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TGF-β1 Regulation of Multidrug Resistance P-glycoprotein in the Developing Male Blood-Brain Barrier

Stephanie Baello, Majid Iqbal, Enrrico Bloise, Mohsen Javam, William Gibb, and Stephen G. Matthews

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P-glycoprotein (P-gp), an efflux transporter encoded by the abcb1 gene, protects the developing fetal brain. Levels of P-gp in endothelial cells of the blood-brain barrier (BBB) increase dramatically during the period of peak brain growth. This is coincident with increased release of TGF-β1 by astrocytes and neurons. Although TGF-β1 has been shown to modulate P-gp activity in a number of cell types, little is known about how TGF-β1 regulates brain protection. In the present study, we hypothesized that TGF-β1 increases abcb1 expression and P-gp activity in fetal and postnatal BBB in an age-dependent manner. We found TGF-β1 to potently regulate abcb1 mRNA and P-gp function. TGF-β1 increased P-gp function in brain endothelial cells (BECs) derived from fetal and postnatal male guinea pigs. These effects were more pronounced earlier in gestation when compared with BECs derived postnatally. To investigate the signaling pathways involved, BECs derived at gestational day 50 and postnatal day 14 were exposed to ALK1 and ALK5 inhibitors and agonists. Through inhibition of ALK5, we demonstrated that ALK5 is required for the TGF-β1 effects on P-gp function. Activation of ALK1, by the agonist BMP-9, produced similar results to TGF-β1 on P-gp function. However, TGF-β1 signaling through the ALK1 pathway is age-dependent as dorsomorphin, an ALK1 inhibitor, attenuated TGF-β1-mediated effects in BECs derived at postnatal day 14 but not in those derived at gestational day 50. In conclusion, TGF-β1 levels at the developing BBB may lead to substantial changes in fetal brain exposure to P-gp substrates, triggering consequences for brain development. (Endocrinology 155: 475–484, 2014)
have previously shown that this rise in P-gp protein occurs in fetal brain microvessels (9). However, the mechanism involved in this up-regulation remains unclear.

Previous studies have implicated TGF-β1 in the regulation of P-gp expression in endothelial cells derived from the adult brain. In vitro, TGF-β1 was found to increase P-gp activity in BECs derived from the adult mouse brain (10). TGF-β1 is secreted by differentiating astrocytes (11, 12) and plays an essential role in brain development (13). Coculture studies of astrocytes and BECs have shown that there is an increase in abcb1 mRNA expression in cocultured BECs compared with monocultured BECs (14). This increase is most likely due to factors secreted by astrocytes because the BECs do not require physical contact with astrocytes for this up-regulation to occur (15–17).

Typically, TGF-β1 binds to TGF-β receptor type 2 (TGFBR2), which phosphorylates and recruits a TGF-β receptor type 1 (TGFBR1) that is responsible for eliciting signal transduction (18). Endothelial cells express two TGFBR1 isoforms: ALK5, which is expressed in a variety of cell types, and ALK1, which is expressed exclusively in endothelial cells (19). These receptors phosphorylate SMAD2/3 and SMAD1/5, respectively, which in turn form a complex with SMAD4 and translocate to the nucleus to effect gene transcription (20, 21). There is a codependency between these TGFBR1 isoforms as membrane-bound ALK5 is required for ALK1 signaling to occur (22). In addition, transmembrane auxiliary receptors (type III receptors) such as betaglycan and endoglin are modulators of cellular responsiveness to TGF-β1. Endoglin potentiates ALK1 signaling. Crosstalk between the ALK1/endoglin route has been shown to inhibit TGF-β1/ALK5/Smad2,3 in endothelial cells (23–25). Similarly, betaglycan regulates cellular responsiveness to TGF-β1. It has been shown to dampen cellular responsiveness to TGF-β1 by disrupting the complex formed by TGFBR2 and TGFBR1 (26, 27).

To date, it is not known how TGF-β1 affects P-gp function in the developing BBB and, if so, which downstream TGF-β1 signaling pathway or pathways mediate the effect of TGF-β1 on P-gp function. Therefore, the objectives of this study were to determine the effect of TGF-β1 on P-gp function and abcb1 expression in BECs during fetal and neonatal development and to investigate the TGF-β1 signaling pathways involved. We hypothesized that TGF-β1 treatment would increase P-gp function and that the magnitude of this effect would vary with the developmental age at which the BECs were derived.

**Materials and Methods**

**Animals**

Twelve-week-old female Dunkin-Hartley-strain guinea pigs were purchased from Charles River Canada, Inc and bred as described previously (28). Pregnant females were untreated during pregnancy. Two-week-old male guinea pigs were purchased from Charles River. All studies were carried out in accordance with protocols approved by the Animal Care Committee at the University of Toronto and in accordance with the Canadian Council on Animal Care.

**Guinea pig primary brain endothelial culture**

BECs were isolated from gestational day (GD)40, GD50, GD65 male fetuses and postnatal day (PND)14 male guinea pigs, as described previously (9). Briefly, guinea pigs were anesthetized using isoflurane (Baxter Corp) and subsequently decapitated. Brains were collected, transferred into biological safety cabinet, and subsequently cut into small pieces and homogenized. The homogenate was centrifuged and the resultant tissue pellet was

<table>
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<th>Table 1. TGF-β1 Regulation of Multidrug Resistance P-gp in the Fetal and Neonatal Male Blood-Brain Barrier</th>
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<td><strong>Target</strong></td>
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<td>β-Actin</td>
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<td>TGFBR2</td>
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<td>Cadherin2</td>
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immersed in dextran solution (17.5%; Sigma). After collagenase
digestion, the mixture was centrifuged and the collagenase-con-
taining supernatant was removed. Cells were resuspended in
DMEM supplemented with 20% fetal bovine serum (Wisent
Inc), plated on 0.5% gelatin-coated 75 mm² tissue culture flasks
(Becton Dickinson Biosciences), and grown at 37°C in 5% CO₂/air.
These cultures have been fully characterized previously (9).
Cell viability following isolation was 99% as assessed by using
trypan blue (Sigma) staining. Cells were then frozen in liquid
nitrogen until use in the following experiments.

**TGF-β1 treatment and P-gp functional assay**

BECs derived from GD40, GD50, GD65, and PND14 guinea
pigs were plated on gelatin-coated 96-well culture plates (Becton
Dickinson Biosciences) at a seeding density of 1 × 10⁴ cells/cm².
Cells were grown 37°C in 5% CO₂/air for 5 days. At confluence,
medium was replaced with phenol-red free DMEM (Wisent Inc)
and 20% charcoal-stripped fetal bovine serum (Wisent Inc).
Twenty-four hours after media change, cells were treated with
TGF-β1 (0.001–10 ng/mL; Invitrogen) for 2, 4, 8, and 24 hours.
Cell viability following TGF-β1 treatment was 99% as assessed
by using trypan blue (Sigma) staining. These TGF-β1 doses were
selected as maternal plasma levels of TGF-β1 range from 1 to 30
ng/mL (29). After treatment, cells were washed twice with warm
Tyrode’s salt solution (Sigma) and P-gp activity was measured
using an established Calcein-AM assay (9).

**TGF-β1 treatment and P-gp specificity**

BECs derived from PND14 male guinea pigs were grown to
confluence in 96-well plates, as described above. Cells were
treated with phenol red-free medium containing stripped fetal
bovine serum and 10 ng/mL TGF-β1 (8 h). Cells were washed
with Tyrode’s and then subsequently incubated for 1 hour with
either 10⁻⁶ M calcein-AM or 10⁻⁶ M calcein-AM with 10⁻⁴ M
verapamil (VPL) (Sigma). Verapamil is an L-type calcium chan-
nel blocker that has been shown to be a competitive inhibitor of
P-gp (30). Cells were then washed and lysed, and calcein was
measured, as described above.

**Figure 1.** P-gp activity in BEC cultures derived at GD40 (A–D), GD50 (E–H), GD65 (I–L), and PND14 (M–P) after treatment with TGF-β1 (0.001–10 ng/mL) for 2, 4, 8, or 24 hours. Activity is displayed as percentage change from untreated control cells mean ± SEM (N = 8/group). A significant difference from control indicated by *, P < .05; **, P < .01; ***, P < .001.
To further validate that the effects of TGF-β1 were specific to P-gp, an alternative P-gp substrate, rhodamine 123 (Sigma), was used. BECs were treated for 8 hours with either phenol red-free medium containing stripped fetal bovine serum or TGF-β1 (10 ng/mL). Cells were washed before incubation with 10 μM rhodamine 123 for 30 minutes. After lysis, rhodamine 123 accumulation was measured (Excitation/Emission: 485/528 nm).

Quantification of mRNA expression

To investigate whether functional changes in P-gp elicited by TGF-β1 corresponded to changes to abcb1 mRNA expression, PND14 BECs were cultured on 10 cm² gelatin-coated tissue dishes (Becton Dickinson Biosciences) at a seeding density of 1 x 10⁴ cells/cm². Cells were grown at 37°C in 5% CO₂/air for 5 days. At confluence, BECs were treated with TGF-β1 (10 ng/mL) for 2, 4, 8, and 24 hours. BECs were washed twice with Hank’s balanced salt solution and total RNA was extracted using TRIzol reagent (Invitrogen) as per the manufacturer’s protocol. Total RNA was subjected to reverse-transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) as per the manufacturer’s protocol. Samples were incubated at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes using the C1000 Thermal Cycler. In addition, RNA was isolated from cultured cells derived at GD40, GD50, GD65, and PND14 to quantify the TGF-β-associated receptors (tgfr2, alk1, alk5, endoglin, betaglycan).

The mRNA levels were quantified using real-time PCR. Primer sequences were designed using Autoprime (Gunnar Wrobel & Felix Kokocinski) based on transcript ID (Ensemble Genome Browser; guinea pig) and synthesized (Integrated DNA Technologies; Table 1). Real-time PCR was performed using a C1000 Thermal Cycler and quantified using the CFX96 Real-Time System (Bio-Rad). Samples were prepared using primer sets (Applied Biosystems) for respective gene and cDNA template using ratios according to manufacturer instructions. For each primer set, a standard curve was generated by serial dilution of a pooled reference sample with a minimum efficiency greater than or equal to 90%. All samples were run in triplicate. Relative mRNA expression was calculated as gene of interest expression normalized (Δct) to reference gene expression (β-actin). β-Actin was not differentially regulated across gestation or altered by TGF-β1, specific inhibitor of smad3 (SIS3), BMP-9, dorsomorphin, or SB-431542 treatment (data not shown).

Figure 2. P-gp activity after treatment with (A) TGF-β1 (10 ng/mL for 8 h) with or without inhibition of P-gp by 10⁻⁴ M VPL; and (B) P-gp activity (using rhodamine 123 as a P-gp substrate) after an 8-hour treatment with TGF-β1 (10 ng/mL). P-gp activity is displayed as percentage change from untreated control cells (zero line). Values displayed as mean ± SEM (N = 8/group). A significant difference from control indicated by **, P < .01; *** P < .001.

Figure 3. The time course (2–24 h) of effect of TGF-β1 (10 ng/mL) treatment on abcb1 expression in BEC cultures derived at PND14. Abcb1 mRNA expression shown relative to β-actin. Values displayed as mean ± SEM (N = 5/group). A significant difference from control indicated by **, P < .01; *** P < .001.

Figure 4. The developmental profile of (A) alk1 mRNA (type I receptor), (B) betaglycan mRNA (type III accessory receptor) in BEC derived at GD40, GD50, GD65, and PND14. Expression was determined as the ratio of target gene over β-actin. Values displayed as mean ± SEM (N = 6/group). A significant difference from GD40 indicated by **, P < .01; *** P < .001.
Signaling pathways involved in TGF-β1 regulation of P-gp

To investigate signaling pathways mediating TGF-β1 effects on P-gp function, BECs derived at GD50 and PND14 were treated with various ALK1 and ALK5 inhibitors and agonists. We investigated signaling on GD50 and PND14 because levels of signal transducing receptor, alk1, varied with gestational age. To investigate the involvement of ALK5 signaling, BECs were pretreated (1 h) with SB-431541 (ALK5 antagonists; 10 μM; Sigma) or SIS3 (30 μM; Sigma). Cells were then treated with TGF-β1 (10 ng/mL) in the presence of the respective inhibitor for 8 hours. To investigate the role of ALK1 signaling, BECs were treated (2, 8, and 24 hours) with BMP-9 (ALK1 agonist; 0.001–10 ng/mL; R&D Systems). In another experiment, BECs were also pretreated (1 h) with dorsomorphin (ALK1 inhibitor; 1, 8, and 40 μM; EMD Millipore) and subsequently treated with TGF-β1 (10 ng/mL) and inhibitor for 8 hours. P-gp activity was assessed after treatment, using Calcein-AM, as described above. The various treatments had no significant effect on cell viability, which was determined using trypan blue (Sigma; data not shown). After treatment, abcb1 mRNA levels were analyzed via qRT-PCR. SMAD3 activation up-regulates cadherin2 mRNA and thus levels were quantified as a positive control.

Statistical analysis

For each experiment, cells were derived from five to eight animals in each age group and cultured independently. All statistical analyses were performed using Prism (GraphPad Software, Inc). TGF-β1 associated receptors (tgfbr2, alk1, alk5, endoglin, betaglycan) and abcb1 mRNA data were analyzed using one-way ANOVA, followed by Newman-Keuls post-hoc test. For RT-PCR experiments all analyses were run in triplicate. Functional P-gp data were analyzed using one-way ANOVA, followed by Dunnett’s (for comparisons against the control group) and Newman-Keuls (for comparisons against other treatment groups) post-hoc analyses. Functional P-gp data are displayed as percentage change in activity from controls. Significance was set at P < .05.

Results

TGF-β1 regulation of P-gp function during development

TGF-β1 significantly increased P-gp function in BECs derived from fetal (GD40, GD50, GD65) and young (PND14) male guinea pigs (Figure 1). This increase in function occurred within 2, 4, and 8 hours of treatment. However, after 24 hours, no effect of TGF-β1 on P-gp activity was detected apart from at the highest concentration (10 ng/mL) with BECs derived from GD40 fetuses. BECs derived from GD40 and GD50 male fetuses were more responsive to TGF-β1 treatment when compared with PND14 BECs (Figure 1).

Effect of TGF-β1 is P-gp specific

To demonstrate that this effect of TGF-β1 was indeed specific to P-gp, BECs derived from PND14 BECs were exposed TGF-β1 in the presence of P-gp inhibitor, VPL. Treatment of BECs derived at PND14 with TGF-β1 in the presence of VPL obliterated the effects of TGF-β1 on P-gp function (Figure 2A). To further demonstrate the effect of TGF-β1 is P-gp specific, we replicated the TGF-β1-induced increase in P-gp function using an alternative substrate of P-gp, rhodamine 123 (Figure 2B).

TGF-β1 regulates abcb1 mRNA in BECs

TGF-β1 increased abcb1 mRNA levels in BECs derived from PND14 guinea pigs (Figure 3). The effect of TGF-β1 (10 ng/mL) on abcb1 mRNA was biphasic. Within 2 hours, abcb1 mRNA increased 3-fold compared with control (P < .001) and returned to baseline levels at 4 hours. However, abcb1 mRNA then increased by 2.5-fold compared with control at 8 hours (P < .01), returning to control levels by 24 hours. The changes in abcb1 mRNA at 8 and 24 hours mirrored the functional changes in P-gp (Figure 1).

Developmental expression of TGF-β1 associated receptors

The relative expression of TGF-β type II receptor (tgfbr2) mRNA (GD40 1.15 ± 0.33, GD50 1.07 ± 0.49,
GD65 1.04 ± 0.19, PND14 1.03 ± 0.27 tgfbr2/β-actin), alk5 mRNA (GD40 1.12 ± 0.25, GD50 0.71 ± 0.29, GD65 1.071 ± 0.20, PND14 0.74 ± 0.2 alk5/β-actin), and the TGF-β1 type III receptor (endoglin) mRNA (GD40 1.35 ± 0.54, GD50 1.49 ± 0.7, GD65 1.11 ± 0.32, PND14 1.20 ± 0.28 endoglin/β-actin) did not change through development. In contrast, alk1 and betaglycan mRNA levels were significantly higher in BECs derived at GD65 and PND14 compared with those derived at GD40 (P < .01; Figure 4A and P < .001; Figure 4B). Because the response to TGF-β1 significantly decreased in PND14 compared with BECs derived at GD50 (Figure 2), whereas alk1 and betaglycan mRNA levels increased, we examined the signaling mechanisms involved in TGF-β1 regulation of P-gp function at GD50 and PND14.

Role of ALK5 in TGF-β1-induced increase in P-gp

BECs derived at GD50 and PND14 were treated with the ALK5 inhibitor SB-431542 to examine if activation of ALK5 is required for P-gp regulation. SB-431542 is a small molecule that inhibits the intracellular kinase domains of ALK5 (31). Both doses of SB-431542 prevented the TGF-β1 induced increase in P-gp activity in BECs derived at GD50 and PND14, indicating that ALK5 is required for TGF-β1 regulation of P-gp function (Figure 5, A and B). To further define the role of ALK5, BECs derived from PND14 male guinea pigs were treated with specific inhibitor of SMAD3 (SIS3). SMAD3 is a signal transduction molecule that is phosphorylated as a result of ALK5 activation, and SIS3 blocks this action (32). Treatment of BECs derived at GD50 and PND14 with TGF-β1 in the presence of SIS3 did not reduce P-gp activity or abcb1 mRNA compared with cells treated with TGF-β1 alone (Figure 6, A, B, D, and E), indicating that SMAD3 was not involved in ALK5-mediated regulation of P-gp. As a positive control, the effect of SIS3 on TGF-β1-induction of cadherin 2 mRNA was determined because TGF-β1 acting via SMAD3 increases cadherin 2 expression (33). As expected, TGF-β1 induction of cadherin 2 mRNA was prevented by SIS3 treatment (Figure 6, C and F).

Role of ALK1 in the TGF-β1-induced increase in P-gp

The contribution of ALK1 activation to changes in P-gp function was investigated. ALK1 signals through SMAD1/5. BECs derived at GD50 and PND14 were treated with the ALK1 agonist BMP-9 (34, 35). Treatment with BMP-9 caused an increase in P-gp activity in BECs derived at both GD50 and PND14 (Figure 7, A–D). However, there was a discrepancy between the effects of BMP-9 (Figure 7) and TGF-β1 (Figure 1). Treatment with BMP-9 (10 ng/mL; 24 h) stimulated an increase in P-gp function in cells derived at GD50 (P < .001) and PND14 (P < 0.05). In contrast, there was no effect of TGF-β1 on P-gp function at 24 hours in BECs derived from PND14 guinea pigs (Figure 1). One explanation is that TGF-β1 is no longer active after 24 hours, in contrast to the agonist BMP-9. To investigate this, BECs derived at PND14 were exposed to TGF-β1 for 24 hours. After 24 hours, this medium was transferred to new BECs derived at PND14 and P-gp activity was accessed. After 2 hours of treatment, p-gp activity remained unchanged.

To confirm that activation of ALK1 is required for TGF-β1-mediated regulation of P-gp, BECs de-
rived at GD50 and PND14 were treated with dorsomorphin (ALK1 inhibitor) (36). Dorsomorphin antagonized the TGF-β1-induced increase in P-gp function in BECs derived at PND14, indicating that ALK1 is required for TGF-β1 regulation of P-gp function (Figure 8B). However, the same doses of dorsomorphin did not inhibit the TGF-β1-induced increase in P-gp function in BECs derived at GD50 (Figure 8A).

Discussion

This is the first study to show that TGF-β1 is a potent modulator of P-gp function in BECs derived in late gestation and the early postnatal period. Effects were greater in BECs derived at earlier stages of development. Moreover, we have shown that the effect of TGF-β1 on P-gp is dependent on ALK5 activation. However, the regulatory effects of TGF-β1 on P-gp function and abcb1 mRNA do not appear to involve classical ALK5/SMAD3 signaling. In addition, activation of the ALK1 pathway mimicked the TGF-β1-induced regulation of P-gp function and we have shown that this pathway is dependent on the maturity of BEC.

There was generally good correlation between P-gp function and abcb1 mRNA following stimulation with TGF-β1. Abcb1 mRNA levels increased at 2-hour and 8-hour time points and decreased at 24 hours, correlating with respective functional data at these time points. The biphasic effect of TGF-β1 on abcb1 mRNA (indicated by the lack of effect of TGF-β1 on abcb1 mRNA at 4 h) may be due to both direct and indirect mechanisms. It is known that TGF-β1 modulates gene expression by affecting transcriptional activation and mRNA turnover rate (37). TGF-β1 has been shown to enhance the stability of COX-2 mRNA in intestinal epithelial cells and human lung fibroblasts (38, 39), and products of this enzyme have potent regulatory effects on P-gp function (40). Moreover, TGF-β1 may stimulate endothelial cells to secrete various factors, potentially affecting P-gp function (10). Thus, the biphasic effect of TGF-β1 on abcb1 mRNA may also result from the production of TGF-β1-induced factors from the endothelium.

The present study has identified the downstream signaling pathways by which TGF-β1 regulates abcb1 mRNA levels and P-gp activity in the developing BBB. Through inhibition of the ALK5 intracellular kinase domain, we have shown that activation of ALK5 is required for TGF-β1 regulation of P-gp. Moreover, by inhibiting SMAD3, we demonstrated that ALK5-associated SMAD3 is not required for TGF-β1 regulation of abcb1 mRNA expression and P-gp function in BECs. However, due to the lack of a commercially available ALK5 inhibitor, we were unable to specifically and completely obliterate ALK5 signaling. Thus, it remains possible that P-gp is regulated through ALK5 non-SMAD signaling pathways such as MAPK and PI3K (41, 42). BECs also express ALK1, a type I receptor activated by TGF-β1. To our knowledge, this is the first study to demonstrate that activation of ALK1 with BMP-9, which induces similar effects on P-gp function to those of TGF-β1. Also, similar to TGF-β1, BMP-9-induced effects on P-gp were reduced in BECs derived near term compared with those derived earlier in gestation. This decreasing cellular responsiveness to BMP-9 may be attributed to an increase in betaglycan mRNA, as betaglycan has been shown to be a negative regulator of BMP signaling (26, 27). Moreover, signaling through the ALK1 pathway is dependent on maturity of BEC because ALK1 inhibitor, dorsomorphin, markedly reduced the TGF-β1-induced increase in P-gp function in BECs derived at PND14, but not those derived at GD50. We speculate that this is may be due to low alk1 mRNA expression in BECs derived at GD50 compared

Figure 7. P-gp activity in BEC cultures derived at (A, B) GD50 and (C, D) PND14 after treatment with BMP-9 (0.001–10 ng/mL) for 8 or 24 hours. P-gp activity is displayed as percentage change from untreated control cells mean ± SEM (N = 8/group). A significant difference from control indicated by *, P < .05; **, P < .01; ***, P < .001.

with those derived at PND14, and that TGF-β1 effects in early development are primarily mediated by ALK5. These findings are consistent with studies demonstrating low alk1 mRNA expression in microvessels derived from mouse forebrain at embryonic day 9 (43).

The balance between alk1 and alk5 mRNA expression is crucial for healthy brain development as aberrations in these receptor levels contribute to the pathogenesis of congenital conditions, such as brain arteriovenous malformations (BVM). The pathogenesis of BVM, the primary cause of intracranial hemorrhage, is poorly understood. Human studies have shown that there is a decrease in ALK1 mRNA expression and an increase in ALK5 mRNA expression in BVM (44). This imbalance of receptor levels correlates with the lower expression of ABCB1 mRNA in BVM when compared with normal human brain samples (45). Therefore, it is possible that compromised brain protection through reduced levels of P-gp activity may contribute to the pathogenesis observed in BVM.

Expression of the TGF-β1 associated receptors, tgfbr2, alk5, and endoglin, did not change in BECs derived from GD40 to PND14, suggesting that these receptors are not responsible for the decrease in BEC responsiveness to TGF-β1 with advancing gestation. However, betaglycan mRNA, an accessory receptor to TGF-β1 signaling, dramatically increased in late gestation. Previous studies have demonstrated that betaglycan-mediated changes in TGF-β1 responsiveness vary with cell type and state (46). Mesenchyme-derived cells, including mesangial cells, are generally poorly responsive to TGF-β1 and express high levels of betaglycan (47). Studies have shown that membrane-bound betaglycan decreases cellular responsiveness to TGF-β1 by preventing TGFBR2 from recruiting and activating TGFBR1 (26, 27). Betaglycan may function through a similar mechanism in BECs, which may explain why increasing levels of betaglycan in late gestation and postnatal BECs correlate with decreasing responsiveness to TGF-β1.

The present study also demonstrated that alk1 mRNA levels were higher in BECs derived from GD65 and PND14 guinea pigs compared with those derived at GD40 and GD50. It has been shown that at earlier stages of development, TGF-β1 acting through the ALK5 receptor is provasculogenic. However, later in development when the endothelial cells have differentiated and both alk1 and alk5 are expressed, there is a shift toward an angiogenic state (48). In terms of the brain vasculature of the developing guinea pig, the highest rate of brain growth occurs from GD40 to PND14 (49), which is accompanied by increasing oxygen demand by this tissue. This demand is met by increasing blood flow to the brain via angiogenesis. Thus, the rise in alk1 mRNA expression BECs derived at PND14 correlates with increasing rates of angiogenesis in the brain vasculature of the neonatal guinea pig. Our studies have shown that TGF-β1, at least partially, mediates P-gp function through ALK1 and so it might be expected that TGF-β1 regulation of P-gp would be more potent in BECs derived from late gestation and neonatal guinea pigs. However, as discussed above, betaglycan expression increases and has been shown to disrupt the interaction between TGFBR2 and TGFBR1. Therefore, an increase in betaglycan may counteract the expected increase in TGF-β1 responsiveness associated with an increase in ALK1 expression. However, the direct relationship between betaglycan and TGF-β1 signaling in BECs requires further investigation.

Based on our data, we can conclude that the timing of TGF-β1 activation at the developing BBB is likely important for brain homeostasis. This activation can occur as a result of TGF-β1 derived from blood or brain extracellular fluid. Increased levels of TGF-β1 in maternal plasma levels have been described in gestational diabetes and pre-eclampsia (50, 51). Because TGF-β1 can cross the placenta (52), this will result in altered TGF-β1 levels in the fetal circulation. In addition, perturbations in TGF-β1 levels...
caused by delayed or early gliogenesis, such as that observed in fetal alcohol syndrome and autism (53, 54), may affect brain protection and consequently contribute to disease pathways. This may, in turn, result in substantial changes in fetal brain exposure to xenobiotics and other P-gp substrates, many of which have teratogenic properties and thus may contribute to disease progression.

In vitro, we have shown that a single dose of TGF-β1 elicits rapid effects on abcb1 mRNA expression and P-gp function, which are no longer present at 24 hours. Our results infer that TGF-β1 contributes to dynamic regulation of P-gp and that short-term perturbations in TGF-β1 do not result in permanent changes in P-gp function. However, in vivo, the release of many astrocyte-derived factors occurs in a pulsatile fashion in response to neuronal activation (55). The release of TGF-β1 from astrocytes may occur in this manner, which may result in a constant regulation of P-gp. BECs and astrocyte coculture studies are required to further investigate this important relationship.

In conclusion, TGF-β1 potently regulates P-gp activity and abcb1 mRNA at the developing BBB, but the magnitude of these effects is age-dependent. We have shown, for the first time, that ALK5 signaling through SMAD3 is not essential for TGF-β1-regulated P-gp function in fetal BECs. Moreover, we have identified that TGF-β1 signaling through the ALK1 pathway represents an important route in the regulation of P-gp function in the developing BBB, particularly near term. P-gp in the fetal BBB protects the developing brain, preventing a wide spectrum of endogenous and exogenous factors from entering the fetal brain. Aberrations in TGF-β1 levels, either as a result of delayed or as early glial cell differentiation, may lead to substantial changes in fetal brain exposure to P-gp substrates, triggering profound consequences with respect to brain development.

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Disclosure Summary: The authors have nothing to declare.

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