Cytochrome P450 2S1 depletion enhances cell proliferation and migration in bronchial epithelial cells, in part, through modulation of prostaglandin E2 synthesis.

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Abbreviations: 12-HHT, 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid; CYP, cytochrome P450; AC, adenylate cyclase; AQ4N (banoxantrone), 1,4-bis[(2-(dimethylamino)ethyl)amino]-5,8-hydroxy-anthracene-9,10-dione bis-N-oxide; AA, arachidonic acid; atRA, all-trans retinoic acid; BEAS-2B, SV40-derived Bronchial Epithelial Cell line; B(a)P, benzo(a)pyrene; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; EdU, 5-ethyl-2′-deoxyuridine; EGFR, epidermal growth factor receptor; ELISA, enzyme linked immunosorbant assay; EP, prostanoid receptors; ILK, integrin linked kinase; LOX, lipoxygenase; MDA, malondialdehyde; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NHBE, normal human bronchial epithelial cells; NSCLC, non small cell lung cancer; PGE2, prostaglandin E2; PGG2, prostaglandin G2; qRT-PCR, quantitative realtime PCR; CPR, NADPH cytochrome P450 reductase; PLC, phospholipase C; SCRAM, scrambled control siRNA; 759, CYP2S1 depleted cell line targeting the 3′UTR; 984, CYP2S1 depleted cell line targeting exon#3; shRNA, short hairpin RNA; siRNA, small interfering RNA; TXA2, thromboxane A2;
ABSTRACT

Cytochromes P450 (CYPs) contribute to the metabolic activation and inactivation of various endogenous substrates. Despite years of research, the physiological role of CYP2S1 remains unknown. Recently, CYP2S1 has demonstrated NADPH P450-reductase independent metabolism of cyclooxygenase (COX)-derived prostaglandins (e.g. Prostaglandin G2 (PGG2)) at nanomolar concentrations. Arachidonic acid is converted to prostaglandin precursors (PGG2 and PGH2), through COX. These precursors are used to synthesize numerous prostanoids, including PGE2. PGE2 promotes cell proliferation, cell migration, and inhibits apoptosis. CYP2S1 metabolism of PGG2 presumably sequesters PGG2 and PGH2, making them unavailable for synthesis of prostanoids like PGE2. Whether CYP2S1 contributes to prostaglandin metabolism and influences cell physiological remains to be determined. The purpose of this study was to evaluate the physiological role of CYP2S1, if any, in human bronchial epithelial cells (BEAS-2B). To do this, we used siRNA to deplete CYP2S1 mRNA and protein by approximately 75% and evaluated the impact of CYP2S1 depletion on cell proliferation and migration. CYP2S1 depletion enhanced both cell proliferation and migration in BEAS-2B. Consistent with the proposed role of CYP2S1 in PGE2 synthesis, the reduction in CYP2S1 expression doubled intracellular PGE2 levels. Interestingly, pharmacological administration of PGE2 enhanced cell proliferation in BEAS-2B cells but failed to promote migration. Our data reveal an important role for CYP2S1 in the regulation of cell proliferation and migration, occurring in part through modulation of prostaglandin synthesis.
INTRODUCTION

Cytochromes P450 (CYPs) are heme-containing monooxygenase enzymes capable of metabolizing various endogenous or exogenous compounds. CYP2S1 is one of the most recently characterized members of the cytochrome P450 family (CYPs) (Rylander et al., 2001). Its expression is restricted to extra-hepatic epithelial cells (Rivera et al., 2002) and is significantly upregulated in response to inflammatory disease. CYP2S1 expression is significantly elevated in psoriatic plaques characterized by inflammation and cell proliferation (Smith et al., 2003).

Expression data also suggest that CYP2S1 may be linked to carcinogenesis: elevated CYP2S1 immunoreactivity is observed in human epithelial colorectal (Kumarakulasingham et al., 2005), metastatic ovarian (Downie et al., 2005), breast (Murray et al., 2010), and squamous cell carcinomas (Saarikoski et al., 2005a) and correlates with poor prognosis in colorectal, ovarian, and breast cancer (Downie et al., 2005; Kumarakulasingham et al., 2005; Murray et al., 2010). Better understanding of how alterations in CYP2S1 expression influence endogenous metabolism, and the cellular consequences associated with this regulation, are essential first steps in determining the impact of elevated CYP2S1 expression, if any, in disease.

Despite the identification of potential endogenous substrates (e.g., all-trans retinoic acid (Bui and Hankinson, 2009), and eicosanoids (Bui et al., 2011), the proposed metabolic mechanism and the relevance of CYP2S1-mediated metabolism remain controversial (Nishida et al., 2010; Xiao et al., 2011). Bui and colleagues identified CYP2S1-mediated metabolism of potential endogenous substrates that include retinoic acid (Bui and Hankinson, 2009) as well as members of the
cyclooxygenase and lipoxygenase-derived eicosanoids (Bui et al., 2011), using a
codon-optimized synthetic CYP2S1 (Bui and Hankinson, 2009; Bui et al., 2009; Bui et
al., 2011). Metabolism of endogenous substrates was shown to be independent of
NADPH P450 reductase, because it requires peroxide utilization for metabolism (Bui
and Hankinson, 2009; Bui et al., 2011). Of the endogenous substrates tested,
cyclooxygenase-derived prostaglandins (e.g., PGG2 and PGH2) were predicted as
likely endogenous substrates for CYP2S1 isomerase activity (Bui et al., 2011).
Arachidonic acid is converted to PGG2 and PGH2 via the cyclooxygenase enzymes.
PGH2 is further metabolized to bioactive prostanoids, including prostaglandin E2
(PGE2). Using peroxide cofactors, CYP2S1 was able to metabolize PGG2
(Km=270nM) and PGH2 (Km=11 μM) into numerous metabolites. The authors predicted
that CYP2S1 expression may effectively divert synthesis of bioactive prostanoids and
demonstrated depletion of PGE2 and PGD2 in mammalian cells over-expressing
CYP2S1 when supplemented with the PGH2 precursor. Although it suggests that
CYP2S1 influences PGE2 synthesis, whether or not this modulation is physiologically
relevant remains to be determined (Nishida et al., 2010; Xiao et al., 2011) and is the
subject of this investigation.

PGE2 is the most well studied COX-derived prostanoids. The physiological
effects of PGE2 are mediated through PGE2 activation of its cognate G-protein coupled
E prostanoid receptors (EP1-4) (Reviewed in (Wang and Dubois, 2006). In epithelial
cells, PGE2 stimulates cell proliferation (Pai et al., 2002) and cell migration (Buchanan
et al., 2003), while inhibiting apoptosis (Munkarah et al., 2002).

The purpose of this study was to identify the physiological significance, if any, of
CYP2S1 in human bronchial epithelial cells (BEAS-2B) by selectively depleting its expression and evaluating the cellular consequences. Our study reveals that the CYP2S1 expression, and presumably changes in endogenous metabolism, alters cell migration and proliferation in human lung cells. Interestingly, cell migration and proliferation observed in CYP2S1 depleted cells appear to be mediated through disparate actions of distinct intracellular pathways. CYP2S1 depleted cells have twice the level of intracellular PGE2. Our results suggest that elevated PGE2 levels may contribute to enhanced cell proliferation, but not migration, in BEAS-2B cells. These data are consistent with the proposed physiological role for CYP2S1 mediated metabolism of PGG2 (Bui et al., 2011), supporting the idea that PGG2 may be a physiologically relevant substrate in bronchial epithelial cells. Additionally, our data hint that CYP2S1-mediated metabolism influences other, as of yet unidentified endogenous pathway(s) that influence cell migration.

MATERIALS AND METHODS

CYP2S1 depletion using short hairpin RNA.

BEAS-2B cells were plated in six well plates and transfected with pLKO.1 shRNA plasmids (Sigma Mission shRNA) bearing 21 nucleotide sequences directed either against CYP2S1 or the non-targeting scrambled control. shRNA sequences targeting CYP2S1 were directed toward Exon 3 (SCH00984, referred to 984), and the 3’ UTR (SCH000759, referred to as 759). The non-targeting scrambled control (SCHSCRAM,
referred to as SCRAM) was utilized as a control. Stable individual colonies expressing the SCRAM, 759 and 984 shRNA were identified and isolated using puromycin (Sigma P8833) selection. Stable colonies were analyzed for CYP2S1 expression using qRT-PCR and western analysis and the colonies with the greatest knockdown (SCRAM#1, 759#7, and 984#1) were used in our study.

qRT-PCR analysis.

Cells were grown in replicates of three wells in 6 well plates until confluent and rinsed with PBS. RNA was isolated according to the manufactures protocol (Qiagen RNeasy) and eluted in 40ul of RNAse-Free water. Total RNA was then quantified using nanodrop spectrometric analysis as well as the Bioanalyzer nano-RNA protocol. cDNA was synthesized from 1µg of total RNA using iScript cDNA synthesis (BioRad) and diluted 1:5 in nuclease free H₂O and stored at -20°C. qRT-PCR was conducted using IQ syber green supermix (BioRad). The RT-PCR reactions were performed in a final volume of 10µL using 1uL of cDNA and 500nM Primers. qRT-PCR was conducted on a BIORAD CFX9600 programming in primer efficiency using standard curves obtained from plasmid amplicons. Primers were designed using Beacon Designer and Roche software suites. The PCR primers for CYP2S1 gene amplification were 5’-AGGCGTTCCCTGCCCCTTCC-3’ (sense) and 5’-CAGTGGGACGGACTTGACGTCAGC-3’ (anti-sense): ACTB gene amplification were 5’-GACAACGGCTCCGCATGTGCA-3’ (sense) and 5’-TGAGGATGCTCCTCTTGCTCTG-3’ (anti-sense). Five additional housekeeping genes were used to normalize CYP2S1 expression, their sequences (Supplemental Table 1) and results (Supplemental Figure 1) are referred to in the table in supplemental materials.
Western Blot Analysis.

BEAS-2B cells (ATCC: CLR-9609) were grown in 75cm² flasks until confluent, rinsed with PBS and isolated using the NE-PER isolation kit (Pierce). Cytoplasmic proteins were quantified using the (bicinchoninic acid) BCA protein assay kit (Pierce), according to manufacturer’s instructions, and frozen at -80°C until analysis. Western analysis was performed using 50μg of protein, which was reduced with DDT and loading buffer, and boiled prior to loading on a 12% Bis-Tris gel (Invitrogen). The protein was run and transferred to a nitrocellulose membrane at 100V for 1hr in transfer buffer. Then membrane was then rinsed, blocked and incubated overnight with CYP2S1 antibody (kindly provided by Dr. Roland Wolf). We also used the commercially available antibody, CYP2S1 (C-19) (Santa Cruz). The protein was then visualized using the Femto chemiluminescent detection kit (Millipore). Dr.Oliver Hankinson kindly provided the CYP2S1 protein loading control. The chemiluminescent signal was visualized using the Chemidoc XRS system (BioRad). Protein loading was controlled using the polyclonal anti-rabbit GAPDH (Sigma G9545) antibody.

Wound Healing Assay.

Bronchial epithelial cells were plated in replicates of 3 in 6 well plates at a 300,000 cells per well for 24hr or until confluence was reached. The horizontal scratch was made in the center of the well using a p10 pipette tip. Immediately following the scratch the cells were washed with PBS and new media was added. Vertical lines were drawn across the scratch as reference markers for imaging. Images were taken at different time points t=0 and 24 hours using the Zeiss Axioscope II. Images from each time point
were aligned using Photoshop CS3 professional Software suite and migrating cells were counted to determine invasive characteristics of cell lines. Cells were counted in replicates of three independent biological experiments.

**Cell proliferation assays.**

Cell numbers were calculated using the hemocytometer. BEAS-2B cells plated at 2,000 to 4,000 cells per well, in replicates, of at least 6 per treatment or genotype, in a 96 well plate. Cells were allowed to incubate overnight at 5% CO₂ at 37°C before the addition of alamarBlue® (Invitrogen) was added to a final concentration of 10%. Media alone and in the presence of fully reduced alamarBlue® (media containing autoclaved alamarBlue®), was used to establish the background and 100% reduced alamarBlue®. Percent reduction of alamarBlue® was calculated and these values were used to compare cells. Care was taken to follow only the wells (n=3 or more) that had similar fluorescent readings between samples at t=0. These experiments were conducted at least 3x in this manner. alamarBlue® data was analyzed using a one-way ANOVA in Prism statistical software, analyzing linear growth rates and differences in growth were assessed at 24 and 48 hours by post-hoc t-tests. All data was normalized to blank media wells (alarBlue® background fluorescence levels). **MTT ASSAYS:** Cells were plated as described above. MTT assays (Sigma) were performed according to manufacturers instructions and the absorbance was monitored at 560nm using the BioTek FL600 plate reader.

**PGE2 ELISA.**
PGE2 level was measured according to the Prostaglandin E2 Biotrak Enzymeimmunoassay (EIA) system (Amersham Biosciences). Briefly, BEAS-2B cells were plated at 50,000 cells/well in a 96 well plate to reach confluence. The next day total cellular PGE2 was assessed according to manufacturers instructions. Following lysis, total protein content was determined using the BCA protein assay kit (Pierce). PGE concentration was normalized to total protein content and represented as pg PGE per ug total protein. Statistical analysis was performed using students t-test.
RESULTS

CYP2S1 shRNA reduced CYP2S1 mRNA and protein expression in bronchial epithelial BEAS-2B cells.

To determine whether changes in CYP2S1 gene expression influences bronchial epithelial cell physiology, we evaluated the short-hairpin RNA (shRNA) sequences targeting the CYP2S1 mRNA. Sigma MISSION shRNA plasmids bearing 21 nucleotide sequences directed toward exon 3 (SCH00984, referred to 984) as well as the 3’ UTR (SCH000759, referred to as 759) of CYP2S1 were used to deplete CYP2S1 expression. (figure 1A). The non-targeting scrambled control (SCHSCRAM, referred to as SCRAM) was utilized as a control. We transfected each of the plasmids into BEAS-2B cells and isolated stable colonies derived from both 759 and 984. Stable colonies demonstrating the most significant difference in CYP2S1 expression relative to SCRAM controls were used for subsequent experiments. CYP2S1 mRNA expression was analyzed using qRT-PCR and normalized to the β-actin (ACTB) housekeeping gene. CYP2S1 mRNA was reduced by approximately 75% in both CYP2S1 depleted 759 (crosshatched bars) and 984 (horizontal lined bar) compared to the scrambled (SCRAM) control (black bar) (figure 1B). This data was also normalized to five additional housekeeping genes with similar reductions in CYP2S1 mRNA (see Supplemental Figure 1). Western analysis was performed using both the human CYP2S1 antibody (provided by Dr. Roland Wolf) as well as the commercially available antibody (see materials and methods). Each CYP2S1 antibody produced similar results (data not shown). BEAS-2B cells displayed a single band at 50kDa, which was consistent with the positive CYP2S1 protein control (2S1, provided by Dr. Oliver Hankinson). Western (figure 1C) and densitometric
DMD#46466

analysis (figure 1D) revealed marked depletion of CYP2S1 protein levels by approximately 75% to 70% in 759 and 984, respectively, when compared to scrambled (SCRAM) control. These CYP2S1 mRNA and protein reductions were stable through multiple passages as well as from older (multiple years) frozen stocks.

Effects of CYP2S1 depletion on bronchial epithelial cell migration and proliferation.

CYP2S1 expression is elevated in epithelial derived cancers (Downie et al., 2005; Kumarakulasingham et al., 2005; Murray et al., 2010), we tested the possibility that altering CYP2S1 expression in human bronchial epithelial cell would influence cellular pathways that either promote or inhibit cell proliferation and/or migration. To determine whether CYP2S1 expression alters cell proliferation and migration in human lung cells, we compared cell proliferation and migration in stably transformed scrambled controls (SCRAM) to cells with significantly reduced CYP2S1 mRNA and protein (759 and 984) (figures 2 and 3).

To assess the effect of CYP2S1 depletion on bronchial epithelial migration, we performed the wound-healing assay. Briefly, the two populations of cells were grown to confluence in six well plates and a wound was created using a 10ul pipet tip. The progression of wound healing was visualized at different time-points (0 hours and 24 hours) and the number of cells invading the wound at 24 hours was quantified (figure 2A, 2B). This assay produced striking results. After 24 hours, CYP2S1-depleted cells nearly covered the entire wound whereas scrambled control hardly migrated. Quantification of these results revealed roughly 3-fold increase in cell migration in
CYP2S1-depleted (~150 cells) over scrambled control (~50 cells) (figure 2B). Our results demonstrate that CYP2S1 depletion promotes cell migration, presumably by reducing CYP2S1-mediated metabolism of currently unknown endogenous substrates.

Differences in cell proliferation were assessed using two independent methods: alamarBlue® and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assays. The alamarBlue® assay detects viable cells using the blue, nonfluorescent dye (resazurin). Under conditions promoting cell proliferation, resazurin is reduced to its fluorescent form, resorufin, and detected by a fluorescent plate reader. MTT is a comparable cell viability detection method that measures metabolic activity in viable cells through the reduction of the yellow tetrazole compound to a purple formazan that can be detected though absorbance changes at 562nm.

CYP2S1 depleted cells (759 and 984) as well as scrambled (SCRAM) controls were plated at equal numbers (~2000 cells) and allowed to proliferate for 24 hours (alarmaBlue® and MTT) or up to 48 hours (alarmaBlue®). Each cell type was assessed for increases in viability. Cell viability in CYP2S1 depleted cells were compared to scrambled controls (SCRAM), which are designated as 100% growth (figure 3A and B). CYP2S1 depleted cells exhibited statistically significant increases of 40% (759, crosshatched bars) and 15% (984, horizontal lined bars) in cell viability compared with scrambled control (SCRAM, closed bars) at 24 hours (figure 3A). After 48 hours, 759 rose an additional 20% to 60% over SCRAM control, while 984 increased by roughly 5% to 20% increase over SCRAM control (figure 3A). Interestingly, the MTT assay demonstrated a more robust and consistent increase between the CYP2S1 depleted cell lines 759 (crosshatched bars) and 984 (horizontal lined bars). Both CYP2S1
depleted cells (759 and 984) exhibited roughly 300% increase over SCRAM control and were not statistically different from one another. We are uncertain as to what factors may contribute to the differences between these assays. However, these data clearly demonstrate that CYP2S1 depletion can enhance cell proliferation in human bronchial epithelial cells.

**Elevated prostaglandin E2 (PGE2) in CYP2S1 depleted cells promotes cell proliferation but not cell migration.**

Recently, CYP2S1 was shown to metabolize bioactive lipids derived from the cyclooxygenase and lipoxygenase pathways in the absence of P450 reductase. Substrates included prostaglandins, PGG2 and PGH2, from the cyclooxygenase pathway as well as numerous hydroperoxyeicosatetraenoic acid derivatives from the lipoxygenase (LOX) pathway (Bui et al., 2011). The authors proposed that CYP2S1 metabolism of PGG2 and PGH2 could effectively divert the AA-derived production of PGE2 to CYP2S1 derived metabolites 12-HHT and thromboxane A2 (TXA2) (Bui et al., 2011). If true, CYP2S1 depletion should increase PGE2 levels (see schematic, figure 5). PGE2 ELISA (figure 4A) was performed to test whether there were differences in PGE2 levels between CYP2S1 depleted cells (759 and 984) and control (SCRAM). PGE2 levels in scrambled controls were approximately 6pg/well or 0.15 pg/ug protein. This is similar to previously reported PGE2 concentrations (Cowan et al., 2006). In contrast, CYP2S1 depleted cells had nearly double the concentration of PGE2 (12pg/well or 0.3 pg/ug protein) in both 759 and 984 cell lines (figure 4A). Since, PGE2 is known to promote cell migration and proliferation in a number of different cell types.
(Sheng et al., 2001), we tested its ability to enhance cell migration (figure 4B) and proliferation (figure 4C) in BEAS-2B. BEAS-2B cells were exposed to PGE2 at varying concentrations of PGE2. However, none of the concentrations of PGE2 tested were able to promote cellular migration. Thus, the migration observed in CYP2S1 depleted cells (figure 2) cannot be attributed to elevated PGE2 levels.

Next, we tested whether PGE2 promotes cell proliferation in BEAS-2B. We performed alamarBlue® studies, which is similar to the MTT assay as they are both indirect measurements of cell viability. Interestingly, BEAS-2B cells grown in the presence of the lowest concentration tested (2 nM) exhibited significant increase (~20%) in alamarBlue® reduction compared to vehicle control (DMSO) at 24 hours. Increased cell proliferation appeared to peak at approximately 200nM (~25% increase) and, although still significantly different, began approaching DMSO control levels at 40μM (figure 4C). The increase in cell viability attributed to PGE2 at 24 hours (~25%) was within the range of alamarBlue® reduction increases observed in response to CYP2S1 depletion in 759 (~40%) and 984 (~15%) (figure 3A). The PGE2 concentration required to promote cell proliferation was in the low nanomolar range. Once this initial increase in cell viability was attained, increasing PGE2 levels failed to promote further statistical increase in cell proliferation, and at μM concentrations appeared to reduce PGE2’s proliferative effects on normal (non transformed) BEAS-2B cells. These results are consistent with PGE2’s biphasic effect on cell proliferation in other cell types (Baylink et al., 1996; Sergeeva et al., 1997), albeit at higher concentrations.

To determine whether the increased levels of PGE2 observed in CYP2S1 depleted cells was sufficient to promote the cell proliferation observed with PGE2, we
evaluated the effects of PGE2 on CYP2S1 depleted cells. Since the MTT assay was shown in our hands to be more robust indicator of differences in cell proliferation after 24 hours (figure 3), we performed the MTT assay to measure cell viability of both scrambled controls (SCRAM) and CYP2S1 depleted (759 and 984) BEAS-2B cells in response to varying concentrations of PGE2 (figure 4D). Consistent with effects observed in nontransformed normal BEAS-2B (figure 4C) the lowest level of PGE2 tested (1nM) was sufficient to enhance cell viability by ~60% in scrambled controls (SCRAM). This increase is MTT reduction was sustained until PGE2 concentrations reached ~100 μM. In contrast, cell proliferation was not enhanced in CYP2S1 depleted cells in response to exogenous PGE2 at any concentration. Interestingly, although not statistically significant, we observe reduced viability in CYP2S1 depleted cells (759 in particular) at 10 μM rather than 100 μM concentrations in scrambled (SCRAM) controls. These data would be consistent with increased concentration of intracellular PGE2 observed in CYP2S1 depleted cells. Taken together, our data suggests that elevated intracellular levels of PGE2 contribute to increased cell proliferation observed in CYP2S1 depleted cells. This data is also consistent with the proposed role of CYP2S1 in metabolizing the PGE2 precursor PGG2, which would divert the cellular production of PGE2, and supports the hypothesis that PGG2 is a physiologically relevant substrate for CYP2S1 in human bronchial epithelial cells.
DISCUSSION

In our current study, we reveal an important contribution of the human CYP2S1 enzyme in the regulation of cell growth and migration in BEAS-2B cells, which are used as surrogates for normal bronchial epithelial cells. Specifically, by depleting CYP2S1 expression, and ultimately CYP2S1-mediated metabolism of endogenous substrates, cell proliferation and migration are enhanced. Interestingly, proliferation and migration appear to be functionally divergent, suggesting that CYP2S1 depletion promotes each through distinct endogenous substrates, metabolites, or perturbation of downstream bioactive molecules.

Heterologous expression and metabolic studies using a synthetic CYP2S1 enzyme (Bui and Hankinson, 2009) have recently demonstrated metabolic activity toward potential endogenous substrates of the CYP2S1 enzyme, including: lipid products derived from the arachidonic acid (AA) cascade (Bui et al., 2011) as well as all-trans retinoic acid (Bui and Hankinson, 2009). Free AA is converted to bioactive eicosanoid metabolites via metabolism through either the lipoxygenase (LOX) enzymes or cyclooxygenase (COX) enzymes. Heterologous expression of the synthetic CYP2S1 enzyme has demonstrated P450 reductase-independent metabolic activity toward both LOX and COX-derived eicosanoids. It is predicted that the most relevant CYP2S1 mediated metabolism may be of the COX-derived prostaglandin intermediate, prostaglandin G2 (PGG2), based on its low Km (270nM). A summary of the AA cascade as well as the proposed role for CYP2S1 in prostanoid metabolism is depicted in figure 5. The first step in the COX pathway is to convert AA to PGG2 through COX peroxidase activity. PGG2 is then converted via COX enzymes or non-enzymatically to
prostaglandins (PGD2, PGF2a, and PGE2 (PGE2), prostacyclin (prostaglandin I2, PGI2) (Reviewed in Kroetz and Zeldin, 2002; Wang and Dubois, 2006; Panigrahy et al., 2010). CYP2S1 is able to convert PGG2 (Km = 270nM) and PGH2 (Km=11μM) to multiple products including: 12S-hydroxy-5Z,8E, heptadecanoic acid (12-HHT), malondialdehyde (MDA), and thromboxane A2 (TXA2) (Bui et al., 2011). If CYP2S1 plays an essential role modulating prostanoid synthesis in human lung cells, we would predict that CYP2S1 depletion would enable more of the PGG2 precursor to be converted to PGH2 and its subsequent prostanoid products, including PGE2. Indeed, our results in BEAS-2B cells show a roughly two-fold increase in intracellular PGE2 synthesis (figure 4A).

Conversely, elevated CYP2S1 expression in mouse hepatocyte (Hepa-1) cells reduced PGE2 and PGD2 levels (Bui et al., 2011). Our CYP2S1 depletion results are consistent with a modulatory role for CYP2S1 in the synthesis of COX-derived prostanoids, and regulation of PGE2, in particular.

Of the prostanoids, PGE2 is the most studied and has been implicated in cell proliferation and cell migration. PGE2 elicits a myriad of cellular effects through binding to and activation of its cognate prostanoid receptors (EP). Four G-protein coupled EP receptor subtypes have been identified: EP1, EP2, EP3, EP4. EP1 stimulates increased intracellular calcium though activation of phospholipase C (PLC). EP2 & EP4 are linked to Gαs and it activates adenylate cyclase (AC) and increase cAMP synthesis. On the other hand, EP3 is linked to Gαi and decreases AC activity and cAMP synthesis. BEAS-2B cells were shown to express each EP receptor mRNA (Tavakoli et al., 2001;
N’Guessan et al., 2007) and protein (N’Guessan et al., 2007) suggesting a full complement of activity mediated through PGE2 activation.

PGE2 stimulates proliferation in epithelial cell lines, including non small cell lung cancer (NSCLC) cell lines. Our data reveal a previously undocumented role for PGE2 in stimulating cell proliferation within BEAS-2B cells (figure 4C). PGE2 stimulated proliferation occurs through both EP1 (Krysan et al., 2005) stimulated activation of ERK signaling, as well as EP4 stimulated signaling and transactivation of the epidermal growth factor receptor signaling (EGFR) (Pai et al., 2002), as well as downstream activation of the integrin linked kinase (ILK) (Zheng et al., 2009). Krysan and colleagues did not observe rapid stimulation of ERK phosphorylation in BEAS-2B cells, suggesting that the PGE2-mediated proliferation of BEAS-2B is not a consequence of EP1 activation of ERK signaling. Our proliferation data appears to be consistent with EP4 transactivation of EGFR, which is was identified to promote cell cycle progression in gastric epithelial cells, which was arrested in Go/G1 in response to a selective EP4 antagonist (Pai et al., 2002). Our results demonstrate that PGE2 is elevated in CYP2S1 depleted cells (figure 4A) and that the elevated PGE2 levels within these cells is sufficient to promote maximal cell viability which, unlike scrambled control (SCRAM) cells, is not further enhanced with exogenous PGE2 (figure 4D). However, the exact mechanism by which elevated PGE2 in CYP2S1-depleted cells promotes cell proliferation, and whether this is the only bioactive molecule responsible for increasing cell proliferation, is a subject of further investigation in our laboratory.
PGE2 promotes cell migration via EP4 activation of src signaling in human alveolar carcinoma cells (A549) (Kim et al., 2010). However, we were unable to phenocopy enhanced migration phenotype observed in CYP2S1 depleted cells by exogenous application of PGE2, even at µM concentrations. These data suggest that although PGE2 levels are increased in CYP2S1 depleted cells (759 and 984), the cellular migration phenotype observed in these cells cannot be attributed to PGE2 mediated activation of EP4 and downstream src signaling. PGE2 promotes cell migration in normal human bronchial epithelial cells (NHBE) (Savla et al., 2001); however, our results suggest that PGE2 alone is not sufficient to promote wound healing in BEAS-2B. This observation is consistent with published results from Cowan and colleagues (Cowan et al., 2006) whereby exogenous application of PGE2 fails to promote cell migration in BEAS-2B. It is possible that CYP2S1 depletion could influence the production of other eicosanoids, or possibly novel endogenous substrate(s) and/or metabolites linked to cell migration. Our laboratory is actively pursuing the mechanism responsible for increased cell migration in response to CYP2S1 depletion.

In conclusion, this study is the first in vitro demonstration that CYP2S1 depletion promotes cell proliferation and migration in human lung cells. Our data provides further evidence supporting a modulatory role for CYP2S1 in regulating prostaglandin (specifically, PGE2) synthesis. It also demonstrates a functional role for PGE2 in enhancing cell proliferation in bronchial epithelial cells. More experiments are required to elucidate the EP receptors and signaling pathways responsible for promoting cell
proliferation in CYP2S1 depleted cells. Future studies should reveal other potentially novel CYP2S1 endogenous substrates and/or metabolites responsible for the observed effects of CYP2S1 depletion on enhanced cell migration. Overall, our data suggest an important physiological role for CYP2S1 in regulating cell migration and proliferation, and may ultimately have implications in carcinogenesis, hyperproliferative disease, and inflammatory disease.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Rowland, AM, Madanayake, TW, and Fidler, TP

Conducted experiments: Madanayake, TW; Fidler, TP; Bajaj, N; Fresquez, TM

Contributed new reagents: N/A

Performed data analysis: Rowland, AM; Madanayake, TW; and Fidler, TP

Wrote or contributed to writing the manuscript: Rowland, AM; Madanayake, TW, and Fidler, TP
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FOOTNOTES

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LEGENDS FOR FIGURES

**Figure 1:** siRNA-mediated depletion of CYP2S1 expression in human Bronchial epithelial (BEAS-2B) cells.  **A.** Cartoon depiction of the CYP2S1 gene. Boxes indicate Exons (1-9) and lines indicate introns as well as the 3'UTR. siRNA target sequences and their position within the gene are indicated by the underlined numbers 759 (targeting the 3'UTR) and 984 (targeting exon 3).  **B.** qRT-PCR quantification of CYP2S1 mRNA transcripts in stably transformed cell lines expressing either the 759 (crosshatched bars) or 984 (horizontal line bars) shRNA plasmids were compared to non-targeting SCRAMBLED shRNA control (SCRAM, closed bars). CYP2S1 mRNA derived from three independent experiments was normalized to the β-actin (*ACTB*) housekeeping gene and is represented as percent knockdown relative to SCRAM control.  **C.** Representative western blot of stably transformed cell lines bearing the control (SCRAM) and CYP2S1 siRNA (759 and 984).  *Top band:* CYP2S1 immunoreactivity.  *Bottom band:* GAPDH immunoreactivity.  **D.** Densitometric analysis for CYP2S1 immunoreactivity was performed on five independent western blots and expressed as a percentage of density corresponding to scrambled control (SCRAM). Differences in protein loading are accounted for by normalization to GAPDH immunoreactivity. Statistical significance was determined using a students t-test in both qRT-PCR (B) and densitometric (D) analysis. Data are represented as mean ± S.D. *** indicates p<0.001.

**Figure 2:** Depleting CYP2S1 expression in human lung cells promotes cell migration in BEAS-2B cells.  **A.** Representative images from the wound healing assay, comparing cell migration in BEAS-2B cells with normal CYP2S1 expression (SCRAM, top set) to
two independent BEAS-2B clones with depleted CYP2S1 expression (759 and 984, middle and bottom sets, respectively). Images were acquired at two independent timepoints: time = 0 hours (directly after wounding, left) and 24 hours after wounding (right). **B.** The number of cells migrating into the wound were quantified 24 hours after wounding in BEAS-2B cells stably transformed with scrambled shRNA (SCRAM, closed bars) as well as CYP2S1 depleted BEAS-2B cells (759 and 984, crosshatched and horizontal line bars, respectively). Cell quantification is derived using three images each from 3 independent experiments. Data represents the mean ± S.D. *** indicates a significant increase p<0.001.

**Figure 3:** Depleting CYP2S1 expression in human lung cells promotes cell proliferation in BEAS-2B cells. **A.** alamarBlue® proliferation assay was used to monitor cell proliferation. Proliferation was significantly increased in CYP2S1 depleted BEAS-2B cells (759 and 984, crosshatched and horizontal line bars, respectively) compared to scrambled control (SCRAM, closed bars). Data represents the mean± S.D. of alamarBlue® reduction at 24 and 48 hours. One way ANOVA was used to determine differences between groups followed by posthoc t-tests. **B.** The MTT was also assessed to evaluate differences in cell proliferation after 24 hours of growth. Cell viability was also found to be significantly increased in CYP2S1 depleted BEAS-2B cells (759 and 984, crosshatched and horizontal line bars, respectively) compared to scrambled control (SCRAM, closed bars. Statistical analysis was performed using the students t-test. * indicates significance of p<0.05, ** indicates significance of p<0.01, and *** indicates a significance of p<0.001.
Figure 4: CYP2S1 depletion enhances PGE2 production, which may account for enhanced proliferation but not migration effects observed in CYP2S1 depleted cells. A. PGE2 ELISA detected elevated intracellular PGE2 in BEAS-2B cells depleted of CYP2S1 (759 and 984, crosshatched and horizontal line bars, respectively) compared to scrambled control (SCRAM, closed bars). PGE2 levels were normalized to cellular protein content (BCA protein assay kit) within each well. Error bars indicate variability between each well. B. PGE2 supplementation alone failed to promote migration of non-transformed (normal) BEAS-2B cells into the wound. Confluent BEAS-2B cells were wounded and allowed to migrate for 24 hours with the addition of 10nM and 1μM PGE2. C. In contrast, PGE2 supplementation at nanomolar concentrations was sufficient to enhance cell proliferation in normal BEAS-2B cells. Data from 3 experiments are represented as percent increases in alamarBlue® reduction 24 hours after t=0 control. D. MTT assay measuring the metabolic activity in the cells reveals an increase in proliferation at 24 hours in response to PGE2 in SCRAM controls (solid line, closed circles), but not CYP2S1 depleted (759 and 984, dashed lines with open circles and open squares, respectively) BEAS-2B cells. Each experiment was performed at least 3 times.

Figure 5: Proposed model for CYP2S1-mediated modulation of prostanoids, including PGE2. Free AA is converted to prostaglandin G2 (PGG2) and H2 (PGH2) via cyclooxygenase (COX) enzymes. PGH2 is converted to prostanoids through thromboxane synthetase (thromboxane A2, TXA), prostaglandin synthetases (prostaglandins D2, F2α, and E2), and prostacyclin synthetase (prostaglandin I2, PGI2). In vitro metabolic studies indicate that CYP2S1 can metabolize PGG2 (Km = 270nM)
and PGH2 (Km=11 μM) to numerous products including: 12S-hydroxy-5Z,8E, heptadecanoic acid (12-HHT), malondialdehyde (MDA), and thromboxane A2 (TXA2) (Bui et al., 2011). This could potentially divert synthesis away from PGE2. Consistent with this proposed role for CYP2S1 in prostaglandin metabolism, CYP2S1 depletion may elevate PGG2 and PGH2 precursors, increasing their availability for synthesis of downstream prostanoids, including PGE2. Enzymes are represented by names enclosed in grey boxes. Open boxes contain either CYP2S1 metabolites (12-HHT, MDA, and TXA2) or PGE2.
**Figure 1**

A. CYP2S1 gene: Mission shRNA targets

![Gene structure diagram]

B. %CYP2S1 mRNA relative to SCRAM

- SCRAM: 100%
- 759: 25%
- 984: 25%

C. CYP2S1

D. CYP2S1/GAPDH relative to SCRAM

- SCRAM: 100%
- 759: 75%
- 984: 75%
**Figure 2**

A. BEAS-2B control (SCRAM)

BEAS-2B CYP2S1-depleted (shRNA#759)

BEAS-2B CYP2S1-depleted (shRNA#984)

Wound @ T=0 hours

Migration @ T=24 hours

B. 

# cells migrating into the wound after 24 hours

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**Note:** This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3:

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B.

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*Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 4

A. PGE2 quantification relative to protein (pg/μg protein)

B. Wound @ T=0 hours vs Migration @ T=24 hours

C. % increase in Alamar reduction relative to DMSO control in BEAS-2B

D. % increase in MTT reduction relative to DMSO control
Supplemental Figure 1: qRT-PCR results using β-actin ACTB as well as five additional housekeeping genes for normalization. Each graph represents CYP2S1 mRNA expression in either CYP2S1 depleted BEAS-2B cell line (759 and 984) relative to CYP2S1 expression in scrambled control (SCRAM). Results from SCRAM were normalized to 1 and CYP2S1 depletion is indicated by normalized fold expression. Statistical analysis was performed using students t-test. ** indicates p<0.01 and *** indicates p<0.001.
Cytochrome P450 2S1 depletion enhances cell proliferation and migration in bronchial epithelial cells, in part, through modulation of prostaglandin E2 synthesis

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Drug Metabolism and Disposition

**Supplemental Table 1**

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**Supplemental Table 1:** Primer sequences for CYP2S1 as well as each of the 5 housekeeping genes used in this study. Sequences are expressed in the 5’ to 3’ direction. Sequences were generated using Beacon Designer (Premier Biosoft International).