at 20% oxygen with and without TGF-β_3 as measured by qRT-PCR ($n = 3$, $P = 0.07$). **C:** Representative Western blot of Eng in villous explants with and without TGF-β_3 in different oxygen conditions. **D:** Eng protein densitometric analysis of explants exposed to 3% oxygen with and without TGF-β_3 ($n = 5$, $^*P < 0.01$). **E:** Eng protein densitometric analysis in explants exposed to 20% oxygen with and without TGF-β_3 ($n = 5$). **F:** Representative Western blot of Eng in villous explants maintained at 3% and 20% oxygen and treated with neutralizing antibodies to TGF-β_3. **G:** Eng protein densitometric analysis of explants exposed to 3% and 20% oxygen with and without TGF-β_3-neutralizing antibodies ($n = 5$, $^*P < 0.05$).

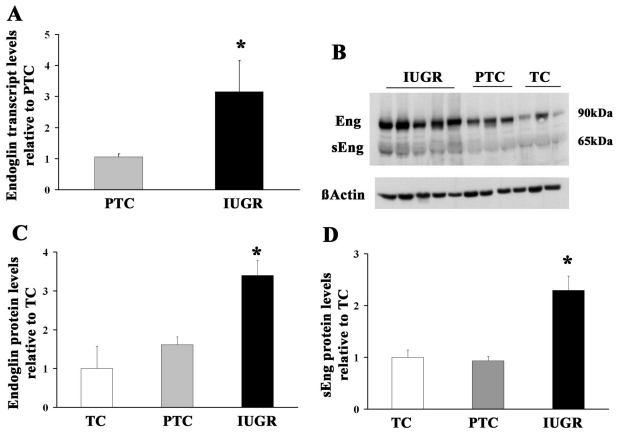

Figure 4. Eng and sEng expression in placentas of IUGR pregnancies. **A:** Eng transcript levels in IUGR placentas versus preterm controls (PTC) measured by qRT-PCR (IUGR, $n = 11$; PTC, $n = 10$; $^*P < 0.05$). **B:** Representative Eng and sEng Western blot in placentas of IUGR, preterm controls (PTCs), and term controls (TCs). **C:** Eng protein densitometric analysis of IUGR placentas ($n = 12$) compared to PTC ($n = 8$) and TC ($n = 8$, $^*P < 0.05$). **D:** sEng protein densitometric analysis of IUGR placentas ($n = 12$) compared to PTCs ($n = 8$) and TCs ($n = 8$, $^*P < 0.05$).

were used as controls. Real-time PCR demonstrated significant elevated transcript levels of Eng in the placentas of IUGR discordant twins compared to their normal co-twins and compared to twin controls (IUGR twin: 1.6 ± 0.3 versus normal co-twin: 0.9 ± 0.14 versus twin control A: 0.98 ± 0.23 and twin control B: 0.93 ± 0.12; $P < 0.05$) (Figure 5A). Analysis of the data according to the chorionicity of the twins showed significantly greater Eng transcript levels in the dichorionic IUGR twin compared to the normal co-twin (1.39 ± 0.2 versus 0.8 ± 0.1, $P < 0.05$) and increased transcript levels, reaching the borderline of statistical significance in the monochorionic IUGR twin compared to the normal co-twin (1.75 ± 0.5 versus 1 ± 0.2, $P = 0.06$) (Figure 5B). Concurrent with the qPCR results, Western blot analysis demonstrated increased expression of both Eng and its soluble form (sEng) in the placentas of dichorionic and monochorionic IUGR discordant twins compared to their normal co-twins and compared to twin controls (Eng: small discordant, 2.09 ± 0.25 versus large discordant, 1.1 ± 0.1, respectively, $P < 0.05$; sEng: small discordant, 1.8 ± 0.2 versus large discordant, 1 ± 0.25, respectively, $P < 0.05$) (Figure 5, C and D). Immunostaining of sections of discordant twins demonstrated a stronger immunoreactivity for Eng in the placentas of the IUGR discordant twin compared to the normal co-twin placenta in both dichorionic and monochorionic discordant twins (Figure 6B).

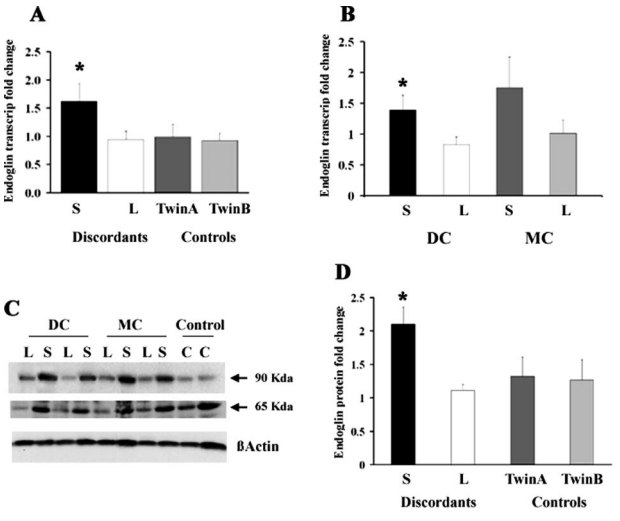

Figure 5. Eng expression in discordant twins' placentas. **A:** Eng mRNA levels in discordant twins (S_s: small IUGR twin; L_s: large normal co-twin; $n = 8$) compared to control twins ($n = 5$), assessed by qRT-PCR ($^*P < 0.05$). **B:** Eng mRNA levels in dichorionic discordant twins (DC, $n = 3$) and in monochorionic discordant twins (MC, $n = 5$) as assessed by qRT-PCR ($^*P < 0.05$). **C:** Representative Eng and sEng Western blot in placentas of discordant dichorionic and monochorionic twins and of normal control twins. **D:** Eng protein densitometric analysis in placentas of discordant twins ($n = 9$) compared to placentas of normal control twins ($n = 4$, $^*P < 0.05$).

Discussion

Herein, we demonstrate that levels of Eng and its soluble form are up-regulated by low oxygen in both *in vitro* (villous explants) and *in vivo* physiological (early gestation) and pathological (singleton IUGR pregnancies and discordant twins) models of placental hypoxia. Moreover, we provide evidence that low O_2-induced Eng production is mediated via TGF-β_3 because neutralizing TGF-β_3 antibodies prevented the increase in Eng levels by low O_2 and TGF-β_3, but not TGF-β_1, stimulated Eng expression at low O_2 conditions.

The development of the placental villous tree during early pregnancy takes place in an environment of relatively low oxygen tension, which is essential for normal embryonic development.[21] At 10 to 12 weeks of gestation, the intervillous space opens to maternal blood, and oxygen levels surrounding the placental villi increases.[22] We found greater levels of Eng at the early stages of gestation, when oxygen tension is low, suggesting that low oxygen positively regulates placental Eng expression. Our explant studies confirmed that low oxygen triggers placental expression of Eng. These findings agree with previous reports regarding increased expression of Eng in pathological tissues associated with hypoxic conditions such as ischemic tissues and tumors.[23,24] Studies with endothelial cells have shown that hypoxia up-regulates Eng expression via hypoxia-inducible factor 1 (HIF-1), which binds to hypoxia-responsive elements in the Eng promoter.[25,26] We have previously shown that HIF-1 expression parallels that of TGF-β_3 during the first trimester of gestation.[9] Their expression is high at 5 to 8 weeks of gestation and decreases at around 10 to 12 weeks when placental oxygen tension increases,[8] mimicking exactly Eng's pattern of expression. In addition, HIF-1, TGF-β_3, and Eng are overexpressed in preeclamptic placentas.[10,17,27–29] These observations together with TGF-β_3 being a HIF-1-responsive gene[30] led us to speculate that TGF-β_3 may be involved in the hypoxia-induced expression of placental Eng. Our finding of neutralizing TGF-β_3 antibodies inhibiting hypoxia-induced Eng expression in placental explants supports this concept. Moreover, our observation that TGF-β_3 increased Eng levels only under hypoxic conditions, suggests that hypoxia and TGF-β_3 have a synergistic effect on placental

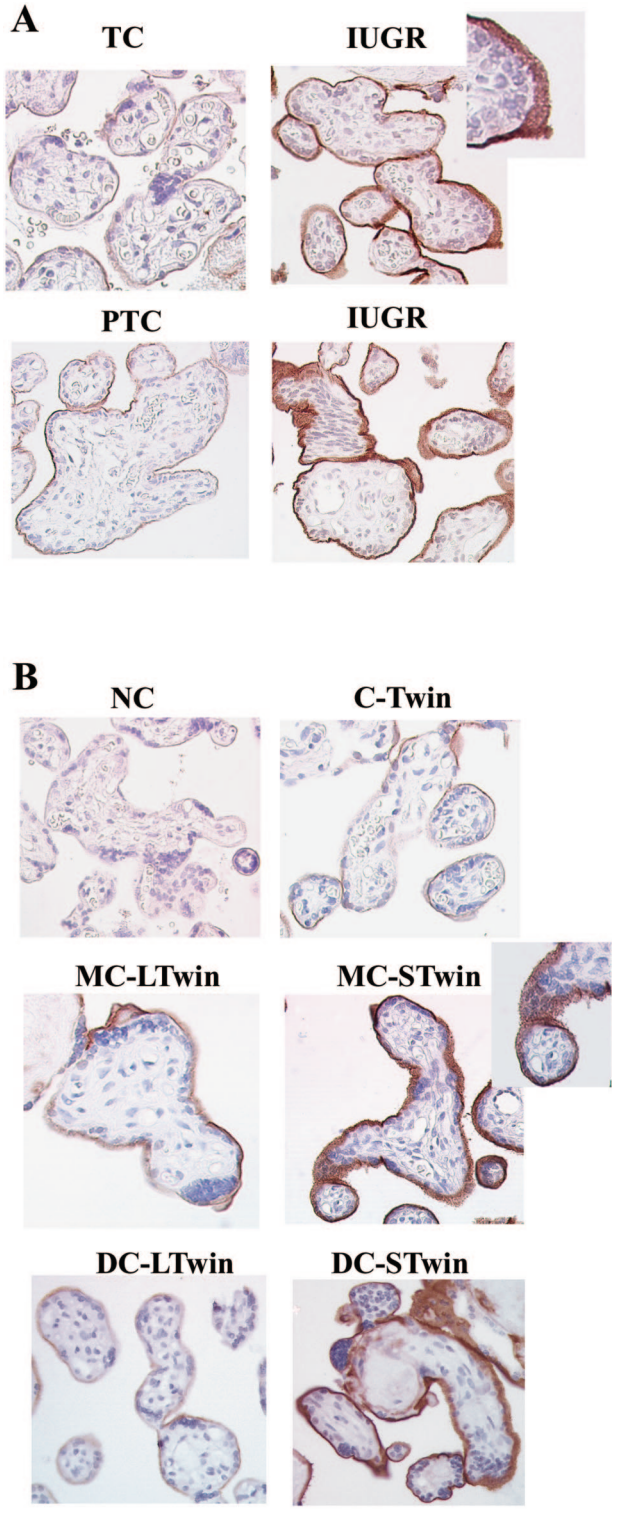

Figure 6. Immunohistochemical staining of Eng in IUGR, discordant twins, and control placentas. **A:** Term control (TC); preterm control (PTC), and IUGR placentas. Brownish staining represents positive Eng immunostaining. **B:** Negative control (NC), control twin (C-Twin), monochorionic large twin (MC-LTwin), monochorionic small twin (MC-STwin), dichorionic large twin (DC-LTwin), dichorionic small twin (DC-STwin). Original magnifications, ×100.

Eng expression. In agreement with our finding, Sanchez-Elsner and colleagues[26] demonstrated a transcriptional cooperation between hypoxia and TGF-β_1 leading to marked stimulation of Eng expression in endothelial cells. They suggested that the cooperative effect between both stimuli on endothelial cells could be an adaptive response aimed at optimizing angiogenesis under special conditions.[26] However, in the human placenta Eng is expressed at high levels by the syncytiotrophoblast cells,[12,31] but at relatively low levels on blood vessels, and herein we demonstrated that TGF-β_3, but not TGF-β_1, stimulated Eng production. The lack of TGF-β_1 effect on placental Eng expression could be explained by the fact that TGF-βs may exert their function in a tissue-specific manner. We have previously reported that blockage of Eng accelerates trophoblast migration and invasion, suggesting that Eng is a negative regulator of this system.[16] Thus, we speculate that in pathological hypoxic conditions Eng is inappropriately up-regulated in part via TGF-β_3, which would lead to abnormal trophoblast invasion and complications such as preeclampsia and IUGR.

Our present data demonstrate that both Eng and soluble Eng are highly expressed in placentas of IUGR pregnancies, including singleton IUGR pregnancies and IUGR discordant twins that do not exhibit signs of preeclampsia. To our knowledge, this is the first report on increased Eng expression in placentas of IUGR pregnancies. Our findings are further strengthened by a recent report showing elevated sEng levels in the circulation of women with IUGR pregnancies.[32] Both Eng and sEng are known to be increased in preeclamptic placentas, and the elevated circulatory levels of sEng in preeclamptic women have been correlated with its severity.[17] It is important to emphasize that all of the IUGR pregnancies included in this study had evidence of abnormal flow in the umbilical artery (either AEDV or REDV), indicating that the growth restriction was because of placental insufficiency. Moreover, all pregnancies were characterized by an early onset of growth restriction, which necessitated delivery of the fetuses before 34 weeks of gestation. None of the patients exhibited clinical signs of preeclampsia and as such represented a pure group of IUGR. Consistent with our findings, Venkatesha and colleagues[17] showed that co-administration of sEng and sFlt to pregnant rats resulted in fetal growth restriction in addition to preeclampsia-like syndrome. Because all our patients had sonographic indication of placental insufficiency and low oxygen stimulates Eng expression, it is plausible that the placental hypoxia because of reduced placental perfusion is responsible for the increased expression of Eng in IUGR pregnancies. Despite our assumption that reduced placental perfusion leading to placental hypoxia initiates the cascade of events leading to IUGR, there is no data in the literature demonstrating low placental pO_2 values in IUGR pregnancies. Pardi and colleagues[33] showed reduced oxygen extraction in IUGR pregnancies implicating a different mechanism leading to fetal hypoxia, although less rigorous clinical criteria than ours were used to classify IUGR because only birth weight and not abnormal umbilical artery flow was used.

Of note, all our IUGR pregnancies were delivered by cesarean section whereas only 44% of the preterm controls were delivered in this way. Mode of delivery can

differently affect gene expression in placental tissue, and increased placental expression of VEGF and VEGFR-2 has been demonstrated in vaginal deliveries accompanied by birth asphyxia.[34] However, none of our preterm control deliveries were complicated by birth asphyxia, and we did not find any difference in Eng expression between normal vaginal deliveries and cesarean sections (data not shown).

Overexpression of Eng in IUGR placentas was also validated in our study of discordant twins, in which the expression of Eng and sEng was elevated in the placenta of the IUGR twin compared to the normal co-twin and compared to twin controls. Discordant twins as a model for IUGR has the advantage of both twins being exposed to the same maternal and uterine environment, and therefore, the normal co-twin can serve as an ideal control for the IUGR twin. All IUGR discordant twins included in the present study had evidence of abnormal flow in their umbilical artery. Thus, the growth restriction was likely because of placental hypoperfusion and villous hypoxia. Although the literature regarding molecular differences between discordant twins is limited, one study showed different expression of hypoxia-regulated genes in the IUGR-derived placenta compared to the placenta of the normal co-twin in dichorionic discordant twins.[4] In the present study, Eng expression was elevated in the IUGR twin compared to its co-twin in both dichorionic discordant twins and monochorionic discordant twins, despite reaching statistical significance only in the dichorionic twins. This could be attributed to the small sample size but also to different mechanisms underlying growth restriction in discordant monochorionic compared to discordant dichorionic twins. Unlike monochorionic twins, in which discordancy has been attributed to placental vascular communication and uneven sharing of the placental disk, the most common accepted mechanism in dichorionic twins is placental insufficiency of the smaller fetus because placentas in discordant dichorionic twins may demonstrate lesions typical of singleton growth restriction.[35,36] However, other studies have found little evidence that anastomoses were a major cause of discordancy among monochorionic twins and suggested that abnormal placentation, rather than placental vascular anatomy, may be responsible for IUGR in monochorionic twins.[37,38]

In summary, our study demonstrates that placental Eng expression is up-regulated by hypoxia via TGF-β_3 and that Eng is overexpressed in IUGR placenta, most likely secondary to placental hypoxia. Overproduction of Eng would cause inhibition of trophoblast differentiation and invasion,[16] which could contribute to the development of IUGR. Furthermore, excessive production of Eng would lead to increased soluble Eng in the maternal circulation, which in turn may cause endothelial dysfunction resulting in preeclampsia and IUGR.[17]

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