OVEREXPRESSION OF THE APICAL SODIUM-DEPENDENT BILE ACID TRANSPORTER TO REPLICATE NECROTIZING ENTEROCOLITIS IN IEC-6 CELLS

By

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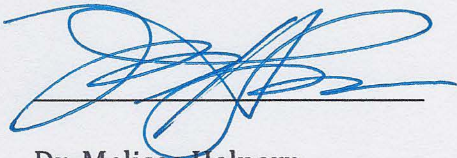

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ABSTRACT

This study tested a method of overexpressing the apical sodium-dependent bile acid transporter (ASBT) in IEC-6 cells to replicate necrotizing enterocolitis (NEC), a gastrointestinal disease prevalent in premature infants. The expression vector pcDNA4/TO was altered to pDR1019, containing the *Rattus norvegicus* ASBT coding sequence. Sequencing confirmed the ASBT sequence in pDR1019 using forward, reverse, and mid-sequence primers (respective E-values: 0.0, 0.0, and $2e^{-174}$). IEC-6 cells were transfected with varying ratios of pcDNA6/TR (tetracycline-controlled repression vector) and pDR1019: 6:1, 15:1, and 30:1 pcDNA6/TR:pDR1019. Using relative quantitative real-time PCR (qrt-PCR), the 30:1 transfection had the greatest fold-change difference of ASBT mRNA expression relative to non-transfected IEC-6 cells (overexpression-induced and non-induced trials: 3120.0-fold and 1445.6-fold, respectively). To test the effects of bile acids and cytokines on ASBT expression in IEC-6 cells with overexpressed ASBT, a 30:1 pcDNA6/TR:pDR1019 transfection was performed, followed by treatments of chenodeoxycholic acid (CDCA), tumor necrosis factor-alpha (TNF-α), and interleukin 18 (IL18). According to a qrt-PCR, the ASBT mRNA expression fold-change of non-transfected trials were: CDCA (2.09×10^{9}-fold), TNF-α (0.39-fold), IL18 (2.12×10^{9}-fold); insufficient cells survived the transfection followed by treatments to yield usable RNA. Using this cell-based model to replicate NEC will aid future molecularly-based investigations of the disease.

STATEMENT OF PURPOSE

The apical sodium-dependent bile acid transporter (ASBT), a membrane protein in ileal enterocytes responsible for sequestering luminal bile acids, has been shown to be increased in NEC. The purpose of this study is to design and test a reproducible method for replicating necrotizing enterocolitis (NEC) in a rat cellular model via ASBT overexpression. This will be accomplished using a two-plasmid inducible expression system with IEC-6 cells; the expression plasmid will be modified to contain the coding region of the rat ASBT gene. The ratio of the dual-plasmid system will be optimized for controllable ASBT overexpression, and the selected model will be tested with cytokine and bile acid treatments.

STATEMENT OF RELEVANCE

Necrotizing enterocolitis (NEC) is a potentially life-threatening condition affecting many premature infants. In order to effectively study NEC, disease models are necessary. Currently, the most widespread models for NEC are neonatal rodents, from which tissue samples and organ cultures are extracted. Using overexpression of the apical sodium-dependent bile acid transporter (ASBT) in IEC-6 cells as an example, this study will provide researchers with a more affordable, faster, and reproducible alternative for investigating NEC.

LITERATURE REVIEW

One of the most common gastrointestinal emergencies in infants, necrotizing enterocolitis (NEC), has high rates of both mortality (10-50%) and morbidity [1-5]. Risk factors for NEC include a young gestational age (<32 weeks), low birth weight (<1500 g), bacterial infection, and enteral feeding [3, 6-10]. Clinically, NEC usually occurs after the first week of life, once enteral feeding has begun [1]. The clinical signs of NEC include abdominal swelling and tenderness, intolerance to feeding, bloody stool, vomiting bile, intestinal inflammation, and elevated gastric residuals [1, 5, 6, 11]. In severe cases of NEC, surgical intervention may be required, which often results in higher morbidity and mortality than medical NEC [2]; both surgical and medical NEC can result in life-long complications and medical expenses [2].

While the exact molecular mechanisms remain unclear, several proteins and cytokines have been associated with the onset or condition of NEC. For instance, elevated intestinal levels of bile acids have been linked with NEC [12, 13]. Therefore, it is not surprising that proteins involved in enterocyte bile acid transport have also been linked with NEC [12, 14-18]. Luminal bile acids are resorbed by enterocytes in the ileum through the apical sodium-dependent bile acid transporter (ASBT) [14, 15, 19]. Bile acids traverse the cells with the aid of the ileal bile acid binding protein (IBABP), and are secreted into the hepatic portal system with the aid of the heteromeric organic solute carrier (Ostα/Ostβ) [14, 15, 19].

Bile acids have been shown to stimulate ASBT expression [20, 21]. Up-regulation of ASBT has been associated with NEC [22]. This increase of active ASBT results in elevated intracellular levels of bile acids in ileal enterocytes, causing damage which could result in

an inflammatory response [22]; such a response can be related to the onset of NEC [11]. In addition,, inflammation and pro-inflammatory cytokines are known to locally decrease ASBT mRNA expression and protein activity, perhaps as a negative-feedback response [23, 24]. Specific cytokines, such as IL18 and TNF-α, have been directly related to NEC [25-29].

The most common laboratory models for NEC include in vivo rat or murine models, and human case studies and chart reviews [30-35]. It is obviously difficult to work directly with neonatal patients, and exhaustive chart reviews will not elucidate the underlying molecular mechanisms of NEC. Likewise, rodent in vivo models and genetic modification experiments can tedious, and may not be distillable to a single molecular conclusion. Such singular points of data are necessary to construct a meaningful pathway for disease development on a molecular level, and thus identify molecular targets for treatment. A cellular model of NEC would be far more conducive to such experimentation because of the relative inexpensiveness of cells, the ability to directly apply treatments to the cells (rather than raising neonatal rats, inducing experimental NEC, and then harvesting ileal segments for treatment), and uniformity which would afford greater reproducibility.

Due to its obvious importance to NEC, ASBT should be examined as a potential initiator of the disease, especially in rodents which serve as common model organisms for the disease [31]. ASBT from *Rattus norvegicus* (rat) was first sequenced by Shneider *et al.* [36]. ASBT has been shown to have variable expression levels throughout early development in a healthy rat, though ASBT is overexpressed in NEC [22, 37]. Therefore, overexpressing ASBT in IEC-6 (rat intestinal epithelial) cells could serve as a model for NEC. Creating and testing such a system, to controllably overexpress ASBT in IEC-6 cells, is the goal of this study.

To overexpress a protein in eukaryotic cells, either inducible plasmids or viral techniques must be used. For ease of later modifications (such as switching the overexpressed gene), a two-plasmid repressor-expresser transfection system was selected as the basis for the ASBT overexpression system. Upon optimizing the use of the plasmid to induce ASBT overexpression in IEC-6 cells, cytokine (TNF-α and IL18) and bile acid (chenodeoxycholic acid; CDCA) treatments similar to those of organ culture studies will be used on ASBT overexpressing cells to determine if the overexpressing cells respond similarly to diseased enterocytes. For example, after transfection with the plasmids, induction of ASBT overexpression, and treatment with CDCA, the cells should experience a buildup of damaging intracellular bile acids, just as in NEC (see Figure 1).

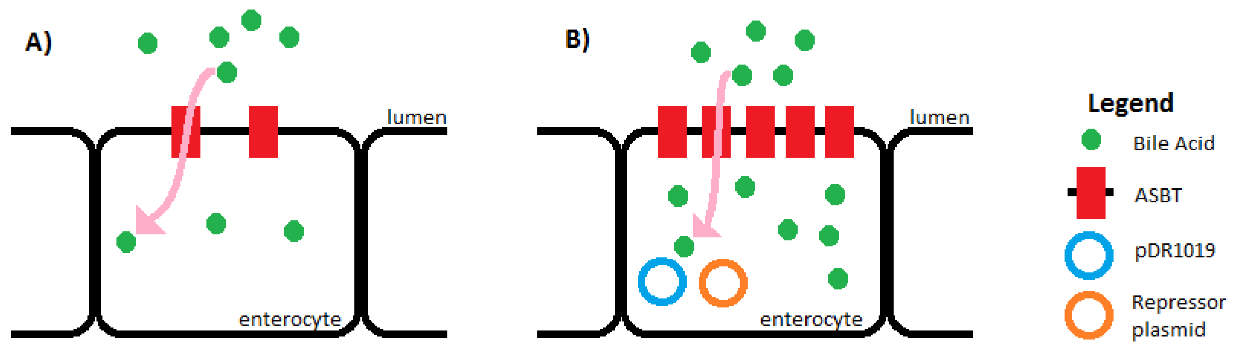


Figure 1. Bile Acid Treatment in the Presence of ASBT Overexpression.
A) Bile acid uptake in the presence of regular ASBT expression. B) Bile acid uptake in the presence of ASBT overexpression, induced by the dual-plasmid system (pDR1019 = expression plasmid).

METHODOLOGY

Cell Maintenance

Cell Culture

IEC-6 cells were cultured in complete media containing DMEM (Dulbecco's Modified Eagle Medium; Mediatech) supplemented with 10% FBS (Fetal Bovine Serum; Atlanta Biologicals), 10 μg/mL insulin (Sigma), and 40 μg/mL gentamicin (Lonza). An antibiotic-free (AB-free) medium was also prepared in the same as complete media, albeit lacking gentamicin. Selection media was the same as complete media, except with 5 μg/mL blasticidin (Invitrogen) and 500 μg/mL Zeocin (Invitrogen) added. Cells were cultured at 37°C in a 5% CO_2 atmosphere. Media was replaced every 2-3 days, and maintenance flasks were passaged twice weekly, when cells were not subject to experimental procedures or selection. All IEC-6 cells were between passage 10 and passage 24 at time of use.

Antibiotic Screening

An antibiotic screen was performed to determine the minimum effective dose of the antibiotics Zeocin and blasticidin when used for selection with healthy wild-type IEC-6 cells, an antibiotic screen was performed; vectors pcDNA4/TO and pDR1019 both confer Zeocin and ampicillin resistance, while vector pcDNA6/TR confers blasticidin and ampicillin resistance. IEC-6 cells were cultured overnight in twelve 10 cm^2 wells (2 X 10^5 cells/well) to attain 25% confluency. In six wells, blasticidin was added in the following concentrations: 0 μg/mL, 1 μg/mL, 2.5 μg/mL, 5 μg/mL, 7.5 μg/mL, and 10 μg/mL. In the other six wells, Zeocin was added in the following concentrations: 0 μg/mL, 125 μg/mL,

250 μg/mL, 375 μg/mL, 500 μg/mL, and 1000 μg/mL. Media and antibiotics were renewed daily, up until day 10 of Zeocin treatment, and day 9 of Blasticidin treatment. Following treatment, cells were trypsinized and counted.

Expression Vector Preparation

Reverse Transcription

RNA was originally extracted from adult rat intestinal tissue on 02/24/2009, using an RNeasy Plus Mini Kit (Qiagen). A Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed to convert the isolated RNA into cDNA, for subsequent amplification of the ASBT gene. 20 μL reaction contents: 3.75 mM $MgCl_2$ (Sigma), 2 mM dNTPs (Sigma), 2.5 μM random hexamers (Applied Biosystems), 8 U RNase inhibitors (Applied Biosystems), 25 U Multiverse Reverse Transcriptase (Applied Biosystems), and 500 ng RNA. Thermocycler parameters: 1) 25°C for 20 min, 2) 42°C for 15 min, 3) 99°C for 5 min, 4) 5°C for 5 min, and 5) 0°C for 0 min. A QIAquick PCR Purification Kit (Qiagen) was used to purify the cDNA product.

Primer	Sequences
ASBTCloningF	5'-GCTA**GAATTC**GCACAAGCAGTGATGGAT-3'
ASBTCloningR	5'-TAA**GCGGCCGC**TAGTGTCTATTTCTC-3'
ASBT-midF	5'-CCCAAACTATGGATTATAGGAACAA-3'
CMV-fwd	5'-CGCAAATGGGCGGTAGGCGTG-3'
BGH-rev	5'-TAGAAGGCACAGTCGAGG-3'
ASBT qrt-PCR primer	5'-TGGACCTCAGTGTTAGC-3'
ASBT qrt-PCR fwd probe	5'-GGCTCCAATATCCTGGCCTAT-3'
ASBT qrt-PCR rev probe	5'-AGCAAGCAGTGTGGAGCAAGT-3'

Table 1. Primer Sequences. *Restriction sites are in bold; the Kozak consensus sequence is underlined.*

ASBT Amplification

For later digestion and subsequent ligation into the expression vector, PCR was used to incorporate *EcoR*I and *Not*I restriction sites into the ends of the ASBT sequence during amplification; the forward primer was also used to add the Kozak consensus sequence for later expression. The 50 μL PCR reaction components were as follows: 150 ng cDNA from RT-PCR, 0.5 μM ASBTCloningF (Sigma), 0.5 μM ASBTCloningR (Sigma), 200 μM dNTPs (Sigma), and 1 U Phusion High Fidelity DNA polymerase (NEB). The following reaction conditions were used: 1) 98°C for 30 sec, 2) 98°C for 10 sec, 3) 50°C for 30 sec, 4) 72°C for 30 sec, 5) go to step two 35 times, 6) 72°C for 10 min, 7) hold at 4°C.

PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). To select samples for a subsequent restriction enzyme digest, samples were analyzed via agarose gel electrophoresis (AGE): 1% agarose, 0.5 μg/mL ethidium bromide (EtBr), 90 V, 50 min; the gel was visualized via ultraviolet (UV) light exposure. Samples were selected for digestion if they showed the expected ~1.1 kb band.

Restriction Digest

The amplified ASBT sample and vector pcDNA4/TO were both subjected to a double restriction enzyme digest, using *Eco*RI-HF and *Not*I-HF; vector pcDNA4/TO was also subjected to single-enzyme digests and a negative control no-enzyme digest. 25 μL reaction conditions: 1 μg DNA sample, 4 U *Eco*RI-HF, 4 U *Not*I-HF, 1X BSA, and 1X Buffer 4 (all restriction digest materials were from NEB). The samples were incubated at 37°C for 16 hr, heat inactivated at 65°C for 20 min, and stored at -20°C.

Restriction digest products were analyzed via AGE (2% agarose, 0.5 μg/mL EtBr, 110 V, 1.5 hr). Both the ~1.1 kb band from the digested ASBT sequence and the ~5.1 kb band from the digest pcDNA4/TO were excised and purified using the QIAEX II Gel Extraction Kit (Qiagen).

Vector Ligation

To ligate the digested ASBT sequence (insert) into the digested pcDNA4/TO (vector), ratios of 1:2 or 1:6 vector:insert were used. 25 μL reaction components: 200 ng digested pcDNA4/TO, 400 ng (1:2 ratio) or 1200 ng (1:6 ratio) digested ASBT sequence, and 10 U E. coli DNA Ligase (NEB). Samples were incubated for 16 hr at 16°C, heat inactivated at 65°C for 20 min, and stored at -20°C.

Expression Vector Amplification and Verification

Transformation

To amplify the ligation products, a transformation was performed using Super Ecos 101 E. coli Competent Cells (Yeastern Biotech). Samples were prepared by adding 2 μL ligation product to 100 μL competent cells; a pUC19 transformation was performed as a positive control. The samples were then vortexed for 1 sec, heat-shocked (42°C, 45 sec), and held on ice for 2 min. 1 mL LB media (antibiotic-free) was added to each sample, then samples were incubated at 37°C for 1 hr. 50 μL and 100 μL aliquots of each sample were separately plated on LB which contained 50 μg/mL Ampicillin (LB/Amp), and incubated for 16 hr at 37°C.

Selecting Samples for Sequencing

Colonies were picked, then cultured in liquid LB/Amp media at 37°C for 16 hr. Pelleted samples were purified using the E.N.Z.A. Endo-Free Plasmid Mini Kit I (Omega Bio-tek). To select samples for sequencing, separate PCR/AGE and restriction digest/AGE analyses were conducted.

Samples were first subjected to PCR to verify the presence of the ASBT sequence. 25 µL reactions were used with the following setup: 50 ng DNA from plasmid purification, 1 µM CMV-fwd, 1 µM BGH-rev, and 1X EconoTaq PLUS GREEN Master Mix (Lucigen). The following reaction conditions were used: 1) 94°C for 2 min, 2) 94°C for 30 sec, 3) 50°C for 30 sec, 4) 72°C for 1.5 min, 5) go to step two 35 times, 6) 72°C for 10 min, 7) hold at 4°C. Samples were analyzed via AGE (1% agarose, 0.5 µg/mL EtBr, 110 V, 45 min); successful ligations were expected to show a ~1.1 kb band, representing the ASBT sequence.

Samples containing the ASBT sequence, as determined via PCR/AGE, were then subjected to a restriction enzyme digest. The reaction parameters from the first restriction digest were used (*Not*I-HF and *Eco*RI-HF digestion, incubated at 37°C for 16 hr). Samples were analyzed via AGE (1% agarose, 0.5 µg/mL EtBr, 110 V, 70 min). Successful ligations were only expected to show the ~5.1 kb vector backbone band and the ~1.1 kb ASBT sequence band. Samples that were deemed successful, based on both the PCR and restriction enzyme digest analyses, were selected for sequencing.

Sequencing

Successful ligation samples were sequenced at the University of Arizona Genetics Core (UAGC) Sequencing Facility, using the CMV-fwd, BGH-rev, and ASBT-midF primers

(see Table 1); sequences were analyzed using FinchTV (Geospiza). The edited sequences were compared, using a nucleotide-nucleotide basic local alignment search tool (BLASTn), to the *Rattus norvegicus* ASBT coding sequence, obtained from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NM_017222.2). 25% glycerol stocks were made for source E. coli colonies of samples with 100% ASBT identity; glycerol stocks were stored at -80°C. The pcDNA4/TO expression vector containing the ASBT sequence (including the Kozak consensus sequence) was renamed pDR1019.

Transfection

Overview

The basis for attempting inducible expression of ASBT was the T-REx System (Invitrogen), which employs a tetracycline-sensitive repressor plasmid (pcDNA6/TR) in conjunction with the expression vector pcDNA4/TO (modified, to become pDR1019, including the Kozak consensus sequence followed by the rat ASBT sequence). IEC-6 cells were transfected with both plasmids (pcDNA6/TR and pDR1019) using the Lipofectamine 2000 (Invitrogen) protocol.

Cell Preparation

On a 12-well plate, 3.5 X 10^5 cells/well were seeded in AB-free media and incubated (37°C, ~24 hr) until confluent. Triplicate wells were used for each treatment. The transfection protocol was adapted from the Lipofectamine 2000 protocol, utilizing the Lipofectamine 2000 Transfection Reagent (Invitrogen; hereafter referred to as

"lipofectamine") as well as OPTI-MEM I + GlutaMAX Reduced Serum Medium (Invitrogen; hereafter referred to as "OPTI-MEM").

Transfection Conditions – Controls

The OPTI-MEM control (C-opti) wells were treated with OPTI-MEM, with no lipofectamine or plasmids; immediately following transfection, the C-opti wells were harvested for RNA using an RNeasy Plus Mini Kit. The lipofectamine control (C-lipo) wells were treated with both lipofectamine and OPTI-MEM, but no DNA; immediately following transfection, the C-lipo wells were harvested for RNA using an RNeasy Plus Mini Kit.

Transfection Conditions – pcDNA6/TR and pDR1019

The ideal ratio of repressor vector to expression vector (pcDNA6/TR:pDR1019) was to be determined to ensure maximal tetracycline-induced expression of ASBT. The ratios 6:1, 15:1, and 30:1 were tested. Both the control ratio (C-6:1, C-15:1, and C-30:1) wells and the tetracycline-treated ratio (Tet-6:1, Tet-15:1, and Tet-30:1) wells were initially treated with OPTI-MEM, lipofectamine, and 1600 ng of the appropriate vector ratios. Immediately following the transfection, the control ratio and tetracycline-treated ratio wells were fed with selection media for 9 days to eliminate all cells not transfected with both vectors. The tetracycline-treated ratio cells were then fed with selection media, supplemented with 1 µg/mL tetracycline, and incubated (37°C, 24 hr); meanwhile, the control ratio cells received the 24 hr incubation in selection media without tetracycline. Following this final 24 hr incubation, both the tetracycline-treated ratio wells and the control ratio wells were harvested for RNA using an RNeasy Plus Mini Kit.

Reverse Transcription

In preparation for the real-time PCR, a reverse transcription reaction was carried out using the extracted RNA (from C-opti, C-lipo, C-6:1, C-15:1, C-30:1, Tet-6:1, Tet-15:1, and Tet-30:1). The same protocol was used as for the previous reverse transcription, except that the new cDNA was not purified following the reverse transcription reaction.

Real-Time PCR

In order to compare the ASBT expression levels between the extracted samples, a relative quantitative real time PCR (qrt-PCR) was performed using the ABI Prism 7000 sequence detection system. Duplicate cDNA samples were used. 25 μL reaction components: 2 μL cDNA template; 12.5 μL 2X TaqMan Universal PCR Master Mix, 1.25 μL 20X rat ASBT primer/probe mix (see Table 1), 1.25 μL 20X Eukaryotic 18S rRNA Endogenous Control primer/probe. All qrt-PCR apparatuses and reagents were supplied by Applied Biosystems. The reactions were heated to 50°C for 2 min, 95°C for 10 min, cycled 45 times between 95°C for 15 sec and 60°C for 1 min, then held at 4°C. The qrt-PCR results were analyzed using the delta-delta-CT method [38].

Treatments of Transfected Cells

Cell Preparation and Transfection

Following the determination of the optimal transfection and ASBT overexpression conditions in IEC-6 cells, the effects on ASBT expression of interleukin 18 (IL18), chenodeoxycholic acid (CDCA), and tumor necrosis factor-alpha (TNF-α) were studied. In 5

12-well plates, IEC-6 cells were seeded at 3.5 X 10^5 cells/well in AB-free media, with 6 wells/treatment condition (see Table 2).

Sample	Transfected	Tetracycline	Treatment
Control	-	n/a	n/a
CDCA-Control	-	n/a	CDCA
CDCA-Tet	+	+	CDCA
CDCA-NoTet	+	-	CDCA
IL18-Control	-	n/a	IL18
IL18-Tet	+	+	IL18
IL18-NoTet	+	-	IL18
TNF-α-Control	-	n/a	TNF-α
TNF-α-Tet	+	+	TNF-α
TNF-α-NoTet	+	-	TNF-α

Table 2. Transfected Cell Treatments.

Controls

24 hr after seeding the cells, the CDCA-Control, IL18-Control, and TNF-α-Control were subjected to the following respective 24 hr treatments (in complete media): 0.25 mM CDCA, 100 ng/mL IL18, and 20 ng/mL TNF-α; Control cells were switched to complete media. After 24 hr, RNA was extracted from the Control, CDCA-Control, IL18-Control, and TNF-α-Control treatment wells using the NucleoSpin RNA II kit (Machery-Nagel).

Treatments

24 hr after seeding the cells, a 30:1 pcDNA6/TR:pDR1019 transfection was performed using the Lipofectamine 2000 protocol (see above) on the following samples: CDCA-Tet, CDCA-NoTet, IL18-Tet, IL18-NoTet, TNF-α-Tet, and TNF-α-NoTet. Following the transfection, the samples were maintained in selection media for 9 days. The Tet samples

(CDCA-Tet, IL18-Tet, and TNF-α-Tet) samples were subjected to 1 μg/mL tetracycline (in selection media) for 24 hrs; the NoTet samples were maintained in selection media. Following the tetracycline treatment, the CDCA samples (CDCA-Tet and CDCA-NoTet), IL18 samples (IL18-Tet and IL18-NoTet), and TNF-α samples (TNF-α-Tet and TNF-α-NoTet) were treated (in selection media, for 24 hr) with 0.25 mM CDCA, 100 ng/mL IL18, and 20 ng/mL TNF-α, respectively.

Real-Time PCR

RNA from all samples (with sufficient cells for extraction) was then isolated using the NucleoSpin RNA II kit; triplicate wells were combined, resulting in two RNA samples per treatment. A Reverse Transcription was performed on the RNA samples extracted from the treated cells, according to the aforementioned protocol. Using cDNA from the reverse transcription, a qrt-PCR was performed (samples used in triplicate), also according to the aforementioned protocol. The qrt-PCR results were analyzed using the delta-delta-CT method [38].

RESULTS AND ANALYSIS

Cell Maintenance

Antibiotic Screening

After ten days of Zeocin treatment, live cells were present in all wells with concentrations of 375 µg/mL and below (Graph 1.A); thus 500 µg/mL, the lowest tested concentration with no live cells remaining, was chosen as the selection dosage of Zeocin. After nine days of blasticidin treatment, live cells were visible in all wells with concentrations of 2.5 µg/mL and below (Graph 1.B); therefore 5 µg/mL, the lowest tested concentration with no live cells remaining, was chosen as the blasticidin selection dosage.

Expression Vector Preparation

Amplification of ASBT from RNA

The ASBT coding sequence was successfully reverse transcribed from RNA, and amplified; this was verified by the presence of a ~1.1 kb band on AGE (see Figure 2).

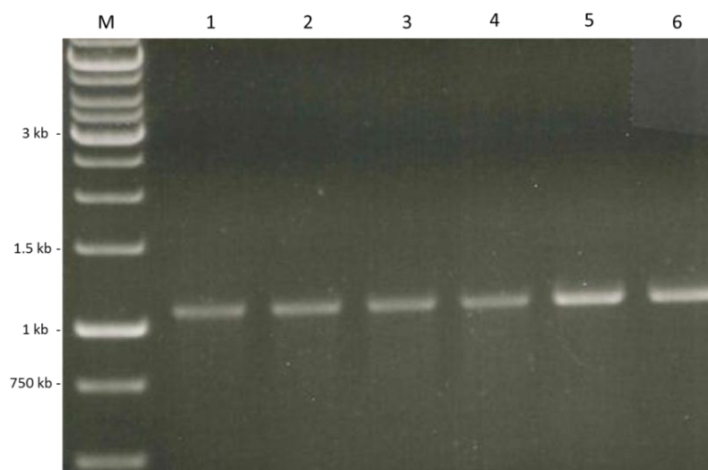
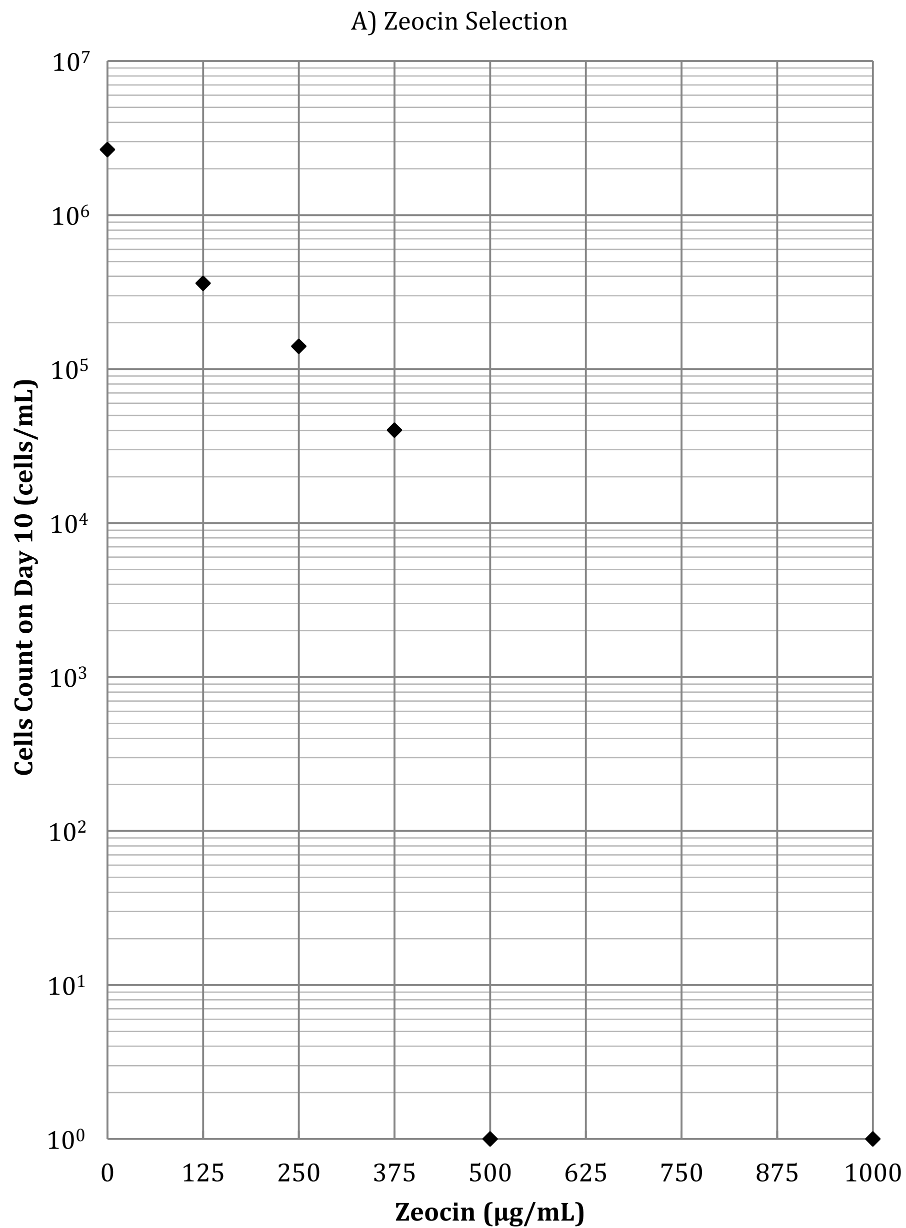
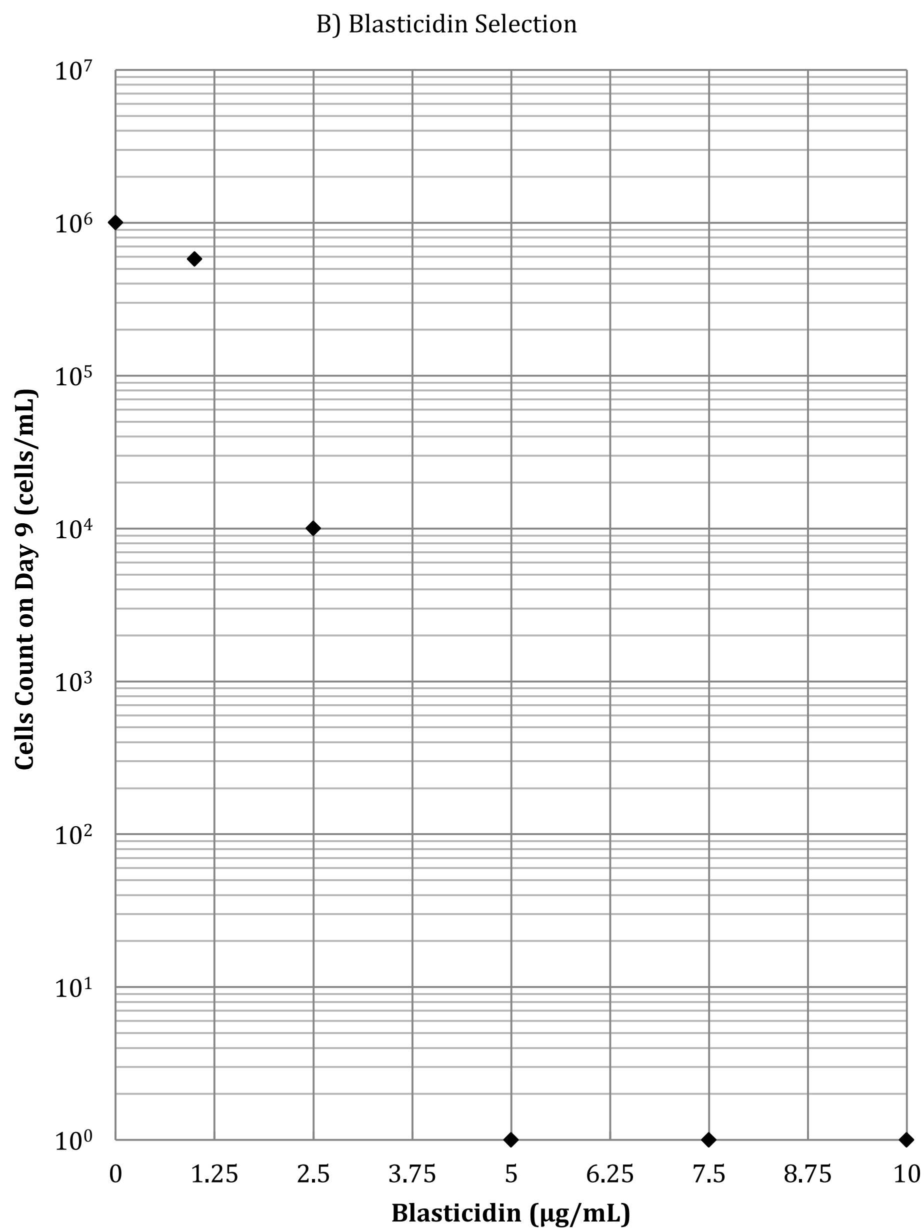


Figure 2. Amplification of ASBT from cDNA. *Samples 1-6 were duplicate amplifications of the same cDNA template.*





Graph 1. *Antibiotic Screening with Zeocin and Blasticidin.*

Restriction Digest

For the vector digest, a modified version of vector pcDNA4/TO was used which contained an imperfect copy of the ASBT gene; this imperfect copy of ASBT was removed via the gel extraction procedure. Compared to the no-enzyme negative control, both the *Not*I-HF and *EcoR*I-HF digests appeared to have successfully linearized the vector (see Figure 3). The *Not*I-HF control shows a faint ~1.1 kb band, corresponding to the aforementioned imperfect ASBT copy; this indicates that the *Not*I-HF digest excised the imperfect ASBT copy, possibly because of star activity.

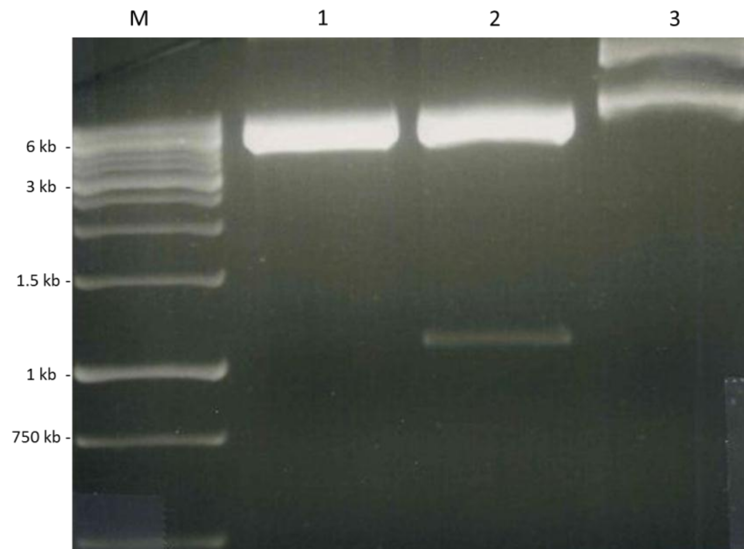


Figure 3. Control Restriction Digests. *1) EcoRI-HF positive control digest of pcDNA4/TO. 2) NotI-HF positive control digest of pcDNA4/TO. 3) no-enzyme negative control of pcDNA4/TO.*

The *Not*I-HF and *EcoR*I-HF double restriction digest of the PCR products and vector pcDNA4/TO (containing the imperfect ASBT insert) was successful (see Figure 4). With the activity of the restriction enzymes confirmed using the vector controls, the PCR products (~1.1 kb bands) were assumed to have been digested appropriately. The pcDNA4/TO digest was successful based on the presence of a ~1.1 kb band; the brightness of this band,

which represents the imperfect ASBT copy, indicates that the double digest effectively cleaved the vector at both restriction sites, fully excising the imperfect ASBT copy.

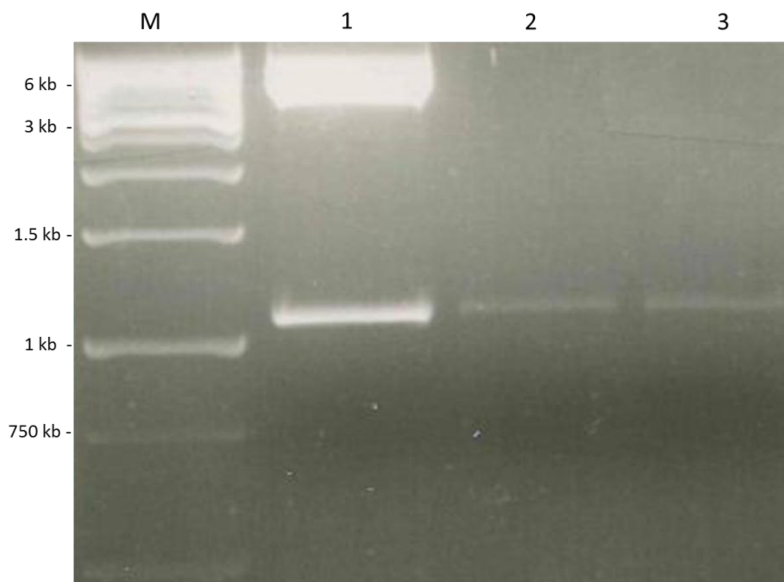


Figure 4. Double Digests of PCR Products and pcDNA4/TO.
1) Double digest of vector pcDNA4/TO. 2) Double digest of PCR product. 3) Double digest of PCR product.

Vector Ligation and Transformation

Plate	Plate	Colonies
Lig 1-100	2:1 insert:vector ligation (100 µL plated)	200+
Lig 2-100	6:1 insert:vector ligation (100 µL plated)	200+
Lig 3-100	digested vector-only ligation (100 µL plated)	~80
pUC19-100	pUC19 control (100 µL plated)	~50
Lig 1-50	2:1 insert:vector ligation (50 µL plated)	100+
Lig 2-50	6:1 insert:vector ligation (50 µL plated)	100+
Lig 3-50	digested vector-only ligation (50 µL plated)	~50
pUC19-50	pUC19 control (50 µL plated)	~25

Table 3. Ligation Plates.

Both the 2:1 and 6:1 insert:vector ligations were successful; this assessment is based on the presence of colonies for both when their respective ligation reaction products were transformed into competent cells, and plated on LB/Amp (see Table 3). The relatively

low number of colonies present for the Lig 3 plates, compared to their Lig 1 and Lig 2 counterparts, indicates that only a low fraction of the colonies on the Lig 1 and Lig 2 plates are likely to contain an empty, self-ligated vector. The pUC19 positive control plates both exhibited colonies, confirming the efficacy of the transformation procedure.

Expression Vector Verification

Selecting Samples for Sequencing

Ten colonies were picked from the ligation plates; plasmid DNA was extracted from these samples. The ligation samples were tested with a PCR amplification of ASBT (see Figure 5) to gauge the presence of the ASBT insert. The ligation samples were also subjected to a *Not*I-HF and *EcoR*I-HF double digest to ensure that only a single insert was present.

Samples 2-9 all show the expected ~1.1 kb bands from both the amplification and double digest (see Figures 5 & 6). Sample 1 contains no insert, as confirmed by the lack of any ~1.1 kb band from either the amplification or the restriction digest (see Figures 5 & 6). The sample 1 vector from the amplification appears to run at ~3.5 kb, indicating a supercoiled form (see Figure 5); the sample 1 vector from the digestion runs at ~6 kb, indicating successful linearization during the restriction digest (see Figure 6). Sample 10 shows the expected ~1.1 kb band from both the amplification and the double digest (see Figure 5 & 6); however, the presence of a ~2.5 kb band from the double digest could indicate the presence of either some supercoiled vector DNA which may not have been successfully linearized during the restriction digest, or a double insertion of the ASBT gene

(see Figure 6). Because of this ambiguity, sample 10 was discarded, along with the empty vector sample 1. Samples 2 through 9 were selected for sequencing.

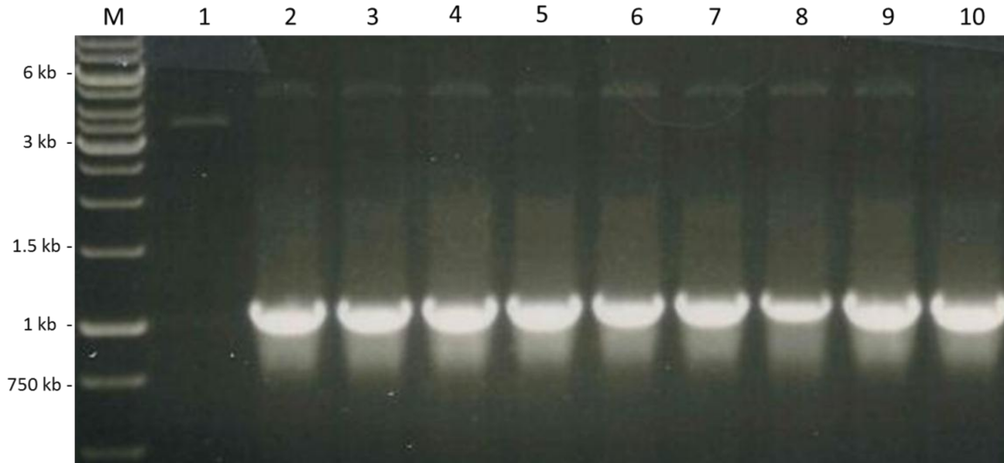


Figure 5. Amplification of ASBT from Ligation Samples. *Samples 1-10 represent ligation product samples from individual colonies, originally picked from the ligation plates; samples correspond to those of Figure 5.*

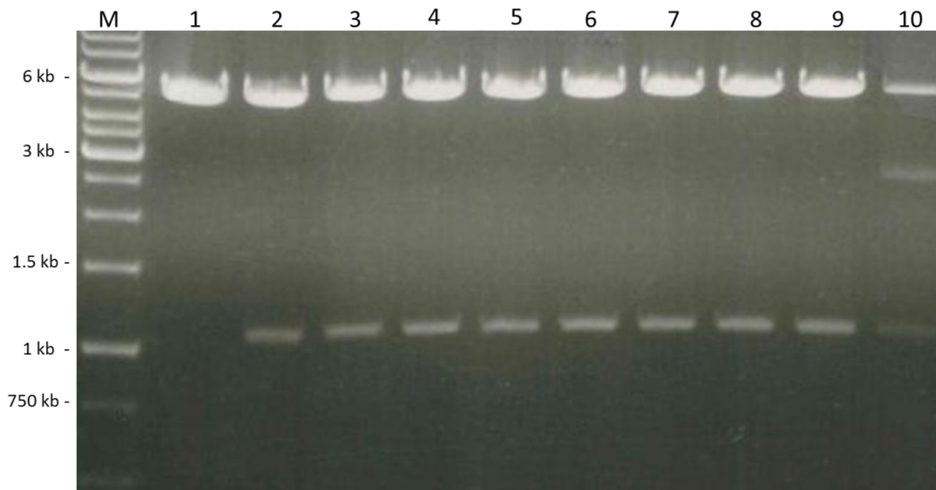


Figure 6. Restriction Digest of Ligation Samples. *Samples 1-10 represent ligation product samples from individual colonies, originally picked from the ligation plates; samples correspond to those of Figure 4.*

Sequencing

Ligation samples 2-9 were sequenced using the CMV-fwd primer (for primer sequences, see Table 2). All sequences were aligned, on NCBI, with the *Rattus norvegicus* ileal sodium-dependent transporter (ASBT) mRNA, complete coding sequence (http://www.ncbi.nlm.nih.gov/nuccore/14719390). While all the samples sequenced with CMV-fwd had low E-values, indicated a high probability of a successful alignment, only Ligation 2 had 100% identity with the rat ASBT gene, with 0% gaps (see Table 4). Therefore, Ligation 2 was subsequently sequenced with both the ASBT-midF and BGH-rev primers to verify the middle and latter parts of the ASBT sequence. The BGH-rev sequence had 100% with the rat ASBT gene, with 0% gaps (see Table 4). The ASBT-midF sequencing data showed 2 mis-identified genes (see Table 4); reviewing the sequence on FinchTV showed that the correct peaks were present, but had been mis-read by the sequencing software and had not been noticed during the manual sequence editing. Based on the sequencing data, the ASBT gene inserted into pcDNA4/TO was confirmed to be the correct rat ASBT gene sequence; pcDNA4/TO containing ASBT was renamed pDR1019.

Sample	Primer	Score	Expect	Identities	Gaps
Ligation 2	CMV-fwd	1528 bits (827)	0.0	827/827 (100.0%)	0/827 (0.0%)
Ligation 3	CMV-fwd	1543 bits (835)	0.0	858/868 (98.8%)	7/868 (0.8%)
Ligation 4	CMV-fwd	1515 bits (820)	0.0	831/836 (99.4%)	3/836 (0.4%)
Ligation 5	CMV-fwd	1576 bits (853)	0.0	860/863 (99.7%)	1/863 (0.1%)
Ligation 6	CMV-fwd	1480 bits (801)	0.0	813/818 (99.4%)	3/818 (0.4%)
Ligation 7	CMV-fwd	1487 bits (805)	0.0	821/828 (99.2%)	4/828 (0.5%)
Ligation 8	CMV-fwd	1537 bits (832)	0.0	839/842 (99.6%)	2/842 (0.2%)
Ligation 9	CMV-fwd	1567 bits (848)	0.0	878/890 (99.7%)	11/890 (1.2%)
Ligation 2	ASBT-midF	616 bits (333)	$2e^{-174}$	336/338 (99.4%)	0/338 (0.0%)
Ligation 2	BGH-rev	1788 bits (968)	0.0	969/970 (99.9%)	0/970 (0.0%)

Table 4. Ligation Sample Sequencing Results.

Transfection

Observations of transfected cells

Immediately before and after transfection, cells appeared healthy and were ~95% confluent. Throughout the selection process, cells appeared progressively sparser. At the end of selection, cells were 5-15% confluent, and grew in patches within the wells.

Real-Time PCR

After reverse transcribing RNA extracted from the transfected cell samples (C-opti, C-lipo, C-6:1, C-15:1, C-30:1, Tet-6:1, Tet-15:1, and Tet-30:1), a qrt-PCR was performed to assess the fold-change in ASBT mRNA expression relative to the C-opti non-transfected control. The C-lipo sample showed a fold-change of 29.96-fold increase compared to the C-opti control, which is minimal relative to the change exhibited by the other tested samples; this indicates that the lipofectamine protocol has only a minute effect on the level of ASBT mRNA expression in the transfected IEC-6 cells (see Figure 7).

C-6:1 and Tet-6:1 showed respective fold-changes of 1581.5-fold and 2018.6-fold (see Figure 7). C-15:1 and Tet-15:1 showed respective fold-changes of 42685.3-fold and 4098.6-fold (see Figure 7); this was an unexpected result because the non-induced sample exhibited more ASBT mRNA expression than the tetracycline-induced sample, and because the magnitude of the overexpression was considerably greater than that of the other transfected samples. C-30:1 and Tet-30:1 showed respective fold-changes of 1445.6-fold and 3120.0-fold (see Figure 7). All transfected samples, induced or not, showed ASBT mRNA expression levels thousands of times greater than C-opti; it is unclear whether this is an artifact of the transfection. With the clearest distinction between the expression-

induced and non-induced cells, the 30:1 pcDNA6/TR:pDR1019 ratio was selected for future transfections.

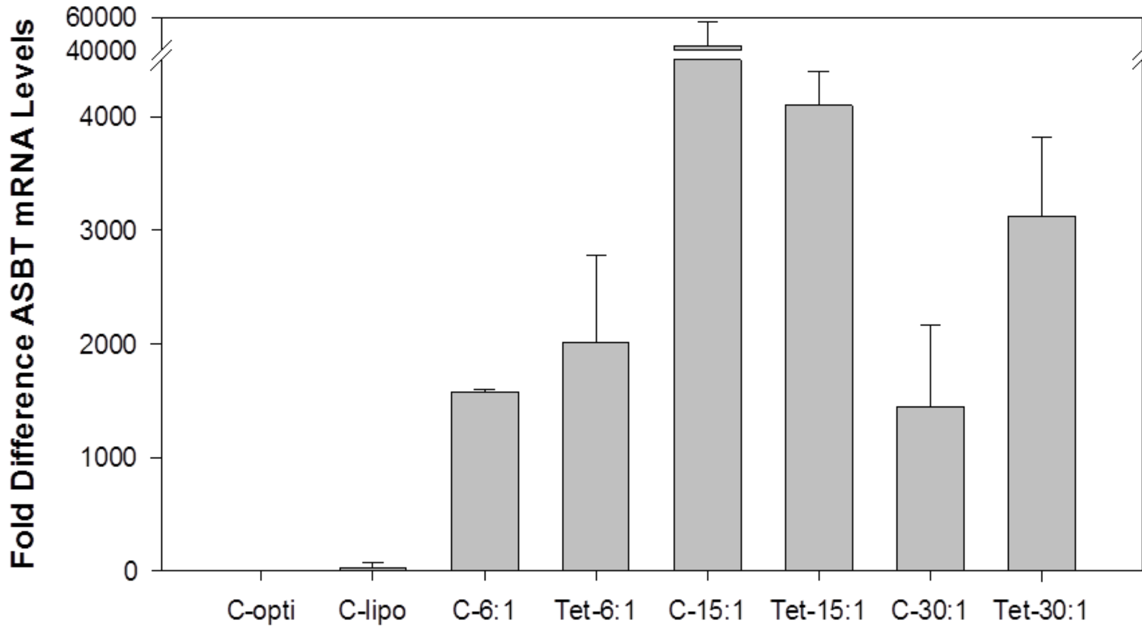


Figure 7. Relative mRNA Levels for ASBT, from Transfections of Varying Ratios. *Fold differences, compared to C-opti, for ASBT mRNA from C-opti controls (n=3), C-lipo (n=4), C-6:1 (n=4), Tet-6:1 (n=4), C-15:1 (n=4), Tet-15:1 (n=4), C-30:1 (n=4), and Tet-30:1 (n=3) samples.*

Treatments of Transfected Cells

Control Treatments

Using cDNA from reverse transcribed RNA (extracted from the Control-1, Control-2, TNF-α-Control-1, TNF-α-Control-2, IL18-Control-1, IL18-Control-2, CDCA-Control-1, and CDCA-Control-2 samples), a qrt-PCR was performed to assess the relative fold-change in ASBT mRNA expression, compared to the non-treated Control samples. The TNF-α-Controls show a fold-change of 0.39X (see Figure 8). The IL18-Controls show a 2.12×10^{9}-

fold difference (see Figure 8). The CDCA-Controls show a 2.09×10^9-fold difference (see Figure 8).

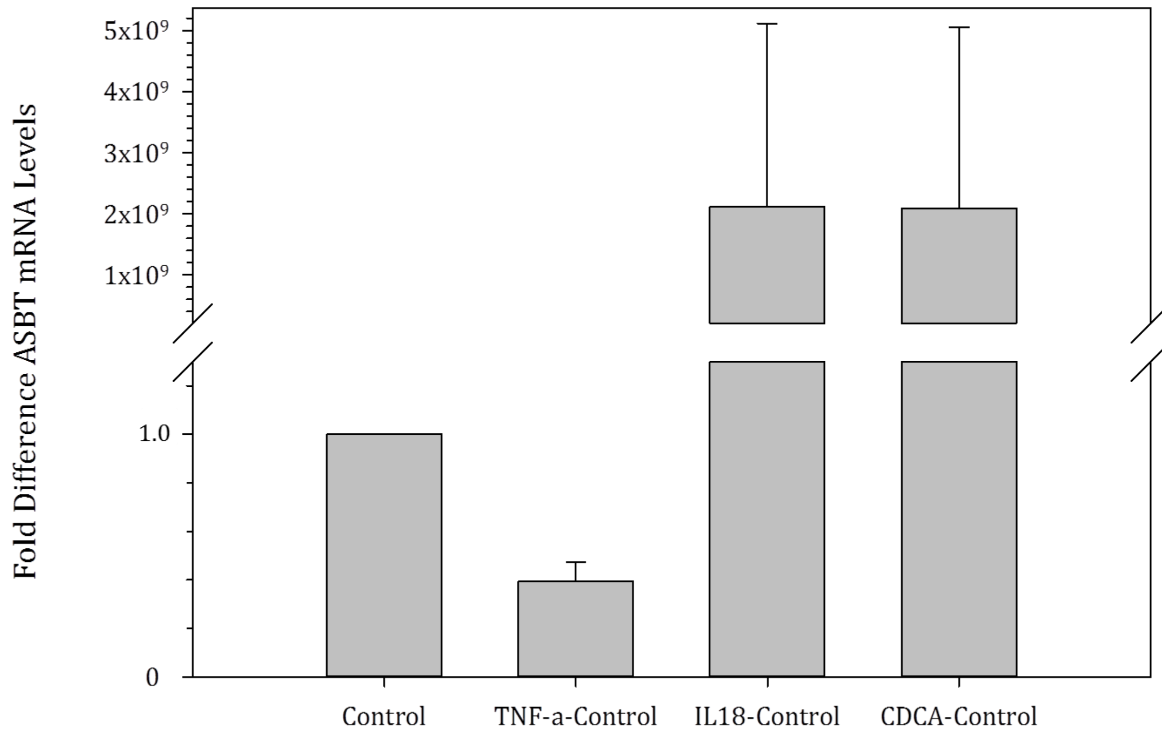


Figure 8. Relative mRNA Levels for ASBT, from Treatment Controls. *Fold differences for ASBT mRNA, compared to Control, from Control (n=1), TNF-α-Control (n=5), IL18-Control (n=5), and CDCA-Control (n=5) samples.*

Transfected Cell Treatments

Upon attempting to extract RNA from the transfected samples, it was found that too few cells survived for usable RNA to be isolated, despite the use of triplicate wells. Therefore, reverse transcription and qrt-PCR were not performed for these treatments.

CONCLUSIONS

The rat ASBT gene was successfully amplified from an adult rat mRNA extract and inserted in the inducible expression vector pcDNA4/TO. The sequence was confirmed, and the mutated pcDNA4/TO containing the rat ASBT sequence was renamed pDR1019 (see Table 4). To prepare for transfection of IEC-6 cells with pDR1019 and the repressor vector pcDNA6/TR, as part of the T-REx System, IEC-6 cells were tested to determine the minimum effective dose of Zeocin and blasticidin, the respective selection markers for pDR1019 and pcDNA6/TR (see Graph 1).

During the first transfection test, the effect of the lipofectamine protocol on ASBT mRNA was compared to similarly cultured non-treated controls. The lipofectamine treatment had a 29.96-fold difference compared to the control (see Figure 7); this is substantially less that any of the transfection results (which register in the thousands; see Figure 7), and so is irrelevant when discussing treatment effects on ASBT mRNA expression in transfected cells. Varied ratios of pcDNA6/TR:pDR1019 were tested to determine the most noticeable difference (between overexpression-induced and non-induced controls) during ASBT overexpression induction; during post-transfection selection with Zeocin and blasticidin, it was noted that only 5-15% of cells survived to the point of tetracycline addition. This testing led to the selection of a 30:1 pcDNA6/TR:pDR1019 ratio as the optimal proportion for later transfections.

Insufficient cells were available for RNA extraction to evaluate the effects of IL18, TNF-α, and CDCA on transfected cells (both overexpression-induced and non-induced transfected controls). However, usable RNA was extracted from the non-transfected cell-

treatment controls; this RNA was used for a subsequent Reverse Transcription and qrt-PCR to determine relative ASBT mRNA expression levels. As expected, the CDCA treatment promoted ASBT mRNA expression, with an increase of 2.09×10^9-fold compared to non-treated IEC-6 cells (see Figure 8). Also as expected, the TNF-α treatment reduced the level of ASBT mRNA expression, with a decrease to 0.39-fold compared to the control (see Figure 8). Surprisingly, the IL18 treatment did not reduce ASBT mRNA expression levels; rather, under this treatment, relative ASBT mRNA expression levels increased to 2.12×10^9-fold compared to the control (see Figure 8).

Since cells were only 5-15% confluent following selection, resulting in insufficient cells for an RNA extraction following certain treatments, a more efficient transfection protocol is needed to ensure more cells contain both plasmids, and thus survive selection. To this end, each plasmid (pcDNA6/TR and pDR1019) should be individually tested to optimize the amount of DNA necessary for efficient transfections. For instance, in a 12-well plate rather than using just 1.6 μg of total DNA, 3.2 μg (2X), 4.8 μg (3X), 9.0 μg (5X), or 16.0 μg (10X) of DNA could be used; after selection with the appropriate antibiotic (Zeocin for pDR1019, blasticidin for pcDNA6/TR), cells could be quantified to determine the most effective amount of DNA for each plasmid. Following this, new ratios for the two-plasmid transfection would need to be determined.

Ideally, a stable cell line of IEC-6 or CaCo2 (human colon carcinoma) cells with overexpressed or inducible-expression ASBT could be established via linearization and recombination into the IEC-6 cell genome. This would prevent the large loss of time and cells during selection, allowing more cells to undergo treatments in a shorter period of time.

Alternative future experiments using this study's methodology to create and test an overexpression system could involve making plasmids specific to other NEC-related proteins such as MUC-2 or Ostα/Ostβ (from both human and rat). This ASBT system could also be tested in human CaCo2 cells to closer replicate the reactions of human NEC patients' cells. This system could also be tested with other cytokines (such as IL-1β or IL-12) or bile acids (cholic acid, dexoxycholic acid etc), or the ASBT inhibitor SC435. It would also be interested to determine the localization of the ASBT protein within the IEC-6 cells, and to compare relative ASBT mRNA expression levels with protein expression levels.

As presented, the ASBT overexpression system has the potential to be a useful molecular model for NEC research. This system, once optimized, will also have the potential to isolate the effects of overexpression of ASBT in response to common cytokine and bile acid treatments.

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