Genetic Variants Associated with Tacrolimus Metabolism in Kidney Transplant Recipients

A Thesis

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Dedications

This Master thesis is dedicated to my mother Darlene Dorr for her support through both tough and smooth times. I further dedicate this to my grandmother Betty Dorr, brother Nathan Dorr and the memories of my father Ronald Dorr, grandfathers Wesley Dorr and Clifford Bahr and grandmother Mabel Bahr.

Abstract

This master's thesis focuses on the work completed during my K01 award Genetic Variants Associated with Tacrolimus (TAC) metabolism in Kidney Transplant Recipients. In **Chapter 1**, I discuss the significance and innovations of this project. In **Chapter 2**, I present reformatted manuscript published in the Pharmacogenomics Journal titled: Identification of genetic variants associated with tacrolimus metabolism in kidney transplant recipients by extreme phenotype sampling and next generation sequencing. In **Chapter 3**, I present a reformatted manuscript published in Drug Metabolism and Disposition titled: CRISPR/Cas9 genetic modification of CYP3A5 *3 in human hepatocytes leads to cell lines with increased midazolam and tacrolimus metabolism. **Chapter 4** is the general conclusions, future directions and take away messages.

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Abbreviations

1-OH MDZ: 1-hydroxyl midazolam
4-OH MDZ: 4- hydroxyl midazolam
13-DMT: 13-O-desmethyl TAC
AA: African American
ABCB1*:* multidrug resistance protein 1
AR: acute rejection
BICB: biomedical Informatics and Computational Biology
bp: base pairs
BT: burden test
CLIA: Clinical Laboratory Improvement Amendment
CTSI: Clinical and Translational Sciences institute
CYB5A: cytochrome b5a
CYP: cytochrome P450
CYP3A: cytochrome P450 3A
dd: double deletion
DDGI: drug-drug-gene interaction
DeKAF: Deterioration of Kidney Allograft Function
DMEM: Dulbecco's Modified Eagle Media
DMSO: dimethyl sulfoxide
EA: European Americans
EPS: extreme phenotype sampling
FPKM: fragments per kilobase per million reads
GATK: Genome Analysis Toolkit's
gRNA: Guide RNAs
GWAS: Genome-Wide Association Study
HDR: Homology directed repair
HHRI: Hennepin Healthcare Research Institute
IPV: intrapatient variability
LC-MS: liquid chromatography – mass spectrometry
mRNA: messenger RNA
LoF: loss of function
MDZ: midazolam
MSB: microsome storage buffer
NCBI: National Center for Biotechnology Information
NGS: next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NHEJ: non-homologous end joining
PAM: Protospacer adjacent motif
PCR: polymerase chain reaction
pm: point mutation
PolyPhen2: Polymorphism Phenotyping Tool 2

POR: cytochrome P450 oxidoreductase
PrIME: Precision health and genomics: Indigenous Mentoring and Ethics
PXR: pregnane X receptor
qRT-PCR: quantitative reverse transcriptase polymerase chain reaction
RTV: ritonavir
sd: single deletion
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIFT: Sorting Intolerant from Tolerant
SNP: single-nucleotide polymorphism
SRA: Sequence Read Archive
TAC: Tacrolimus
UTR: untranslated region
VEP: Ensembl Variant Effect Predictor

Chapter 1

Significance and Innovations in Study to Investigate Genetics of Variants Associated with Tacrolimus (TAC) Metabolism in Kidney Transplantation

Although immune suppressants have improved outcomes for solid organ transplantation, there remain significant challenges with toxicity and acute rejection (AR). Studies show African Americans (AAs) have higher rates of AR[2] and worse kidney allograft survival than European Americans (EAs)[3]. TAC, the primary immune suppressant and cytochrome P450 3A (CYP3A) substrate is used in about 90% of solid organ transplantation[4]. TAC has a narrow therapeutic window[5] with blood concentrations (troughs) that are highly associated with efficacy[6] and toxicity[7]. The trough is the lowest drug concentration in the patient's blood stream prior to the next drug dose. There is high interpatient variability in doses to achieve therapeutic concentrations[8, 9]. On average, AAs have higher rates of TAC metabolism and require greater doses than EAs[8] for optimal outcomes. A high TAC trough intrapatient variability (IPV) predicts fibrosis and tubular atrophy lesions in renal transplant recipients[10]. This supports the association between high TAC IPV and poor long-term outcomes after kidney transplantation[11] including AR[12, 13] and renal function decline[14]. It has been shown that nonadherence does not completely explain the high IPV of TAC troughs[12, 15]. In AAs it is beneficial to reach target TAC troughs early to prevent the risk of AR and graft loss[16]. Thus, genotype-based dosing, that accounts for clinical factors, could reduce TAC IPV. There may also be differential TAC transport into the cell[17] or metabolic genes activation such as cytochrome P450 oxidoreductase (*POR*) or multidrug resistance protein 1 (*ABCB1*)[18, 19] that alter TAC troughs. We showed that about half of the variation in TAC metabolism in AAs remains unexplained after accounting for clinical factors and common *CYP3A5* alleles[20] (*3, *6 or *7) that are associated with TAC blood concentrations[21].

Hypothesis

We hypothesize that low-frequency genetic variants are vital contributors to variation in metabolism of TAC and other medications that are substrates of CYP3A. These variants that alter metabolism, may explain interpatient and intrapatient variability in TAC response and/or toxicity. ***Thus, there is a critical need to determine the role of low-frequency genetic variants in drug metabolism leading to a model, which***

includes clinical factors, for optimal dosing; especially since nearly half of all medicinal compounds are CYP3A substrates[22]. Identifying these variants will lead to a safer and highly effective TAC dosing model for kidney transplant recipients via precision medicine.

The long-term goal of these studies is to develop a genotype-driven TAC dosing models that include clinical factors for transplant patients to improve outcomes through precision medicine. For this Master's project, I aimed to identify genetic variants associated with TAC blood concentrations in kidney transplant recipients, and develop a cell culture model to functionally validate genetic variants effect on TAC metabolism.

We have access to DNA, TAC troughs and clinical outcome data from >5000 kidney transplant recipients (1,015 AAs) prospectively enrolled in the Deterioration of Kidney Allograft Function (DeKAF grant: AI U19 AI070119)[20, 21, 23-32], GEN03[33, 34] and iGeneTRAiN[35, 36] genetic epidemiology studies. We leveraged these unique resources to identify clinically-relevant, low-frequency genetic variants that impact TAC troughs in AAs. A next-generation sequencing (NGS) study shows that 86% of genetic variants have a frequency lower than 0.5%[37] indicating that low-frequency variants are important to explain phenotypic differences between individuals. In **Chapter 2** of this thesis, using an extreme phenotype sampling (EPS) model accounting for known, *CYP3A* variants and clinical factors, we identified low-frequency variants using NGS of genomic DNA, in 5% of AAs with extremely high and low TAC troughs over time. In Chapter 3 of this thesis, genetic variants in the CYP3A5 gene were engineered into human hepatocytes using, CRISPR biotechnology[38]. Drug metabolism assays[39, 40] with these cell lines validate the effect of each genetic variant on TAC metabolism. In **Chapter 4** of this thesis, we will show summarize conclusions, future directions and take away messages.

SIGNIFICANCE

1. The immune suppressant TAC is used in 90% of solid organ transplants (**Figure 1.1**) **and the TAC blood concentrations are highly variable between patients.** TAC is the most frequently used immune suppressant to prevent kidney AR following transplant[1]; TAC is also used in about 90% of all solid organ transplants[41]. The variability in TAC troughs and TAC's efficacy are correlated with TAC metabolism[21, 42] and low TAC troughs can lead to increased risk for AR[24]. It is common clinical practice

to measure blood troughs and change doses to achieve a therapeutic trough targets. This practice is great for making TAC a model system for precision medicine as it can correlate dosing with troughs which are not routinely monitored for other drugs. However, this trial and error approach of TAC dosing is inefficient and better approaches are needed, such as genotype-based dosing that accounts for clinical factors. ***Our preliminary data analysis shows high dosing variations, especially in AAs, are associated with AR, so the trial and error approach is not ideal.*** The primary enzymes responsible for TAC metabolism are the cytochrome P450 3A enzymes CYP3A4 and CYP3A5[43-46]. Genetic variability in the CYP3A enzymes leads to altered TAC troughs[47-49]. Common variants in drug metabolism genes account for variability in TAC metabolism, but do not fully explain why TAC is metabolized differently by individual people. Particularly, AAs compared to EAs, have differential TAC metabolism. Thus, we proposed to do a targeted sequencing approach, using an EPS model[50, 51], to identify low-frequency genetic variants associated with TAC metabolism which may lead to a refined TAC dosing model. ***<u>Relevance</u>: Although immune suppressant therapies exist, patients require personalized doses to reach therapeutic troughs due to variability in TAC metabolism. Our precision approach may lead to individualized genotype-based TAC dosing while accounting for clinical factors.***

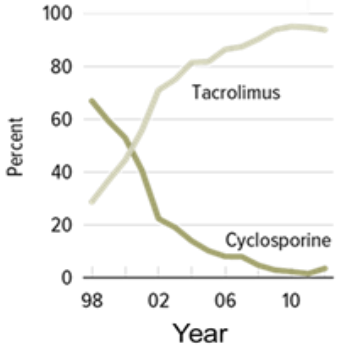

Figure 1.1: Calcineurin Inhibitor Use in Kidney Transplantation by Year[1]

2. TAC trough intrapatient variability (IPV) within renal recipients' blood leads to poor outcomes and genotype-based dosing, accounting for clinical factors, may solve this problem. There have been studies that show TAC troughs do not correlate with AR after kidney transplant[7, 52]. However, these studies did not factor in important genetic variants and did not include adequate number of AAs for population diversity. Research has shown increased risk of AR in AAs[16, 53]. TAC troughs in the first month post-transplant of <5 ng/mL were correlated with AR, while troughs around 10 ng/mL were correlated with better outcomes[54]. Studies show that high TAC trough IPV were associated with poor outcomes[10, 11, 55, 56]. Two studies have shown that high TAC IPV associated with increased rates of late AR in kidney allografts[14, 57]. Some potential

causes of high TAC IPV could be: meal-fat content, diarrhea, drug-drug interactions, genetic factors, non-adherence or generic TAC substitution[58]. Also, one dose daily TAC formulation, despite potential of improving adherence, did not reduce the high IPV compared to standard twice daily dosing[59]. A retrospective study on adherence found that TAC IPV was associated with AR, but could not be explained by nonadherence alone[12]. Similarly, a prospective study on adherence found that high TAC IPV was not explained by nonadherence alone[15]. Additionally, TAC IPV was significantly associated with kidney AR, but the IPV was not explained by the *CYP3A5 *3* (rs776746) genotype alone[13] suggesting that other genetic variants associated with TAC metabolism are important. A study that investigated the role of *CYP3A4*, *CYP3A5* and *ABCB1* allele combinations, with 47% subjects of African descent, found that 2 or more of these alleles had major impact on TAC IPV[60]. Another study, in mainly EA subjects, found that the effects of *ABCB1* variants on TAC troughs, were strongly accentuated by *CYP3A4* and *CYP3A5* genotype[19]. Thus, combinations of genetic variants associated with TAC metabolism are important in explaining high TAC IPV. ***Relevance: This thesis research set out to identify genetic variants associated with TAC metabolism in AAs that can be implemented clinically to reduce TAC IPV through precise personalized dosing.***

3. AA kidney allograft recipients have worse outcomes post-transplant than other populations due to variations in TAC metabolism even after accounting for clinical factors. AAs have a 42% higher incidence of AR and lower kidney allograft survival when compared to EAs[2]. Reaching TAC troughs rapidly is critical in reducing the risk for early AR events[2]. We, and others, have reported that TAC troughs for AAs are on average much lower than in EAs; thus AAs often require higher doses of TAC for adequate immune suppression[21, 61]. However, ***the need for increased TAC dosing is due to genotype and not race,*** and other clinical factors. Sub-therapeutic immune suppression is associated with an increase in AR particularly in AA[16]. The expression of *CYP3A5* has shown to be a major factor in the metabolism of TAC. Lower TAC levels are in part due to the functional *CYP3A5*1* allele, opposed to the loss of function (LoF) *3 allele, resulting in higher TAC metabolism in AAs. The *1 allele is more frequent in the AAs (allele frequency = 0.85) than in EAs (allele frequency = 0.14). However, there remains significant variability in AA TAC troughs after accounting for this genotype. We

identified via a Genome-Wide Association Study (GWAS), two additional, common LoF *CYP3A5* variants *6 (rs10264272) and *7 (rs41303343) in AAs that are associated with TAC troughs[20, 62]. ***We hypothesize that other less-frequent variants in AAs are associated with TAC metabolism.*** These variants would not be identifiable by GWAS because GWAS is limited to common variants that are present on the GWAS DNA chip array. ***Relevance: By using TAC as a model for CYP3A and associated genes, this Master's of Science project aimed to understand the effect of genetic variants on drug metabolism, after accounting for clinical variables. Understanding the genetic and clinical factors associated with TAC metabolism may lead to better TAC dosing models and improvement in kidney transplant outcomes.***

4. Frequency of genetic variants differs between populations but race as a variable is an inferior predictor of TAC troughs compared to genotype. We determined that the common LoF single-nucleotide polymorphisms (SNPs) in *CYP3A5* are **3*, **6*, and **7* and account for 53% of the variation in TAC troughs, after accounting for clinical factors in AAs[20]. Similarly, we found that in EAs, high-frequency SNPs, explain 41%, of the variability in TAC troughs, after accounting for clinical factors[63]. Our GWAS data from EAs shows that SNPs in *CYP3A5*3*, *CYP3A4*22* and *CYP3A4*3* were associated with TAC troughs[63]. These data show that we can identify variants in EAs and AAs that are associated with TAC troughs and that important, common variants differ by self-identified race. However, GWAS is not comprehensive for low-frequency or race-specific SNPs and NGS strategy is necessary to find additional variants associated with drug metabolism. ***Relevance: Genetic variability must be understood at the individual, genotypic level and not solely at the race level.***

5. TAC provides an exceptional model system for creating precision medicine tools. Nearly 50% of all medications are metabolized by this CYP3A pathway[22]. TAC is metabolized primarily by CYP3A4 and CYP3A5 localized in the liver, kidney, and intestine[46, 64]. CYP3A5 has approximately 1.5 times the metabolic activity on TAC than CYP3A4[65]. At least 15 metabolites of TAC have been identified[66]. Additionally, TAC troughs are monitored regularly in transplant recipients to make precise genotype and phenotype associations. The major transporter for TAC is the efflux transporter P-glycoprotein (ABCB1)[67]. Other genes that may impact TAC metabolism are the

pregnane X receptor (PXR) and CAR receptors (NR1/2 and NR1/3) that regulate the expression of *CYP3A4* and *CYP3A5*. Reports show that 25-hydroxy vitamin D up-regulates *CYP3A4* in the intestine[68, 69]. PPAR alpha (PPARA) may be involved in cytochrome P450 regulation, while POR, and cytochrome b5 (CYB5A) are necessary redox partners involved in the P450 metabolic cycle[70, 71]. Additional genes that may impact TAC metabolism are shown in **Chapter 2**. This metabolic pathway is also used to breakdown the drug midazolam (MDZ), which we use as a standard in this **Chapter 3**. Since TAC is also routinely monitored in patients, any dosing equations based on TAC associated genetic variants, can be tested. ***Relevance: TAC, and MDZ, are metabolized by complex pathways involving many genes that are important in metabolism of many drugs. This study can provide insight into dosing models, as a foundation for dosing for many drugs.***

INNOVATION

Currently the *status quo* is to start treating all kidney transplant patients with the 2-5 mg of TAC twice a day or use a weight-based dose, then adjust doses by trial and error based on periodic TAC trough measurements until a therapeutic dose is attained. These regimens are primarily based on EA pharmacokinetic data which does not apply to all populations due to differences in TAC metabolism. My mentors have developed a TAC dosing model using high-frequency SNPs and clinical factors[62], but the model lacks information regarding low-frequency variants which likely account for additional and important variation in TAC blood concentrations[20]. This genetic epidemiological study investigated a cohort of AA kidney allograft recipients with repeated measures of TAC concentrations from Clinical Laboratory Improvement Amendment (CLIA) certified labs over the first six months post-transplant, banked DNA, clinical covariates, medication history and clinical outcomes. Our approach to identify genetic variants, through NGS, and an EPS model associated with TAC metabolism is a pioneering method of developing personalized dosing models to address the outcome disparities in AA transplant recipients.

Additionally, we have developed Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease gene editing technology in my lab, accompanied by cell culture-based metabolism assays, to functionally validate genetic variants identified

in transplant patients. It is my goal to use my genetics background along with functional validation, the CRISPR/Cas9 technique, and cell culture to address race disparities in drug response[72, 73]. This project provides me opportunity to be at the forefront of biotechnology and pharmacogenomics while leading to an understanding of the impact of the low-frequency genetic variants compared with known high-frequency genetic variants on TAC metabolism. Furthermore, this work is translatable and may allow us to refine TAC dosing models as we learn more about genetic variants associated with TAC metabolism identified in this study[9, 62]. Someday, refined drug dosing models will incorporate low-frequency variants, to determine the precise dose needed to achieve a therapeutic TAC concentration for any individual. Moreover, the development of these cell lines, with engineered genetic variants in pharmacogenes, will lead to a bank of cell lines useable to understand the metabolic processing of other drugs that are metabolized via the same CYP3A enzyme system. Throughout this project, we are developing a bank of genetically unique cell lines for drug metabolism studies while improving strategies to identify and validate, genotypes for providing precision medicine to transplant recipients leading to improved outcomes.

Premise of this Thesis

Although I earned my PhD from the University of Minnesota in 2011 majoring in Molecular Cellular Developmental Biology and Genetics, this Master program in Biomedical Informatics and Computational Biology was a core training component for my K01. The K01 grant, K01AI130409, from the National Institute of Allergy and Infectious Diseases (NIAID), titled Genetic Variants Associated with Tacrolimus Metabolism in Kidney Transplant Recipients had project period from 2018 to 2023. The point of this additional training was to expand my quantitative and modeling skills and enhance my abilities as a clinical and translational scientist.

Chapter 2

Identification of genetic variants associated with tacrolimus metabolism in kidney transplant recipients by extreme phenotype sampling and next generation sequencing

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Abstract

An EPS model with targeted NGS identified genetic variants associated with TAC metabolism in subjects from the Deterioration of Kidney Allograft Function (DeKAF) Genomics cohort which included 1,442 European Americans (EA) and 345 African Americans (AA). This study included 48 research participants separated into 4 groups of 12 (AA high, AA low, EA high, EA low). Groups were selected by the extreme phenotype of dose-normalized TAC trough concentrations after adjusting for common genetic variants and clinical factors. NGS spanned >3 Mb of 28 genes and identified 18,661 genetic variants (3,961 previously unknown). A group of 125 deleterious variants, by SIFT analysis, were associated with TAC troughs in EAs (burden test, p=0.008), *CYB5R2* was associated with TAC troughs in AAs (SKAT, p=0.00079). In *CYB5R2*, rs61733057 (increased allele frequency in AAs) was predicted to disrupt protein function by SIFT and PolyPhen2 analysis. The variants merit further validation.

Introduction

TAC, the primary immune suppressant used in >90% of solid organ transplants[4], is a substrate of cytochrome P450 (CYP) enzymes CYP3A4 and CYP3A5. TAC has a narrow therapeutic window[5] with troughs that are highly associated with efficacy[6] and toxicity[7]. TAC troughs are routinely monitored to maintain a therapeutic range and guide dosing adjustments. Most AAs often have higher rates of TAC metabolism generally due to higher active CYP3A5 expression. Consequently, AAs often require higher TAC doses than European Americans (EAs)[8]. High TAC clearance and low troughs in kidney transplant recipients during the first 90 days post-transplant is a risk factor for AR[75].

There is large interpatient variability[76] in TAC metabolism which is partially due to genetic variants and clinical factors that alter clearance. TAC dosing equations including common genotypes for CYP3A single-nucleotide polymorphisms (SNPs) and clinical factors have been developed to personalize TAC dosing[9, 77]. However, dosing equations have not allowed for low-frequency variants (<5% frequency in population) because few studies have been sufficiently powered to identify them. Thus, further understanding the genetics of TAC disposition, especially the low-frequency variants, could be translated into more precise TAC dosing strategies.

Genetic variants impact TAC troughs[42] by altering CYP3A4 and CYP3A5 enzyme expression, activity[78], transcription factors, co-enzymes or transporters. Previously, with

a diverse cohort of kidney allograft recipients and a custom SNP array, we found that 52.5% of TAC trough variation in research participants was explained by a set of common SNPs and clinical variables[21]. Upon further investigation of TAC troughs using a genome-wide association study (GWAS), three loss-of-function (LoF) genetic variants, *CYP3A5*3* (rs776746), *CYP3A5*6* (rs10264272) and *CYP3A5*7* (rs41303343), in AAs were highly associated with TAC troughs[76]; these three genetic variants and clinical factors, accounted for 53.9% of the TAC trough variability in AAs. In a cohort of EAs, *CYP3A5*3* and*CYP3A4*22* (rs35599367) were significantly associated with TAC troughs[79]. A limitation of GWAS SNP arrays is that the genotypes are typically restricted to known common genetic variants requiring alternative methods to identify low-frequency, and unknown, variants associated with TAC disposition.

We hypothesized that TAC trough variation between subjects is, in part, due to low-frequency genetic variants which may not be present on a GWAS SNP array. To identify low-frequency genetic variants associated with TAC metabolism, we used an EPS model and NGS. The EPS model allows increased statistical power with fewer specimens for analysis[51] and can account for known clinical factors and common genotypes[76]. Our EPS model investigated subjects with either the highest or lowest dose-normalized TAC troughs in our cohort. Because AAs often have different allele frequencies than EAs, we conducted a separate analysis for EAs and AAs. After selecting the research participants with extreme dose-normalized TAC troughs, and adjusting for clinical and common genetic variants, we used targeted NGS to identify low-frequency genetic variants. The variants may be useful for improving TAC dosing and understanding TAC trough variability.

Methods

Subjects and Tacrolimus Measurements

The 48 subjects identified for EPS and NGS were selected from 345 AAs and 1,443 EAs enrolled in our multi-center prospective, observational trial Deterioration of Kidney Allograft Function (DeKAF) Genomics (clinicaltrials.gov NCT00270712). The study was approved by the Institutional Review Board and informed consent was obtained from each subject prior to entering the study. Although race status was collected by self-reporting, GWAS principal components were used to select research participants for the EPS model. GWAS was previously conducted with all research

participants. Research participants in the analysis were AA or EA kidney transplant recipients, ≥18 years who received TAC maintenance immunosuppression from 7 centers: University of Minnesota, Hennepin County Medical Center, University of Alabama, Mayo Clinic-Rochester, University of Iowa, University of Manitoba and University of Alberta. Recipient characteristics, clinical outcomes, TAC troughs and doses and concomitant medications were prospectively collected. Oral TAC was initiated around time of transplant using twice daily dosing. Doses were adjusted to achieve each institution's target trough. TAC troughs were measured at each center approximately 12-hours following the last dose, at steady state with the current dose. Generally, troughs of 8–12 ng/mL were targeted for the first 3 months and 6–10 ng/mL for 3–6 months post-transplant. Median (range) of 18 (1–24) troughs were obtained for each subject in the first 6 months post-transplant. TAC trough whole blood measurements were clinically measured at each site and analyzed in CLIA approved laboratories with >95% measured by liquid chromatography-mass spectrometry.

Genotyping of Research Participants

Before this study, genotyping on all subjects was performed on recipient DNA isolated from peripheral blood with the Affymetrix Transplant GWAS array that has been previously described[80]. The EPS model adjusted for common SNPs and principal components data from this array to assign race.

Selection of Research Participants for EPS

To select subjects with the extreme phenotype of TAC troughs, 48 kidney transplant recipients with the 12 highest and 12 lowest TAC troughs from the EA or AA cohorts, after accounting for clinical factors, known common genetic variants and enrolling transplant center, were selected for this study (**Figure 2.1**). To select these individuals, linear mixed-effects models (LMMs) were used to test for associations between natural log (ln)–transformed dose-normalized TAC troughs and the LoF genotypes *CYP3A5 *3* (rs776746), *CYP3A5 *6* (rs10264272) and *CYP3A5 *7* (rs41303343) in AAs. For EAs, we adjusted for LoF genotypes *CYP3A5*3* and *CYP3A4*22* (rs35599367). Log transformation was used to ensure that the outcome was normally distributed. Our prior analyses found that dose-normalized troughs initially start low, rise quickly until day 9 after transplant and then plateau in the early weeks

after transplant[21, 81]. Therefore, a simple spline method was used to model the effect of time on all trough concentrations, with the change in slope occurring at day 9. The longitudinal LMMs included a random intercept, random slopes for days after transplant, and days after post-transplant day 9. Confounding fixed clinical factors were retained in the EPS model and were selected by performing backward selection with retention p-value of 0.10. For EAs, we adjusted for factors: time post-transplant, transplant recipient age, weight, diabetes status, living vs. deceased donor, donor gender, and antibody induction type; and time-varying covariates included estimated glomerular filtration rate, steroid use, calcium channel blocker use, angiotensin-converting enzyme inhibitor use and antiviral use. For AAs, we adjusted for time post-transplant, transplant recipient age, simultaneous pancreas and kidney transplant (SPK), and antibody induction type and time-varying covariates glomerular filtration rate (GFR) and antiviral use. The multivariable models were used to determine residuals which were then used to identify the subjects with the extreme phenotypes of adjusted TAC troughs. Analyses were conducted with SAS version 9.2 software (SAS Institute, Cary, NC).

Targeted Next Generation Sequencing (NGS)

Hybridization-based capture was performed with 1 µg of genomic DNA with NimbleGen SeqCap EZchoice kit (Roche, NimbleGen). Sequencing spanning the entire length of 28 genes (**Table 2.1**) was performed and extended ~20,000 base pairs upstream and downstream of these genes. Thus, the extended sequencing length included 42 partial genes adjacent to the 28 genes for a total of 70 genes (**Table 2.2**) spanning 3,123,443 base pairs. These 28 genes were selected because they were hypothesized as associated with TAC disposition. We used a custom relaxed coverage probe design (Roche NimbleGen) allowing up to 20 close matches in the genome that increased the coverage across all regions. Standard SeqCap EZ gDNA libraries were developed and hybridized with the custom EZ choice probes following standard protocols. The captured libraries were multiplexed and sequenced using MiSeq V2 chemistry (2x150 bp).

Figure 2.1: Extreme Phenotype Sampling (EPS) Model to Detect Genetic Variants Associated with Tacrolimus Metabolism from African American (AA) or European American (EA) Kidney Transplant Recipients. The figures represent the mean dose-normalized TAC troughs on the y-axis and the distribution of subjects on the x-axis. The 12 recipients with highest or lowest TAC troughs, after adjusting for clinical variables and common genetic variants, from each group were selected for targeted next generation sequencing (NGS). **A.** The model used to select AA kidney transplant recipients was adjusted for genetic variants *CYP3A5 *3*, **6*, and **7*. The 12 AA subjects with the highest (3.5%) or 12 with the lowest (3.5%) TAC troughs were used for NGS from a cohort of 345 total subjects. **B.** The model used to select EA kidney transplant recipients was adjusted for genetic variants *CYP3A5 *3* and *CYP3A4 *22*. The 12 EA subjects with the highest (0.8%) or 12 with the lowest (0.8%) dose-normalized TAC troughs were used for NGS from a cohort of 1,443 total subjects.

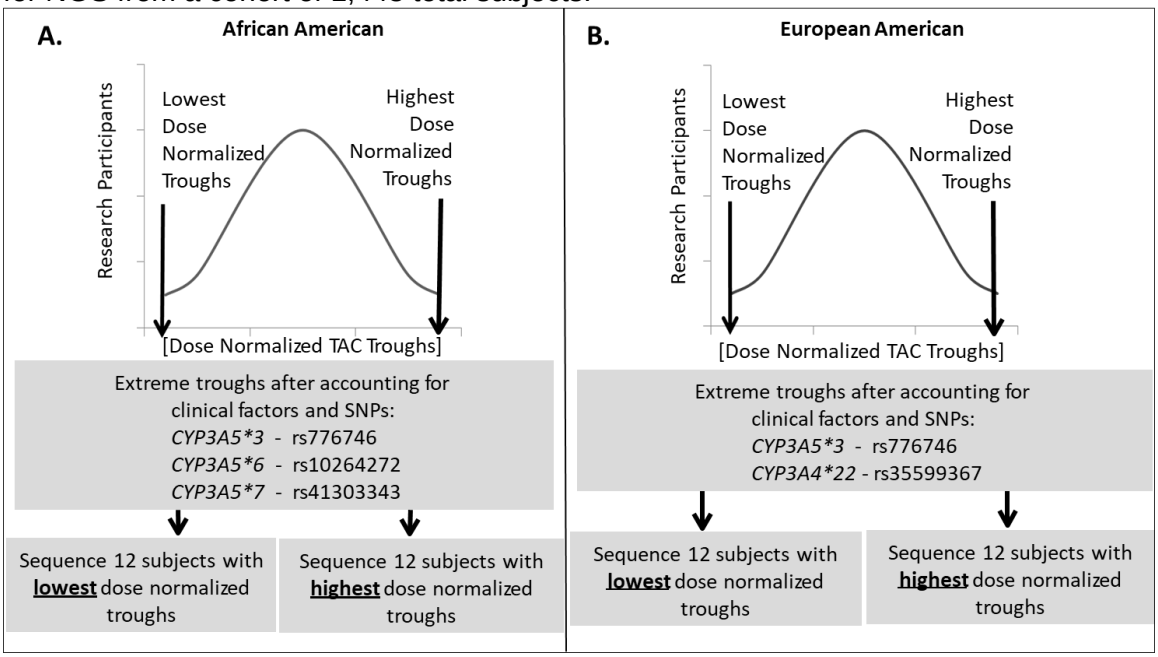


Table 2.1: Full Genes Sequenced in this Study

Note: Each gene was sequenced 20 kilobases upstream and downstream of the gene.

Gene	Protein Name	Function and Relevant References Showing Association with TAC Disposition
CYP3A locus	Cytochrome P450 subfamily: CYP3A4, CYP3A5, CYP3A43, CYP3A7, CYP3A51P	Metabolism of TAC [46, 82-84]
CYP2J2	Cytochrome P450, subfamily 2J polypeptide 2	P450 enzyme expressed in intestine, heart. Drug metabolism. Metabolizes arachidonic acid promoting kidney homeostasis, TAC has inhibitory effect nephrotoxicity [85, 86]
Co-enzymes		
POR	cytochrome P450 oxidoreductase	P450 oxidoreductase and reduced cytochrome b5 supply electrons into the P450 cycle. Addition of cyt b5 stimulates CYP3A4 activity *in vitro*. Oxidoreductase responsible for electron transfer from NAD to CYP450, (POR*28 is associated with increased CYP3A activity and increase TAC clearance) [87-89]
CYB5A	Cytochrome B5, TypeA	Participant in the CYP450 cycle as an electron donor for cytochrome b5. Drug metabolism
CYB5R1	NADH-Cytochrome B5 Reductase	Reduces cytochrome b5. Cytochrome b5 donates second electron in P450 cycle and enhances CYP3A activity.
CYB5R2	NADH-Cytochrome B5 Reductase-2	Bifunctional reductase that contains cytochrome b5 and reductase domains in same protein. Cytosolic enzyme. Unclear if it associated with P450.

CYB5R3	Cytochrome B5 Reductase 3	Participant in CYP450 cycle as electron donor for cytochrome b5. Drug metabolism [90], Present in endoplasmic reticulum membrane
CYB5R4	NADH-Cytochrome B5 Reductase-4	Reduces cytochrome b5. Cytochrome b5 supplies second electron in P450 cycle and stimulates CYP3A activity.
CYB5RL	NADH-Cytochrome B5 Reductase-Like	Reduces cytochrome b5
CYB5D1	Cytochrome B5 Domain-Containing Protein-1	Serves as an electron donor for cytochrome b5 and thus participates in CYP450 cycle. Thus, play a role in drug metabolism
Transporters		
ABCB1	ATP-Binding Cassette, Subfamily B, member 1	Efflux transporter known as Multi Drug Resistance1 or P-glycoprotein. TAC is a substrate. Actively transports TAC into the intestinal lumen as a counter-transport pump [67, 91, 92]
ABCC1	ATP-Binding Cassette, subfamily C, member 1	Efflux transporter. Also known as Multidrug resistance associated protein 1 (MRP1). Findings suggest that MDR1 polymorphisms has effect on TAC pharmacodynamics [17, 93, 94]
ABCC2	ATP-Binding Cassette, subfamily C, member 2	Efflux transporter also known as Multidrug resistance associated protein 2 (MRP2) [95].
ABCG2	ATP-Binding Cassette, Subfamily G, member 2	Efflux transporter, also named Breast Cancer Resistance Protein. TAC is a inhibitor, variants in ABC transporter gene may also associate with TAC pharmacokinetics [92]

ABCE1	ATP-Binding Cassette, Subfamily E, member 1	Efflux transporter also known as ribonuclease 4 inhibitor
SLCO1B3	Solute Carrier Organic anion transporter family, member 1B3	Uptake transporter for organic anions. Also known as OATP1B3.
Transcription Factors		
VDR	Vitamin D Receptor	Ligand activated transcription factors) that control gene expression). Highly expressed in intestine, but not in liver. Affects intestinal expression of CYP3A [96]
NR3C1 (GR)	Nuclear Receptor Subfamily 3, group Member 1	Glucocorticoid Receptor. Glucocorticoid-activated transcription factor that controls gene expression (several drug metabolizing genes contain GR response elements) [97]
NR1I2 (PXR)	Nuclear Receptor Subfamily 1, group 1, Member 2	Pregnane X Receptor. Ligand activated transcription factors) that control gene expression Regulates expression of drug metabolizing enzymes and drug transporters in liver [88, 98, 99]
NR1I3 (CAR)	Nuclear Receptor Subfamily 1, group 1, Member 3	Constitutive Androstane Receptor. Ligand-activated transcription factors) that control gene expression. Alters expression of CYP3A genes. Key regulator of drug metabolizing enzymes and drug transporters [100]
HNF4A	Hepatocyte Nuclear Factor-4-α	Transcription factor for hepatic gene expression regulation, Regulates PXR and CAR expression and CYP3A expression[101]

CEBPA	C/EBP-Alpha	Co-factor (activator) for gene regulation. Especially transporters ABBC2 and ABCB1 [102, 103]
CEBPB	CCAAT/Enhancer Binding Protein, Beta	Co-factor (activator) for gene regulation. Especially transporters ABBC2 and ABCB1
PPARA	Peroxisome Proliferator-Activator Receptor Alpha	Has regulatory effect on CYP3A4 expression [88, 104, 105]
FOXA2	Forkhead Box protein A2	Transcription factor also named HNF3-β, has effect on hepatic *CYP3A4* expression[106]
NCOR1	Nuclear Receptor Corepressor 1	Co-factor (repressor) for gene regulation. Associated with transporters ABBC2 and ABCB1
YY1	Transcriptional Repressor Protein	Downregulates Cytochrome c Oxidase and *CYP3A4* and *CYP3A5*

Table 2.2: List of all 70 genes used in the gene based statistical test
Since we sequenced 20 kb upstream and downstream, and spanning the entire length of 28 genes in **Table 2.1**, this led to partial sequencing of 42 genes adjacent to these 28 genes and thus 70 total genes.

ABCB1	*CYB5D2*	*LOC401980*	*PPFIBP2*
ABCC1	*CYB5R1*	*LSMD1*	*R3HDML*
ABCC2	*CYB5R2*	*MAATS1*	*RIPPLY2*
ABCC6	*CYB5R3*	*MRPL37*	*RNU12*
ABCE1	*CYB5R4*	*NCOR1*	*RUNDC3B*
ABCG2	*CYB5RL*	*NDUFS2*	*SLC25A29*
ADIPOR1	*CYP2J2*	*NR1I2*	*SLCO1B3*
ANAPC10	*CYP3A4*	*NR1I3*	*STYXL1*
ANKFY1	*CYP3A43*	*NR3C1*	*TMEM120A*
APOA2	*CYP3A5*	*OR2AE1*	*TMEM88*
CDCP2	*CYP3A7-CYP3AP1*	*OTUD4*	*TOMM40L*
CDPF1	*FCER1G*	*OVCH2*	*TTC19*
CEBPA	*FOXA2*	*PIGL*	*VDR*
CEBPA-AS1	*GSK3B*	*PKD2*	*YY1*
CEBPB	*HNF4A*	*PKDREJ*	*ZSCAN25*
CHD3	*HOOK1*	*POLDIP3*	*ZZEF1*
YB5A	*KDM6B*	*POR*	
CYB5D1	*LINC00261*	*PPARA*	

Bioinformatics Analysis of NGS Data

The raw Illumina sequences were evaluated for quality with FASTQC[107]. Sequenced reads were aligned to University of California Santa Cruz's human reference genome (GRCH 37/hg 19) with a Burroughs-Wheeler Aligner[108]. Depending on the reporting group[109, 110], recommended sequence depth is at least 10X-30X; we targeted >20X depth for making variant calls. Genome Analysis Toolkit's (GATK) best practices[111-116] pipeline was used to identify and call variants[117]. The final list of variants obtained were annotated with the snpEff tool[118] and the Ensembl Variant Effect Predictor (VEP)[119]. Variants were evaluated *in silico* by Sorting Intolerant from Tolerant (SIFT)[120, 121] and Polymorphism Phenotyping Tool 2 (PolyPhen2)[122-124] to assess potential impact on protein.

Statistical Analysis of Extreme Phenotype Individuals

A logistic regression model was applied, as cases and controls were identified as low versus high dose-normalized TAC trough subjects respectively, in either AA or EA groups. Due to small sample size, tests for association were performed by permutation testing and p-values were calculated. A continuous trait test was performed by regressing the dose-normalized TAC troughs on the genetic variants accounting for the selection procedure. Thus, two types of analyses were performed: **A)** Association tests for each of the single genetic variants with both EA and AA groups to determine SNPs associated with TAC metabolism in each group. **B)** Gene based test on 70 genes (**Table 2.2**) with burden test (BT) (linear sum of variant scores test), sequence kernel association test (SKAT) (sum of squared variant based test), and an optimal combination of BT and SKAT (SKATO)[125]. P-values were further Bonferroni corrected with significance level of 0.0007 for the gene-based test. Focused-SNP set analyses, with each subject group, were performed on SIFT and PolyPhen2 selected variants which were predicted to impact protein function.

Manual Curation of Genetic Variants to Identify Genetic Variants

We manually inspected variants identified by GATK analysis in the TAC related genes *CYP3A4*, *CYP3A5, POR* and *CYB5A* for previously unidentified genetic variants

in the 5'-untranslated regions (UTR), exons and 3'-UTR regions of these genes in our EPS model cohorts.

Results

Subject characteristics are described in **Table 2.3**. The EPS model in **Figure 2.2** shows the natural-log of dose-normalized TAC troughs from the groups over the first 6-months post-transplant. As expected, the EA high group had the highest dose-normalized TAC troughs over time. The AA high group had the next highest TAC troughs, third was the EA low group and the AA low group had lowest TAC troughs. The subjects with 2 known LoFs could have any combination of *CYP3A5 *3*, **6*, **7* or *CYP3A4 *22* alleles. After adjusting for *CYP3A5 *3*, **6*, and **7* in the AA high group 6 subjects had 2 known LoFs, while the AA low group had 1 subject with 2 known LoFs (**Table 2.3**)**.** After adjusting for *CYP3A5 *3* and *CYP3A4 *22* alleles*,* the EA high group had 11 subjects with 2 known LoFs and the EA low group had 10 subjects with 2 known LoFs. Since the EPS model adjusted for common LoF genetic variants, these groups may have complex genetics associated with TAC disposition.

Table 2.3: Clinical and Genetic Characteristics of the Extreme Phenotype Subjects in African American (AA) and European American (EA) Groups. The High groups had the highest dose-normalized TAC troughs, while the Low groups had the lowest dose normalized TAC troughs. AA cohort N=345 and EA cohort N=1443.

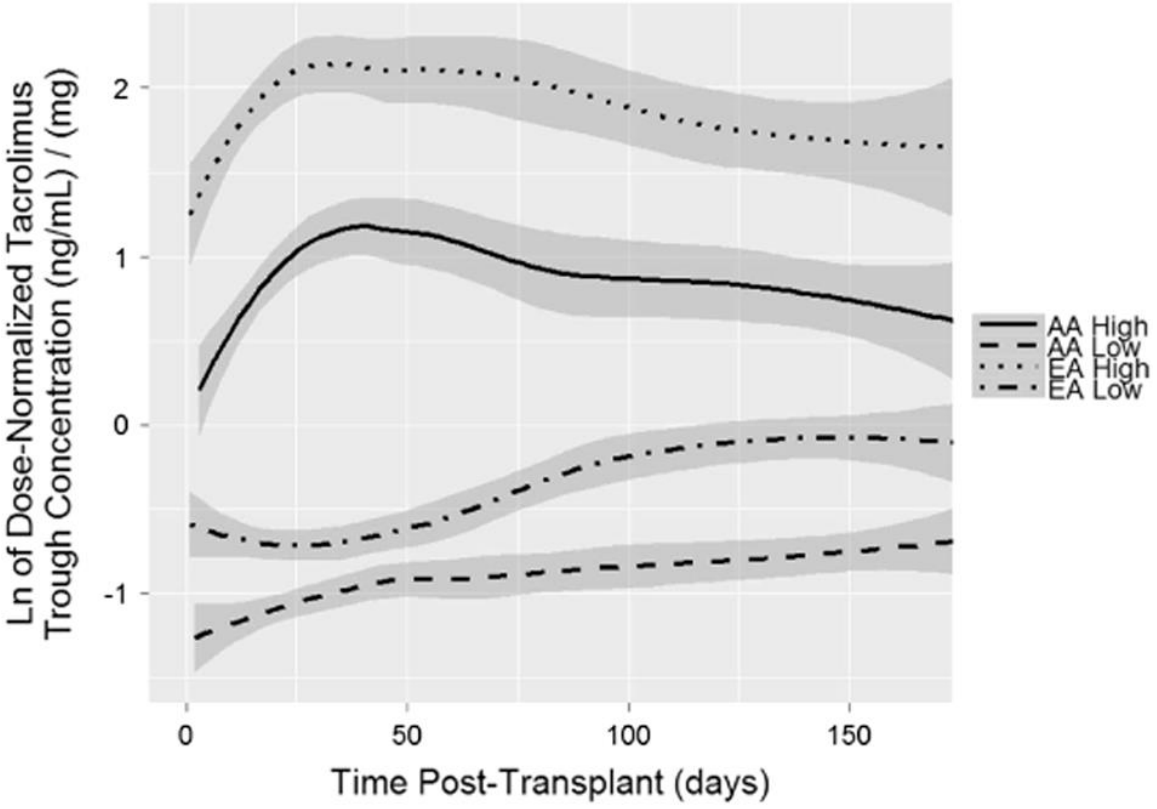
Variable		Dose-Normalized TAC Trough Groups			
		AA High	AA Low	EA High	EA Low
N		12	12	12	12
Age	**18-34**	2	2	1	0
	35-64	9	10	8	10
	65-84	1	0	3	2
Diabetes	**yes**	6	9	9	7
	no	6	3	3	5
Donor Status	**Living**	2	8	11	7
	Deceased	10	4	1	5
Donor Gender	**Male**	7	4	7	10
	Female	5	8	5	2
Number of subjects with *CYP3A5 *3* Alleles rs776746_G	**0**	5	4	0	0
	1	6	7	1	2
	2	1	1	11	10
Number of subjects with *CYP3A5 *6* Alleles rs10264272_T	**0**	10	10	12	12
	1	1	2	0	0
	2	1	0	0	0
Number of subjects with *CYP3A5 *7* Alleles rs41303343_TA	**0**	8	11	12	12
	1	3	1	0	0
	2	1	0	0	0
Number of subjects with *CYP3A4 *22* Alleles rs35599367_A	**0**	11	12	10	11
	1	1	0	2	1
	2	0	0	0	0
Number of subjects with known CYP3A Loss of Function Alleles (*CYP3A5 *3*,*6*,*7* or *CYP3A4 *22*)	**0**	2	1	0	0
	1	4	10	1	2
	2	6	1	11	10
Estimated Glomerular Filtration Rate* (mL/min)	**< 54.9**	19.9%	9.1%	19.0%	31.6%
	54.9-67.9	11.7%	45.7%	28.8%	27.0%
	67.9-83.5	24.5%	17.8%	22.3%	20.9%
	>83.5	43.9%	27.4%	29.9%	20.5%

Weight (kg)*	**< 69.4**	26.5%	4.6%	56.5%	20.5%
	69.4-80.9	20.9%	12.8%	28.3%	49.3%
	80.9-94.6	32.7%	21.0%	12.0%	0.9%
	>94.6	19.9%	61.6%	3.3%	29.3%
Steroid Use in First 6 Months	**Yes**	11	11	12	12
	No	1	1	0	0
Simultaneous Pancreas and Kidney Transplant	**Yes**	1	0	0	1
	No	11	12	12	11
Antibody Induction	**Monoclonal**	8	5	5	3
	Polyclonal	4	7	7	8
Calcium Channel Blocker in First 6 Months	**Yes**	8	9	5	9
	No	4	3	7	3
ACE Inhibitor in First 6 Months	**Yes**	4	4	5	2
	No	8	8	7	10
Antiviral Use in First 6 Months	**Yes**	12	9	12	11
	No	0	3	0	1
TAC Daily Dose (mg) Median (range)		4.0 (0.5 – 12.0)	14.0 (1.0 – 36.0)	1.0 (0.1 – 6.0)	14.0 (2.0 – 36.0)
TAC Trough Concentration (ng/mL) Median (range)**		7.5 (1.0 – 21)	5.1 (1.0 -18)	8.9 (2.4 – 26)	8.1 (1.3 – 29)
Dose Normalized TAC Trough Concentration (ng/mL) Median (range)		2.4 (0.3-31)	0.38 (.083-1.4)	7.7 (1.0-82)	0.57 (0.13-4.8)

* Estimated Glomerular Filtration Rate and Weight are for time point closest to the corresponding TAC trough measurement

**TAC troughs, and dose normalized TAC troughs, were measured periodically for each subject, up to 24 times per subject.

Figure 2.2: Dose Normalized TAC Troughs of Subjects from Extreme Phenotype Sampling (EPS) Model used for Next Generation Sequencing (NGS). The figure shows natural log transformed TAC dose-normalized troughs over time, in high and low AA or EA TAC groups. Data lines represent smoothed conditional means and gray areas represent 95% confidence intervals. The 12 EA subjects with the highest (0.8%) or 12 with the lowest (0.8%) TAC troughs were used for NGS from a cohort of 1,443 total subjects. The 12 EA subjects with the highest (3.5%) or 12 with the lowest (3.5%) TAC troughs were used for NGS from a cohort of 345 total subjects after adjustment for known genotypes and clinical factors.



Data Availability

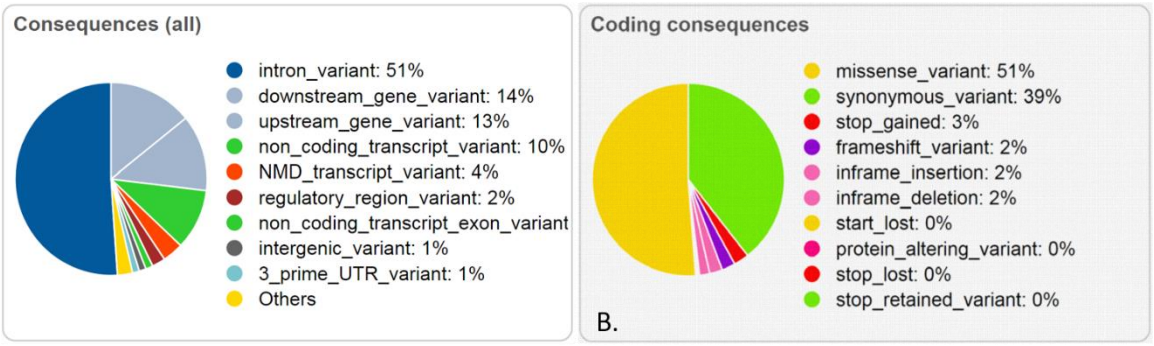
Raw sequence data, in FASTQ file format, are available at the United States National Center for Biotechnology Information's (NCBI) Sequence Read Archive (SRA) with SRA accession number: SRP156752. The associated phenotype and covariate data are available at NCBI's Database for Genotypes and Phenotypes with dbGaP accession number: phs001670.v1.p1.

Variants Identified from Sequencing

The estimated coverage of NimbleGen sequencing was 86.6% of the total bases across the entire genetic distance of 28 genes. The remaining 13.4% were not covered

because of the repetitive nature of the genomic regions. The sequencing depth was 60X across these 28 genes and 42 adjacent partial genes after mapping and quality control filtering of the sequences. The sequencing of the 42 partial genes did not span the entire length of those genes. A total of 18,661 variants in 48 extreme phenotype subjects were identified and processed for quality. With the Variant Effect Predictor (VEP) tool [119], out of the 18,661 total variants identified, 3,961 variants (21.2%) were unknown and 14,700 (78.8%) were previously identified. The VEP analysis of these variants, and the coding variants, based on their predicted consequences are described in **Figure 2.3**. Although many of the genetic variants had unspecified significance, we identified 15,948 variants in the AA cohort and 11,074 variants in the EA cohort that were different (alternative allele) than the reference genome GRCH 37/hg 19.

Figure 2.3: Variant Effect Predictor (VEP) results based on genetic variants identified A. Predicted consequences of the 18,661 genetic variants identified in this sequencing study. **B.** Predicted gene expression consequences from coding sequences in the VEP analysis.



*NMD_transcript_ variant: Nonsense mediated decay transcript variant.

Statistical Association of Variants Identified through Sequencing with TAC Troughs

The association testing identified 397 and 297 variants that were associated with dose-normalized TAC troughs in AA and EA, respectively, with p<0.05 by either case-control or continuous trait tests. However, 15 (**Table 2.4**) and 9 (**Table 2.5**) variants in AA or EA, respectively had a p<0.005. Variants identified in the EA cohort with p<0.005 were in *ABCC1, ANAPC10, NR3C1* and *OTUD4*. Variants identified in the AA cohort with p<0.005 were in *ADIPOR1*, *CYB5R2*, *OVCH2* and *POR*.

Table 2.4: Single Variants Associated with Dose- Normalized Tacrolimus Troughs, identified in African American Kidney Transplant Recipients (p<0.005). The table indicates the chromosome location of the variants based on GRCH37 assembly, the variant alternate allele, the consequence effect of the variant on the Ensembl transcripts, the gene symbol, the exon number out of the total number of exon in that gene, the intron number out of the total number in that gene, Existing known variants' rs number if available and the allele frequencies from 1000 Genomes project as given by VEP software. AF = global, AFR = African population, AMR = American population, EUR = European population, EAS = East Asian population, SAS = South Asian population, AA = Allele Frequency from in African American population from Lung and Blood Institute-Exome Sequencing Project (NHLBI-ESP), EA = Allele Frequency in European American population from NHLBI-ESP. Also shown are the related test p-values for association with TAC troughs.

Location	Allele	Consequence	Symbol	Exon	Intron	Existing_variation	Allele frequencies								Pvalb[1]	Pvalc[2]
							AF	AFR	AMR	EAS	EUR	SAS	AA	EA		
7:75552252-75552252	A	intron_variant	*POR*	-	1/14	-	-	-	-	-	-	-	-	-	0.001	0.002
7:75558027-75558037	C	intron_variant	*POR*	-	1/14	rs6681105 6	-	-	-	-	-	-	-	-	0.001	0.002
7:75573951-75573956	GTGTTTGT	intron_variant	*POR*	-	1/14	rs6767595 9	0.26	0.52	0.24	0.25	0.09	0.10	-	-	0.001	0.002
7:75576956-75576956	T	intron_variant	*POR*	-	1/14	rs239955	0.26	0.52	0.24	0.25	0.09	0.10	-	-	0.001	0.002
7:75565740-75565740	A	intron_variant	*POR*	-	1/14	rs239960	0.25	0.49	0.24	0.25	0.09	0.10	-	-	0.002	0.003
11:77101 78-77101 78	T	downstream_gene_variant	*OVCH2*	-	-	rs4501973	0.46	0.18	0.57	0.58	0.61	0.49	-	-	0.002	0.001
11:77118 72-77118 72	C	downstream_gene_variant	*OVCH2*	-	-	rs1083984 2	0.47	0.18	0.57	0.59	0.62	0.50	-	-	0.002	0.001

11:7712471-77124771	T	stop_gained	*OVCH2*	15/15	-	rs4509745	0.48	0.23	0.58	0.59	0.62	0.49	0.31	0.62	0.002	0.001
1:20293183 9-20293 1839	A	upstream_gene_variant	*ADIPOR1*	-	-	rs2232854	0.31	0.18	0.40	0.43	0.35	0.28	0.23	0.34	0.002	0.002
7:75544455-75544455	C	upstream_gene_variant	*POR*	-	-	rs3823884	0.48	0.94	0.42	0.27	0.27	0.35	-	-	0.002	0.004
11:7687305-76873 05	T	intron_variant	*CYB5R2*	-	8/8	rs1279450 7	0.26	0.44	0.18	0.14	0.25	0.22	-	-	0.003	0.001
7:75586536-75586536	C	intron_variant	*POR*	-	2/14	rs4728533	0.73	0.48	0.76	0.74	0.91	0.84	-	-	0.003	0.003
7:75563682-75563682	G	intron_variant	*POR*	-	1/14	rs1253323 5	0.26	0.52	0.24	0.25	0.09	0.10	-	-	0.003	0.004
11:7687517-76875 17	C	intron_variant	*CYB5R2*	-	8/8	rs1104152 3	0.49	0.30	0.49	0.68	0.51	0.53	-	-	0.004	0.004
11:7686602-76866 06	TGTTTGTT	stop_retained_variant, 3_prime_UTR_variant	*CYB5R2*	9/9	-	rs5365125 97, rs16411	0.47	0.26	0.49	0.64	0.51	0.53	0.28	0.51	0.004	0.004

-TAC troughs were adjusted in the extreme phenotype model for clinical variables and genotypes *CYP3A5*3*, *CYP3A5*6,* and *CYP3A5*7*.

[1] Pvalb: Logistic regression with permutation applied to calculate p-value in the case-control trait test.

[2] Pvalc: Linear regression applied to obtain p-values in the continuous trait test

Table 2.5: Single Variants Associated with Tacrolimus Adjusted Troughs, Identified in European American Kidney Transplant Recipients of (p<0.005). The table indicates the chromosome location of the variants based on GRCH37 assembly, the variant alternate allele, the consequence effect of the variant on the Ensembl transcripts, the gene symbol, the intron number out of the total number in the gene, Existing known variants' rs numbers if available and the allele frequencies from 1000 Genomes project as given by VEP software. AF = global, AFR = African population, AMR = American population, EUR = European population, EAS = East Asian population, SAS = South Asian population. Also shown are the related test p-values for association with Tac troughs.

Location	Allele	Consequence	Symbol	Intron	Existing_variation	Allele frequencies						Pvalb[1]	Pvalc[2]
						AF	AFR	AMR	EAS	EUR	SAS		
4:146068652-146068652	T	intron_variant	*OTUD4*	13/20	rs12502109	0.31	0.37	0.31	0.47	0.13	0.26	0.001	0.002
20:43074372-43074372	C	downstream_gene_variant	*14kb 3' of HNF4A*	-	rs1321826	0.16	0.32	0.16	0.03	0.11	0.15	0.002	0.004
20:43075161-43075161	A	downstream_gene_variant	*14kb 3' of HNF4A*	-	rs7272694	0.16	0.32	0.16	0.03	0.11	0.15	0.002	0.004
20:43075280-43075280	C	downstream_gene_variant	*15kb 3' of HNF4A*	-	rs7267639	0.16	0.32	0.16	0.03	0.11	0.15	0.002	0.004
5:142803548-142803548	G	intron_variant	*NR3C1*	1/8	rs72802815	0.25	0.23	0.42	0.10	0.34	0.23	0.005	0.004
16:16203559-16203559	T	intron_variant	*ABCC1*	21/29	rs35090860	0.21	0.06	0.14	0.40	0.21	0.25	0.005	0.003
16:16208172-16208172	T	intron_variant	*ABCC1*	22/29	rs4544399	0.20	0.04	0.14	0.40	0.19	0.25	0.005	0.003
16:16208173-16208173	C	intron_variant	*ABCC1*	22/29	rs4562453	0.20	0.04	0.14	0.40	0.19	0.25	0.005	0.003
4:146001613-146001613	T	intron_variant	*ANAPC10*	3/4	rs35098431	0.35	0.51	0.32	0.47	0.13	0.26	0.005	0.004

-Tac troughs were adjusted in the extreme phenotype model for clinical variables and genotypes *CYP3A5*3* and *CYP3A4*22*.

[1]Pvalb: Logistic regression with permutation applied to calculate p-value in the case-control trait test.

[2]Pvalc: Linear regression applied to obtain p-values in the continuous trait test

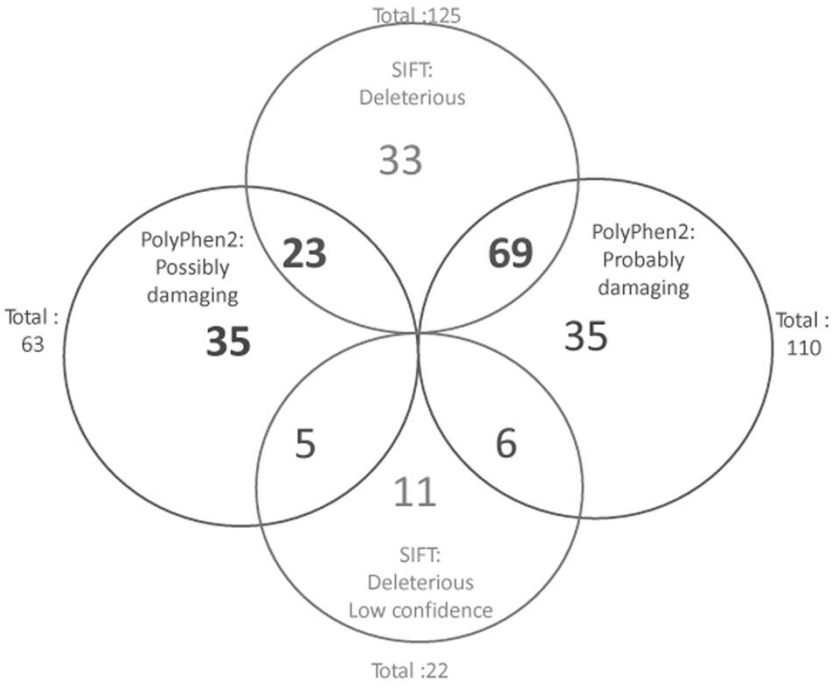
SIFT and PolyPhen2 analysis of identified genetic variants

SIFT[120, 121] analysis was conducted on all the genetic variants identified in the 48 subjects. Of the 18,661 identified genetic variants, 125 were determined to be deleterious, 22 were deleterious-low confidence while the remaining variants were tolerated.

PolyPhen2[122-124] analysis was also performed on genetic variants. Of the 18,661 genetic variants, 110 were determined to be probably damaging, 63 of the variants were determined to be possibly damaging and the remaining variants were benign.

Figure 2.4 shows a Venn diagram of the SIFT and PolyPhen2 results of variants predicted to impact protein function. We discovered 69 genetic variants classified as both deleterious by SIFT and probably damaging by PolyPhen2. These 69 genetic variants (**Supplemental Table 1[74]**) have the highest likelihood of affecting protein function and thus may also affect TAC disposition.

Figure 2.4: SIFT and PolyPhen2 Results of all 18,661 variants in a Venn diagram
SIFT and PolyPhen2 are bioinformatics analytic tools that predict the affect specific genetic variants may have on protein function. Of the 18,661 variants, 125 were deleterious and 22 were deleterious with low confidence by SIFT while the remaining variants were tolerated. Polyphen2 analysis found 110 of the variants were probably damaging, 63 were possibly damaging while the remaining variants were benign to impacting protein.



SKAT Gene Based Test Identified CYP5R2 Association with TAC Disposition in African American Cohort

By using SKAT[126-129] to test the gene-level association with the continuous trait of dose-normalized TAC troughs, the most significant gene associated with TAC troughs in AA subjects was *CYB5R2* by SKAT after Bonferroni correction ($p=7.9x10^{-4}$). *CYB5R2* was also significant by SKAT by case-control test ($p=8.5x10^{-4}$). None of the genes were significantly associated with the EA cohort. Of the 525 variants identified in *CYB5R2* (including upstream and downstream), 4 of these variants were found within the *CYB5R2* gene that were predicted to functionally impact protein function according to SIFT or PolyPhen2 (**Table 2.6**). The genetic variant identified in *CYB5R2* most likely to disrupt protein function was rs61733057 (Leu163Trp) because it is predicted as deleterious by SIFT and probably damaging by PolyPhen2. As seen in **Table 2.6**, the missense A to C variant rs61733057, in *CYB5R2*, has a global allele frequency of 0.05, but has increased allele frequency in both Africans (0.106) and AAs (0.119) compared with EAs (0.048). Likewise, rs61733056 is more frequent in AAs. CYB5R2 is a possible co-enzyme that may supply reducing equivalents to P450, although it is generally thought that CYB5R3 functions by supplying the second electron into the P450 cycle.

Table 2.6: Genetic Variants in the *CYB5R2* Gene Associated with Dose Normalized Tacrolimus Troughs in African American Kidney Transplant Recipients. The table indicates the location of the variants in the *CYB5R2* gene, consequences, the codon changes, rs numbers and predicted protein effect from SIFT and PolyPhen2 analysis (with prediction scores), chromosome location of the variants based on GRCH37 assembly, the variant allele used to calculate the consequence, the consequence effect of the variant on the Ensembl transcripts, the Exon number out of the total number, Existing known variant rs numbers. Also shown are the allele frequencies from 1000 Genomes project as given by VEP software. AF = global, AFR = African population, AMR = American population, EUR = European population, EAS = East Asian population, SAS = South Asian population, AA = Allele Frequency from in African American population from Lung and Blood Institute-Exome Sequencing Project (NHLBI-ESP), EA = Allele Frequency in European American population from NHLBI-ESP.

							Allele Frequencies							
Location	**Allele**	**Consequence**	**Exon**	**Existing_variation**	**SIFT**	**PolyPhen2**	**AF**	**AFR**	**AMR**	**EAS**	**EUR**	**SAS**	**AA**	**EA**
11:76871466-76871146	A	missense_variant	9/9	rs67173996	deleterious(0.03)	Benign(0.019)	0.161	0.035	0.052	0.504	0.128	0.089	-	-
11:76877155-76877155	C	missense_variant	8/9	rs12801394	deleterious(0.03)	Benign(0)	-	0.853	0.679	0.817	0.763	0.766	0.852	0.771
11:76890229-76890229	C	missense_variant	7/9	rs61733057	deleterious(0)	probably_damaging(0.947)	0.050	0.106	0.043	0.002	0.060	0.016	0.119	0.048
11:76908733-76908733	T	missense_variant	4/9	rs61733056	Tolerated(0.08)	possibly_damaging(0.875)	0.075	0.213	0.035	0.014	0.004	0.053	0.192	0.003

Focused SNP-set Analysis for Association in the EA and AA Cohorts

Genetic variants were further analyzed to detect association with dose-normalized TAC troughs within the AA or EA cohorts. The SNPs analyzed by SIFT and Polyphen2, with predicted impact on protein function, were grouped into 4 categories: **1.)** Polyphen2: probably damaging (N=110) (**Supplemental Table 2**[74]) **2.)** PolyPhen2: possibly damaging (N=63) (**Supplemental Table 3**[74]) **3.)** SIFT: deleterious (N=125) (**Supplemental Table 4**[74]) **4.)** SIFT: deleterious–low confidence (N=22) (**Supplemental Table 5**[74]). These 4 categories of variants were tested by burden test (BT), SKAT, and SKATO for association with the EA and AA cohorts, separately. The group of 125 predicted deleterious variants (**Supplemental Table 4**[74]) from SIFT had significant association with the EA cohort (BT, p=0.008) by case-control test.

Variants Observed During Manual Inspection of Variants

We examined SNPs in *CYP3A4*, *CYP3A5, POR* and *CYB5A genes*. We found several SNPs in the 5'-UTR of *CYP3A4* and *CYP3A5* that could affect protein expression. Surprisingly, we identified synonymous and non-synonymous SNPs with no reported rs numbers in dbSNP database in *POR*. In contrast, only a single previously unreported non-synonymous variant, His44Asn in exon 2 was identified in *CYB5A*, along with 16 previously unreported SNPs in the first 2,300bp upstream in the 5'-UTR. These variants are shown in **Supplemental Table 6**[74] (*CYP3A4*)**, Supplemental Table 7**[74] (*CYP3A5*)**, Supplemental Table 8**[74] *(POR*) and **Supplemental Table 9**[74] (*CYB5A*).

Discussion

This study showed that an EPS model and NGS identified 18,661 genetic variants associated with TAC disposition in 48 extreme phenotype subjects. VEP analysis determined 3,961 variants (21.2%) were unknown and 14,700 (78.8%) were previously known. We found 125 genetic variants that were predicted as deleterious of protein function by SIFT analysis and were significantly associated with TAC disposition in the EA group (BT, p=0.008). We further found 110 genetic variants that were probably damaging to protein function by PolyPhen2. Of these variants, 69 were also deleterious according to SIFT analysis and would represent the genetic variants most likely to affect protein function, and thus TAC disposition. For our studies, individual variant analysis lacks power due to small sample size with very limited number of

genotype counts. Though some individual variants in a gene have weak signals, combining them can lead to a significant result as done in SKAT. Thus, a major finding was the significant association of *CYB5R2* with TAC troughs in AAs by SKAT analysis. The genetic variant, rs61733057, in the *CYB5R2* gene, was identified and predicted to be deleterious by SIFT and probably damaging by PolyPhen2. Thus, we have identified variants associated with TAC troughs in kidney transplant recipients that require future *in vitro* assessment or validation in another cohort.

At the time of this study, it was not feasible to determine low-frequency variants, by sequencing all subjects because that required NGS of thousands of subjects. Therefore, we used an EPS approach that was successful to identify low-frequency variants in other diseases[130,131,132,50,133,134]. Previous research suggests sampling from both high and low extremes is important to identify variants associated with a particular phenotype[134]. This EPS approach allows for smaller sample sizes to identify low-frequency genetic variants associated with a phenotype[51]. Thus, our study corroborates other studies showing that the EPS approach can identify genetic variants, or genes, which are associated with a phenotype. This EPS approach can save time and money by sampling fewer subjects.

Genetic Variants associated with TAC metabolism were identified and shown in **Table 2.4** (AA) and **Table 2.5** (EA). **Table 2.4** shows single genetic variants associated with TAC metabolism in AAs. Many of these variants were in *POR* which encodes for a coenzyme involved in cytochrome P450 metabolism. The variants in *OVCH2* are upstream, and likely in the promotor, of *CYB5R2*. A single variant found in *ADIPOR1*, was likely identified since it is downstream of the gene *CYB5R1*. Additionally, **Table 2.5** has genetic variants associated with TAC metabolism in EAs. The variants identified were in the genes *OTUD4*, *NR3C1*, *ABCC1*, upstream of *HNF4A* and *ANAPC10*. The variant in *ANAPC10* was also located in the 5'-UTR of *ABCE1* gene. The *OTUD4* variants are located in the 3'-UTR of *ABCE1*. *ABCE1* is an ATP-binding cassette protein but lacks the transmembrane domain needed for transporter function. *ABCE1* functions as a ribonuclease L inhibitor where it associates with the ribosome and initiation factors eIF3 and eIF5. We speculate that this would lead to less mRNA transcription, and reduce protein expression but that may be non-specific for *CYP3A4* and *CYP3A5*. The variant found in the glucocorticoid receptor *NR3C1*, a transcription factor that can influence the expression of PXR, which in turn regulates *CYP3A4* and *CYP3A5*. The other variant

found in AAs was in the *ABCC1* gene which may be involved in TAC transport. In general, functional assays will be needed to validate the association of these variants with TAC metabolism.

Table 2.6 shows 4 variants found in *CYB5R2* and indicates *CYB5R2* to be associated with TAC metabolism in AAs. *CYB5R2* has not previously been associated with TAC troughs, disposition or metabolism, but was unexpectedly identified in AAs with extreme TAC troughs. *CYB5R2,* (in chromosome 11), differs from its homolog *CYB5R3* (in chromosome 22), but share high sequence identity and there is limited literature regarding *CYB5R2*. Both CYB5R2 and CYB5R3 can reduce cytochrome b5 and act as co-factors for cytochrome P450 function (supply electrons into the P450 cycle). While CYB5R2 is located in the nucleus, CYB5R3 is present in the endoplasmic reticulum in liver. CYB5R3 exists in two forms as a membrane-bound variant in membranes including in erythrocytes where low activity variants have been associated with methemoglobinemia and a truncated soluble cytoplasmic form containing the FAD catalytic domain. *CYB5R2* has been identified as a tumor suppressor that is epigenetically regulated[135]. *CYB5R2* negatively regulates vascular endothelial growth factor[136] which could contribute to its tumor suppressor activity. Furthermore, *CYB5R2* is epigenetically regulated through promoter methylation[137], associated with patient survival of glioblastoma, and functions in collagen maturation, immunoregulation via toll-like receptor pathways, and osmotic stress[138]. The *CYB5R2* variant, rs61733057, that likely impacts *CYB5R2* protein function, was identified. It has elevated frequency in the AAs compared with EAs. According to 1000 genomes database (**Table 2.6**), the identified variants associated with TAC troughs in AAs rs61733057 and rs61733056 are primarily in people of African descent, which would corroborate our finding of this variant in AAs. However, with limited *CYB5R2* literature it is difficult to determine its function in TAC disposition.

This study identified genetic variants in *CYP3A4*, *CYP3A5*, *POR* and *CYB5A*. Although many of these variants (**Supplemental Tables 6-9[74]**) did not show significant association with TAC troughs in our analysis, numerous naturally occurring genetic variants were identified that have not been reported. Many of these variants were in the 5' and 3'-UTR regions of *CYP3A4* and *CYP3A5*. We identified 1 exonic SNP in *CYP3A4* with gene position 1022 A>G which would lead to amino acid substitution Lys341Arg. We identified multiple non-synonymous SNPs in *POR* without rs numbers. More than

160 *POR* variants have been described to be associated with altered steroid metabolism and Antley-Bixler syndrome and disordered steroidogenesis. Five of the novel *POR* variants appeared only in single individuals with high TAC troughs, namely Arg186Val, Asp473Tyr, Gly589Val and Ala661Ser in the EA high group and Arg453Ser in the AA high group. If these SNPs result in lowered transfer of electrons into the P450 cycle, one would expect reduced clearance via CYP3A4 and CYP3A5.

We recently developed an *in vitro* method to validate the association of genetic variants with drug metabolism[139]. Variants are genetically engineered into cell lines, using CRISPR/Cas9, and then the cells are assayed to determine the effect of the specific variant on drug metabolism. This method was successfully used to validate the effect of *CYP3A5 *1* vs. *CYP3A5 *3* (rs776746) alleles on TAC metabolism[139]. This method can be used to engineer variants, identified in this study, into a hepatocyte cell lines to study TAC metabolism.

This study had limitations. Although we sequenced numerous genes expected to be associated with TAC troughs, whole genome sequencing would have been more complete. However, there were considerable cost differences between whole genome sequencing and targeted NGS. A *FOXP3* genetic variant, rs3761548, was reported to be associated with TAC troughs[140] and we did not sequence *FOXP3*. Another limitation of this study is that SIFT and PolyPhen2 are not completely accurate prediction algorithms. One study found, for missense variants in G protein couple receptor genes, that SIFT and PolyPhen2 were 83% and 85% accurate, respectively; while the LoF prediction was over 90% accurate for both, predicting non-functional variants was 54 or 57% accurate, respectively[141]. One study investigated the accuracy of SIFT and PolyPhen2 for predicting missense mutations in *BRCA1*, *MSH2*, *MLH1* and *TP53* genes that resulted in area under the curve of receiver operating characteristic curves for both algorithms to be between 78 and 79%[142]. Another study has shown that SIFT, PolyPhen2 and other predictive *in silico* tools' accuracy is gene dependent and also best when used in combination[143]. Thus, we focused on the identified variants in this study that were identified to disrupt protein function by both SIFT and PolyPhen2. A further limitation that we did not consider was TAC adherence because adherence data was not collected. Due to the limited number of subjects in each group (N=48, 4 groups of 12), additional statistical power would be gained by sequencing more subjects. Although, there are

limitations to this study, this model was effective at identifying genetic variants associated with TAC metabolism in kidney transplant recipients.

We envision expanding this study with more subjects to identify more genetic variants. We foresee these genetic variants being translated into refined TAC dosing equations[9, 77]. Refined dosing equations could be used to reduce variability in TAC troughs while reaching optimal therapeutic TAC troughs quickly post-transplant to reduce poor outcomes.

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Chapter 3

CRISPR/Cas9 genetic modification of *CYP3A5 *3* in human hepatocytes leads to cell lines with increased midazolam and tacrolimus metabolism

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Abstract:

CRISPR/Cas9 engineering of the *CYP3A5 *3* locus (rs776746) in human liver cell line HuH-7 (*CYP3A5 *3/*3*) led to three *CYP3A5 *1* cell lines by deletion of the exon 3B splice junction or point mutation. Cell lines *CYP3A5 *1/*3 sd* (single deletion), *CYP3A5 *1/*1* dd (double deletion) or *CYP3A5 *1/*3* pm (point mutation) expressed the *CYP3A5 *1* mRNA, had elevated *CYP3A5* mRNA ($p<0.0005$ for all engineered cell lines) and protein expression compared with HuH-7. In metabolism assays, HuH-7 had less TAC (all p-values < 0.05) or MDZ (all p-values < 0.005) disappearance than all engineered cell lines. HuH-7 had less 1-hydroxyl MDZ (1-OH MDZ, all p-values < 0.0005) or 4-hydroxyl MDZ (4-OH, all p-values < 0.005) production in metabolism assays than all bioengineered cell lines. We confirmed CYP3A5 metabolic activity with the CYP3A4 selective inhibitor CYP3CIDE. This is the first report of genomic *CYP3A5* bioengineering in human cell lines with drug metabolism analysis.

Introduction:

About 75% of the oral drugs in the United States are enzymatically metabolized by the cytochrome P450 (CYP) family of enzymes [145]. The CYPs, and other drug metabolizing enzymes, are polymorphic resulting in large variability in metabolic clearance of drugs. *In vitro* systems to study drug metabolism and genetic variation include: cloned and expressed enzymes, human and animal microsomes from individual or pooled donors, freshly isolated and cultured or cryopreserved hepatocytes. However, primary hepatocytes are not an optimal option because they require harvesting liver, are expensive, are not immortalized and are highly variable from specimen to specimen. To study genetic variants' association with metabolism, a genotyped bank of liver microsomes [146], from individual donors, can be examined but cannot sustainably be

engineered to study newly identified genetic variants such as rare variants or those found in minority populations. Also, microsomes are difficult to use to study combinations of genetic variants, especially rare variants or those found in minority populations. Liver microsomes are often from Caucasians limiting their use to understand metabolism in minority populations. Furthermore, since microsomes come from various individuals they are genomically heterogeneous, and from uncontrolled environments, while cell line models are, for the most part, genomically identical except for any specifically altered genetic variant. Thus, we developed genetically modified human liver cell lines that are a sustainable option to investigate the impact of genetic variants on drug metabolism.

Recent reports showed, in rats, that knockout of *CYP2E1* [147] or *CYP3A1/2* [148] using CRISPR/Cas9 could be used in drug metabolism studies. However, using CRISPR/Cas9 to modify human cell lines to study association of genetic variants with drug metabolism has not been reported. We hypothesized that human liver cell lines can be engineered with CRISPR/Cas9 [38, 149] to evaluate the effect of genetic variants on drug metabolism. Single genetic variants can be engineered into cell lines that result in altered enzyme activity, gene regulation or protein expression for drug transport or metabolism studies. This paper provides evidence of this concept to study genetic variants in *CYP3A5* and their effect on metabolism of two CYP3A4 and CYP3A5 enzymatic substrates: MDZ a sedative or anesthetic, and TAC, an immune suppressant. Among the CYP enzymes, CYP3A4 and CYP3A5 are the most abundant in the liver and their expression is highly variable. The *CYP3A5 *3* (rs776746) loss of function allele is highly prevalent in people of Caucasian descent [150] (allele frequency = 0.94) and leads to low metabolism rates of Tac [151] compared with individuals with *CYP3A5 *1* genotype. However, the *CYP3A5 *1* (expresser) allele is enriched in AAs [152] and leads to rapid metabolism of MDZ, Tac and other drugs. Approximately, 50% of oral drugs are

metabolized by CYP3A4 and CYP3A5 [22, 153]. Consequently, the *CYP3A5* genotype is an important factor in determining appropriate doses of drugs. People of African ancestry are often under-dosed initially with Tac following organ transplantation [21], in part, due to the high prevalence of the *CYP3A5*1* allele in the AA population (allele frequency 0.85). Carriers of the *CYP3A5 *1* allele, often need higher doses of drugs, that are CYP3A5 substrates, to achieve therapeutic drug levels in blood. Therefore, there is a need to develop an *in vitro*, cell culture based, system to understand the effects of genetic variants on drug metabolism prior to clinical use of new drugs or to improve dosing of existing drugs.

The first step in development of a suitable liver cell line was to find a clinically relevant parental cell line. To date, there is not a commercially available liver cell line that is diploid at chromosome 7 and expresses *CYP3A5* *1. The Caco-2 cell line [154] is a human intestinal cell line that metabolizes drugs, but it has five copies of chromosome 7[155155155] and thus is not suitable for studying the diploid *CYP3A5* seen in most patients. The HuH-7 cell line [156, 157] was derived from a hepatic carcinoma that can convert the substrate MDZ, primarily through CYP3A4 activity, in cell culture to its metabolite products hydroxylated 1-OH MDZ and 4-OH MDZ [39, 40, 158]. However, HuH-7 cells are not very efficient at MDZ metabolism because they are homozygous for the slow metabolizing *CYP3A5 *3* allele. Thus, there is a need to develop a liver cell line that mimics the rapid drug metabolism associated with the *CYP3A5 *1* genotype in cell culture.

We hypothesized that by genetically modifying the HuH-7 cell line to the more metabolically active *CYP3A5 *1/*1* or *1/*3* genotypes, the cells would have increased MDZ and Tac metabolic activity. To test the hypothesis, we used CRISPR/Cas9 bioengineering [38, 149] to develop and characterize new cell lines then phenotypically

evaluate the genotypes' effects on MDZ and TAC metabolism. These newly engineered cells can be used as a parental cell line in future studies to assess association of additional genetic variants with drug metabolism. This is the first report of genomic *CYP3A5* bioengineering in human cell lines and functional analysis of associated drug metabolism phenotypes.

Materials and Methods:

Selection of HuH-7 hepatocyte cell line as Parental Cell line

We selected HuH-7, liver carcinoma cells from the Japanese Cell Research Bank (cat. number: JCRB0403) because: **A.**) HuH-7 cells metabolized MDZ in cell culture [40, 158]. **B.**) HuH-7 cells were diploid, at chromosome 7 where both *CYP3A4* and *CYP3A5* are located; thus, clinically relevant. **C.**) We sequenced the cells at the *CYP3A5 *3*, *6*, and *7* loci and HuH-7 cells carried *3/*3 alleles at the rs776746 locus, a defined variant to change via CRISPR/Cas9 bioengineering to the more functional *1/*1* or *1/*3* alleles. **D.**) These HuH-7 cells were also *1/*1* at the loss of function *CYP3A4 *22* (rs35599367) allele. **E.**) We confirmed *CYP3A4* and *CYP3A5* mRNA expression by qRT-PCR.

Parental Cell line and Characterization

HuH-7[156, 157], hepatoma cells from a 57 year old Japanese male, were purchased from the Japanese Cell Research Bank and used as parental cell line for genetic modification. Cells were grown in Dulbeco's Modified Eagle Media (DMEM) with high glucose and pyruvate supplemented with 10% Fetal Bovine Serum and Antibiotic-Antimycotic (Gibco). We refer to this as "media" throughout rest of manuscript.

Genotyping of Cell lines

Genomic DNA was isolated from HuH-7 cells using the Roche High-Pure PCR template preparation kit. PCR and sequencing primers (**Supplementary Table 1**), surrounding SNPs *CYP3A4 *22* (rs35599367, C>T), *CYP3A5 *3* (rs776746, 6986A>G), **6* (rs10264272, 14690G>A) and **7* (rs41303343, nonfunctional) were designed using the NCBI primer-BLAST primer design tool. The sequences surrounding the SNPs in the genomic DNA from the HuH-7 cells were PCR amplified using AccuPrime™ Pfx DNA Polymerase kit and then the PCR products were characterized on 1% agarose gel or purified with the Qiagen PCR clean up kit and sequenced. PCR products were then Sanger sequenced using the primers listed in **Table 3.1** by UMGC.

Table 3.1: Primers used in this study for PCR, RT-PCR, and Sequencing.

Gene/Locus	Primer	forward Primer Sequence 5'->3'	Purpose
*CYP3A4 *22*	**22F*	AATTCTGCTGTCAGGGCAAC	PCR Amplification and Sequencing
	**22R*	TTGAGAGAAAGAATGGATCCAAAAA	PCR Amplification and Sequencing
	**22-1F*	GGCATAGAGTCTGCAGTCAGG	Sequencing
	**22-1R*	TCACCTTCTATCACACTCCATCA	Sequencing
	**22-2F*	TCAGTGTCTCCATCACACCC	Sequencing
	**22-2R*	GGATTGTTGAGAGAGTCGATGTT	Sequencing
*CYP3A5 *3*	*8F*	CTGTCAGAGGGGCTAGAGGT	PCR Amplification and Sequencing
	8R	CCTCCCAGGTTCAAGCGATT	PCR Amplification and Sequencing
	7853 F	GCATTTAGTCCTTGTGAGCACTTG	PCR Amplificatio

			n and Sequencing
	8303 R	CATACGTTCTGTGTGGGGACAAC	PCR Amplification and Sequencing
	7884F	ACCTGCCTTCAATTTTTCACTG	Sequencing
	8267R	CTTCACTAGCCCGATTCTGC	Sequencing
	3A5 ex2F	GTCACAATCCCTGTGACCTGAT	PCR Amplification and Sequencing
	3A5 ex5R	TTGGAGACAGCAATGACCGT	PCR Amplification and Sequencing
	3A5 ex2F Seq	CTGTTTCACTTTGTAGATATGGGAC	Sequencing
	3A5 ex5R Seq	AATCCCACTGGGCCTAAAGAC	Sequencing
*CYP3A5*6*	*4F*	TCTGCCCATCTGTCACCAAT	PCR Amplification and Sequencing
	4R	TTGGCCACATGTCCAGTACT	PCR Amplification and Sequencing
	15488F	GGCACCAGATAACCACCTTC	Sequencing
	15989R	GGGCTCTAGATTGACAAAAACA	Sequencing
*CYP3A5*7*	*12F*	TCCTCCACACATCTCAGTAGGT	PCR Amplification and Sequencing
	12R	TAAGGCCTGACCTTGTCCCT	PCR Amplification and Sequencing
	28064F	ACTTCACGAATACTATGATCATTTACC	Sequencing
	28351R	CATTGACCCTTTGGGAATGA	Sequencing
	28448R	CATTGACCCTTTGGGAATGA	Sequencing
GAPDH	*GAPDHF*	GCATCCTGCACCACCA	qRT-PCR
	GAPDHR	GGATGACCTTGCCCACA	qRT-PCR

Plasmids, Guide RNA construction and Transfection

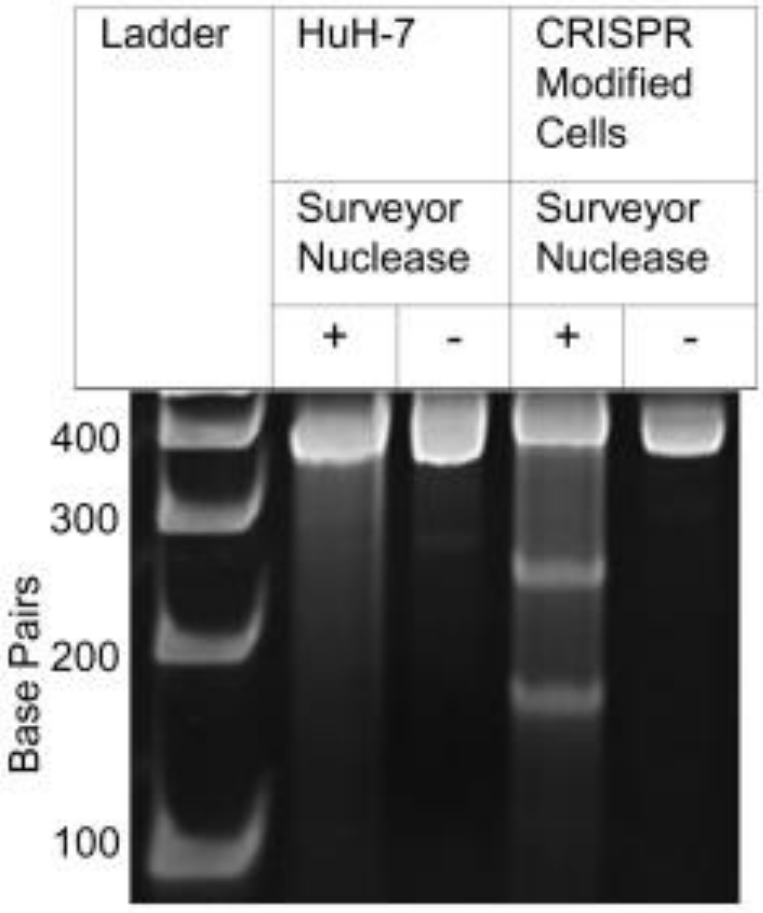
A plasmid that expressed a human codon-optimized Cas9 [149, 159, 160] nuclease was purchased from Addgene. Guide RNAs (gRNAs) targeting the *CYP3A5*3* locus were designed using the CRISPR design tool at http://crispr.mit.edu/. DNA gBLOCKS were designed, synthesized, and purchased from Integrated DNA Technologies combining the gRNA from the CRISPR design tool with the gRNA synthesis protocol[159] from Addgene. The gBLOCKs were TOPO cloned using the Zero Blunt® TOPO® PCR Cloning Kit into pCR™Blunt II-TOPO® vector. Plasmids were expanded in One Shot® *Stbl3*™ Chemically Competent *E. coli* bacteria purchased from Thermo Fisher. Plasmids were sequence verified. Plasmids were then prepared for transfection following the Qiagen Plasmid Maxi Kit. Plasmids were quantified and assessed for purity using a NanoDrop 2000 UV/Vis spectrophotometer. Newly designed gRNAs and hCas9 plasmid DNA were transfected into the HuH-7 cells using a Neon® Transfection System.

Surveyor Assay to select guide RNAs

Genomic DNA was isolated from transfected cells and then we performed Surveyor assay to screen gRNAs for ability to cut at *CYP3A5* *3 locus using a modified protocol[161] along with Surveyor enzyme from Surveyor® Mutation Detection Kit for Standard Gel Electrophoresis from Integrated DNA Technologies. Briefly, DNA was extracted from bulk transfected cells using Roche High-Pure PCR template preparation kit. PCR was performed using AccuPrime™ Taq DNA Polymerase, high fidelity with *CYP3A5* specific primers Cel1F*3 (5'-CAACTGCCCTTGCAGCATTT-3') and Cel1R*3 (5'- ACCCAGGAAGCCAGACTTTG-3') to produce a 397 bp product (if no deletions). Bio-Rad thermocycler was programed as: **1.** 94 °C for 5 minutes, **2.** 94 °C for 15 seconds, **3.** 56°C for 30 seconds, **4.** 68 °C for 0.5 minutes, **5.** Go to step 2, 34 times, **6.**

68 °C for 0.5 minutes, **7.** 4 °C indefinitely. PCR products were denatured and re-annealed following a published protocol[161] and visualized in a 10% Bio-Rad Criterion™ TBE Polyacrylamide Gel to determine DNA heteroduplexes from heterogeneous cell cultures caused by CRISPR/Cas9 and gRNA targeting (**Figure 3.1**).

Figure 3.1: Surveyor Assay used to select guide RNA. Surveyor nuclease assay indicated HuH-7 parental cells transfected with hCas9, guide RNA 2, and HDR template ssODN successfully mutated the *CYP3A5 *3* locus. Parental and CRISPR modified genomic DNA was used for PCR and surveyor nuclease detection of genome modification at **3* locus. Parental cell PCR products treated with surveyor nuclease had a 397 bp band, while the CRISPR modified cells' PCR products had bands of 397, 236 and 161 bp indicating genetic modification at the **3* locus in bulk transfected cells. These cells were then single-cell cloned to isolate cell line *CYP3A5 *1/*3* pm.



Transfection with selected gRNA and hCAS9

To create cell lines that delete the *CYP3A5*3* splice junction, via non-homologous end joining (NHEJ), two selected gRNAs (gRNA1 and gRNA2) and hCas9 plasmids were transfected into the HuH-7 cells using Neon® Transfection System. The two gRNAs target each side of the *CYP3A5**3 locus. The resultant cells were then single cell cloned to produce homogenous cell lines.

To create the single-nucleotide polymorphism (SNP) cell line, gRNA2 was transfected into cells, with homology directed repair (HDR) template. HDR single-stranded DNA template ssODN 3A5*3_E+. The sequence of ssODN 3A5*3_E+ is 5'-gcttaacgaatgctctactgtcatttctaaccataatctctttaaagagctcttttgtctttcaaTATCTCTTCCGTGTTTGGACCACATTACCCTTCATCATATGAAGCCTTGGGTGGCTCC-3'. The underlined a indicates the *3 base that is changed in from a guanine (g). The lower-case letters are the intron sequence, while the upper-case letters are the exon 3B sequence. The cells were treated with 1 µM SCR7 (Xcessbio) and 5 µM L755,507 (Xcessbio) at the time of transfection and during the 7 days following transfection until single-cell cloning.

Single cell cloning and cell line screening via PCR and DNA Sanger Sequencing

Transfected cells were plated in media/soft agar mixture as previously described [72, 162], and propagated to become homogenous cell lines. Specifically, in 150 mm^3 15 ml of a 0.6% solution of UltraPure™ Low Melting Point Agarose (Thermo Fisher Scientific, cat. number: 16520-100) in media was plated at 38.5°C and cooled until solid. Next, 15,000 transfected cells in 15 mL of 38.5°C media with 0.3% UltraPure™ Low Melting Point Agarose was layered on top and cooled. The plate was covered with 10 mL media and incubated at 37°C with 5% CO_2 for about 3-5 weeks until cell colonies were visible. Colonies were then picked with a sterile 200 µL pipette tip and transferred to individual

wells of a 96-well Collagen I coated plate and cultured until confluent (approximately 3-4 weeks).

For large scale screening, we dissociated the cells using Trypsin-EDTA (0.25%) and transferred half the culture to fresh 96-well plates with media and grown. The remaining cells in the 96-well plate were centrifuged at 350 x g for 5 minutes. Trypsin was removed and the cell pellets were lysed using the QuickExtract™ DNA Extraction Solution from Epicentre. Lysates were used as PCR template and then PCR amplified with an AccuPrime™ Pfx DNA Polymerase kit in 96-well PCR plates. The primers for amplification of the *CYP3A5*3* region were designed using NCBI Primer design (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are: 7853F (5'-GCATTTAGTCCTTGTGAGCACTTG-3') and 8303R (5'-CATACGTTCTGTGTGGGGACAAC-3'). Thermocycler was programed as follows: **1.** 94 °C for 5 minutes **2.** 94 °C for 15 seconds **3.** 55 °C for 30 seconds **4.** 68 °C for 30 seconds **5.** Go to step 2, 34 times **6.** 68 °C for 7 minutes **7.** 4 °C indefinitely. PCR products were purified using the MinElute 96 UF PCR Purification Kit (Qiagen), characterized by electrophoresis through a 2% agarose gel and by sequencing of the PCR products. Sequencing was performed by the UMGC using sequencing primer 7884F (5'- ACCTGCCTTCAATTTTTCACTG-3') and 8267R (5'-CTTCACTAGCCCGATTCTGC-3'). Sequence data were analyzed using DNA Star Lasergene and Geneious software.

RNA Splicing Assay

RNA was isolated from the confluent cells using the Qiagen RNeasy Mini Kit. RNA was quantified using the Qubit® 2.0 Fluorometer and the Qubit® RNA BR Assay Kit. RNA was then converted to cDNA using oligo-dT primer and ThermoScript™

Reverse Transcriptase kit. *CYP3A5* *1/*3 or *1/*1 genotyped human liver RNA was also used as controls and cDNA made by same method. PCR primers were developed using the NCBI primer design tool and mRNA sequence file of *CYP3A5* (Genbank accession # BC025176.1) of the 500 base pairs surrounding the *3 nucleotide locus. The cDNA was then used as PCR template with primers: *CYP3A5* cDNA ex2F (5'-GTCACAATCCCTGTGACCTGAT-3') and *CYP3A5* cDNA ex5R (5'-TTGGAGACAGCAATGACCGT-3'). Thermocycler settings were as follows: **1.** 94°C for 5 minutes **2.** 94°C for 15 seconds **3.** 50.5°C for 30 seconds **4.** 68°C for 30 seconds **5.** Go to step 2, 34 times **6.** 68°C for 5 minutes **7.** 4°C forever using the AccuPrime™ Pfx DNA Polymerase kit. PCR products were purified using the QIAquick PCR Purification Kit then characterized by electrophoresis through a 2% agarose gel and imaged using ethidium bromide staining and a Bio-Rad ChemiDoc™ Touch Imaging System.

Quantitative RT-PCR to detect CYP3A5 transcripts

CYP3A5 mRNA was quantified using cells from the MDZ and Tac assays. The primers were the same as for the mRNA splicing assay (**Table 3.1**). The RNA was the same as the RNA splicing assays and GAPDH primers were used as the reference control. 2 µg of RNA was converted to cDNA and 5 µl of 1 in 20 diluted cDNA was used in a 20µl reaction mix for SYBR Green assay based quantitative RT-PCR. We used a Roche lightcycler and made graphs using Graphpad Prism software.

Immunoblot Analysis for CYP3A5 *1 and *3 variants in engineered cell lines

CYP3A4 and CYP3A5 protein expression was determined by immunoblot analysis. Total lysates were recovered from HuH-7 cells and the new derivative cell lines. For microsome preparation, cells were centrifuged at 1500 rpm for 5 minutes and

washed immediately in 1x PBS. This was followed by homogenization using a glass-Teflon homogenizer and a microsome storage buffer (MSB) containing 100 mM potassium phosphate, pH 7.4, 1.0 mM EDTA 20% glycerol with protease inhibitor cocktail. Following differential centrifugation (12,000 × g for 30 minutes; 34,000 × g for 120 minutes), the pellet was resuspended in MSB.

Protein was estimated by using the Bio-Rad protein assay for microsomes and Pierce™ BCA Protein Assay Kit assay for lysates with bovine serum albumin as the standard. 60 µg and 40 µg of total lysate and microsomes, respectively, were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with 1:10000 dilution of monoclonal anti-CYP3A4 K03 [163], or 1:250 dilution of WB-3A5 (Corning, Corning, NY), followed by 1:10000 dilution of secondary antibodies HRP-conjugated anti-mouse and anti-rabbit (Jackson ImmunoResearch Inc., West Grove, PA), respectively. β-Actin protein expression was determined by monoclonal anti-actin (Sigma, St. louis, MO), followed by 1:10000 dilution of secondary antibodies HRP-conjugated anti-mouse. The blot was developed with the ECL™ Western Blotting Reagents (GE Healthcare). Bands on film were optically scanned.

Midazolam and Tacrolimus Metabolism Assays

HuH-7 and engineered cells were grown to confluence in 12 well Corning™ BioCoat™ Collagen I Multi-well Plates for 3-4 weeks in media. Media was refreshed 2-3 times a week. Cells were then overlaid with Corning Matrigel® Matrix and then induced for 3 days by addition of 100 µM phenytoin Sodium (USP), diluted in methanol, and 10 µM rifampicin (Sigma), diluted in methanol, in cell culture. Media was changed daily with inducers rifampicin and phenytoin added. On the fourth day, 500 µl of media was added to the cells with 100 µM phenytoin and 10 µM rifampicin with either equal volumes of

methanol, as a negative control, or MDZ from Cerriliant diluted in methanol so that MDZ final concentration was 5 µM (1628 ng/mL) in cell culture media. Cells were incubated overnight, and media was collected and assayed for MDZ, 1-OH MDZ and 4-OH MDZ by high performance liquid chromatography mass spectrometry. To determine the metabolic function of the engineered cell lines on Tac (Toronto Research Chemicals Inc.) the same process except we used 6 well collagen coated plates and 1.5 mL with 13 ng/mL Tac reaction volume.

Detection methods for Tac, MDZ, 1-OH MDZ, and 4-OH MDZ

Detection and quantification of midazolam, 1-OH MDZ and 4-OH MDZ in cell culture media was performed using a high-performance liquid chromatograph (Agilent 1200 Series, Santa Clara CA) coupled with a TSQ Quantum triple stage quadrupole mass spectrometer (Thermo-Electron, San Jose, CA). Detection and quantification of tacrolimus was performed using chromatographic separation (Agilent 1100 - High Performance Liquid Chromatography Agilent Inc., Santa Clara, CA) and mass spectrometry (API 4000, Sciex Inc., Redwood City, CA). These detailed methods are in the supplemental files.

CYP3CIDE Experiments

CYP3CIDE [153, 164] (Sigma-Aldrich) was used as a selective CYP3A4 inhibitor in cell culture and diluted in DMSO. To determine the concentration of CYP3CIDE to use in cell culture we performed a dose response in Huh-7 and the *CYP3A5 *1/*1* dd cell line between 100 nM and 1 mM in cell culture using MDZ as the substrate. For further analysis, we used 50 µM CYP3CIDE in our experiments with all cell lines. Dose response curves were assessed using Graphpad Prism software.

Statistical Analysis

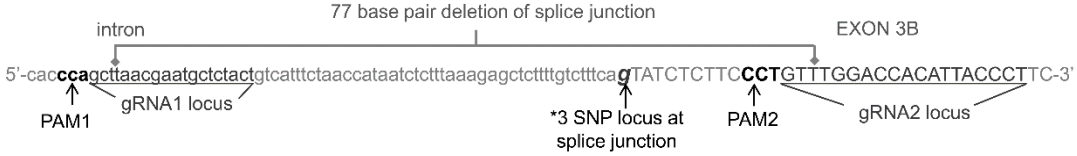
All comparisons were conducted using t-test for continuous variables.

Results:

Genotyping of single-cell clones following CRISPR/Cas9 bioengineering of CYP3A5

To create *CYP3A5 *1* cells from the HuH-7 cells that were *CYP3A*3* we altered *CYP3A5* based on CRISPR proto-spacer adjacent motifs (PAM) near the exon 3B splice junction (**Figure 3.2**). To identify the cell line that had the exon 3B 5' splice junction deleted, we screened single-cell clones by PCR amplifying, then forward and reverse Sanger sequencing of a 451 base pair (bp) region flanking the splice junction (**Figure 3.2**).

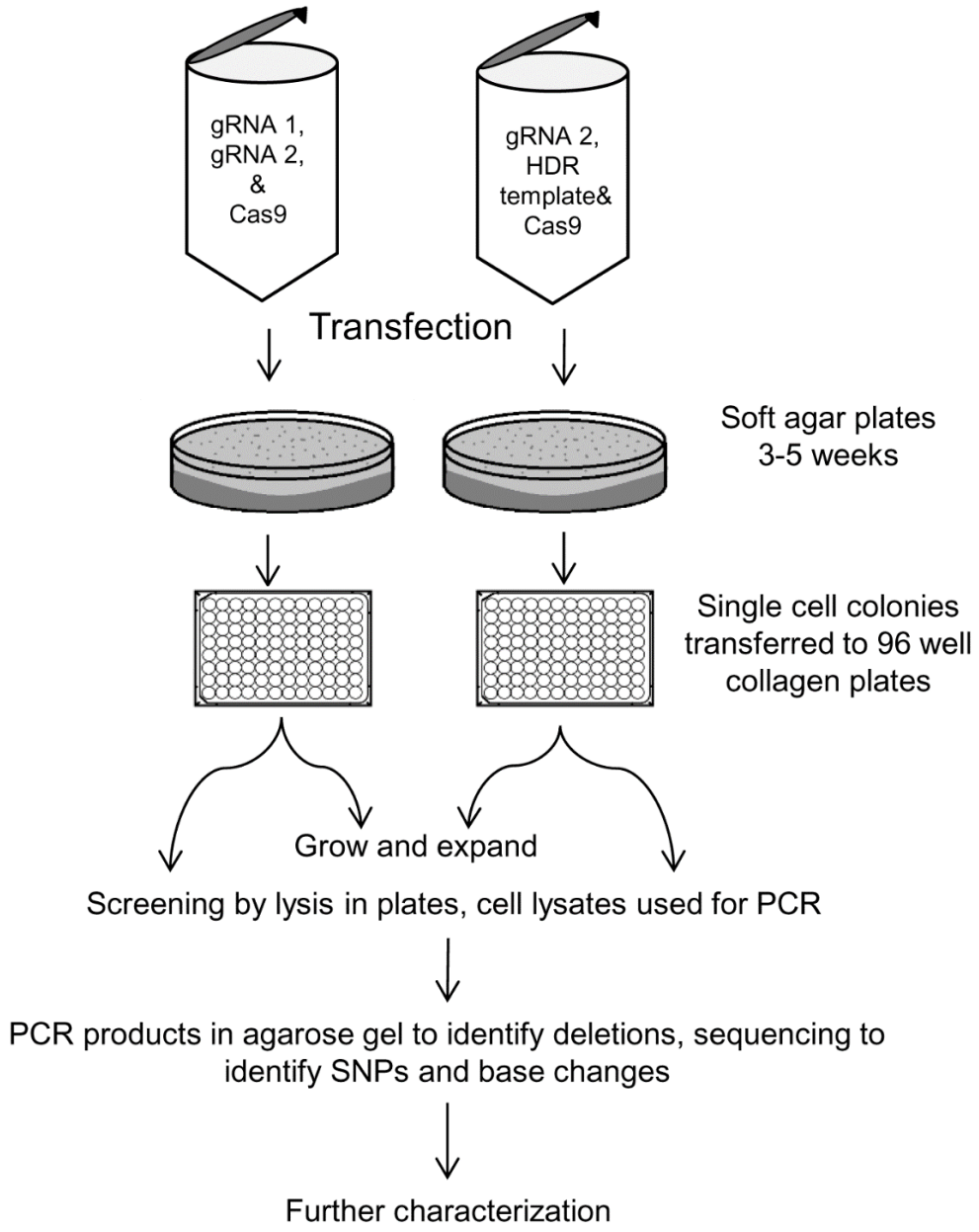
Figure 3.2: *CYP3A5 *3* locus (rs776746) and guide RNA targeting strategy. Guide RNAs (gRNA) were targeted to proto-space adjacent motifs (PAM) sequences on each side of the *CYP3A5 *3* SNP (gRNA1 or gRNA2 locus). Exon 3B sequence is in capital letters while the upstream intron sequence is in lower case letters. There are 77 base pairs between the gRNA guided Cas9 cut sites.



The splice junction contains the *3* locus (rs776746) and we deleted this junction to express *CYP3A5 *1* mRNA via alternative splicing. These cells were transfected with gRNA1, gRNA2, and hCas9 plasmids (**Figure 3.3**). We screened 235 single cell clones and 74 (32%) were mutated. Mutations in modified cell lines were as follows: 22 (9.4%) had a heterozygous frameshift near one of the two gRNA cut sites, 23 (9.8%) were

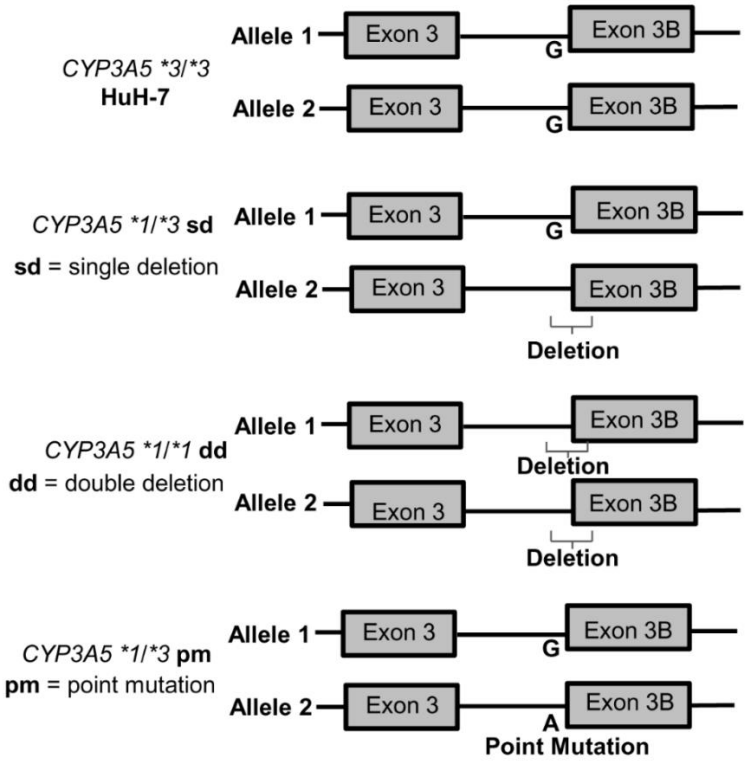
heterozygous for the 77 base pair deletion between gRNA sites, 15 (6.4%) cell lines were homozygous for the 77 base pair deletion, and 14 (6.0%) cell lines were classified as “other”. The “other” mutations in cell lines include cell lines that had multiple frameshifts, or were heterozygous for a deletion and frameshift or had other mutations. The heterozygous deletion cell line was designated *CYP3A5 *1/*3* sd (sd = single deletion) and the homozygous deletion cell line was designated *CYP3A5 *1/*1* dd (dd = double deletion) (**Figure 3.4**).

Figure 3.3: Workflow for development of *CYP3A5* genetically modified cell lines using CRISPR/Cas9 and clonal selection. The *CYP3A5 *3* splice junction was deleted with gRNA1, gRNA2 and Cas9 or the *CYP3A5 *3* SNP was point mutated using gRNA2 and a homology directed repair (HDR) template to convert the **3* guanine to a **1* adenine. Following transfection, the cells were single cell cloned by plating in soft agar. The single cell clones were transferred to collagen I coated plates until confluent. Cells were then expanded or screened by PCR of the *CYP3A5 *3* locus and sequencing of the PCR products.



To identify the cell line *CYP3A5 *1/*3* pm (pm = point mutation) (**Figure 3.4**) we screened 212 single cell clones using DNA sequencing that were transfected with hCAS9, gRNA2 and homology-directed repair (HDR) template (**Figure 3.3**). We found that 33 (16%) were mutated. Mutation in cell lines were as follows: 23 (11%) had a heterozygous frameshift near the gRNA2 cut site, 2 (0.9%) had homozygous frameshift, 1 (0. 5%) had heterozygous point mutation at *3 locus, 0 (0.0%) had homozygous point mutations at *3 locus, and 8 (4.0%) were classified as other. These other mutants include cell lines that had multiple frameshifts, were heterozygous for a deletion and frameshift or had other mutations.

Figure 3.4: *CYP3A5* maps of cell lines used in this study. The cell lines used in this study include HuH-7 (*CYP3A5 *3/*3*), *CYP3A5 *1/*3* sd which has a deletion of *3* splice junction at one allele, *CYP3A5 *1/*1* dd which has a deletion of *3* splice junction at two alleles, and the *CYP3A5 *1/*3* pm which has a guanine to adenine point mutation converting one allele from *3 to *1*.



PCR characterization of genomic DNA in new cell lines

Of the cell lines that were sequenced, select cell lines that were *CYP3A5 *1/*3* or *1/*1* by Sanger sequencing screening were validating by PCR characterization of genomic DNA at the *CYP3A5* *3 locus (**Figure 3.5A and 3.5B**). The 77 base pair deletions were characterized by PCR amplification of genomic DNA and visualized by 2% agarose gel electrophoresis (**Figure 3.5B**). Both heterozygous (C*YP3A5 *1/*3* sd) and homozygous (*CYP3A5 *1/*1* dd) cell lines were developed with the 77 base pair *CYP3A5* exon 3B 5' splice junction deleted. The point mutation heterozygous cell line, *CYP3A5*1/*3* pm, did not have a deletion at the splice junction (**Figure 3.5B**). These cell lines were then further validated with Sanger sequencing (**Figure 3.6**).

Figure 3.5: Characterization of genomic DNA *at CYP3A5 *3* loci in genetically modified cell lines. A.) Map of genomic DNA spanning *CYP3A5* exons 3, 3B and 4. **B.)** Electrophoresis through 2% agarose gel of PCR products spanning the *CYP3A5 *3* locus (rs776746) of genomic DNA from the cell lines. Data show HuH-7 genomic DNA as the reference. *CYP3A5 *1/*3* sd had two different alleles, one allele being the same as the HuH-7 reference and one allele being 77 base pairs shorter than the reference. The 77 base pair deletion indicated deletion of the Exon 3B splice junction. *CYP3A5 *1/*1* 1dd showed no reference allele and thus deletion of the Exon 3B in both alleles. *CYP3A5 *1/*3* pm has one PCR product the same size as the reference which is expected in a point mutant.

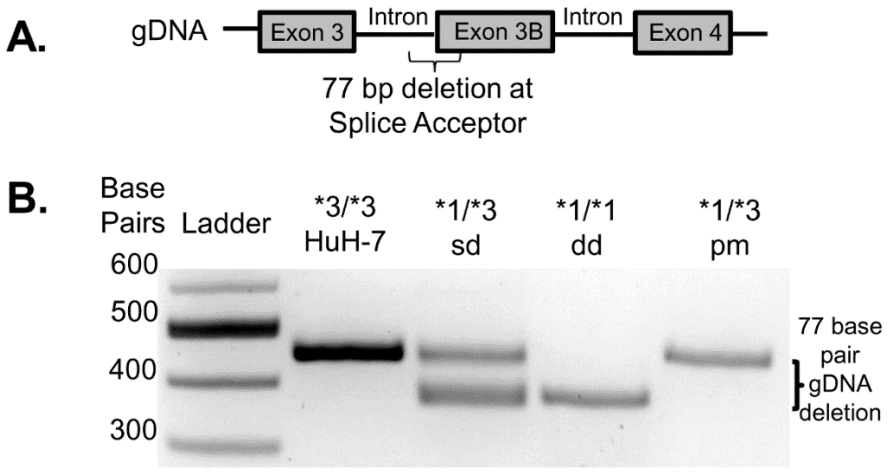
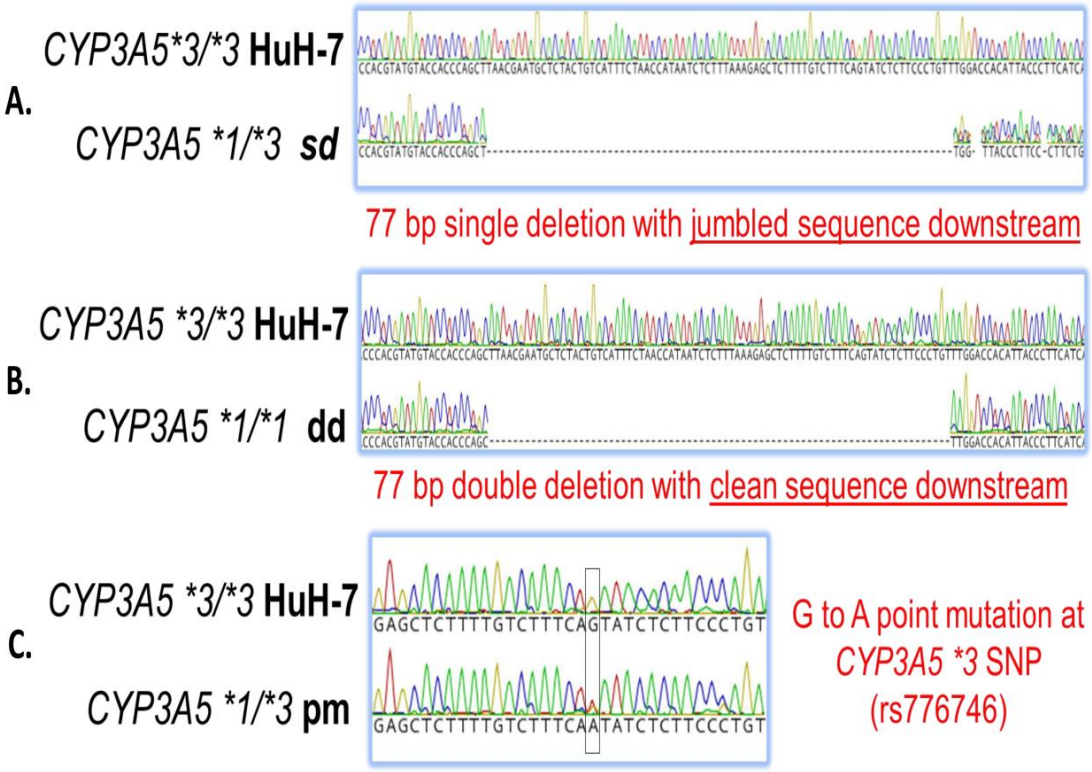


Figure 3.6: DNA Sequence Chromatograms of *CYP3A5 *3* locus in Cell Lines
The sequences were aligned to reference sequence from the HuH-7 cell line that had genotype *CYP3A5 *3/*3*. **A.)** Heterozygous deletion of splice junction in cell line *CYP3A5 *1/*3* sd showed jumbled sequence downstream of deletion indicating heterozygosity. **B.)** Homozygous deletion of splice junction in cell line *CYP3A5 *1/*1* dd showed clean sequence downstream of deletion indicating homozygosity. **C.)** Heterozygous point mutation seen in cell line *CYP3A5 1*/*3* pm.



CYP3A5 mRNA splicing assay and sequencing of engineered cells

CYP3A5 mRNA splice variants were evaluated by gel electrophoresis at the **3* locus to ensure the deletion, or point mutation, of the *CYP3A5* exon 3B 5' splice junction changed the cells to express the **1* mRNA instead of the **3* mRNA (**Figure 3.7A and 3.7B**). Keuhl et al. have previously shown that the 131 base pair exon 3B was present in the *CYP3A5 *3* mRNA and absent in the **1* mRNA [165], which was confirmed by Busi and Cresteil [166, 167]. Total mRNA from the cell lines was converted to cDNA then PCR

amplified with primers that flanked the *CYP3A5* exon 3B to determine if the exon 3B was absent in the engineered cell lines. RNA from human liver cDNA genotyped as **1/*3* were used as the control (**Figure 3.7B**). This mRNA splicing assay confirmed the absence of the **3* mRNA in the newly developed cell line *CYP3A5 *1/*1* dd. Additionally, the **1/*3* heterozygote cell lines, CYP3A5*1/*3 sd and CYP3A5*1/*3 pm, expressed both the **1* and the **3* mRNA splice variants as compared to the human liver cDNA controls (**Figure 3.7B**). To further validate the identity of the *CYP3A5* splice variants in the cell lines we sequenced the *CYP3A5* mRNA via Sanger sequencing of the *CYP3A5* RT-PCR products (**Figure 3.8**). The sequences confirmed that the exon 3B was absent in the *CYP3A5 *1/*1* dd cell line when aligned to a *3 sequence. The *CYP3A5 *1/*3* cell lines *CYP3A5 *1/*3* sd and *CYP3A5 *1/*3* pm sequences became jumbled at exon 3B as is expected when sequencing a heterozygote (**Figure 3.8**). However, sequencing in forward and reverse directions confirmed the *CYP3A5 *1/*3* heterozygote engineered cells expressed both the *CYP3A5 *3* and **1* mRNAs.

Figure 3.7: Characterization of *CYP3A5* RNA expression in genetically modified cell lines. A.) mRNA map of *CYP3A5* exons 3, 3B and 4. **B.)** mRNA splicing assay showed expression of *CYP3A5 *1* mRNA in modified cell lines. Genotyped human liver from a *CYP3A5 *1/*3* genotyped patient was used as control. **C.)** Quantitative RT-PCR showed expression levels of *CYP3A5* in cell lines relative to *GAPDH*. P-value comparing *CYP3A5* mRNA expression between cell lines are from a paired two-sample T-test.

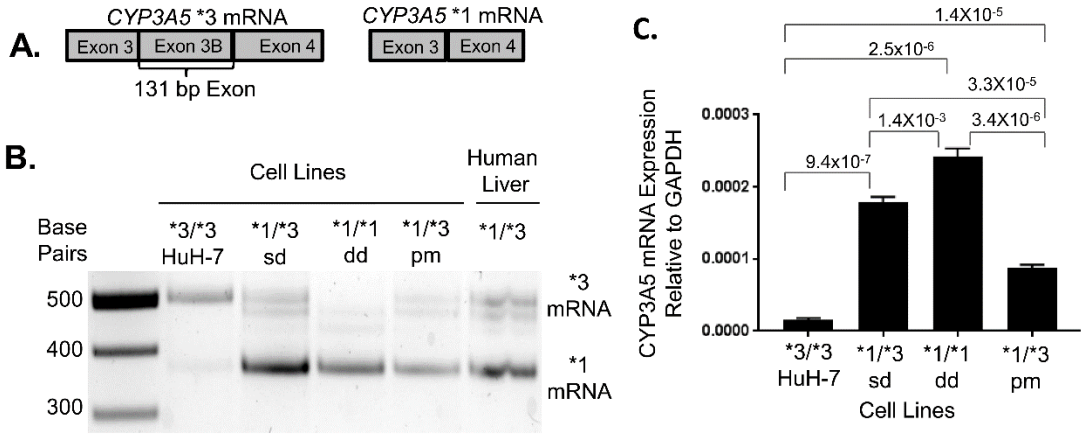
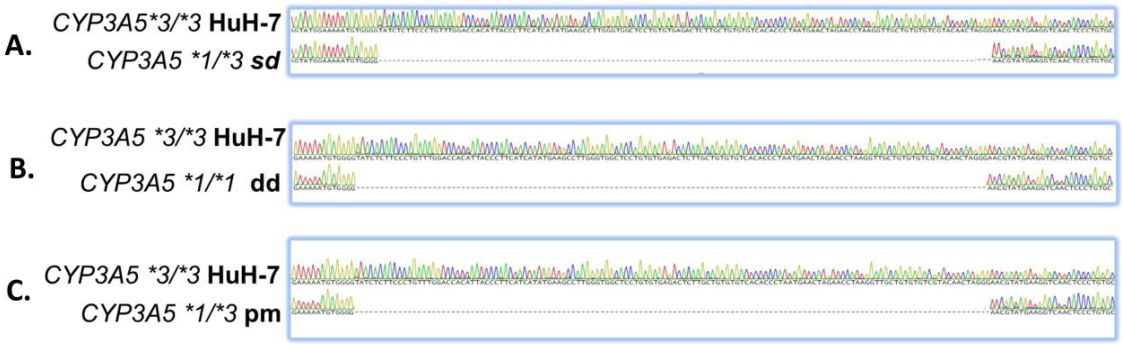


Figure 3.8: Sequence Chromatograms of cDNA from CYP3A5 *3 locus mRNA in Cell Lines. Total RNA was isolated from the cell lines, reverse transcribed with oligo dT primer and the *CYP3A5* mRNA cDNA was sequenced with primers that flank the 131 base pair exon 3B. All cell sequences were aligned to HuH-7 reference control that was *CYP3A5 *3/*3* genotype. **A.)** Absence of exon 3B in cell line *CYP3A5 *1/*3* sd. It is possible we only saw the **1 CYP3A5* in this sequence because previous reports show that *CYP3A5 *3* mRNA is targeted for non-sense mediated decay and would be more difficult to identify in a heterozygote. **B.)** Absence of exon 3B in cell line *CYP3A5 *1/*1* dd. **C.)** Absence of exon 3B in cell line *CYP3A5 1*/*3* pm.



Quantitative RT-PCR resulted in elevated CYP3A5 transcripts in *1 expressing cell lines

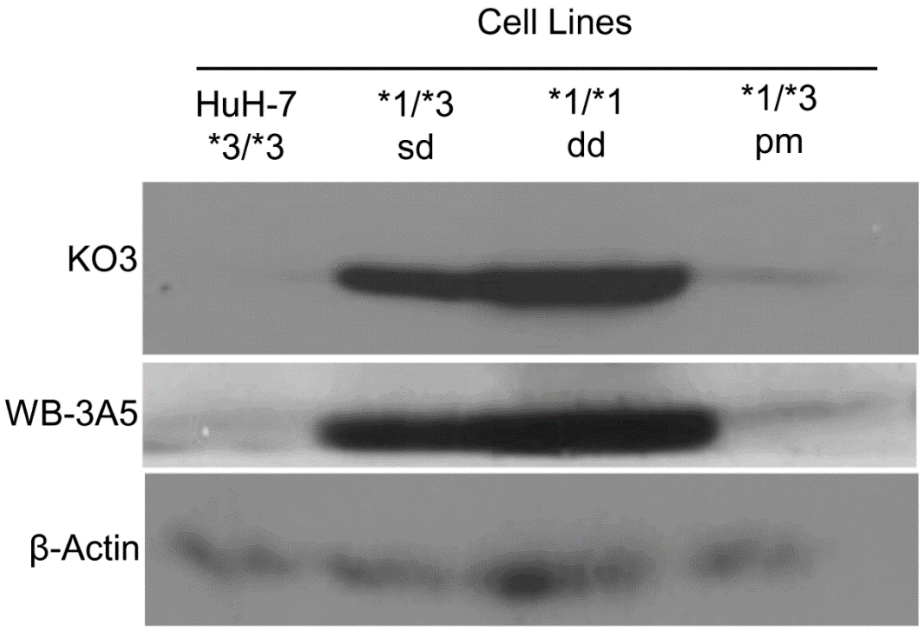
Quantitative RT-PCR showed significantly elevated *CYP3A5* mRNA expression in *CYP3A5 *1/*1* dd compared with HuH-7 *CYP3A5 *3/*3* cells (p-value = 2.5×10^{-6}). All engineered *CYP3A5 *1* cell lines had elevated *CYP3A5* mRNA compared with HuH-7 (p-value $\leq$ 0.0001) (**Figure 3.6C**). The *CYP3A5 *3* mRNA, expressed in cell lines, was targeted for nonsense-mediated decay[30], as the likley cause of reduced *CYP3A5* mRNA in **3* cell lines. The *CYP3A5 *1/*3* cell lines had intermediate *CYP3A5* mRNA expression compared to HuH-7 *CYP3A5 *3/*3* and *CYP3A5 *1/*1* dd (**Figure 3.6C**).

Immunoblot confirms for CYP3A5 expression in engineered cell lines

We immunoblotted for the CYP3A5 protein expression in the cell lines using two separate primary antibodies: KO3 [163], that detects CYP3A family (including CYP3A4 and

CYP3A5), or the CYP3A5 specific WB-3A5 [168] (**Figure 3.9**). The *CYP3A5*1/*3 sd* cell line visually expressed less CYP3A5 protein expression than *CYP3A5 *1/*1* dd, but higher expression than the *CYP3A5 *3/*3* HuH-7 cells. The *CYP3A5*1/*3* pm cell line had poor CYP3A5 protein expression compared to the *CYP3A5 *1/*3* sd cell line (**Figure 3.9**). These results were consistent with both the KO3 and WB-3A5 antibodies. It is likely that the K03 antibody did not detect much CYP3A4 protein due to low *CYP3A4* expression in the HuH-7 cell line. Thus, the bioengineered cell lines express CYP3A5 protein.

Figure 3.9: Characterization of *CYP3A5* protein expression in genetically modified cell lines. Immunoblot with primary (1º) antibodies K03 antibody [163] that recognizes CYP family proteins including CYP3A4 and CY3A5, WB-3A5 [168] that is specific for CYP3A5 or β-actin as a reference.



Metabolism assays show CYP3A5 *1 expressing cells have increased MDZ and Tac metabolism compared with CYP3A5 *3/*3 HuH-7 cells

As shown in **Figure 3.10A**, we performed an MDZ metabolism assay to determine if the new cell lines metabolized MDZ to the products 1-OH MDZ and 4-OH

MDZ. We also quantitated Tac disappearance using the metabolism assay. As expected, the *CYP3A5* *3/*3 (HuH-7) cells had higher levels of Tac (**Figure 3.10B**) and MDZ (**Figure 3.10C**) in cell culture after overnight incubations than *CYP3A5 *1/*3* sd, *CYP3A5 *1/*3* pm or *CYP3A5 *1/*1* dd cell lines because of the decreased metabolism by the *CYP3A5 *3/*3* cells. Furthermore, increased production of 1-OH MDZ (**Figure 3.10D)** and 4-OH MDZ (**Figure 3.10E**) was observed by the engineered cell lines compared with the parental HuH-7 cell line. Significant metabolic differences between each of the cell lines were found comparing substrate disappearance or product formation between the engineered cell lines and the HuH-7 parental cell line (all p-values < 0.05) (**Table 3.2**). Thus, the engineered *CYP3A5 *1* expressing cell lines were more efficient at converting MDZ to its hydroxylated metabolites compared with the *CYP3A5 *3/*3* parental HuH-7 cell line. The engineered *CYP3A5* expressing cells are also more active at metabolizing Tac than the HuH-7 cells which coincides with previous studies with cloned, expressed, *CYP3A4* and *CYP3A5* that demonstrated that the intrinsic clearance of TAC is higher for *CYP3A5* than *CYP3A4* [65].

Figure 3.10: MDZ and TAC metabolism assays confirm that *CYP3A5 *1* expressing cells have increased metabolic activity compared with HuH-7 *CYP3A5 *3/*3* expressing cells. A.) Metabolism assay used in this study. Cells were plated on collagen I coated plates, and grown at confluence for 2-3 weeks then layered with Matrigel®. Cells were then induced with rifampicin and phenytoin for 3 days then substrate of Tac or MDZ was added over night. Cell culture media was collected and assayed for Tac, MDZ or the MDZ products 1-OH MDZ or 4-OH MDZ by liquid chromatography-mass spectrometry. **B.)** Tac was used as the substrate and was assayed to assess its disappearance. Each column represents 5 biological replicates of a representative experiment and shows the disappearance of the Tac caused by the cells' metabolism. **C.)** MDZ was used as the substrate to assess its metabolism. Each column represents 6 biological replicates of a representative experiment and shows the disappearance of the MDZ caused by the cells' metabolism. **D.)** The corresponding 1-OH MDZ products from the MDZ experiments are shown and the **E.)** corresponding 4-OH MDZ products.

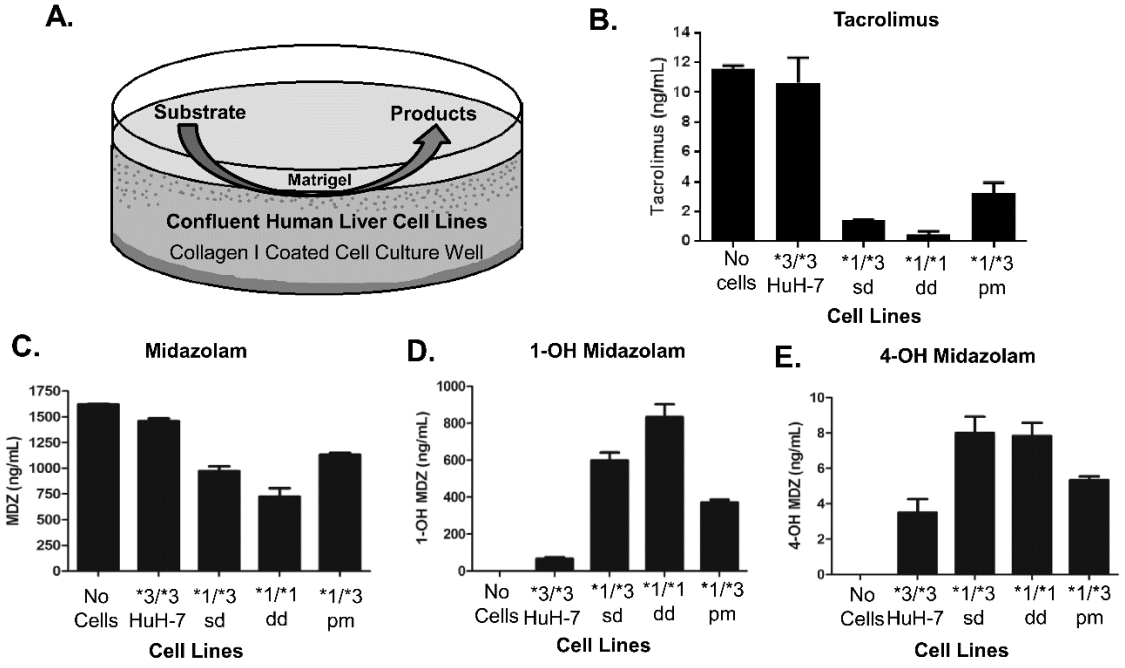


Table 3.2: P-Values Comparing Tacrolimus or Midazolam Metabolic activity between cell lines. P-values were calculated based on the paired two-sample T-test comparing between each cell line. Substrates and Products were quantified by liquid chromatography of the media from the cell cultures.

Cell lines Compared	P-Values			
	Substrates		Products	
	Tac	MDZ	1-OH MDZ	4-OH MDZ
[+]HuH-7 **3/*3* vs. [++]*CYP3A5 *1/*3* sd	5.4×10^{-3}	2.4×10^{-5}	2.3×10^{-6}	7.3×10^{-4}
HuH-7 **3/*3* vs. [+++]*CYP3A5 *1/*1* dd	2.7×10^{-3}	5.3×10^{-4}	8.6×10^{-5}	2.7×10^{-4}
HuH-7 **3/*3* vs. [++++]*CYP3A5 *1/*3* pm	2.7×10^{-2}	5.0×10^{-7}	3.8×10^{-8}	1.3×10^{-3}
*CYP3A5 *1/*3* sd vs. *CYP3A5 *1/*1* dd	1.0×10^{-2}	3.6×10^{-1}	3.5×10^{-2}	1.6×10^{-2}
*CYP3A5 *1/*3* sd vs. *CYP3A5 *1/*3* pm	5.3×10^{-2}	5.3×10^{-1}	1.8×10^{-3}	9.4×10^{-1}
*CYP3A5 *1/*1* dd vs. *CYP3A5 *1/*3* pm	3.1×10^{-2}	4.1×10^{-1}	2.0×10^{-3}	2.4×10^{-2}

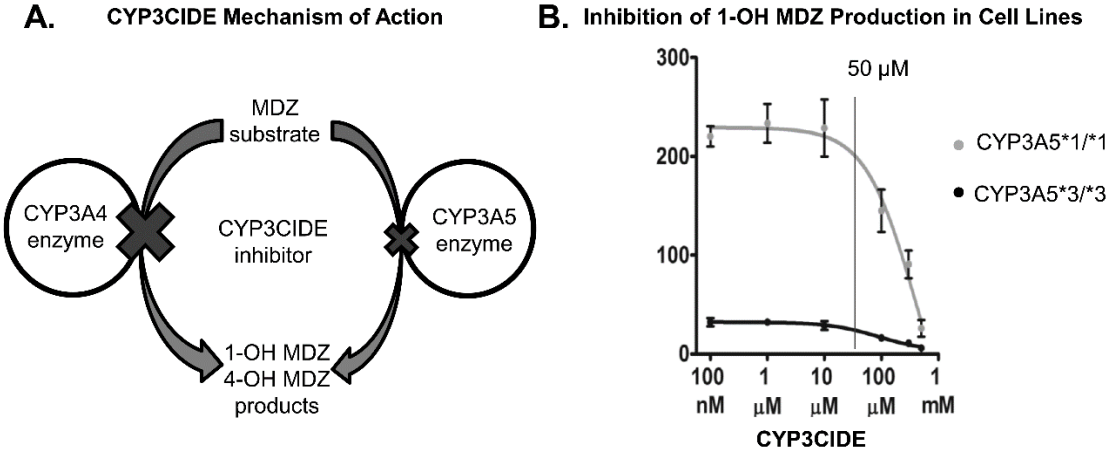
[+]*HuH-7 was the parental cell line with CYP3A5 *3/*3 alleles*
[++]*CYP3A5 *1/*3 sd was a bioengineered cell line with the CYP3A5 *1 allele made by deletion of a splice acceptor on one of the alleles.*
[+++]*CYP3A5 *1/*1 dd was a bioengineered cell line with both CYP3A5 *1 alleles made by deletion of a splice acceptor on two of the alleles.*
[++++]*CYP3A5 *1/*3 pm was a bioengineered cell line with the CYP3A5 *1 allele made by a point mutation of a splice acceptor on one of the alleles.*

CYP3CIDE as a selective CYP3A4 inhibitor in MDZ assays

CYP3CIDE [153, 164] is a selective CYP3A4 inhibitor that also inhibits CYP3A5 at higher concentrations. The concentration dependent effects of CYP3CIDE are shown in **Figure 3.11**. To determine the concentration of CYP3CIDE to use in cell culture we did a dose response study with the HuH-7 (*CYP3A5 *3/*3*) and *CYP3A5 *1/*1* dd cell lines

(**Figure 3.11B**). Following the dose response study, the cell lines were incubated with MDZ with or without 50 µM CYP3CIDE to assess CYP3A5 activity in modified cell lines.

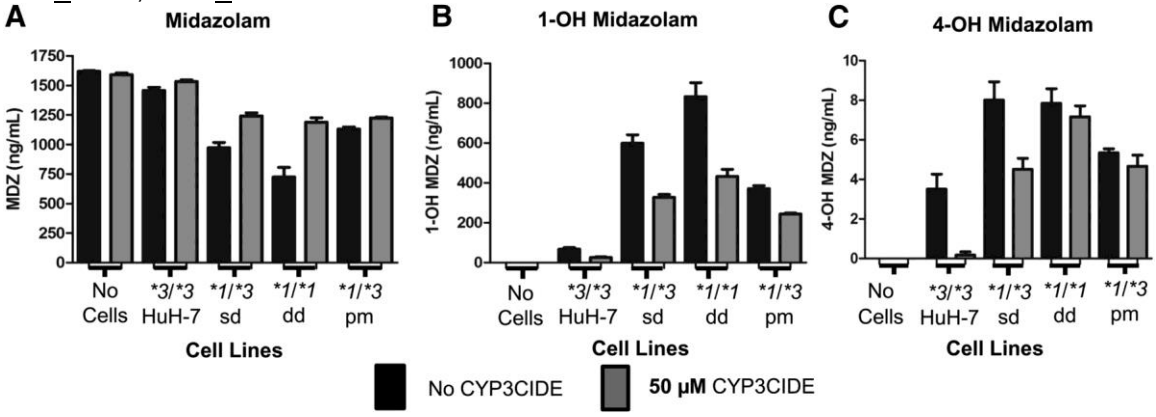
Figure 3.11: CYP3CIDE selective inhibition of CYP3A4 and CYP3A5 and dose response in cell lines A.) CYP3CIDE inhibition of CYP3A4 and CYP3A5 enzymatic activity. CYP3CIDE has a higher affinity on CYP3A4 inhibition than CYP3A5 inhibition. The substrate in this experiment was MDZ and the products of the reactions were 1-OH MDZ and 4-OH MDZ. **B.)** CYP3CIDE dose response curve in HuH-7 (*CYP3A5 *3/*3*) and *CYP3A5 *1/*1* dd cell lines with the 1-OH MDZ product as metabolite. The concentration of 50 µM CYP3CIDE was chosen for further study in other cell lines.



When CYP3CIDE was present, there was slight difference in the MDZ reduction by the HuH-7 cell line ($p = 0.044$). The MDZ reduction was more pronounced in all the *CYP3A5 *1* expressing cell lines comparing with or without CYP3CIDE ($p \leq 0.005$) (**Figure 3.12A**). The 1-OH MDZ production by HuH-7 cells was lower with CYP3CIDE ($p<0.05$), while all 3 *CYP3A5 *1* expressing cell lines had even more significant reduction of 1-OH MDZ production (all p-values ≤ 0.005) (**Figure 3.12B**). Further analysis of MDZ metabolism by the cell lines with 4-OH MDZ as the minor metabolic product (**Figure 3.12C**) showed that CYP3CIDE almost completely halted 4-OH MDZ production by HuH-7 cells ($p = 0.007$). **Figure 3.12C** also showed HuH-7 and *CYP3A5 *1/*3 sd cell lines had less 4-OH MDZ production with CYP3CIDE ($p \leq 0.01$ and $p \leq 0.05$, respectively). Neither *CYP3A5 *1/*1* dd nor *CYP3A5 *1/*3* pm had significant 4-

OH MDZ production differences with CYP3CIDE ($p > 0.05$). These CYP3CIDE experiments showed that these cell lines had differential activity when a selective CYP3A4 inhibitor was present; indicating phenotypically active *CYP3A5* which was not present in the parental HuH-7 (*CYP3A5 *3/*3*) cell line.

Figure 3.12: Effect of CYP3CIDE on MDZ metabolism in *CYP3A5* genetically modified cells. Cells were treated with (grey bars) or without 50 µM CYP3CIDE (black bars) and assayed for MDZ metabolism. Each column in **C-E** represents 6 biological replicates of a representative experiment. **C.)** MDZ disappearance by cell lines after incubation of MDZ in presence of CYP3CIDE. **D.)** Production of 1-OH MDZ by cell lines in the presence of CYP3CIDE. **E.)** Production of 4-OH MDZ by cell lines in the presence of CYP3CIDE. P-values in **C-E** were calculated based on the paired two-sample T-test comparing with or without CYP3CIDE are: ns = not significant and $P > 0.05$, * $P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.



Discussion:

This study showed the successful CRISPR/Cas9 bioengineering of a human liver cell line, HuH-7, to create new cell lines that express the common *CYP3A5 *1* variant that is known to be highly relevant towards drug metabolism. Unlike recent reports using CRISPR/Cas9 to knock out *CYP2E1* [147] or *CYP3A1/2* [148] function in rats, we used CRISPR/Cas9 to activate *CYP3A5* expression in human cells lines by conversion of **3* to **1* genotype. This is the first report of engineered cell lines for both heterozygous and homozygous *CYP3A5 *1* expression in human liver cell culture and phenotypic analysis.

This study showed it is possible to use two methods of CRISPR/Cas9 biotechnology to modify the HuH-7 cells to express *CYP3A5 *1* by splice junction deletion using two gRNAs or with one gRNA and a homology directed repair template. Without the need for fluorescent activated cell sorting or less precise limiting dilution techniques, a soft agar clonal selection with expansion on collagen I coated plates technique was used to isolate unique human hepatocyte cell lines. This technique is important in isolating hepatocyte cell lines because the cells do not grow well as single cells on standard plastic cell culture dishes. Also, growing the cell lines at confluence for 2-3 weeks, layering with Matrigel® and inducing the cells with rifampin and phenytoin increased the hepatocytes metabolic activity. We determined the impact of induction while developing the MDZ metabolism assay. Since induction increased MDZ metabolism in the HuH-7 cells, we did not change the protocol for the genetically modified cell lines or when using Tac as the substrate. In a previous study, 1-OH MDZ production by HuH-7 cells was low, ~2.5 picomol/mg protein/min with rifampicin induction and lower at ~1 picomol/mg protein/min without induction [158]. In comparison, our induction method in HuH-7 parental cell line produced ~67 ng/mL 1-OH MDZ in media. Although these units are not the same, our method does have higher MDZ metabolism. In our genetically modified cell lines such as, *CYP3A5 *1/*1* dd, 833 ng/ml 1-OH MDZ was formed. Relative to the unmodified HuH-7 (*CYP3A5 *3/*3*) cells, these new cell lines express elevated *CYP3A5* mRNA and led to significantly higher metabolism of Tac and MDZ which are well-known substrates for CYP3A5. The genetically modified cells also produce the expected MDZ metabolites (1-OH MDZ and 4-OH MDZ). Thus, these cells show promise as cell lines for drug metabolism assessment. Since these cells are diploid at chromosome 7 by karyotype [169], and by copy number of the *CYP3A5* locus [170, 171], and these new cells have increased drug

metabolism, the cells would be ideal to use as parental cell lines to functionally study other genetic variants. Newly discovered variants can also be genetically engineered into the cells to study combinations of genetic variants.

Differential response to drug therapy is common. These differences are in part due to the presence of genetic variants in CYP enzymes that alter drug metabolism and pharmacokinetics. Ethnic minority populations may carry alternative variants or the same variant as Caucasians but with a different allele frequency [172]. Because ethnic minority populations are generally underrepresented in clinical trials in the United States, differences in pharmacokinetics and response rates are often not detected until the drug has reached the market [173]. Therefore, efficient preclinical methods to study how metabolism may be altered in the presence of alternative alleles would be extremely useful. The cell lines that we developed show the potential of the CRISPR/Cas9 biotechnology in the drug metabolism field. We chose *CYP3A5 *3* and **1* as model alleles to genetically engineer since the effect of these alleles on metabolism is well-established *in vitro* by traditional methods and clinically. Specifically, this technology will allow for the creation of cell lines with varying combinations of common alleles, a platform to study rare alleles and those occurring in populations not represented in clinical trials.

A limitation of this study is that we did not investigate the metabolic activity of the newly developed cell lines in the absence of induction. We know induction increases MDZ metabolism in HuH-7 cells[158]; thus the induction process is necessary to study robust metabolism in the genetically modified cells. Another limitation of this study is that there are possibly off-target effects of the CRISPR/Cas9 engineering that are common in this type of genetic engineering[174]. We addressed this by selecting gRNAs with the least amount of potential off targets, using the Massachusetts Institute of

Technology CRISPR algorithm, and confirmed with sequencing that the DNA was cut at the desired site surrounding the *CYP3A5*3* locus. Additionally, this study showed that we can produce the *CYP3A5 *1* mRNA in human liver cell lines as seen in human samples from previous studies by Kuehl and colleagues and by Busi and Cresteil[165, 167]. We showed that these cells definitively express *CYP3A5 *1* mRNA, and active CYP3A5 enzyme, as either heterozygous or homozygous compared with control HuH-7 cells. However, the two *CYP3A5 *1/*3* heterozygote cell lines either have a 77 bp splice junction deletion or a point mutation and these cell lines have different *CYP3A5* expression and activity than HuH-7. The *CYP3A5 *1/*1* dd was vastly more metabolically active than the other cell lines and would be the most useful for comparative studies with the *CYP3A5 *1* genotype.

Future work with these methods and developed cell lines include a number of directions. We envision panels of cell lines developed using genetic modification that can be used to study genetic variants associated with drug metabolism. These panels could include variants of particular metabolism genes, gene families, common variants, or rare variants. Common variants or combinations of common variants could be engineered into cell lines and used in preclinical drug metabolism screens to predict pharmacokinetics. Rare variants could also be engineered into the cell lines. If rare alleles were found to alter metabolism it may predict subjects at risk for drug failure or toxicity. It may also allow for early testing of alternative doses for trial subjects carrying combination of variants or rare alleles. There are substantial challenges in studying rare alleles in human clinical trials due to inadequate sample size; therefore, engineered cells could bring extreme value to drug development. In addition, drugs already on the market could be rapidly screened. Since the new cells in this study express *CYP3A5*, the cells are especially useful to study genetic variants that effect *CYP3A5* expression.

Finally, it may also be possible that multiple variants could be engineered into a single cell line which would more closely emulate the specific human populations. This technology can potentially lead to faster preclinical development that can save time and money. As the use of this technology expands, we will be able to more accurately predict substrate metabolism, pharmacokinetics, toxicities and efficacy, especially in minority patients with rare genetic variants.

Authorship Contributions:

Participated in research design: Dorr, Remmel, Muthusamy, Fisher, Moriarity, Kazuto, Wu, Guan, Schuetz, Oetting, Jacobson, Israni

Conducted experiments: Dorr, Muthusamy, Fisher, Kazuto

Contributed new reagents or analytic tools: Dorr, Remmel, Muthusamy, Fisher, Kazuto, Schuetz, Jacobson, Israni

Performed data analysis: Dorr, Muthusamy, Fisher, Kazuto, Wu, Guan

Wrote or contributed to the writing of the manuscript: Dorr, Remmel, Muthusamy, Fisher, Moriarity, Kazuto, Wu, Guan, Schuetz, Oetting, Jacobson, Israni

Footnote:

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Chapter 4 –Conclusions, Future Directions and Take Away

The Biomedical Informatics and Computational Biology (BICB) Master's program has been an important part of my scientific journey. As I finished my Doctorate of Philosophy (PhD) in Molecular Cellular Developmental Biology and Genetics at University of Minnesota in 2011, this Masters of Science in BICB was a core component of my K01 career development grant from the National Institute for Allergy and Infectious Diseases (NIAID). The K01 has the same title as this thesis, Genetic Variants Associated with Tacrolimus Metabolism in Kidney Transplant Recipients. This BICB program has enhanced my career by giving me essential skills in biostatistics, computational coding and bioinformatics analysis that I can now use as I go forward in my career as a scientist.

In **Chapter 1** of this thesis, I showed the significance of understanding the genetics of TAC metabolism in kidney transplant recipients and how this can be used for precision dosing of immune suppressants to improve clinical outcomes. We further have identified, there are differences in TAC metabolism based on genotypes in the CYP3A5 gene, specifically the CYP3A5 *1, *3, *6, and *7 and the CYP3A4 *22 common variants. As part of the K01 study, we aimed to identify and validate low-frequency genetic variants associated with TAC metabolism.

In **Chapter 2**, we showed that we can use an EPS model, adjusted for the aforementioned common variants in AA or EAs, coupled with NGS to identify genetic variants associated with TAC metabolism. We did identify the genetic variant in CYB5R2 as significant.

In **Chapter 3**, we showed that genetic variants can then be introduced into human liver cell lines, with CRISPR biotechnology, then assayed to validate the variants effect on TAC metabolism. In this study, we developed the CYP3A5 *1 expression cell lines in TABLE X. I further show the RNAseq expression of these cell lines in fragments per kilobase per million reads (FPKM). This data was produced as part of the Computational Biology course I took with Dr. Chad Myers. During the BICB program, my K01 primary mentor, Dr. Ajay Israni and I patented the development of these cells and the methodology to assay drug metabolism in cell lines (US Patent and Trademark Office No. 20200032269, "Genetically Modified Cell Lines for Metabolic Studies").

Table 4.1: Cell lines developed in this study and RNA expression by RNAseq		
Cell line	**CYP3A5 FPKM**	**Cell Source** Cat. number
HuH-7 (CYP3A5 *3/*3)	**0**	JCRB 0403
*CYP3A5 *1/*1* DD	**120**	ABM T6346
*CYP3A5 *1/*3* SD	**77**	ABM T6347
*CYP3A5 *1/*3* PM	**27**	ABM T6348
JCRB = Japanese Cell Research Bank ABM = Applied Biological Materials, made in Dorr Lab		

Conclusions:

The major conclusions from this thesis are that we can use the EPS and NGS methodology to identify genetic variants associated with TAC metabolism coupled with cell line engineering and cell culture drug metabolism assays to validate the variants.

Future Directions:

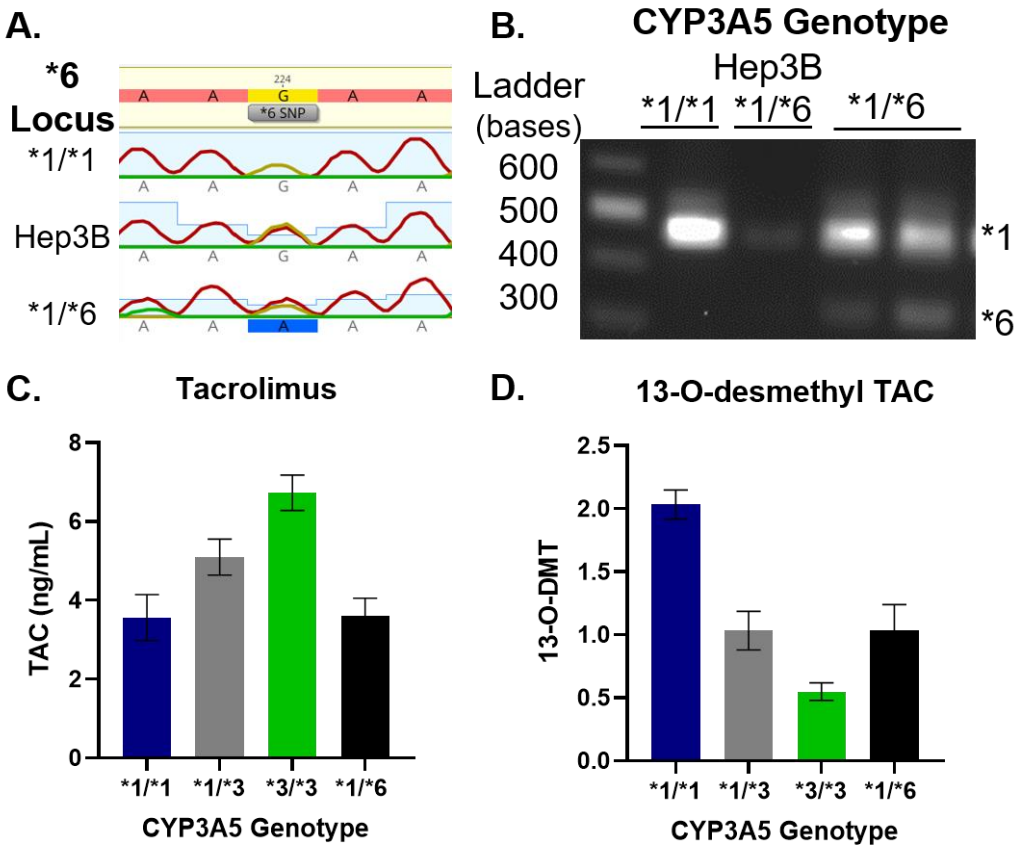
Our team has performed extensive follow up on the work presented in this thesis. We have further applied the EPS and NGS methodology to a cohort of 515 AA kidney transplant recipients in the DeKAF and GEN03 studies and sequenced 118 of the patients using an expanded targeted sequence strategy. This data was presented, by me, at the 2020 American Transplant Congress as a plenary talk and was further analyzed and presented at the 2023 American Transplant Congress by Experimental and Clinical Pharmacology PhD student Moataz Mohamed, with mentorship from my K01 co-mentor Dr. Pamala Jacobson and me. This expanded experimental data was further analyzed by recent University of Minnesota Biostatistics PhD graduate Dr. Bin Guo. In this study, we did not validate the *CYB5R2* variant, but we did identify 4 variants in the genes *PPP3CA*, *PCCA* and *SLC28A1* associated with TAC troughs. We aim to publish this data in the future and I will be the senior author. This BICB program has been essential to my ability to mentor students in Biostatistics, Bioinformatics and Computational Biology.

My lab at Hennepin Healthcare Research Institute is in the process of extensive follow up on the cell line development and genetic variants validation in cell culture. My lab assistants, Sarah Elmer and Duy (Tin) Vo, have worked to develop a new CYP3A5

*1/*6 cell line using CRISPR technology and single cell cloning. We show CRISPR modification of the CYP3A5 *1/*1 DD cell line (CRISPR modified from HuH-7) to develop CYP3A5 *6 cells. We used Integrated DNA Technologies CRISPR reagents to modify the CYP3A5 *6 locus in CYP3A5 *1/*1 DD cells. After single cell cloning, initial characterization was by *6 DNA locus sequencing and RT-PCR that spanned Exons 5-8 of CYP3A5 for an expected 469 base product in CYP3A5 *1/*1 expressers, followed by the cell culture TAC metabolism assay.

CYP3A5 *1/*6 cell line appears to function intermediate between CYP3A5 *1/*1 and *1/*3. In **Figure 4.1**, we show development of a *1/*6 cell line that expresses a new mRNA by RT-PCR (~300-350 bases). This cell line metabolized TAC similar to *1/*1, but produced 13-DMT similar to *1/*3.

Figure 4.1: CRISPR Development of a CYP3A5 *1/*6 cell line from the CYP3A5 *1/*1 DD cell line. A. Sanger sequencing of the *6 locus in CYP3A5 *1/*1 DD, Hep3B (*6 control) and *1/*6 cells. **B.** RT-PCR across Exons 5-8, spanning the *6 loci in exon 7, showing a new RNA transcript in *6 cells (*1/*6 in duplicate). **C.** TAC and **D.** 13-O-desmethyl TAC (13-DMT) quantification by LC-MS.



Our preliminary data supports our hypothesis that ritonavir (RTV) inhibits TAC metabolism in both *CYP3A5 *1/*1* and **3/*3* cell lines, but more so in **1/*1*. Figure 4.2 shows a TAC metabolism assay with the inhibitor RTV in *CYP3A5*1/*1* DD or HuH-7 *CYP3A5*3/*3* cell lines. The *CYP3A5*1/*1* cell line, although rapidly metabolizes TAC, was converted to a poor metabolism phenotype in the presence of RTV, and metabolism was not significantly different than that by *CYP3A5*3/*3*. The *CYP3A5*1/*1* cell line produced more 13-DMT metabolite (p<.0001) and metabolized TAC (p<.0001) more than the *CYP3A5*3/*3* cell line. In presence of RTV, neither cell line produced the 13-DMT metabolite. **Figure 4.3** shows the DDGI effect comparing the difference between X_1 and X_2. Whereas X_1 is the effect of **1/*1* vs **3/*3* genotype on TAC metabolism, X_2 is the effect of RTV between cell lines. The resultant DDGI effect is 5.9. **Relevance: Our cell culture assay can be used to investigate TAC DDGIs.**

Figure 4.2: Cell culture assay to investigate DDGI. Cells with either *CYP3A5*1/*1* or **3/*3* genotype were exposed to TAC with or without RTV. **A.** TAC and **B.** 13-*O*-desmethyl TAC quantification in cell culture media by LC-MS. Analysis by one-way ANOVA with Tukey posttest for multiple comparisons.

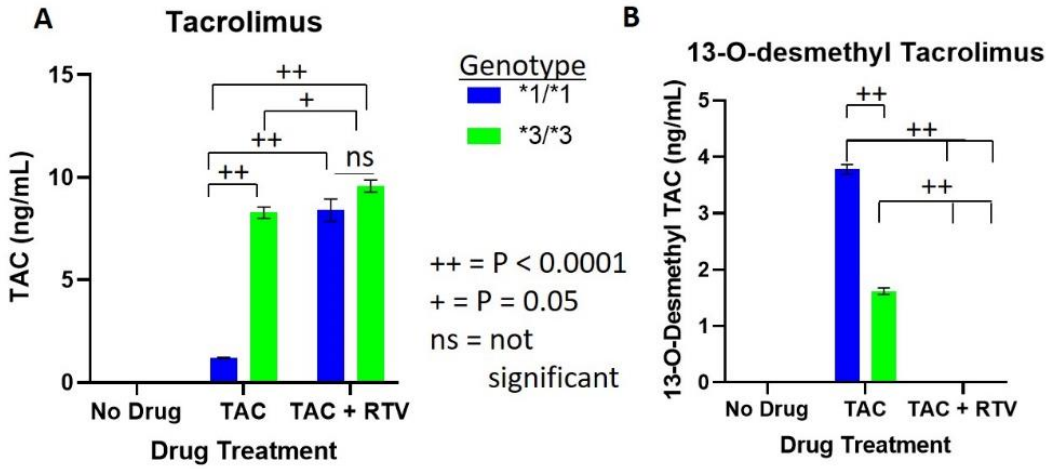
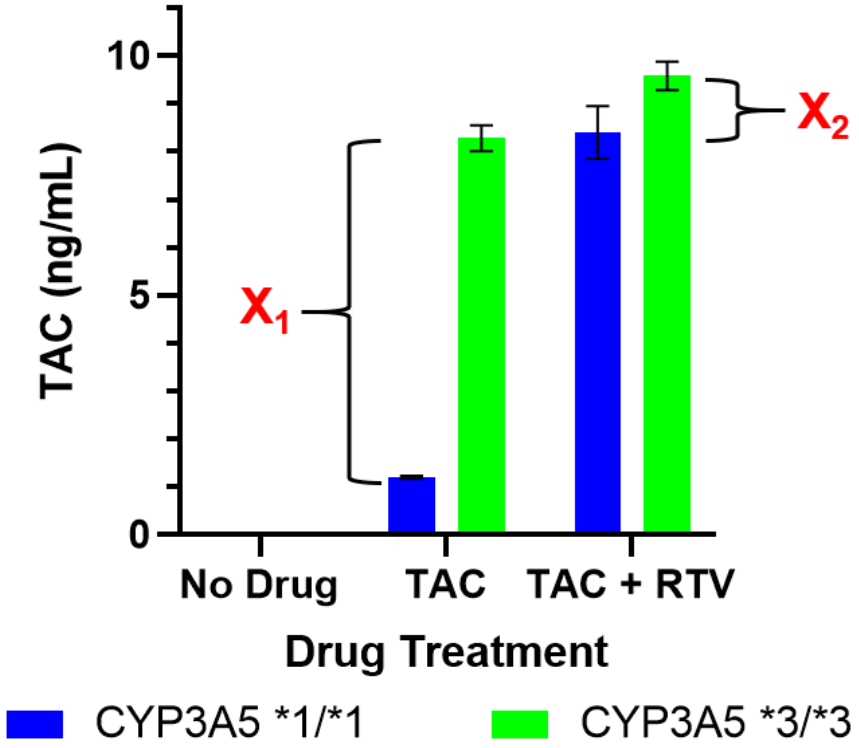


Figure 4.3: DDGI effect of RTV and *CYP3A5*1/1* vs **3/*3* genotype. The DDGI effect can be determined in this graph as the difference in X_1 and X_2. X_1 = 7.1, X_2 = 1.2, with the resultant DDGI effect in this preliminary experiment as 5.9.



Take Away

In closing, this BICB program has enriched my journey as a scientist by training me in important statistics and computational approaches to doing clinical and lab-based science. I use these skills daily in my scientific pursuits. I attained an R21 titled "Molecular and Cellular Analysis of Allograft Loss in Kidney Transplant Recipients" from NIAID in 2022. With the computational and biostatistics skills learned in BICB, I was able to perform power calculations for that grant on my own with the guidance of my K01 advisory team members Baolin Wu and Weihua Guan. I have worked closely with Dr. Wu and Guan over the past decade and they have trained me in computational biology, statistics and bioinformatics. Dr. Israni and Jacobson have also put enormous amounts of time training me in clinical and translational sciences. Dr. Yuk Sham has guided me through the BICB program and been a strong advisor. My main takeaway, besides the science presented in this thesis is that I now have skills that will be useful for the rest of my life to do science, and live, with a strong statistical mindset.

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