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Objective—Biglycan (BGN) has reduced expression in placentae from pregnancies complicated by fetal growth restriction (FGR). We used first trimester placental samples from pregnancies with later small for gestational age (SGA) infants as a surrogate for FGR. The functional consequences of reduced BGN and the downstream targets of *BGN* were determined. Furthermore, the expression of targets was validated in primary placental endothelial cells isolated from FGR or control pregnancies.

Approach and Results—*BGN* expression was determined using real-time polymerase chain reaction in placental tissues collected during chorionic villous sampling performed at 10 to 12 weeks' gestation from pregnancies that had known clinical outcomes, including SGA. Short-interference RNA reduced *BGN* expression in telomerase-immortalized microvascular endothelial cells, and the effect on proliferation, angiogenesis, and thrombin generation was determined. An angiogenesis array identified downstream targets of BGN, and their expression in control and FGR primary placental endothelial cells was validated using real-time polymerase chain reaction. Reduced *BGN* expression was observed in SGA placental tissues. *BGN* reduction decreased network formation of telomerase-immortalized microvascular endothelial cells but did not affect thrombin generation or cellular proliferation. The array identified target genes, which were further validated: angiopoetin 4 (*ANGPT4*), platelet-derived growth factor receptor α (*PDGFRA*), tumor necrosis factor superfamily member 15 (*TNFSF15*), angiogenin (*ANG*), serpin family C member 1 (*SERPIN1*), angiopoietin 2 (*ANGPT2*), and CXC motif chemokine 12 (*CXCL12*) in telomerase-immortalized microvascular endothelial cells and primary placental endothelial cells obtained from control and FGR pregnancies.

Conclusions—This study reports a temporal relationship between altered placental *BGN* expression and subsequent development of SGA. Reduction of *BGN* in vascular endothelial cells leads to disrupted network formation and alterations in the expression of genes involved in angiogenesis. Therefore, differential expression of these may contribute to aberrant angiogenesis in SGA pregnancies.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37:1168-1179. DOI: 10.1161/ATVBAHA.117.309422.)**

Key Words: biglycan ■ cell proliferation ■ gene expression ■ pregnancy ■ thrombosis

Fetal growth restriction (FGR) is a serious pregnancy complication defined as a neonatal birth weight <10th percentile for gestation together with evidence of a compromised fetal conditions, such as reduced amniotic fluid volume, increased head-to-abdominal circumference ratio, and abnormal umbilical artery blood flow patterns.[1] FGR greatly increases the risk of perinatal complications, including fetal compromise in labor, fetal death in utero, neonatal morbidity, and neonatal death.[2–4] Live born infants from pregnancies complicated by FGR have an increased risk of pediatric disorders, such as cerebral dysfunction and learning difficulties, and of developing chronic adult onset diseases, including, cardiovascular disease, type II diabetes mellitus, and hypertension.[5–7] Identifiable causes of FGR account for ≈30% of

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Nonstandard Abbreviations and Acronyms

ANG	angiogenin
ANGPT2	angiopoietin 2
ANGPT4	angiopoietin 4
BGN	biglycan
CVS	chorionic villous sampling
CXCL12	CXC motif chemokine 12
FGR	fetal growth restriction
NC	negative controls
PDGFRA	platelet-derived growth factor receptor α
PG	proteoglycan
PLEC	primary placental endothelial cell
SERPINC1	serpin family C member 1
SGA	small for gestational age
siRNA	small interference RNA
TIME	telomerase-immortalized microvascular endothelial
TNFSF15	tumor necrosis factor superfamily member 15

all cases, whereas the remainder are idiopathic.[1] Idiopathic FGR is often associated with uteroplacental insufficiency,[8] abnormal umbilical artery Doppler velocimetry,[9] oligohydramnios,[10] and fetal growth asymmetry.[11] Placental insufficiency may result from various factors, including constriction of the placental blood vessels because of reduction in vasodilator activity,[12] incomplete cytotrophoblastic invasion of the maternal spiral arteries,[13] or maldevelopment of the placental villous structures.[14] Morphological differences include a decrease in villous number, diameter, and surface area, as well as a decrease in arterial number, lumen size, and branching.[14] At the cellular level, the FGR-affected placenta is characterized by reduced extravillous trophoblast proliferation, migration, and invasion into the placental bed.[13] These factors result in increased resistance to blood flow within the placenta in both the maternal and fetal circulations, ultimately resulting in fetal hypoxia and acidosis.

Normal pregnancy is characterized by a dominant hypercoagulable state highlighted by profound changes in hemostasis, such as increased concentration of procoagulant factors, decreased anticoagulant activity, and diminished fibrinolytic activity.[15]

Regulation of thrombin at the endothelial surface is achieved by proteoglycans (PGs) that are macromolecules comprising a core protein and at least 1 negatively charged polysaccharide glycosaminoglycan side chain. The small leucine-rich PG family represents a network of signal regulators that are responsible for many upstream signaling cascades and forms major conduits of information for cellular responses and modulators of distinct pathways.[16] Biglycan (BGN) and decorin belong to the class I small leucine-rich PGs and can be substituted with either chondroitin or dermatan sulfate glycosaminoglycan side chains.

Angiogenesis is the physiological process whereby new vessels are formed from pre-existing ones. Dysregulated blood vessel formation may also contribute to the pathogenesis of disease, including cancers and pregnancy diseases, such as FGR. We have recently demonstrated that reduced expression of decorin in microvascular endothelial cells results in altered cellular functions, including angiogenesis.[17] We have also previously demonstrated an association between reduced placental *BGN* expression and FGR,[18] and we have hypothesized that this reduction contributes to the pathogenesis of FGR.[19]

BGN is considered to have an organizing role in the assembly of the extracellular matrix and is structurally related to decorin, fibromodulin, and lumican PGs. Targeted disruption of the *BGN* gene results in abnormal collagen fibril morphology.[20] *BGN* can affect signal transduction pathways during cell growth and differentiation through induction of the cyclin-dependent kinase inhibitor p27Kip1.[21] Moreover, *BGN* is expressed both in the cell surface and in the pericellular space in various tissues of mesenchymal origin mainly.[22] It is released from the extracellular matrix by proteolytic digestion or secreted by activated macrophages.[22] Soluble *BGNs* are capable of interacting with cell surface receptors, thereby causing downstream signaling events.[22] The interaction of cells with the extracellular matrix has been shown to be vital in the regulation of many basic cellular functions, including proliferation, migration, and survival, as well as differentiation and tissue morphogenesis. Because disturbances in many of these biological functions have been demonstrated in the pathogenesis of FGR, *BGN* may also play a major role in the pathogenesis of FGR.

Borbely et al[23] have reported that BGN protein expression was localized to extravillous trophoblasts in the first trimester placentae of normal and of molar pregnancies, suggesting a role for BGN in the regulation of invasive properties of extravillous trophoblasts. However, the temporal relationship between reduced BGN expression and FGR is yet to be investigated. Therefore, in this study, it was hypothesized that reduced BGN expression early in first trimester contributes to the pathogenesis of FGR. Small for gestational age (SGA) is often considered as a surrogate for FGR and is defined as a birth weight <10th percentile of a birth weight curve.[1] Therefore, we investigated the expression of *BGN* mRNA and protein in SGA placentae collected early in gestation to determine whether reduced BGN expression is temporally associated with subsequent SGA/FGR development.

Aberrant angiogenesis and abnormal endothelial function in the placental villous microvasculature are associated with FGR pregnancies.[14] However, the role of placental *BGN* in microvascular endothelial functions is largely unknown. We hypothesized that reduced *BGN* in placental microvascular endothelium contributes to abnormal angiogenesis in SGA pregnancies. We went on to investigate the effect of reduced *BGN* expression on the function of cultured microvascular endothelial cells in vitro and on aortic ring sprouting ex vivo. The downstream target genes of *BGN* were also determined and further validated in placental microvascular endothelial cells obtained from pregnancies complicated with FGR and from uncomplicated control pregnancies.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Biglycan mRNA and Protein Expression in Chorionic Villous Sampling Samples

Relative quantification of *BGN* mRNA to *18S* rRNA in human chorionic villous sampling (CVS) samples using real-time polymerase chain reaction is shown in Figure 1A. The level of expression of *BGN* mRNA was significantly decreased in CVS samples obtained from SGA-affected pregnancies (n=15) compared with those obtained from CVS-control pregnancies (n=50; P=0.0003; Mann–Whitney U test).

A representative immunoblot for BGN protein expression in control and CVS samples from 3 pooled groups of CVS-SGA (n=5 in each group) and 3 groups of CVS-controls (n=12 in each group) is shown in Figure 1B. The bottom panel shows immunoreactive GAPDH protein as a loading control.

Semiquantitative analyses of BGN protein relative to GAPDH was determined using scanning densitometry. Figure 1C shows the result from 3 groups of pooled CVS-control (n=50) and CVS-SGA (n=15). The expression of BGN protein was significantly decreased in CVS-SGA (n=15) compared with CVS-control pregnancies (P=0.02; Mann–Whitney U test).

Reduced *BGN* mRNA Expression After Small Interference RNA Transfection in TIME Cells

Four independent small interference RNA (siRNAs; designated as S2, 3, 5, and 6) were designed to reduce *BGN* expression in telomerase-immortalized microvascular endothelial (TIME) cells. A transfection reagent alone control (Mock) and a nontargeted siRNA-transfected control were used as negative controls (NC). Figure 2 show that treatment with all 4 siRNAs significantly reduced *BGN* mRNA expression compared with both the controls at 48 hours after transfection (Mock: 1.24±0.01 and NC: 1.09±0.09 versus S2: 0.29±0.02, S3: 0.17±0.01, S5: 0.40±0.001, and S6: 0.20±0.004; all P<0.05; n=6; 1-way ANOVA). As S3 and S6 produced the greatest decrease in *BGN* expression, these 2 siRNAs were used in all other experiments.

A qualitative image of BGN protein relative to GAPDH was determined using Western immunoblotting. Figure 2E shows a representative immunoblot of 42 kDa BGN protein treated with Mock, NC, S3, and S6 (n=3 pooled samples). The expression of BGN protein was qualitatively decreased in BGN siRNA-treated cells compared with controls (decrease of 45% for S3- and 48% for S6-treated cells compared with NC [100%] or Mock [100%] using scanning densitometry). GAPDH was used as a loading protein.

Reduced *BGN* Expression Does Not Increase TIME Cell Apoptosis

To determine that the reduction in *BGN* expression was not because of cellular toxicity leading to apoptosis after treatment with siRNAs, the mRNA expression of 3 common apoptotic markers was analyzed. Real-time polymerase chain reaction demonstrated that the mRNA expressions of *BCL2*, *p53*, and *CASPASE3* were not significantly different compared with the Mock or the NC controls (P>0.05; n=18; 1-way ANOVA; data not shown).

Reduced *BGN* Expression Does Not Affect TIME Cell Proliferation and Thrombin Generation but Decreases Network Branch Formation

The effect on TIME cell proliferation after 48 hours incubation with *BGN* S3 or S6 was determined using the xCELLigence system. Optimization experiments confirmed that 5000 cells per well was the optimal density of cells to allow uninhibited cell proliferation (data not shown). Figure 3A is a representative graph showing the cell index of TIME cells treated with S3 and S6 compared with Mock or NC controls for 48 hours in culture. At 48 hours post-siRNA transfection, the proliferation potential of the TIME cells was unchanged compared with both controls (Mock: 3.20±0.73 and NC: 3.75±0.45 versus S3: 2.67±0.83 and S6: 2.62±0.79; P>0.05; n=9; 1-way ANOVA).

The endogenous thrombin potential of the TIME cells after reduction in *BGN* mRNA expression by siRNA was determined using the CAT (calibrated automated thrombogram) system. Reduction in *BGN* expression did not alter the endogenous thrombin potential of the TIME cells compared with the Mock and NC controls (Mock: 1583±25.44 and NC: 1636±50.57 versus S3: 1599±42.37 and S6: 1409±189.2; P>0.05; n=9; 1-way ANOVA; Figure 3B).

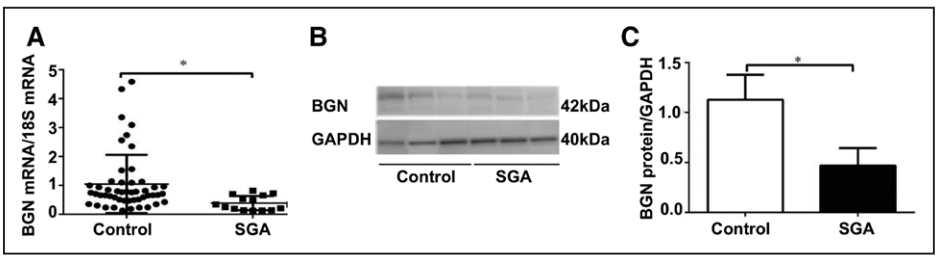

Figure 1. Reduced biglycan (BGN) mRNA expression in small for gestational age (SGA)-affected chorionic villous sampling (CVS) samples. **A**, Real-time polymerase chain reaction (PCR) analyses for *BGN* mRNA relative to *18S* rRNA. *BGN* mRNA relative to *18S* rRNA in SGA (n=15) and control (n=50) in early gestation was determined using real-time PCR. Gene expression differences between SGA and control placental tissues were calculated according to $2^{-\Delta\Delta CT}$ method.[23a] Data presented as mean±SEM. A P<0.05 was considered to be statistically significant as denoted by *. **B**, Western immunoblot for BGN and GAPDH. Immunoblot for BGN protein was performed in control and SGA samples as described in the Materials and Methods in the online-only Data Supplement. A representative immunoblot shows immunoreactivity for placental protein from 3 pooled groups of SGA (n=5 in each group) and 3 groups of control (n=12 in each group). Immunoreactive protein for BGN and GAPDH was detected in all samples at 42 and 40 kDa, respectively. **C**, Semiquantitative analyses of BGN protein. A semiquantitative analysis of BGN protein relative to GAPDH was determined using scanning densitometry software, ImageJ 1.44p. The graphs depict results from 3 groups of pooled control (n=50) and SGA (n=15). Data presented as mean±SEM. *P<0.05 was considered to be statistically significant.

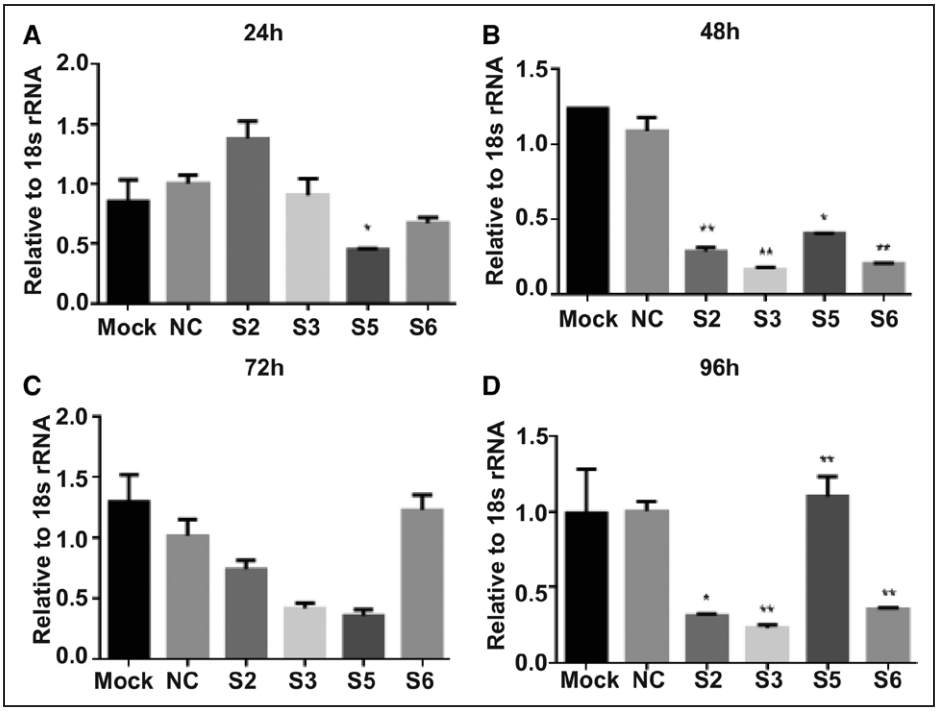

Figure 2. Reduced biglycan (*BGN*) mRNA expression after small interference RNA (siRNA) transfection in telomerase-immortalized microvascular endothelial (TIME) cells. **A**–**D**, Real-time polymerase chain reaction was performed on TIME cells transfected with Mock and negative controls (NC) control, and *BGN* S2, 3, 5, and 6 oligonucleotides, for 24, 48, 72 and 96 h. Relative quantification of *BGN* mRNA expression relative to the housekeeping gene *18S rRNA* was calculated using the $2^{-\Delta\Delta CT}$ method (*significance; $P<0.05$; n=9; 1-way ANOVA).

The ability of TIME cells to form branching networks after *BGN* gene reduction by siRNA was determined using the μ-slide Angiogenesis system by IBIDI. Optimization experiments to determine the optimal Growth-Factor Reduced Matrigel concentration and cell density were performed (data not shown). After incubation for a total of 48 hours, the cells were stained with calcien-AM. Figure 3C shows representative images at ×100 magnification taken after 48 hours *BGN* S3 and S6 transfection. A qualitative decrease in TIME cell network formation in S3- and S6-transfected cells compared with Mock or NC-treated cells was observed. Network formation potential of TIME cells, as quantified by the number of branch points, was significantly decreased after *BGN* S3- and S6-treated cells compared with Mock and NC controls (Mock: 96±9.01 and NC: 127.7±8.09 versus S3: 54±3.79 and S6: 54±12.58; $P<0.05$; n=9; 1-way ANOVA; Figure 3D). The effect of *BGN* downregulation on tube length and total number of tubes formed was also determined (Figure 3D). No significant differences were observed ($P>0.05$; n=6; 1-way ANOVA).

In addition, the consequence of reduced BGN on angiogenesis was further examined using a recently described mouse aortic ring sprouting assay (Figure 3E).[24] BGN siRNA treatment significantly inhibited angiogenic sprouting (assessed as the average number of sprouts/aortic ring) compared with NC controls ($P<0.001$; n=3; 1-way ANOVA).

Identification of Downstream *BGN* Target Genes in TIME Cells

TIME cells were transfected with S3 or NC control, and the Human Angiogenesis Taqman polymerase chain reaction array was used to identify potential downstream targets of *BGN*. The relative mRNA expression of the 84 genes after *BGN* mRNA and protein downregulation is shown in Figure 4. The *y* axis represents the fold change for each of the 84 genes normalized to the average expression of the 5 housekeeping genes included in the array. Genes that had an expression level >2 as indicated by a red line were classified as genes with a fold increase. Conversely, genes that had an expression level below the red line were classified as genes with a fold decrease in gene expression. The genes that showed marked fold changes (highest or lowest changes) and have proangiogenic roles involved in migration, invasion, and growth of endothelial cells were selected for further validation: angiopoetin 4 (*ANGPT4)* with a fold increase of 13×10^3, platelet-derived growth factor receptor α (*PDGFRA*) with a fold increase of 18×10^8, tumor necrosis factor superfamily member 15 (*TNFSF15*) with a fold increase of 41×10^4, angiogenin (*ANG*) with a fold increase of 2763.41, serpin family C member 1 (*SERPINC1*) with a fold increase of 352, angiopoetin 2 (*ANGPT2*) with a fold decrease of 0.02, and CXC motif chemokine 12 (*CXCL12*) with a fold decrease of 5.51×10^{10}.

The mRNA expression of *ANGPT4*, *PDGFRA*, *TNFSF15*, *ANG*, *SERPINC1*, and *CXCL12* was further validated in TIME cells transfected with *BGN* S3 or S6 independently (Figure 5). Increased expression of *ANGPT4* (Mock: 1.10±0.26 and NC: 1.00±0.12 versus S3: 125±27.0 and S6: 149±32.0; $P<0.01$; n=6; 1-way ANOVA), *PDGFRA* (Mock: 1.01±0.10 and NC: 1.09±0.16 versus S3: 5.21±0.07 and S6: 4.97±0.72; $P<0.05$; n=6; 1-way ANOVA), *TNFSF15* (Mock: 1.00±0.04 and NC: 1.80±0.31 versus S3: 147±11.0 and S6: 42±6.90; $P<0.05$;

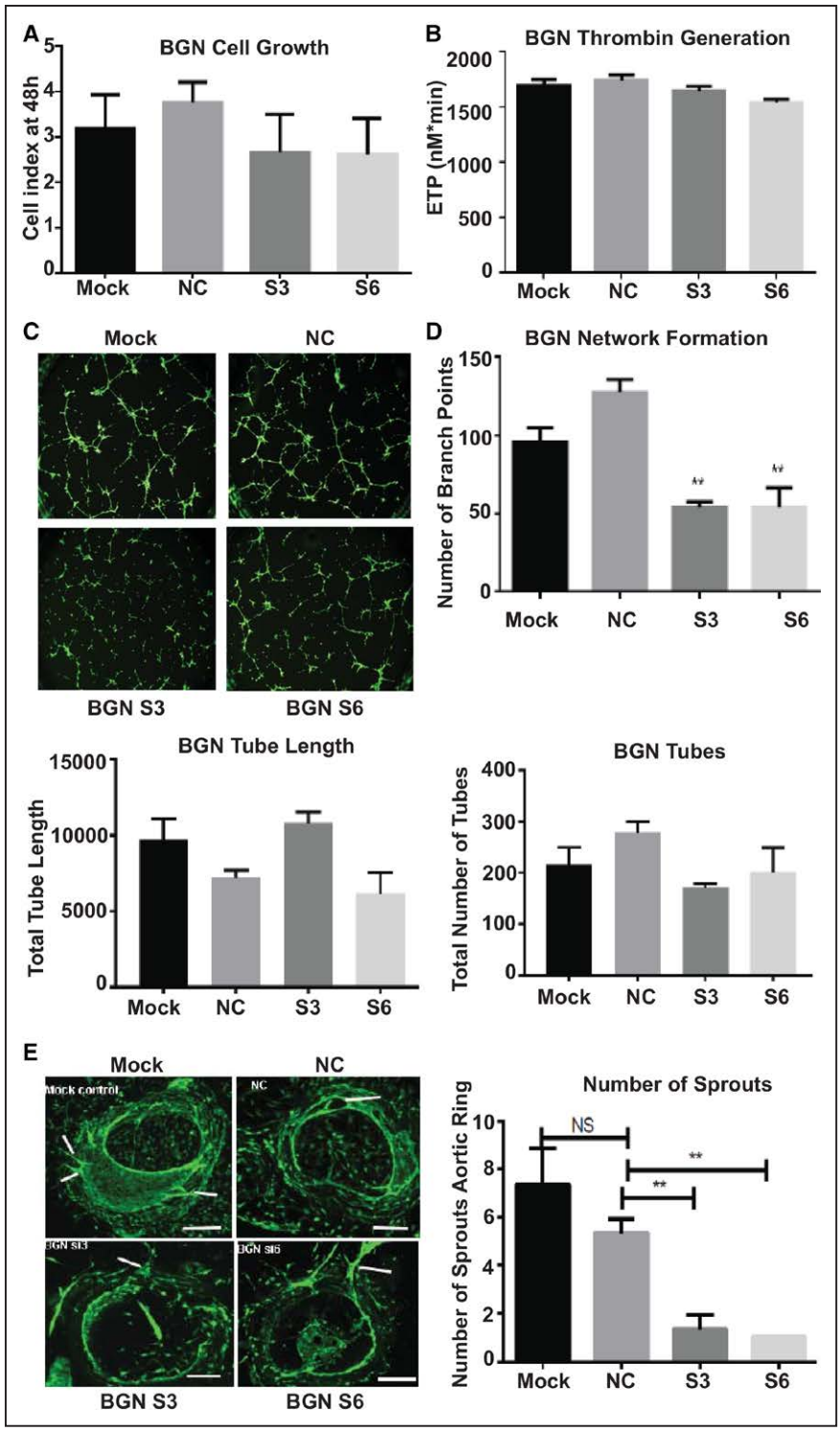

Figure 3. Reduced biglycan (*BGN*) expression does not alter telomerase-immortalized microvascular endothelial (TIME) cell growth and thrombin generation but decreases network formation. **A**, Time cell growth was determined using xCELLigence (Roche Diagnostics). *BGN* downregulation did not alter the growth of TIME cells ($P<0.05$; n=18; 1-way ANOVA). The *y* axis represents the cell index over time. **B**, *BGN* gene reduction in TIME cells does not affect thrombin generation, ($P<0.05$; n=18; 1-way ANOVA). The *y* axis represents the amount of thrombin generated relative to time. **C**, Representative images of TIME cell network formation after 48 h Mock, negative controls (NC), and *BGN* S3 or S6 transfection. Cells were stained with calcien-AM, and images were taken using a fluorescent microscope (CellR, Olympus, Japan). Magnification of all images is at ×100, scale bars represent 50 μm. **D**, The ability for TIME cells to form networks and form tubes of vessels after transfection with Mock, NC, and BGN S3 or S6 for a 48-h time period was determined using IBIDI angiogenesis slides. The total number of branch points, tubes, and tube lengths was determined using the Wimasis Image Analysis tool (*significance; $P<0.05$; n=9; 1-way ANOVA). The *y* axis represents the number of branch points at 48 h. **E**, Reduced BGN mRNA inhibits angiogenic sprouting from mouse aortic rings. Murine mouse aortas were treated with BGN S3 or S6, and NC control. Mock control contained media supplemented with 5% FCS as a positive control. In the presence of S3 or S6, angiogenic sprouting was significantly reduced. Representative images of calcein-stained aortic rings are shown (*significance; $P<0.01$; n=3; 1-way ANOVA).

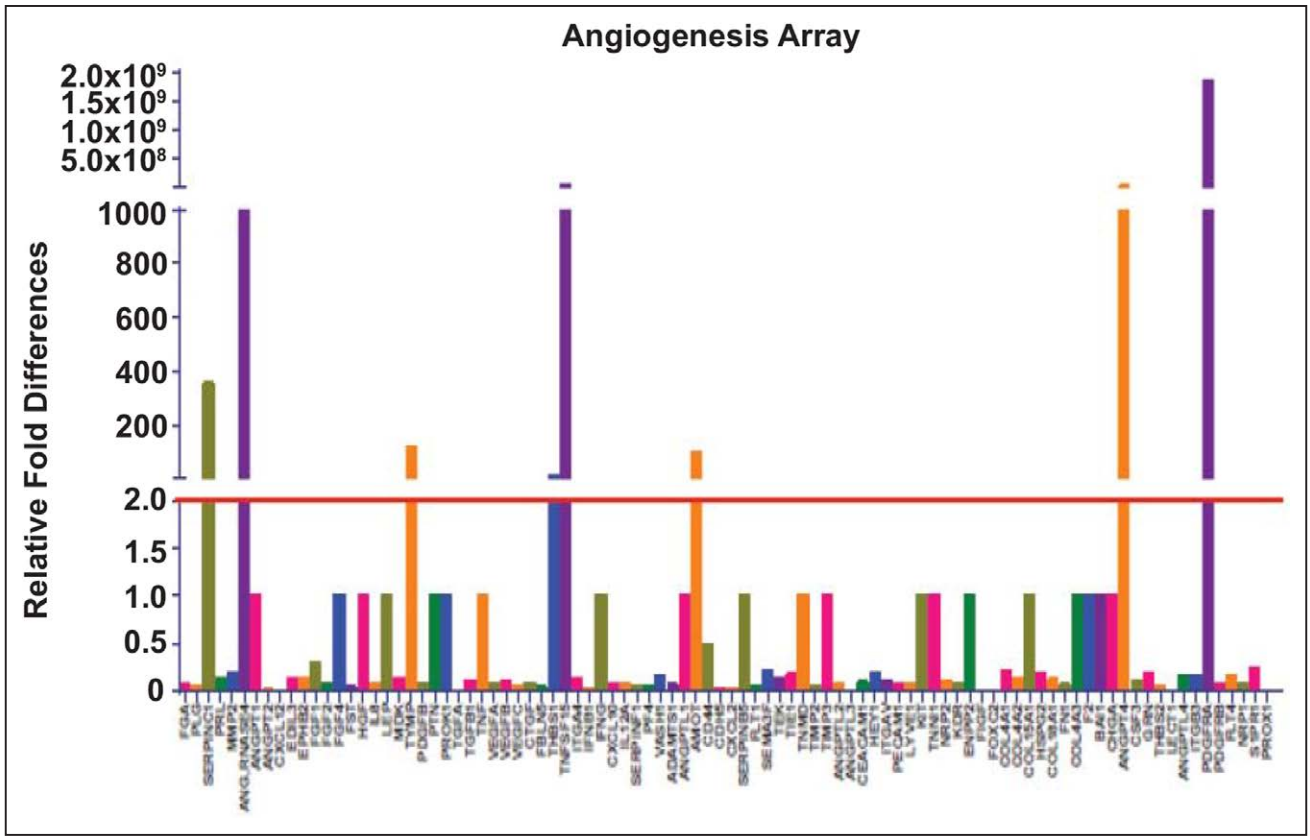

Figure 4. Identification of biglycan (*BGN*) downstream target genes after small interference RNA (siRNA) transfection. RNA was extracted from telomerase-immortalized microvascular endothelial cells transfected with *BGN* siRNA, transcribed into first strand cDNA, and the Taqman Angiogenesis real-time polymerase chain reaction array was performed for gene profiling. The 84 pre-dispensed genes, which included a panel of housekeeping genes, were amplified for 40 cycles of denaturation and primer extension. Gene expression values (fold change above or below threshold value of 1) were subsequently calculated for the *BGN* siRNA-treated plate, relative to the negative controls (NC) and normalized to the housekeeping gene panel (*x* axis). The red line shows the threshold value at 1. Values >1 were termed a fold increase, and those <1 were considered a fold decrease.

n=6; 1-way ANOVA), and *ANG* (Mock: 1.06±0.25 and NC: 0.71±0.11 versus S3: 6.01±1.28 and S6: 4.87±0.18; $P<0.05$; n=6; 1-way ANOVA) was confirmed. Conversely, decreased expression of *ANGPT2* (Mock: 1.01±0.08 and NC: 0.90±0.05 versus S3: 0.29±0.03 and S6: 0.40±0.05; $P<0.05$; n=6; 1-way ANOVA) and *CXCL12* (Mock: 1.02±0.16 and NC: 0.85±0.12 versus S3: 0.29±0.09 and S6: 0.30±0.08; $P<0.05$; n=6; 1-way ANOVA) also was confirmed. These results are consistent with the array analysis; however, the expression of *SERPINC1* was not significantly different when validated in TIME cells (graph not shown, $P>0.05$; n=6; 1-way ANOVA).

Validation of *BGN* and Its Downstream Genes in Placental Villi Obtained From Pregnancies Affected With FGR and From Uncomplicated Control Pregnancies

The mRNA expression of the candidate downstream targets of *BGN* was validated in the placental villi obtained from control and FGR-affected pregnancies. A significant increase in mRNA expression was observed for *PDGFRA* in placental villi from FGR-affected pregnancies compared with controls (control: 1.81±0.44 versus FGR: 6.52±1.72; $P<0.05$; n=15 each; Mann–Whitney *U* test), as shown in Figure 6. The mRNA expression of *ANGPT2* was significantly decreased in placental villi from FGR-affected pregnancies compared with controls (control: 2.04±0.46 versus FGR: 0.27±0.08; $P<0.005$; n=15 each; Mann–Whitney *U* test). However, the gene expression of *ANG* and *CXCL12* was not significantly different ($P>0.05$).

Validation of *BGN* and Its Downstream Genes in Primary PLEC Isolated From FGR-Affected Pregnancies and From Uncomplicated Control Pregnancies

The mRNA expression of the *BGN* and its candidate downstream targets was then validated in freshly isolated primary placental endothelial cells (PLECs) from FGR-affected pregnancies and from uncomplicated control pregnancies. A significant decrease in *BGN* mRNA expression in FGR PLECs was observed (control PLEC: 2.20±0.28 versus FGR PLEC: 0.32±0.07; $P<0.05$; n=3 each; Mann–Whitney *U* test), as shown in Figure 7. In addition, increase in mRNA expression was also observed for *PDGFRA* in FGR PLEC compared with control PLEC (control PLEC: 1.43±0.41 versus FGR PLEC: 30.02±12.82; $P<0.05$; n=3 each; Mann–Whitney *U* test). Although the mRNA expression of *ANGPT2* (control PLEC: 1.02±0.09 versus FGR PLEC: 0.26±0.08; $P<0.05$; n=3 each; Mann–Whitney *U* test) and *CXCL12* (control PLEC:

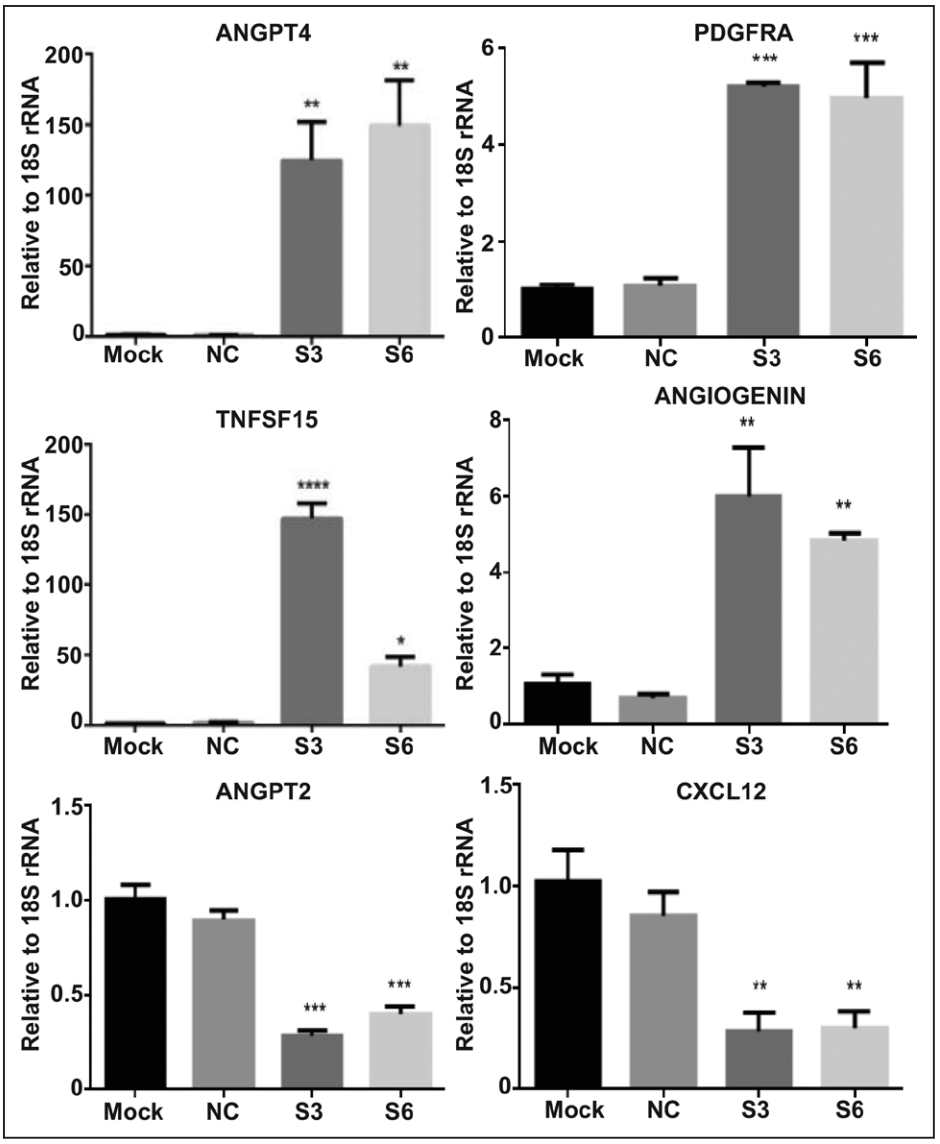

Figure 5. Validation of candidate biglycan (*BGN*) downstream target genes in telomerase-immortalized microvascular endothelial (TIME) cells. cDNA from TIME cells transfected with Mock and negative controls (NC), and *BGN* S3 or S6 was amplified for 40 cycles using pre-validated Taqman gene expression assays for angiopoietin 4 (*ANGPT4*), platelet-derived growth factor receptor α (*PDGFRA*), tumor necrosis factor superfamily member 15 (*TNFSF15*), angiogenin (*ANG*), angiopoietin 2 (*ANGPT2*), and CXC motif chemokine 12 (*CXCL12*). The *18S rRNA* housekeeping gene was used for relative quantification according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001). The NC control was used as the calibrator (*significance; $P<0.05$; n=6; 1-way ANOVA). The *y* axis represents the gene expression relative to *18S rRNA*.

1.51±0.60 versus FGR PLEC: 0.22±0.12; $P<0.05$; n=3 each; Mann–Whitney *U* test) was significantly decreased in FGR PLEC compared with controls, *ANG* mRNA expression was not significantly different in PLEC isolated from FGR and control (control PLEC: 1.08±0.17 versus FGR PLEC: 1.55±0.26; $P>0.05$; n=3 each; Mann–Whitney *U* test).

Discussion

Abnormal placental development at the beginning of pregnancy may result in complications in late pregnancy.[25] Previous studies have reported that it is possible to assess gene expression differences in first trimester placentae from pregnancies with known outcomes, well before clinical disease symptoms or developmental abnormalities are apparent.[26–32] The results of this study correlate with our previous findings in which we have demonstrated a significant reduction in placental *BGN* from human pregnancies complicated by FGR collected at third trimester gestation compared with gestation-matched controls.[18] The results from this study support a temporal relationship between reduced *BGN* expression and SGA/FGR and, therefore, suggest a causal role for placental *BGN* in the success of pregnancy outcome.

Fetoplacental development during early gestation requires complex modulation and coordination between growth factors, cytokines, and transcription factors.[33] These aim to modulate villous cytotrophoblast differentiation or extravillous cytotrophoblast migration and invasion during this crucial time. We have shown that *BGN* expression is decreased in CVS samples collected from SGA pregnancies. Therefore, *BGN* may have a direct regulatory role in early placentation by modulating

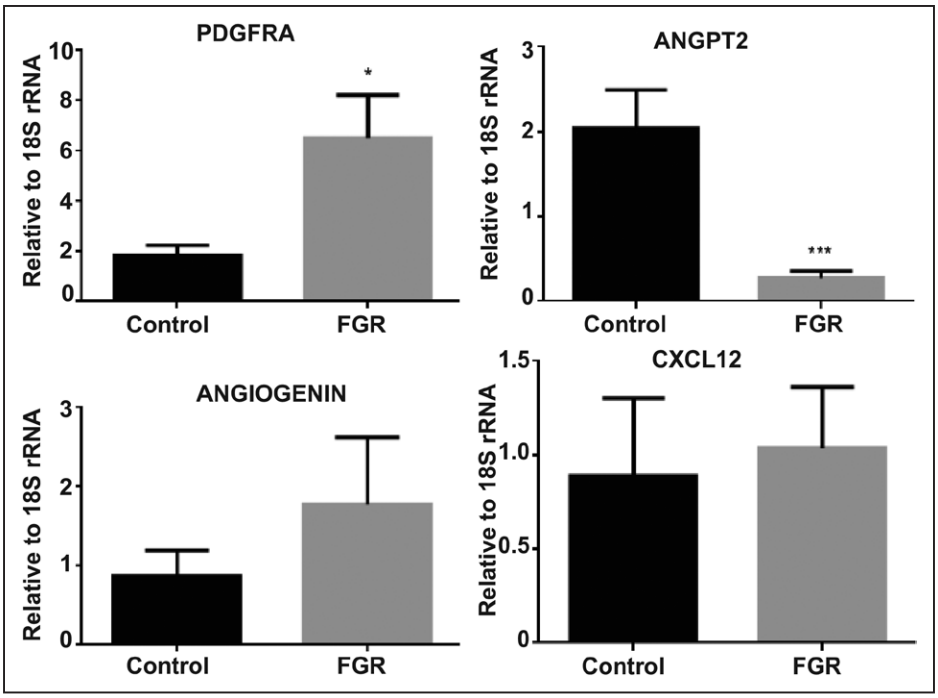

Figure 6. Expression of biglycan (*BGN*) target genes in control and fetal growth restriction (FGR)-affected placentae. The mRNA expression of platelet-derived growth factor receptor α (*PDGFRA*), angiogenin (*ANG*), angiopoietin 2 (*ANGPT2*), and CXC motif chemokine 12 (*CXCL12*) was determined by real-time polymerase chain reaction in placentae obtained from control and FGR-affected pregnancies according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001). The average values of the control placentae were used as the calibrator (*significance; $P<0.05$; Mann–Whitney *U* test). The *y* axis represents the gene expression relative to *18S rRNA*.

villous cytotrophoblast differentiation or extravillous cytotrophoblast migration and invasion during first trimester. Furthermore, because endothelial cell functions become more prominent only after 16 to 18 weeks of pregnancy, it could be that less placental BGN during early gestation may contribute to abnormal trophoblast function. As a consequence, the cross-talk between trophoblasts and endothelial cells may become impaired leading to altered basic cellular functions.

BGN is a protein with many distinct functions depending on its localization. This study has demonstrated that a reduction in the expression of BGN in microvascular endothelial cells results in a decrease in the potential for those cells to form the branches required for networks and, thus, the ability to form new communicating blood vessels without affecting the tubular formation of the vessels or the actual number of vessels formed. We have also further assessed this using the novel ex vivo mouse aortic ring angiogenesis assay to confirm that the reduction in BGN expression significantly reduced number of sprouts formed. However, the reduction in BGN expression in the TIME cells did not affect endothelial cell growth or thrombin generation as previously hypothesized. This may be because of the fact that the primary targets of BGN, presented in this study, include those that are highly involved in vascular remodeling, matrix remodeling, and cell migration.

Our array data have demonstrated that reduction in BGN expression resulted in various upregulated and downregulated target genes. As TIME cells are not endothelial cells specific to the human placenta, we wanted to confirm whether the increase or decrease in expression observed in key candidates in the array remained consistent in endothelial cells cultured from placental tissue, as well as in whole placental tissue. For this reason, we chose candidate genes specifically in the context of their expression and function in the human placenta, in particular their role in angiogenesis, and potential roles in the pathogenesis of pregnancy disorders.

The multitude of genes in the array can be classified as either antiangiogenic or proangiogenic. Successful placentation is dependent on the proper establishment of a vascular network involving the formation of new vessels and remodeling of the pre-existing vessels.[34] A balance between the proangiogenic and antiangiogenic functions is essential for proper placental development, and any alterations can cause major pregnancy complications, such as preeclampsia and FGR, and lead to poor obstetric outcomes.[35,36] The majority of genes that we chose to further validate, including *ANG, ANGPT4, PDGFRA, ANGPT2*, and *CXCL12*, have proangiogenic roles and are involved in migration, invasion, and growth of endothelial cells.[37–44]

The expression of *ANG* rises exponentially from first to third trimester, which corresponds with the growth trajectory of both the placenta and fetus.[45] In addition, placental explants from patients with FGR secreted, in vitro, significantly elevated amounts of *ANG* compared with control explants. *ANG* was found to be localized to both villous syncytiotrophoblast and fetal endothelial cells.[46] Our data show that the expression of *ANG* is increased in cultured TIME cells after reduction in *BGN* expression. However, *ANG* expression was not significantly different in either FGR placental tissue or PLECs compared with controls. This difference could be because of

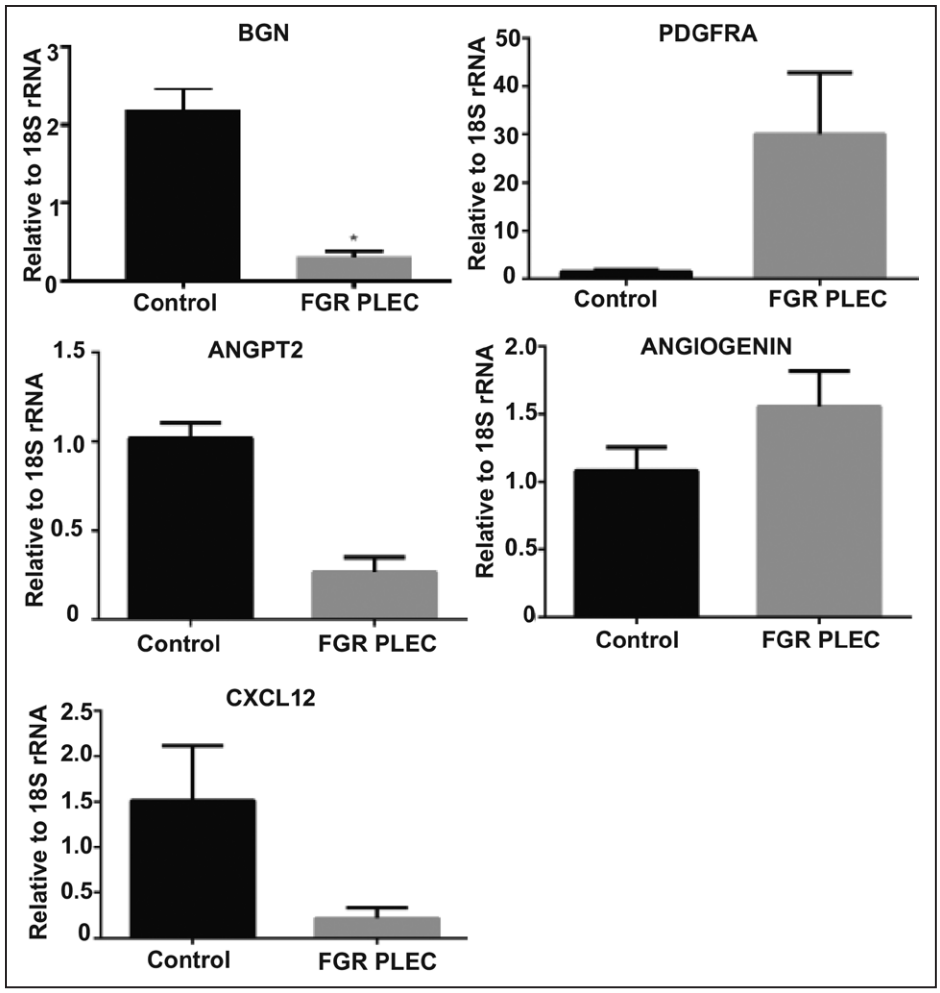

Figure 7. Expression of biglycan (*BGN*) target genes in control and fetal growth restriction (FGR)-affected primary placental endothelial cells. The mRNA expression of *BGN*, platelet-derived growth factor receptor α (*PDGFRA*), angiopoietin 2 (*ANGPT2*), angiogenin (*ANG*), and CXC motif chemokine 12 (*CXCL12*) was determined by real-time polymerase chain reaction in primary placental endothelial cells (PLECs) according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001). The average of the control PLECs was used as the calibrator (*significance; $P<0.05$; Mann–Whitney *U* test). The *y* axis represents the gene expression relative to *18S rRNA*.

the use of placental tissue (whole and isolated) versus using explants and the subsequent testing of secreted versus endogenous *ANG*.

Jarvenpaa et al[47] demonstrated that the expression of *PDGFRA* was decreased in women with preeclampsia (PE) associated with FGR by using a real-time polymerase chain reaction–based profiling array. Our results show that reduction in *BGN* resulted in an increase in *PDGFRA*. This could potentially be because of a difference between pregnancies complicated by both preeclampsia and FGR, or FGR alone as in this study. Nevertheless, this increase in *PDGFRA* after *BGN* reduction could represent a compensatory mechanism in trying to protect the cells from more damage by minimizing the decrease in endothelial cell migration during angiogenesis.

The angiopoietins are endogenous ligands for the vascular endothelium–specific receptor tyrosine kinase Tie2. This angiopoietin/tie system has well-established roles in vascular remodeling and angiogenesis during placentation.[48,49] ANGPT2 is the better characterized of the 2 proteins, and it seems that it can act as a context-dependent agonist or antagonist of Tie2.[50] In other words, it can either be proangiogenic or antiangiogenic depending on the exposure time or concentration of ANGPT2. Limited data are available on the expression of both ANGPT2 and ANGPT4 in pregnancy disorders. Conflicting data on expression of ANGPT2 in PE and FGR are available; however, the study numbers are small and require further investigations.[51] Our study shows decreased expression of ANGPT2 in term control placentae compared with those complicated by FGR, and in PLECs.

CXCL12 is expressed constitutively and ubiquitously in most tissues and plays a critical role in vasculogenesis and angiogenesis.[42,43] During migration and invasion of cells in heterogenous tissues, they may encounter different oxygen levels because of poor or altered vascularization, such as in pregnancy disorders, and studies have suggested that chemotaxis is a cell function that may be affected by oxygen availability. The expression of *CXCL12* is increased in placentae of women with PE compared with controls.[52] In this study, we have shown that *CXCL12* expression is decreased in PLECs from placentae affected by FGR, as well as after *BGN* reduction in TIME cells. It could be that although there are definite similarities between the pathogenesis of PE and FGR, they

Table. Demography of Chorionic Villous Sampling Samples Used in This Study

Description	FGR (n=15)	Control (n=50)	*P* Values
Maternal age	39.24±2.04	38.64±2.45	0.08
Smoker	2/15	0/50	
Preeclampsia	0/15	0/50	
Parity	1.86±1.5	1.2±0.67	0.29
Birth weight	2551±506	3461±225	<0.05
Gestation at delivery	40.06	40.60	0.10
Male sex	7 (46.7%)	29 (58%)	
Birth weight <10th centile for gestation and sex	15 (100%)	0	
Birth weight <5th centile for gestation and sex	11 (73.3%)	0	
Birth weight <3rd centile for gestation and sex	6 (40%)	0	

FGR indicates fetal growth restriction.

are still considered separate diseases and treated differently clinically. In fact, the expression of *BGN* is decreased in placentae affected by FGR,[18] but not in PE.[53] Therefore, it is plausible that the decrease in *CXCL12* we observed in this study is unique to the pathogenesis of FGR, either as a result of a multitude of changes contributing to the disease or simply by the reduction in *BGN* expression alone.

In contrast, our array also showed altered fold changes in *SERPINC1* (otherwise known as antithrombin III) and *TNFSF15* (or vascular endothelial growth inhibitor). Both of these genes play antiangiogenic roles in endothelial cells.[54,55] Because the network formation ability of TIME cells was altered after *BGN* gene downregulation, we speculate that this downregulation may either directly or indirectly contribute to an increased expression of genes responsible for antiangiogenic roles in endothelial cells. Future experiments will aim to explore this further by determining the function of the downstream targets in the presence or absence of *BGN*. However, the increase in expression of genes with proangiogenic effects in endothelial cells may indicate either directly contributing to a compensatory mechanism to counteract aberrant angiogenic signaling in the endothelial cells or an indirect regulation in the complex signaling pathways governing basic endothelial cell proliferation, network formation, and angiogenesis of endothelial cells. In addition, the proangiogenic genes also have roles in vascular development, maintenance, and cell–cell adhesion.[56–64] This is promising because a defect in any of these fundamental endothelial cell functions may contribute to abnormal angiogenesis and placental microvascular dysfunction, which are characteristic of FGR pathogenesis.

In this study, we have shown that reduction in the expression of the PG BGN resulted in reduced network formation in cultured microvascular endothelial cells. Furthermore, reduction in BGN resulted in altered expression of growth factors and chemokines that are highly involved in the regulation of angiogenesis in many vascular systems. Moreover, we have recently shown that a reduction in decorin (another member of the small leucine-rich PG family of PGs) also contributes to alterations in cell function in microvascular cells.[17] Therefore, the possibility of a combined deficiency between these 2 closely related PGs should be explored further because it may lead to clues about the complex interconnection between PGs and their target genes, and the manifestation of FGR symptoms.

In this study, the cohort of SGA samples considered as proxy for FGR demonstrated a significantly smaller mean fetal birth weight delivered at term compared with the term control group. Currently, CVS is the only available means to study a temporal relationship between altered placental gene expression in pregnancies with subsequent development of SGA/FGR at term and uncomplicated control pregnancies. CVS is used as a diagnostic procedure mainly for fetal karyotyping in women of advanced age at delivery, or women who have a family or personal history of genetic abnormality. Therefore, the use of the SGA samples in this study limits our ability to relate our research findings to all pregnant women.

In summary, our study reports that placental *BGN* expression is decreased early in gestation in pregnancies that may subsequently go on to develop FGR. Furthermore, reduced expression of *BGN* in a microvascular endothelial cell line results in altered network formation as a consequence of altered downstream growth factor expression or via a direct local effect of reduced *BGN* in the microvascular environment.

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Disclosures

None.

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Highlights

- This study demonstrates that altered biglycan expression during early pregnancy results in alteration to the proliferative, angiogenic, and thrombin-generating potential of human microvascular endothelial cells.
- The angiogenic changes observed is mirrored in ex vivo rat aortas resulting in a decreased number of sprouts.
- The target genes of biglycan include genes that play important roles in angiogenesis, such as *ANG*, *ANGPT2*, *SERPINC1*, and *CXCL12*.
- The expression of these target genes is altered in primary placental endothelial cells and in fetal growth restriction–affected whole placentae.
- It is therefore plausible that differential expression of biglycan, as well as these targets, may contribute to the pathogenesis of fetal growth restriction and represent an important step to understanding the mechanism of regulation of human microvascular endothelial cells by biglycan which is likely to have important implications in the placenta, and those which extend beyond the field of obstetrics.