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Controlled Delivery of Bioactive Molecules for Improvement of Endothelial Progenitor Cells in Gestational Diabetes Mellitus

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Abstract

Endothelial Colony-Forming Cells (ECFCs) are a subtype of endothelial progenitor cells (EPCs) from circulating adult and human cord blood that express characteristics of putative EPCs. Due to their robust clonal proliferative potential and ability to form *de novo* blood vessels *in vivo*, ECFCs have been used in cell-based therapies and tissue engineering to treat cardiovascular diseases. However, in diabetic conditions, ECFCs are subject to stress-induced premature dysfunction that limits their therapeutic use. Previous studies showed that ECFCs from gestational diabetes mellitus (GDM) exhibit upregulation of transgelin (TAGLN), lower cell migration, and impaired angiogenic potential. Here, we hypothesize that cell surface engineering of a targeted small molecule delivery system can help rejuvenate GDM-ECFCs and restore their therapeutic potential.

This study reports GDM-ECFC rejuvenation through controlled delivery of the small molecule SB-431542 (SB). SB inhibits transforming growth factor-β (TGFβ**)** via targeted delivery to ECFCs, inhibiting the TGFβ-ALK1 signaling pathway and regulating TAGLN levels. Via controlled delivery of SB, progenitor phenotypes of GDM-ECFCs can be restored, as suggested by decreased TAGLN expression. This is further confirmed by improved cellular migration and proliferation, as well as enhanced angiogenic potential of GDM-ECFCs *in vitro* and *in vivo.* The ability of this engineered system to achieve targeted SB delivery and yield reparative effects for ECFCs offers a promising novel therapeutic method for treatment of cardiovascular complications in diabetic patients.

Contents

1 Introduction

Diabetes mellitus can lead to subsequent detrimental health disorders, most of which are related to cardiovascular complications. Major factors include that endothelial progenitor cells are rare (Gian Paolo Fadini et al., 2005; Hamed, Brenner, & Roguin, 2011; Tepper et al., 2002), lack angiogenic potential at the site of injuries, and have impaired abilities to restore blood perfusion (Wils, Favre, & Bellien, 2017). In gestational diabetic pregnancy, which accounts for 10% of all pregnancies, infants are also at higher risk of developing chronic obesity, type 2 diabetes, and hypertension later in their lives (Blue et al., 2015). Studies conducted with cord blood endothelial progenitor cells, called endothelial colony-forming cells (ECFCs), showed that ECFCs obtained from gestational diabetes mellitus (GDM) pregnancy were fewer in number and demonstrated reduced capability to form networks *in vitro* (Ingram et al., 2008). Recently, Varberg *et al.* found that dysregulation of Transgelin (TAGLN) expression in GDM-ECFCs affected the migration and network-forming ability of cells *in vitro* (Varberg et al., 2018).

As conventional drugs for treating cardiovascular diseases in diabetic patients are non-specific and may result in pleiotropic effects, strategies have been developed to increase the number of progenitor cells and reverse their altered functions (G. P. Fadini & Avogaro, 2010). In the current study, we focus on improving GDM-ECFC functions by targeted delivery of a small molecule called SB-431542 (SB), which is an inhibitor of transforming growth factor-β (TGFβ). Many bioactive molecules have been used to improve functions of endothelial progenitor populations, such as growth factors (VEGFs, PDGFs, bFGFs), NO donors, and other endothelial-derived factors (Wils, Favre, and Bellien, 2017). TGFβ-inhibitors serve as potent regulators of angiogenesis by regulating downstream expression of angiogenic factors, specifically vascular endothelial growth factors, such as Transgelin and extracellular matrix components such as integrins. The SB-431542 nanoparticle used in this experiment exhibits a compartmental nanoparticle delivery system, releasing molecules that interfere with TGFβ stimulation through the ALK5/SMAD3 pathway. This reduces downstream effects known to inhibit vessel remodeling.

This study assesses the angiogenic impact of treated cells and inhibition of the TGFβ-ALK1 signaling pathway. Controlled delivery of SB431542 molecules (TGFβ-inhibitor) was accomplished by release from nanoparticles via diffusion. The initial concentration of molecules or number of nanoparticles used for cell conjugation was adjusted to control delivery. We observed that controlled delivery of TGFβ inhibitor to ECFCs improves migration capabilities and network formation of ECFCs *in vitro* (data not shown). In this paper, we particularly demonstrate the effect of treatment on regulating TAGLN and SERPINE expression in order to address one of the causes that alters the functions of GDM-ECFCs.

TGFβ operates through two different receptors, ALK5 and ALK1. When TGFβ stimulation operates through ALK5, SMAD3 is activated to yield downstream effects on specific transcription factors, ultimately inhibiting vessel remodeling. Through the ALK1 pathway, however, TGFβ signaling leads to activation of the SMAD1/5/8 complex, operating on different transcription factors from the ALK5 pathway and leading to promotion of vessel remodeling. The SB used in this experiment is thought to inhibit the ALK5/SMAD3 pathway, thus interfering

with the aforementioned mechanism and decreasing the downstream effects that typically inhibit vessel remodeling (Patterson, 2014).

Transgelin (TAGLN) is a gene target of the TGFβ/SMAD3 pathway. Through ALK5-dependent TGFβ signaling, SMAD3 can be induced to bind the TAGLN promoter (Yu et al., 2008). The TAGLN promoter region from -162 to +41 is up-regulated by the SMAD3/4 complex and AP-1 elements following TGFβ stimulation (Chen, Kulik, & Lechleider, 2003). Prior studies have investigated the role of TAGLN, the TGFβ-dependent gene, on cellular activities including vasculature formation and repair in human pulmonary arterial smooth muscle cells (Huang et al., 2018).

In addition to TAGLN, it was shown that TGFβ regulates SERPINE expression and is capable of leading to overexpression of SERPINE (Mo, Zhang, Ji, Liu, & Fan, 2015). SERPINE inhibits plasmin generation, thus progressing diseased conditions in vascular disorders. SERPINE transcription is induced by TGFβ activation via EGFR and Rho signaling mechanisms. In the EGFR pathway, TGFβ stimulation activates EGFR/pp60$^{c\text{-}src}$, which activates MEK/ERK1/2 and leads to SERPINE expression. The EGFR/MEK pathway operates through USF-dependent transcriptional controls. Through the Rho mechanism, TGFβ activation stimulates Rho/ROCK, which activates SMAD2/3 and leads to SERPINE expression. Specific promoter regions within SERPINE, such as PE1/2 (E5/E4) and HRE, are thought to act as molecular switches regulating SERPINE transcription during activation of fibrogenic factors, such as TGFβ. SERPINE acts as a factor causing and marking the development of cardiovascular disease; thus, SERPINE inhibition has been shown to slow disease progression (Samarakoon & Higgins, 2008).

2 Methods

1. Cell Culture

The human ECFCs were courteously provided by the AngioBio Core Facility of Indiana University School of Medicine at South Bend with an approved IRB protocol. ECFC cells were isolated and cloned from human cord blood of newborn babies from either normal or GDM pregnancy based on established protocol (Mead et al., 2008). Progenitor phenotypes were mainly identified by their colony-forming property and characterized by their expression of specific cell surface markers such as CD31, CD45, CD144, and CD146. For both normal and GDM phenotypes, 4 different cell lines were utilized. Normal ECFCs included E1-CB-111, E1-CB-150, E1-CB-153, E1-CB-157; GDM-ECFC lines included E1-CB-36, E1-CB-37, E1-CB-71, and E1-CB-74. Complete medium for growing ECFCs consisted of endothelial cell growth medium 2 (Promocell C-22011), supplement Mix (Promocell, C-39216), and 0.2% mycoZap^{TM} Prophylactic (Lonza). The cells were maintained at 37^{o}C, 5% CO_2 and passaged as needed using DetachKit (Promocell, C-41222).

2. Nanoparticle Delivery System

Delivery via liposomal nanoparticle used in this experiment was developed in the Hanjaya-Putra lab, and it includes multilamellar liposomes under 200nm in size. This method has been confirmed to provide a biocompatible delivery system. Details of this technique are pending publishing -- in this experiment, the system was used to investigate gene expression of cells under study.

3. Gene Expression

RNA Extraction

Cellular RNAs were isolated and purified using TRIzol® Reagent (Life technologies) and RNeasy® Plus Mini Kit (Qiagen). Adherent cells were homogenized in TRIzol reagent for 10 minutes. Homogenates sat at room temperature for 5 minutes, followed by addition of 140µL of chloroform to each tube. Tubes were shaken for 15 seconds and sat at room temperature for 2-3 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C. Following centrifugation, samples separated into 3 phases: an upper aqueous phase containing RNA, a white interphase, and a lower organic phase. RNAs were extracted by transferring 200µL of the upper aqueous phase into a new collection tube. 1.5 volumes (300µL) of 100% ethanol were added to each tube and mixed by pipetting repeatedly. All of this sample was transferred to an Rneasy spin column in a 2mL collection tube and centrifuged at 8000g for 15 seconds.

After discarding flow-through, 700µL of Buffer RW1 was added to the spin column to wash the column membrane. Columns were centrifuged at 8000g for 15 seconds and flow-through was discarded. 500µL of Buffer RPE with ethanol was added to the column membrane, centrifuged at 8000g for 15 seconds, and flow-through was discarded. This step for Buffer RPE was repeated, now with a centrifugation time of 2 minutes to wash the column membrane. In new 2mL collection tubes, spin columns were centrifuged for 1 minute at full speed to eliminate carryover of Buffer RPE. Columns were placed in 1.5mL collection tubes with 20µL of Rnase-free water added to each tube, and the tubes were centrifuged for 1 minute at full speed. RNA concentrations and purification were determined using NanoDrop™ 2000c Spectrophotometer (Thermoscientific) with Rnase-free water as the blank.

cDNA Synthesis

RNA volume needed to yield 500ng of RNA in a 10µL RNA/H_2O solution was calculated from RNA concentration. A 2x RT MasterMix with Rnase inhibitor was made from a High-Capacity cDNA Reverse Transcription Kit (Appliedbiosystems) with the following ratios: 2.0µL 10x RT buffer, 0.8µL 25x (100mM) dNTP mix, 2.0µL 10x RT random primers, 1.0µL reverse transcriptase, 1.0µL Rnase inhibitor, 3.2µL nuclease-free H_2O. 10µL of MasterMix was added to each well of a PCR strip tube, followed by 10µL of the RNA sample, with pipetting to mix samples. Tubes were sealed, spun to eliminate bubbles, and loaded into a Mastercycler (Eppendorf) with the following thermal cycling program: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and 4°C on hold until the tube was unloaded. Synthesized cDNA

was preserved at -80°C for further use.

mRNA Expression (RT-qPCR)

Quantitative real-time PCR (RT-qPCR) was conducted to determine gene expression at mRNA level. The TaqMan assay was used, which typically had specific PCR primers and a FAM label. Four TaqMan assays used in the experiment included two target genes (TAGLN, SERPINE) and two endogenous control genes (GAPDH, HPRT-1). A cocktail for one assay included: 5.0µL TaqMan MasterMix, 0.5µL assay, 3.5µL Rnase-free water. Each well of a 384-well plate (Appliedbiosystems) was filled with 9.0µL of cocktail and 1.0µL of cDNA. Negative control included 1.0µL of Rnase-free water instead of cDNA solution. The plate was sealed, spun to eliminate bubbles (Fig. 1), loaded into an RT-qPCR machine (QuantStudio™ 5 System, Appliedbiosystems) and run by a fast-mode cycle (Fig. 2). Experiment of each condition was performed in triplicates.

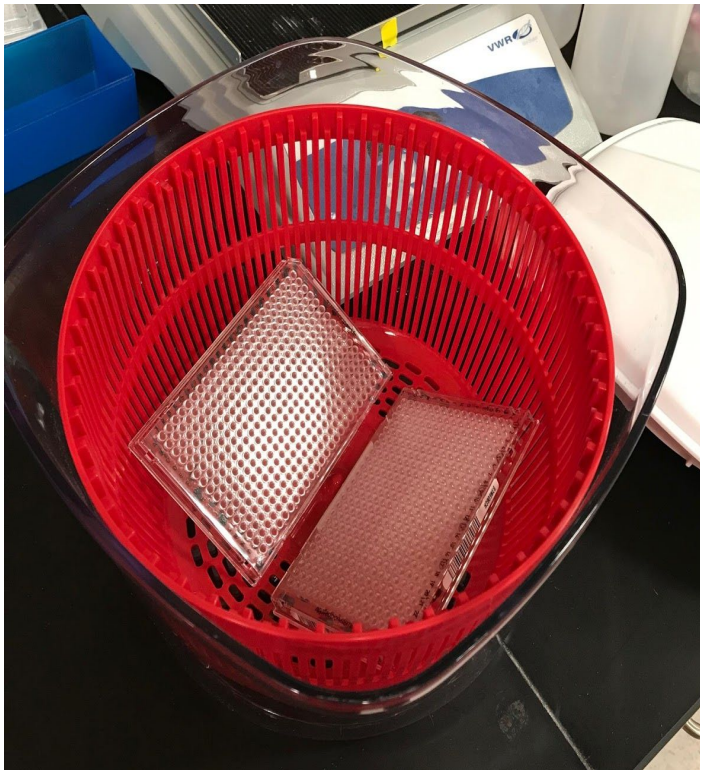

Figure 1: Spinning apparatus for 384-well plate (with balance) prior to RT-qPCR. Sample wells frequently contained bubbles after pipetting; spinning was necessary to eliminate bubbles, as RT-qPCR is affected by bubble presence.

Figure 2: QuantStudio™ 5 System (Appliedbiosystems). Samples in a 384-well plate were run by the following fast-mode cycling program (repeated 40 times) for RT-qPCR analysis: 50.0°C for 2 minutes, 95.0°C for 20 seconds, 95.0°C for 1 second, and 60.0°C for 20 seconds.

3 Results and Discussion

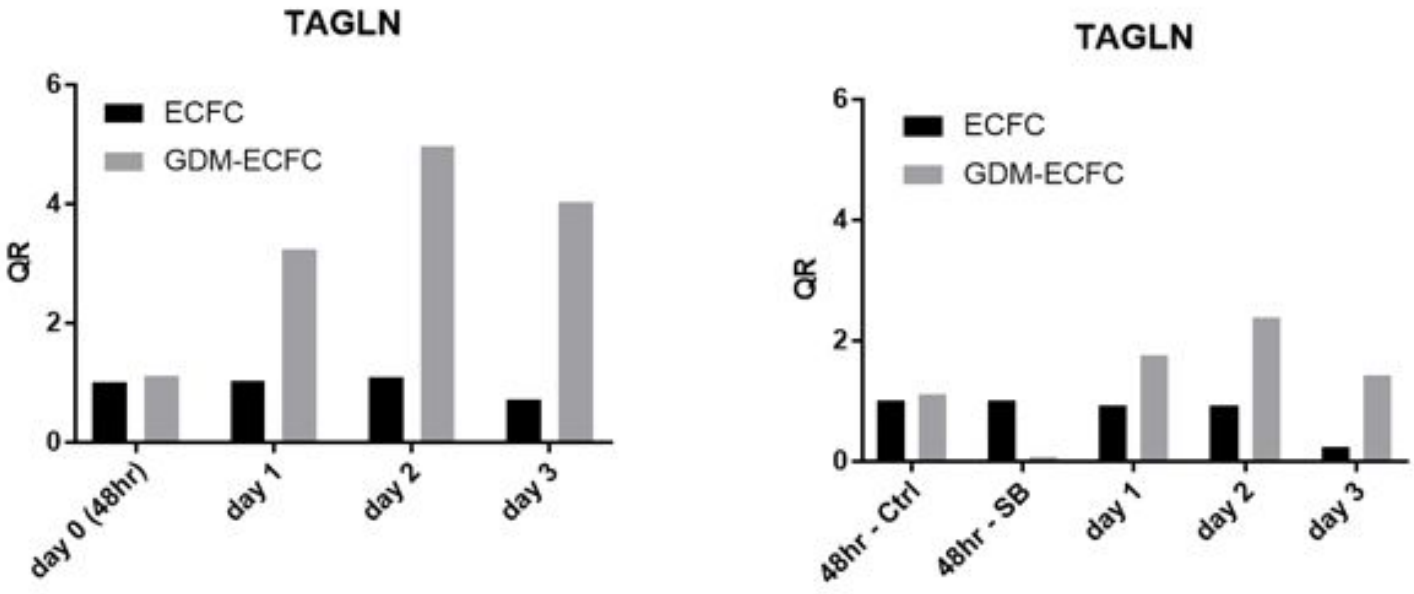

Figure 3A: TAGLN gene expression of normal ECFCs and GDM-ECFCs over multiple time periods was studied in control (left) and SB-treated (right) conditions. A kinetics experiment was performed to determine TAGLN gene expression in normal ECFCs vs. GDM-ECFCs for non-SB (left) vs. SB-treated (right) conditions. TAGLN expression was shown to be relatively constant in normal ECFCs from day 0-3, with an increase in TAGLN gene expression for GDM-ECFCs from days 0-3 (left). Day 0 includes a 48-hour incubation period in control medium. After confirming the expected increase in TAGLN gene expression for GDM-ECFCs over normal ECFCs, TAGLN expression was measured in SB-treated normal ECFCs and GDM-ECFCs (right). While in normal ECFCs expression of TAGLN remained relatively constant over time, GDM-ECFC TAGLN expression showed a slight increase relative to ECFCs. Reduction occurred in TAGLN expression for GDM-ECFCs in the SB-treated (right) vs. non-SB treated (left) condition, confirming delivery of SB to GDM-ECFCs reduces TAGLN expression.

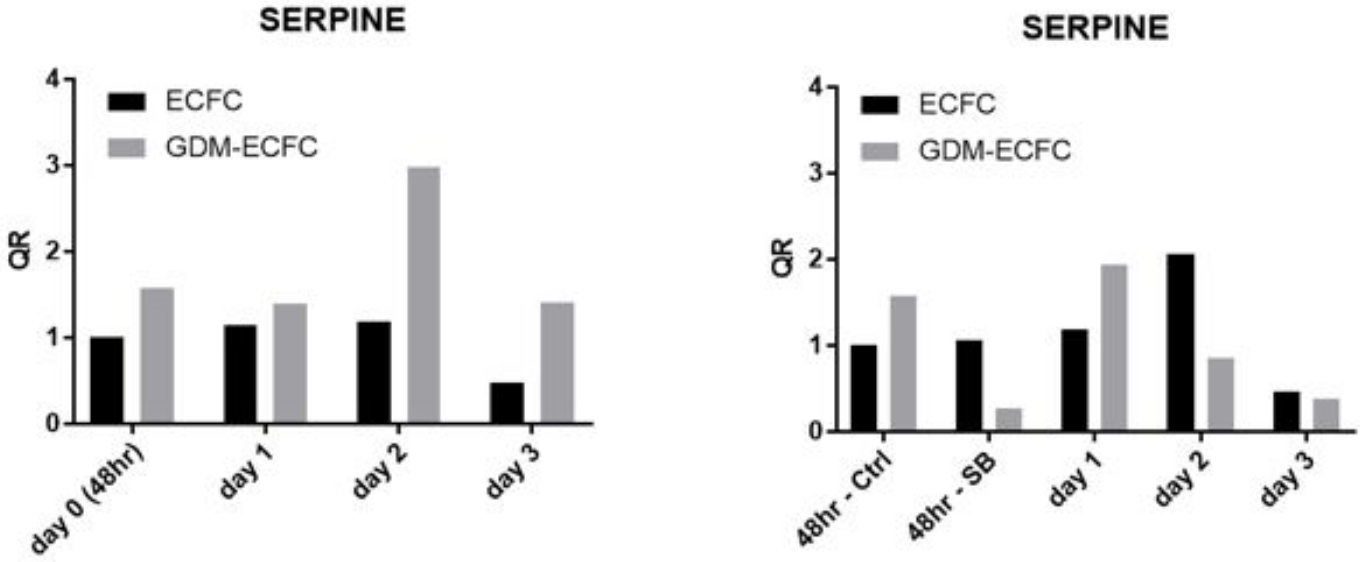

Figure 3B: SERPINE expression in normal ECFCs and GDM-ECFCs over 3-day trial period. SERPINE expression was measured in normal ECFCs and GDM-ECFCs in non-SB treated conditions (left) over 3 days. Day 0 includes a 48-hour incubation period in culture medium. The left panel confirms relatively constant SERPINE expression over time in normal ECFCs versus an increase in SERPINE expression (day 2) observed for GDM-ECFCs. The right panel shows reduced SERPINE expression of SB-treated GDM-ECFCs starting at day 2 (right).

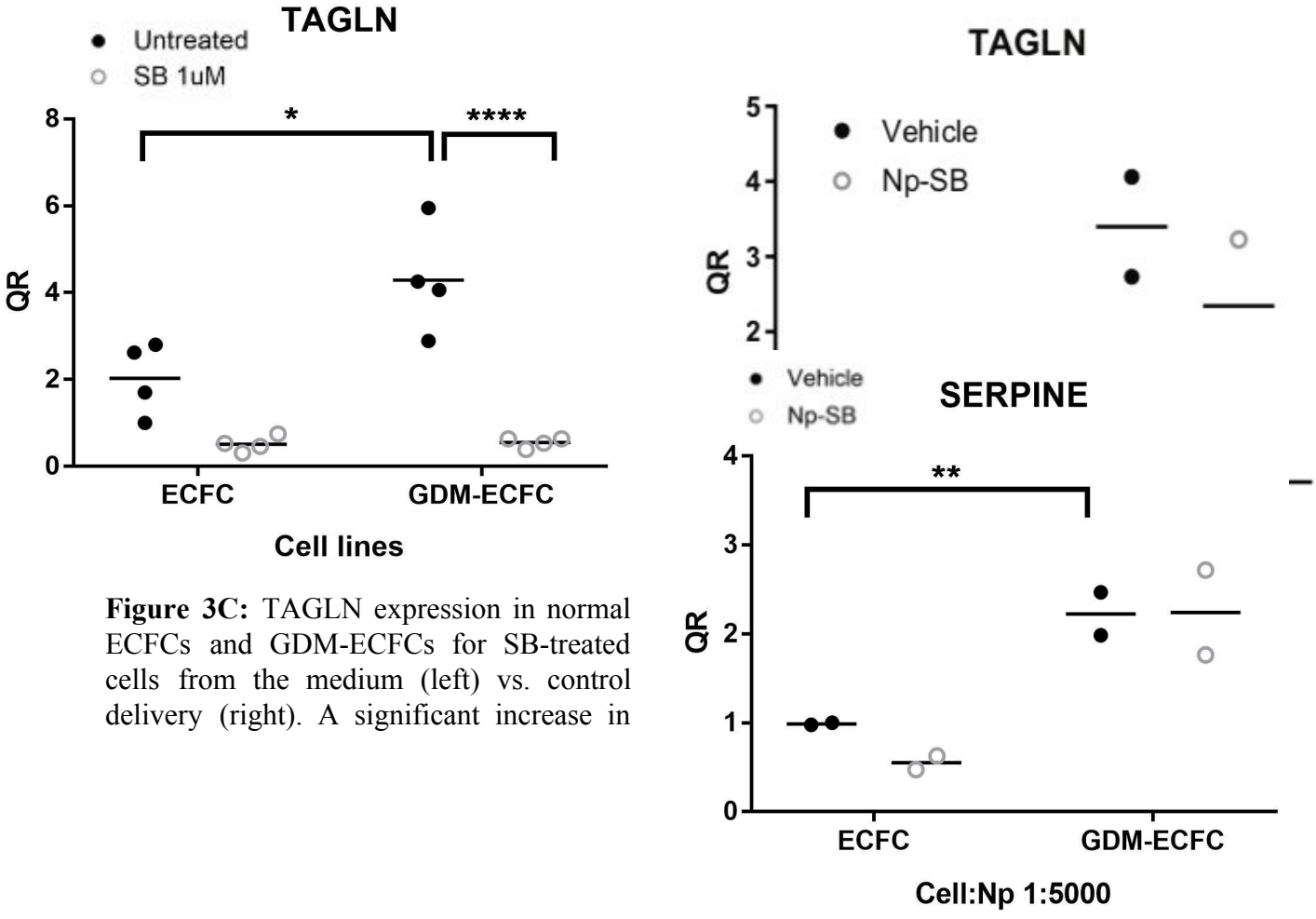

Figure 3C: TAGLN expression in normal ECFCs and GDM-ECFCs for SB-treated cells from the medium (left) vs. control delivery (right). A significant increase in

TAGLN expression occurs for untreated (left) GDM-ECFCs compared with normal ECFCs (p<0.05). An increase in TAGLN expression occurs for vehicle-treated (right) GDM-ECFCs compared with normal ECFCs. Upon SB treatment via both SB medium and vehicle (left and right), TAGLN expression decreases for both normal ECFCs and GDM-ECFCs. GDM-ECFCs show a significant decrease in TAGLN expression between untreated and SB medium-treated cells (left, p<0.0001). This figure reveals the possibility of using the controlled delivery system as the replacement of SB diluted in medium. While the effectiveness of SB in lowering TAGLN decreases over time due to natural excretion of SB by the body, controlled delivery of SB can encourage sustained presence and longer-lasting effects of SB. Fig. 3C shows controlled delivery of SB (Np-SB) reducing TAGLN expression in treated vs. non-treated endothelial cells, confirming the success of the delivery system's desired effects to decrease TAGLN expression.

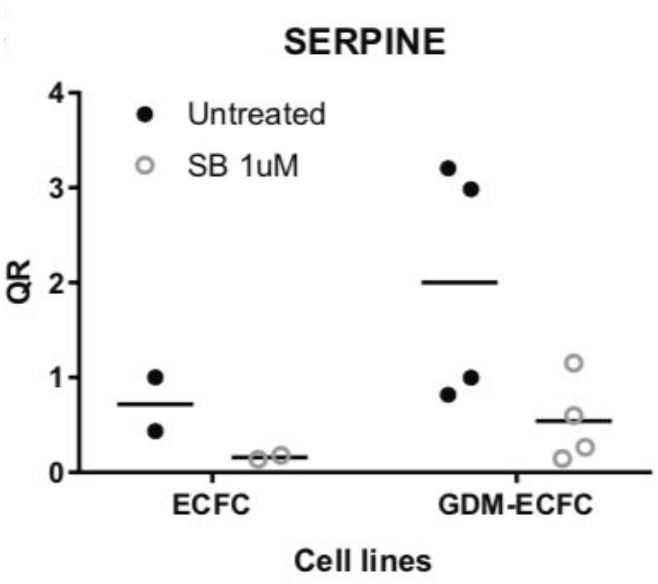

Fig. 3D: SERPINE expression in normal ECFCs and GDM-ECFCs for SB-treated cells from the medium (left) and from control delivery (right). Like Fig. 3B, Fig. 3D confirms increased SERPINE expression in GDM-ECFCs vs. normal ECFCs, with a significant increase observed for SERPINE expression in vehicle-treated GDM-ECFCs vs. normal ECFCs (p<0.01). Decreased SERPINE expression is observed in SB-treated GDM-ECFCs vs. non-treated GDM-ECFCs, making SB-treated GDM-ECFCs comparable to the non-diseased condition. Fig. 3D is expected to confirm a similar trend as observed for TAGLN in Fig. 3C: treatment of GDM-ECFCs via controlled delivery (Np-SB) should produce lowered SERPINE expression compared to untreated (vehicle) GDM-ECFCs. Though this effect is not strongly observed for SERPINE, its pattern is predicted from observable effects of TAGLN expression in Np-SB-treatment for GDM-ECFCs (Fig. 3C). The experiment can be repeated to observe if expected trends arise in SERPINE, or if SERPINE operates by a different mechanism and yields unanticipated outcomes.

In the experimental results, three conditions were tested to evaluate the effects of GDM vs. normal ECFCs on expression of two target genes (TAGLN, SERPINE):

1) Kinetic expression: measured genetic expression over a set time period following a 48-hour day 0 incubation period. While experiments for this condition confirmed increased gene expression in GDM-ECFCs and the ability of SB medium to counteract this increase, the question arose how the effects of treatment with SB medium change over time. Kinetic properties were investigated in Figs. 3A and 3B by measuring gene expression over multiple time points following a 48-hour incubation period. It was revealed that positive effects of SB medium treatment – reduction in gene expression levels in GDM-ECFCs compared to normal ECFCs – were blunted over time. That is, the ability of SB medium to produce desired decreases in TAGLN and SERPINE levels in GDM-ECFCs vs. normal ECFCs decreased as the SB medium was replaced by fresh medium after 48 hours (day 0). This decrease is similar to natural excretion of SB in the body, such as through urination. As the body metabolizes and excretes substances continuously, SB medium becomes involved in this process, decreasing its effects in the body as natural metabolic processes occur. It has been shown that long-term administration of SB-431542, with quantifiable activity at tyrosine kinases, has wide effects throughout a number of tissues that can lead to potential off-target, deleterious effects (Patterson, 2014). Additionally, current methods of SB delivery require intraperitoneal or intravenous dosing twice daily due to natural metabolism of the molecule, making therapeutic uses largely impractical (Patterson, 2014). Thus, while SB medium was confirmed to decrease gene expression levels in GDM-ECFCs, the next point of study became how to sustain SB's positive effects on GDM-ECFCs for longer time periods.

2) Normal ECFCs vs. GDM-ECFCs (SB in medium): measured gene expression in normal ECFCs vs. GDM-ECFCs. From these results, effects of the GDM condition on gene expression of TAGLN (Figs. 3A and 3C) and SERPINE (Figs. 3B and 3D) were observed. The GDM condition increased TAGLN and SERPINE expression compared to normal ECFCs (Figs. 3A and 3B, left), confirming gene expression was a variable of interest for reduction in GDM-ECFCs. Figs. 3A, 3B, and 3C demonstrated decreased expression of TAGLN and SERPINE (respectively) for SB 1 μM medium treatment, confirming the expected decrease in gene expression and reduction toward levels closer to the normal condition for SB medium-treated GDM-ECFCs. These results confirm that ECFCs and GDM-ECFCs differ based on TAGLN and SERPINE expression, with higher expression in GDM-ECFCs. The increase in gene expression can be combated by treating GDM-ECFCs with SB 1μM medium, leading to reduced gene expression in GDM-ECFCs and assumption of gene levels more representative of normal ECFCs.

3) Control Delivery: Vehicle vs. Treated: measured genetic expression in control (Vehicle) vs. treated (SB-Np) ECFCs and GDM-ECFCs. To address the possibility of sustaining SB's positive effects on GDM-ECFCs for longer time periods, a control delivery system was developed for direct SB delivery into GDM-ECFCs. Targeted delivery of SB via a controlled delivery method was designed to protect SB from interaction with and excretion by natural bodily processes, sustaining SB's presence and beneficial effects for longer time periods in GDM-ECFCs. Figs. 3C and 3D confirmed that SB treatment via targeted delivery produced desired effects of SB to

lower gene expression in GDM-ECFCs vs. normal ECFCs. This targeted delivery system would maintain the sustainable delivery of molecules to cells over time, especially during cell culture when medium was changed. Thus, targeted delivery reduces the number of molecules needed for the experiment. Both GDM and normal ECFCs were treated with Vehicle as a control, confirming the targeted carrier method did not significantly affect gene expression levels. Thus, the observed effect was due to SB treatment via controlled delivery methods. SB-Np treatment was effective in reducing TAGLN levels as desired (Fig. 3C, right), with gene expression showing a similar pattern as treatment with SB medium. Though significant reduction of SERPINE expression was not observed in SB-Np treated GDM-ECFCs, reduction was observed for normal ECFCs. Since SB-431542 lacks perfect targeting of the SERPINE pathway, it is suspected that GDM triggers other pathways that hinder SERPINE regulation. Further experiments involving GDM-ECFCs are needed to investigate this hypothesis and its potential for future improvements to the regulation system. Overall, treatment of SB-Np produced comparable beneficial effects as those known with SB medium treatment for both cell types treated with TAGLN and normal ECFCs treated with SERPINE, confirming the potential effective targeted delivery mechanism of SB in treating diseased GDM-ECFCs.

4 Conclusion

Gestational diabetes mellitus (GDM) impairs endothelial cell health and compromises functions of endothelial colony-forming cells (ECFCs). This warrants investigation of therapeutic mechanisms reducing negative effects of GDM on endothelial cell health. Our study addresses the concern that while SB treatment reduces expression of genes harming ECFCs (TAGLN and SERPINE), more sustainable treatment and lasting effectiveness of SB are required to promote long-term GDM-ECFC rejuvenation.

By engineering a targeted delivery system for SB, this study aims to sustain SB's beneficial effects in the body. The controlled delivery system encapsulates SB during distribution to GDM-ECFCs, protecting SB from natural metabolism and excretion to preserve its bodily presence. Targeted delivery lowers expression of TAGLN and SERPINE in SB-treated GDM-ECFCs compared to the vehicle control. Thus, controlled SB delivery via this engineered system effectively combats effects of GDM on endothelial cell health. Targeted SB delivery leads to comparable increases in ECFC rejuvenation (lowered gene expression) in vitro as treatment of GDM-ECFCs with SB-containing medium. Besides TAGLN, this study shows evidence of SB's effect on SERPINE expression for normal ECFCs. Failure to observe a similar effect on GDM-ECFCs for SERPINE indicates that GDM likely triggers other pathways affecting SERPINE levels, offering future areas for investigation into these cellular mechanisms. However, because SERPINE is a less commonly investigated gene, our result serves to broaden current knowledge of SB treatment's potential benefits on ECFC health and rejuvenation in diseased conditions.

5 Future Studies

While this study confirmed the ability of a controlled SB delivery method to combat negative effects (increased harmful gene expression) of the GDM condition on endothelial progenitor cells, the ability of targeted delivery to increase the presence of these effects for longer time periods remains a possibility for further investigation. This will prove not only the ability of SB to promote rejuvenation of ECFCs in the GDM condition, but will also distinguish the targeted SB delivery system as a preferential treatment over treatment with SB medium due to sustained effects of SB. Through this lab's work, it has been shown (data not included) that kinetic effects of targeted delivery of SB last up to 7 days. A future area of investigation will be how to develop methods for more sustained delivery of bioactive molecules *in vivo* that can last up to 2 weeks or longer, thus addressing the chronic nature of many cardiovascular conditions. Despite this potential for future study, the controlled delivery method demonstrated here shows improvement from current delivery models that require twice-daily administration of the molecule as it is excreted through natural metabolism. For future *in vivo* applications, targeted delivery can reduce the number of nanoparticles needed by directly introducing more treatment molecules and minimizing the effects of excretion from the body over time. In addition, further uncovering the mechanism of action for SERPINE and its effects on ECFCs, as well as effects of controlled SB delivery on SERPINE expression, will offer greater insight into potential targets for combating the GDM condition, thus promoting clinical therapy development for such a widely prevalent and debilitating disease.

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