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Crypt organoids culture as an *in vitro* model in drug metabolism and cytotoxicity studies.

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Abbreviations used in this paper:

CAR, constitutive androstane receptor; IECs, intestinal epithelial cells; ISC, intestinal stem cells; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; CYP, Cytochrome P450; UGT, UDP-glucuronosyltransferase; XPGs, xenobiotic processing genes; XNR, xenobiotic nuclear receptors, DMEs, drug metabolism enzymes; GI, gastrointestinal;

Abstract

The gastrointestinal tract is enriched with xenobiotic processing proteins that play important roles in xenobiotic bioactivation, metabolism, and detoxification. The application of genetically modified mouse models has been instrumental in characterizing the function of xenobiotic processing genes (XPG) and their proteins in drug metabolism. Here, we report the utilization of 3D crypt organoid cultures from these animal models to study intestinal drug metabolism and toxicity. With the successful culturing of crypt organoids, we profiled the abundance of Phase I and Phase II XPG expression, drug transporter gene expression and xenobiotic nuclear receptor (XNR) gene expression. Functions of XNRs were examined by treating crypt cells with XNR prototypical agonists. Real-time quantitative PCR demonstrated that the representative downstream target genes were induced. These findings were validated from cultures developed from XNR-null mice. In crypt cultures isolated from *Pxr*^{-/-} mice, PCN failed to induce *Cyp3a11* gene expression; similarly, WY14643 failed to induce *Cyp4a10* in the *Ppara*^{-/-} crypts. Crypt cultures from control (*Ugt1*^{F/F}) and intestinal epithelial cell (IEC) specific *Ugt1* null mice (*Ugt1*^{ΔIEC}) were treated with camptothecin (CPT)-11, an anticancer pro-drug with severe intestinal toxicity that originates from insufficient UGT1A1 dependent glucuronidation of its active metabolite SN-38. In the absence of *Ugt1* gene expression, *Ugt1*^{ΔIEC} crypt cultures exhibit very limited production of SN-38 glucuronide (SN-38G), concordant with increased apoptosis in comparison to *Ugt1*^{F/F} crypt cultures. This study suggests crypt organoid cultures as an effective *in vitro* model for studying intestinal drug metabolism and toxicity.

Introduction

The gastrointestinal (GI) tract is lined with epithelial cells that express an abundance of xenobiotic or drug metabolizing enzymes (DMEs) in addition to efflux and influx transporters. Thus, the absorption of xenobiotics and drugs are subjected to metabolism by DMEs localized in the mature enterocyte. This process contributes significantly to bioactivation, metabolism, and detoxification of drugs, all of which are associated with the outcome of drug efficacy and safety. A greater appreciation attributed to the GI tract and drug metabolism has been achieved by examining drug metabolism and toxicity in knockout animal models or fully humanized animal models. For example, human CYP3A4 is considered by many to be one of the most versatile of the CYPs involved in drug metabolism because it accounts for the metabolism of almost half of all prescribed medications (Zanger et al., 2008). Van Herwaarden et al (van Herwaarden et al., 2007; van Waterschoot et al., 2008; van Waterschoot et al., 2009). generated intestine- and liver-specific expression of human CYP3A4 in a *Cyp3a*-null background demonstrating intestinal CYP3A4 activity had a profound impact on systemic exposure of the anticancer drug docetaxel following its oral administration. Camptothecin (CPT)-11 (Irinotecan), is a prodrug that is hydrolyzed by carboxylesterase (CES) in different tissues to form the active topoisomerase inhibitory metabolite SN-38 (Kaneda et al., 1990; Sugimoto et al., 1990), which is further metabolized by UGT1A1 dependent glucuronidation to form an SN-38 glucuronide (SN-38G) (Iyer et al., 1998). Classically, the bioactivation and detoxification of CPT-11 have been credited primarily to hepatic metabolism (Alimonti et al., 2004; Michael et al., 2006). However, recent studies have confirmed that intestinal CES2 has a greater affinity toward CPT-11 in comparison to liver CES1 (Hatfield et al., 2011). With the development of *Ugt1^{F/F}* mice, deletion of the *Ugt1* locus in intestinal epithelial cells (*Ugt1^{ΔIEC}*) produced severe CPT-11-induced intestinal toxicity in comparison to *Ugt1^{F/F}* mice or mice with a liver specific *Ugt1* deletion (*Ugt1^{ΔHEP}*) (Chen et al.,

2013). This result highlighted that UGT1A1 expression in intestinal tissue is critical in preventing SN-38 induced toxicity.

Given the importance of the GI tract and the beneficial application of genetically modified mouse models to examine drug metabolism and toxicity, the development of primary cells cultured from these mice to examine gene expression and toxicity will provide an important tool to complement *in vivo* animal experiments. Sato et al (Sato et al., 2009). established long-term culture conditions in which one single Lgr5+ stem cell embedded in matrix gel can independently generate villus-like epithelial domains with the presence of all differentiated IECs. The 3-D culture is a continuously expanding, self-organizing structure which is reminiscent of normal gut. This result has been popularly applied in the study of crypt-villus biology, regenerative medicine, and gene therapy (Sato and Clevers, 2013; Grabinger et al., 2014; Ranga et al., 2014), but its applications to investigate intestinal epithelial cell specific xenobiotic metabolism has been lacking. In this article, we report the application of crypt organoid cultures from genetically modified mice as a model to examine gene expression and drug metabolism.

Material and methods

Reagents: WY14643, pregnenolone 16 α -carbonitrile (PCN), 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), phenobarbital, docosahexaenoic acid (DHA), and β -naphthoflavone (BNF), N-acetylcysteine, and CPT-11 standard were from Sigma-Aldrich. SN-38 and T0901317 were purchased from Cayman Chemical Company. SN-38 glucuronide was from Santa Cruz Biotechnology. Internal standards deuterated d10-CPT-11 and d3-SN-38 were purchased from Toronto Research Chemicals. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from the National Cancer Institute, National Institutes of Health, Chemical Carcinogen Reference Standard Repository. Matrigel basement membrane matrix was purchased from BD biosciences. Advanced DMEM/F12 medium and the supplements including GlutaMax, hepes, penicillin streptomycin, mouse EGF, N2, and B27 supplements were purchased from Thermo Fisher Scientific; mouse Noggin was from Peprotech. HEK-293 cells producing R-Spondin 1 protein (293T-HA-Raspol_Fc cell line) was a generous gift from Dr. Calvin Kuo laboratory at Stanford University (Ootani et al., 2009). The cells were cultured and passaged per standard protocol. For collecting R-Spondin 1 conditioned media, 293T-HA-Raspol_Fc cells at approximately 75% confluency were changed to advanced DMEM/F12 medium and cultured for another week. Medium was then collected by passing through 0.22 μ m sterile filter.

Animals: C57BL/6J mice were purchased from Charles River Laboratories. Intestinal specific *Ugt1* deletion mice (*Ugt1* ^{Δ IEC}) were generated in our laboratory (Chen et al., 2013). *Ppara*^{-/-} mice were purchased from Jackson Laboratories (Lee et al., 1995). *Pxr*^{-/-} and *Car*^{-/-} mouse models were a kind gift from Drs. Ron Evans (Laboratory at Salk Institute, La Jolla) (Xie et al., 2000) and Masahiko Negishi (Ueda et al., 2002; Yamazaki et al., 2005) (NIEHS), respectively. The proposed mouse experiments have been approved per the procedures as set by the University of California San Diego (UCSD) Institutional Animal Care and Use Committee (IACUC). All the proposed

mouse strains were housed in a pathogen-free facility with automated temperature control and a 12 h x 12 h light/dark cycle.

Isolation of crypt cells and organoid culture. Intestinal crypt cell isolation and organoid culturing were carried out according to previous publications (Sato et al., 2009; Sato and Clevers, 2013) with several modifications. Briefly, mouse small intestine was dissected, cut longitudinally and washed briefly by ice-cold DPBS. The tissue was further dissected into small pieces and incubated in DPBS containing 2 mM EDTA at 4°C with gentle shaking. One hour later, the EDTA solution was removed and 10% FBS in DPBS buffer added followed by vigorously shaking to release villi and crypt cells. The cell solution was passed through tea filter to remove tissue debris, and then further filtered through a 70 µm cell strainer. The filtrate was centrifuged at 1,000 g for 10 min at 4°C and the cell pellet washed twice by DPBS. Crypt cells were counted. Approximately 1,000 crypts were suspended into 50 µl ice-cold Matrigel and plated into pre-warmed 24-well tissue culture plates. Ten minutes after the incubation, 0.5 ml of complete growth medium (advanced DMEM with GlutaMax, HEPES, Penicillin/streptomycin, N2 and B27 supplements, with 2.5 mM N-acetylcysteine, 0.1 µg/ml mNoggin, 0.05 µg/ml mEGF, and 10% of R-Spondin 1 conditioned medium) was added. The growth of crypt organoids was monitored. Mouse EGF was added every other day. Fresh medium was added every four days. Cells were normally ready for passage every 7 days. Crypt cells were exposed to various chemicals on day four and after 24 h the cells were collected in triplicate for further analysis. The results were described as the average ± SEM, which represent multiple independent experiments with compatible data. Student's t-test was used for statistical analysis.

RNA preparation and Reverse Transcription Quantitative PCR (RT-qPCR) analysis. Culture medium was removed and the plates were kept on ice. Ice-cold DPBS was added to break up the Matrigel and the organoids released by pipetting several times through P1000 tips. Crypts cells were collected by centrifugation. Cell pellets were then incubated with 1 mL of TRIzol reagent and

RNA isolated as outlined by TRIzol kit. RNA concentrations were determined with a Nanodrop Lite spectrophotometer (Thermo Scientific). Reverse transcription was performed by using 1 µg of RNA and following instructions from the iScript cDNA Synthesis Kit (Bio-Rad). Real time PCR (RT-qPCR) experiments were carried out on a CFX96 QPCR system (BioRad) by using Ssoadvanced SYBR Green reagent (BioRad). Primers were designed through mouse primer depot (<https://mouseprimerdepot.nci.nih.gov/>). Transcription levels were quantified with the Ct value normalized to mouse cyclophilin (*Cph*).

Protein Preparation and Western Blots. Cells were released from matrigel. Cell lysates were prepared in a RIPA buffer. After protein concentration determination, protein samples were subjected to gel electrophoresis by utilizing Nupage 4–12% (wt/vol) Bis-Tris gradient gel (ThermoFisher). Western blots were developed and imaged using a ChemiDoc Touch Imaging System (BioRad). The following antibodies were used: goat anti UGT1A (Santa Cruz, sc-27418), rabbit anti-cleaved caspase 3 (Cell Signaling, #9661), and anti- α -Tubulin (Sigma, T9026).

LC/MS studies of CPT-11, SN-38, and SN-38G. Sample preparation was performed as previously described (Marangon et al., 2015). Media or cell lysates were centrifuged, the supernatants were added with 0.1% acetic acid (v/v 1;3) containing the internal standards deuterated d10-CPT-11 and d3-SN-38 at 15 ng/ml. Standards were prepared by spiking 10 µL of working solutions in 90 µL of blank medium which sustains subsequent treatments as samples. Samples were centrifuged and one microliter of the supernatants was injected for analysis by a Prominence HPLC (Shimazu, Tokyo, Japan) coupled with and API4000 mass spectrometer (Sciex, Massachusetts, USA). Separation was achieved using a Gemini C18 column (100X4.6mm, 3µM; Phenomenex, Torrance, CA, USA), and MS analysis was achieved using positive mode with ion spray voltage set at 5000V and a source temperature of 600°C. The following transitions were used for quantification in MRM mode: CPT-11: m/z 587.4→124.2; SN-38: m/z 393.3→349.3; SN-

38G: m/z 569.3 \rightarrow 392.3, and the internal standards d10-CPT-11: m/z 597.4 \rightarrow 133.3, and d3-SN-38: m/z 396.2 \rightarrow 352.2.

Results

Development of crypt organoids and the expression of drug metabolism enzymes. Each crypt culture contained multiple cells and normally formed spherical structures on the first day after the incubation (**Fig 1A**), followed by budding. Small buds usually could be visualized in two to four days after the incubation. The crypt region underwent continuous budding events; by day 5 after incubation, the lumen of organoids became distorted and dark, which were filled with matured enterocytes or apoptotic cells (**Fig 1A**). Well-developed organoids were collected between day 7-10 after cells were passaged, and RT-qPCR was conducted to investigate gene expression patterns. As demonstrated in **Figure 1B**, Phase I and Phase II XPGs along with transporter genes were identified in mature crypt organoids. Cytochrome P450 (*Cyp*) gene expression for *Cyp2b10* and *Cyp3a11* along with other XPGs including *alcohol dehydrogenase Adh1*, *aldehyde dehydrogenase Aldh1a1* and *Aldh1a7* were actively expressed, all of which have been known to exhibit high expression levels in intestine (Vaglenova et al., 2003; Huang et al., 2009; Renaud et al., 2011). In comparison to *carboxylesterase (Ces) 1*, *Ces2* is known as the intestinal specific isoform (Hatfield et al., 2011), which is consistent with our results, although RT-qPCR results cannot provide absolute gene expression levels. Phase II XPGs were highlighted by gene expression of *Gst* in addition to the *Ugts* encoded by the *Ugt1* and *Ugt2* gene families, both of which are intestinal enriched genes (Gibbs et al., 1998; Strassburg et al., 1999; Shin et al., 2009). Efflux drug transporters were also identified in crypt organoids, such as *P-glycoprotein (Pgp, Abcb1a)* and *Mrp2 (Abcc2)*, both of which are important transporters of many drugs and xenobiotics in enterocytes (Sparreboom et al., 1997; Mottino et al., 2000), along with intestinal specific transporters *Abcg5/8* (Berge et al., 2000), which are important sterol efflux pumps.

Functional nuclear receptors observed in organoids that respond to xenobiotic treatment.

Fundamental to understanding the regulatory events associated with DMEs has been a clear vision characterizing the role of the xenobiotic nuclear receptors (XNRs) in control and expression

of the xenobiotic processing genes (XPGs) (Rushmore and Kong, 2002; Staudinger et al., 2010; Chai et al., 2013; Staudinger et al., 2013; Hoffmann and Partridge, 2015). Xenobiotic and environmental toxicant exposure has the potential to modify XPG expression through interactions with XNRs. Altered gene expression through activation of XNRs affects drug metabolism as well as drug-drug interactions, leading to changes in drug efficacy and safety. The gene expression levels of XNRs in crypt organoids were determined, as shown in **Figure 2A**. We examined expression of the genes encoding the aryl hydrocarbon receptor (*Ahr*), pregnane X receptor (*Pxr*), constitutive androstane receptor (*Car*), peroxisome proliferator-activated receptors (*Ppar*), retinoid X receptor (*Rxr*), and the liver X receptor (*Lxr*), and RNA encoded by each gene was detectable. These gene expression patterns are relative to *Cph* gene expression and do not reflect comparative expression values of RNA between the genes, since the primers for each gene may have differences in their annealing properties. To determine their functionality, crypt organoids were then treated with specific XNR ligands and downstream target gene expression evaluated. TCDD treatment, the prototypical agonist of the AhR (Quattrochi and Tukey, 1993), induced *Cyp1a1* gene expression (**Fig 2B**); BNF, an AhR agonist (Sinal et al., 1999), also induced *Cyp1a1* (**Fig 2B**). Similarly, when crypt organoids were treated with TCPOBOP, a mouse specific CAR agonist (Tzamei et al., 2000), *Cyp2b10* was induced (**Fig 2C**). *Cyp3a11* gene expression was induced by PCN (**Fig 2D**), a well-studied PXR ligand (Guo et al., 2002). We observed that *Cyp3a11* was also induced by TCPOBOP and the LXR agonist T0901317, implying that *Cyp3a11* gene expression can also be regulated by activated CAR and LXR. *Cyp4a10* was largely induced by the PPAR α agonist WY14643 (**Fig 2E**). Drug transporter genes were also inducible, as shown in **Fig 2F** with induction of *Abca1* gene expression by the LXR agonist T0901317.

Crypt organoids isolated from XNR-knockout mice for XPG expression studies. Knockout (KO) mouse models have been used to understand the physiological and pharmacological role of a specific gene. Knockout mouse models have also been exploited in drug metabolism studies,

particularly in elucidating XNR-mediated gene regulation of XPGs. Here we have utilized both global and intestinal specific KO mouse models to investigate the utilization of organoid cultures in drug metabolism studies. Crypt cells were isolated from both wildtype and *Ppara*^{-/-} mice, and after 5 days the organoids were treated with breast milk (1:100 dilution), the fatty acid DHA at 5 μ M, and the PPAR α agonist WY14643 at 100 μ M. Twenty-four hours after treatment, cells were collected for RT-qPCR analysis. In wildtype cells, breast milk, DHA and WY14643 activated gene expression of the PPAR α targeted gene *Cyp4a10*; however, these inductions were completely diminished in crypt organoids isolated from *Ppara*^{-/-} mice (**Fig 3A**). Similarly, PCN also lost its capability in mediating the *Cyp3a11* induction in *Pxr*^{-/-} organoids (**Fig 3B**). These results confirm that crypt organoids maintain the genetic characteristics that are exhibited in KO mice following activation of XNRs.

Intestinal crypt cultures metabolize CPT-11. Stable crypt organoids express CES1 & 2, UGT1A1, and the transporters ABCC2 and ABCB1A based upon gene expression analysis. CPT-11 enters intestinal epithelial cells and is cleaved by CES to the active topoisomerase inhibitor SN-38, a product that is toxic and damaging to tissues if not metabolized further by glucuronidation. To determine if intestinal crypt cultures could metabolize CPT-11 and culminate with the formation of the SN-38 glucuronide (SN-38G), cells were first treated with a range of CPT-11 concentrations (**Fig 4A**). After exposure for 24 h, culture media (extracellular) and cells (intracellular) were harvested for quantitation of SN-38 and SN-38G by HPLC-MS analysis. In culture media, as the concentration of CPT-11 was increased there was a concordant increase in SN-38 and SN-38G (**Fig 4A**). The concentrations of SN-38 fit a linear model with R-squared at 0.99, but the SN-38G levels showed poor linearity with R-squared at 0.84 indicating enzymatic saturation (**Fig 4A**). As the concentration of CPT-11 is increased, there is the rapid appearance of intracellular SN-38 at 10 μ M CPT-11 that is nearly triple the concentration of intracellular SN-38G (**Fig 4B**), supporting the extracellular values that implicate UGT1A1 saturation. When the

ratios of SN-38G/SN-38 were determined in both compartments, the ratios were much higher in the extracellular compartment, indicating that both SN-38 and SN-38G are removed from the cell by efflux transporters (**Fig 4C**). The highest concentration of CPT-11 used resulted in a sharp decline in both the extracellular and intracellular SN-38G/SN-38 ratios, indicating that enzymatic cleavage of CPT-11 to SN-38 and the resulting glucuronidation of SN-38 to SN-38G were declining. Because SN-38 is toxic, this result corresponded to an increase in cell death, as demonstrated by induction of cleaved caspase 3, a marker of cell apoptosis (**Fig 4D**).

In the absence of glucuronidation, the intestinal epithelium is sensitive to the toxic actions of SN-38 (Chen et al., 2013). When we targeted the deletion the *Ugt1* locus and the *Ugt1a1* gene in *Ugt1^{ΔIEC}* mice and challenged *Ugt1^{ΔIEC}* mice with CPT-11, they were highly susceptible to diarrhea in comparison to wild type *Ugt1^{F/F}* mice (Chen et al., 2013). Apoptosis was not observed in *Ugt1^{F/F}* crypt cells when treated with CPT-11 at 1 μM, but apoptosis was apparent in crypt organoids from *Ugt1^{ΔIEC}* mice at this concentration (**Fig 4E**), in which cells showed no expression of UGT1 protein (**Fig 4E**) and failed to form the metabolite SN-38G (**Fig 4F**). Therefore, crypt organoids from *Ugt1^{ΔIEC}* mice were at least 10-fold more sensitive to the toxic actions of CPT-11 than *Ugt1^{F/F}* mice. Crypt organoid cultures can be employed to examine the metabolic outcome of drug and xenobiotic metabolism, the outcome of metabolism following XNR activation, and the disposition of metabolism under conditions of altered pharmacogenetics.

Discussion

Recent advances in establishing long-term culture conditions of crypt-villi that propagate into functional organoids with intact luminal surfaces have made it possible to investigate intestinal epithelial cell functionality in tissue culture (Sato et al., 2009; Sato and Clevers, 2013). As intestinal epithelial cells turn over in adults, for example, every 5 days in mice, new epithelial cells are formed from long-lived intestinal stem cells (ISCs) that sit at the base of the crypts (Barker et al., 2008). These stem cells produce the rapidly proliferating progenitor cells called transit-amplifying (TA) cells that migrate up the crypt-villus axis and eventually differentiate into mature epithelial cells. With discovery of the signaling pathways that are necessary to maintain stemness leading to epithelial cell proliferation, environmental cues have been employed to promote the crypt ISCs to form mini-gut cultures that are composed of budding epithelial cells (Sato et al., 2009; Sato et al., 2011; Sato and Clevers, 2013). These 3D mini-gut organoid cultures resemble *in vivo* intestinal epithelial structures, with a single ISC producing the different types of differentiated cells needed to support the development of the mature enterocytes (Sato et al., 2009; Sato et al., 2011). It has also been demonstrated that the organoids maintain absorptive and digestive functions making the tissue an excellent model to examine drug uptake and metabolism and the impact of xenobiotic exposure on epithelial cell function. Taking advantage of this unique physiology, we explored the use of 3D-mini-gut organoids to examine XPG expression, inducibility and metabolism.

Within 8 days of culturing crypt stem cells, the expanding organoids show well-structured epithelial cell projections. Constitutive expression of XPGs was abundant, with those encoding the classically identified Phase I, Phase II and Phase III (transporters) genes well represented. The culture medium contains high concentrations of N-acetylcysteine, which is a substrate in the synthesis of glutathione (Weinander et al., 1994). Elevated glutathione concentrations provide a rich reductive capacity for the cell, indicating that organoid cultures may be an excellent model to

examine receptor mediated induction of XPGs by drugs and xenobiotics without producing excessive oxidative stress. The major XNRs that are targeted for activation by drugs and xenobiotics are also expressed, and include the AhR, PXR, CAR, PPAR α and LXR, which are key modulators of gene expression that often control the balance between exposures resulting in a toxic or efficacious outcome. Expression of the XNRs is functional in the proliferating organoids, as we demonstrated by receptor mediated induction of key genes linked to drug transport and metabolism. Similar results have recently been demonstrated (Bijmans et al., 2017). There was high specificity for activation of the AhR by TCDD and induction of *Cyp1a1* gene expression (Strom et al., 1992). While BNF is an agonist of the AhR, it also induces *Cyp1a1*, but the response is not as potent as TCDD (Sinal et al., 1999). Interestingly, we did not notice any unusual growth inhibition properties of our proliferating organoids following TCDD treatment, as was noted in a recent publication examining growth properties following activation of the AhR (Park et al., 2016). These differences may result from the concentrations of TCDD used; while we found excellent induction using 50 nM, Park et al (Park et al., 2016). treated their organoid preparations with 0.1 to 1.0 μ M. The mouse *Cyp2b10* gene is selectively induced by mouse specific CAR ligands (Honkakoski et al., 1998). Activation of PXR by PCN generates robust induction of *Cyp3a11* gene expression. Activation of CAR has been shown previously to induce *Cyp3a11* (Hernandez et al., 2009), corroborating our results with crypt organoids demonstrating induction of *Cyp3a11* gene expression by the CAR activator TCPOBOP. This is further supported when crypt organoids deficient in PXR exhibit elevated *Cyp3a11* gene expression when treated with TCPOBOP. In addition, activation of LXR by T0901317, which targets activation of *Abca1* gene expression (Wagner et al., 2003), can also support induction of *Cyp3a11* gene expression. These findings indicate that *Cyp3a11* can be regulated following activation of PXR, CAR and LXR. We have shown previously that oral administration to mice with the PPAR α agonist WY14643 induces intestinal CYP4A protein expression (Senekeo-Effenberger et al., 2007). Significant activation of

Cyp4a10 gene expression in organoid cultures is also documented following exposure with WY14643, a response which is eliminated in cultures derived from *PPAR α -null* mice.

The functionality of the crypt organoid cultures can be exploited for metabolism and toxicology studies by implementing cultures derived from XNR and XPG knockout mice. We demonstrated this capability using *Ugt1 ^{Δ IEC}* mice, where we have targeted the specific deletion of the *Ugt1* locus in intestinal epithelial cells. These mice are deficient in the UGT1A proteins, including UGT1A1. Characteristic of crypt organoids and intestinal epithelial cells, they express CES2, necessary to cleave the prodrug CPT-11 to SN-38, the active topoisomerase 1 inhibitor. The high expression levels of CES2 is an excellent example that documents the maintenance of tissue specific expression markers that can be exploited to examine drug metabolism. It supports previous reports from Clevers (Sato et al., 2009; Sato and Clevers, 2013) and more recently the van Mills group (Bijsmans et al., 2017) that gene expression profiles in intestinal organoids resemble patterns observed in normal tissue. We had shown previously in *Ugt1 ^{Δ IEC}* mice that treatment with CPT-11 led to severe intestinal damage, resulting from the efficient generation of SN-38 coupled with the inability to generate UGT1A1 dependent SN-38G (Chen et al., 2013). Organoids generated from *Ugt1^{F/F}* mice efficiently generated SN-38 and SN-38G, with cell apoptosis being evident at 10 to 100 μ M CPT-11. When organoids from *Ugt1 ^{Δ IEC}* mice were treated with the same concentrations of CPT-11, remarkable cell apoptosis was evident at 1 μ M CPT-11. These findings provide evidence that examining drug metabolism in crypt organoids can be efficiently linked to specific XPGs while confirming a mechanistic link between gene expression and toxicity.

In conclusion, crypt organoids maintain functional, tissue specific gene expression encoding DMEs, and can be further regulated following activation of XNRs. Crypt organoids developed from stem cells isolated from genetically modified mice maintain their engineered phenotypical features, as determined both at the gene expression and functional levels. It is

argued that the use of organoid cultures may eventually reduce the need for animal models in scientific research, since the cultures maintain tissue specific gene expression which can be regulated following activation of XNRs (Clevers, 2013; Bijsmans et al., 2017). While the organoid cultures are a superior model when compared to primary cells or long term differentiated cells, there will always be the need for drug testing in vivo. An example of this need is to define the mechanisms leading to regulation of intestinal *UGT1A1* gene expression during development. Neonatal jaundice and severe forms of neonatal hyperbilirubinemia develop because of delayed expression of the *UGT1A1* gene during development, and its expression in the GI tract is critical in preventing bilirubin toxicity. Agents that activate many of the XNRs when given orally to neonatal *hUGT1* mice induce intestinal *UGT1A1* and reduce serum bilirubin. These same agents can induce the *UGT1A1* gene in tissue culture. However, we have discovered that intestinal *UGT1A1* gene expression is very responsive to agents that induce mild oxidative stress (Liu et al., 2016). This new mechanism, which can be replicated only in vivo, may lead to the identification of new agents or therapeutics that may be considered as new therapy in the future to prevent the substantial mortality and permanent morbidities resulting from severe neonatal hyperbilirubinemia, which is estimated to impact over 1-million newborns every year (Wong et al., 2011; Bhutani et al., 2013). Thus, crypt organoids could become a valuable tool in drug metabolism and toxicity studies to complement those efforts being conducted with humanized and genetically modified mice.

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Authorship Contribution

Participate in research design: Chen.

Conduct experiments: Lu, Rettenmeier, Paszek, Trottier, Barbier, Chen.

Contributed new reagents or analytic tools: Trottier, Barbier.

Performed data analysis: Lu, Rettenmeier, Trottier, Barbier, Chen.

Wrote and contributed to the writing of the manuscript: all authors.

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Legends for Figures

Figure 1. The growth of small intestinal crypt organoids and the expressions of XPGs in mature crypt organoids.

(A) Shown are mouse small intestinal crypt organoid cultures at various times after primary culture. Images were taken with a Nikon Eclipse Ts2 inverted microscope, with the scale bar shown at 90 μm . (B) Mature crypt organoids (in triplicate) were collected 8 days after plating and RNA prepared for RT-qPCR analysis. Gene expression of Phase I and Phase II drug metabolism genes and transporters are expressed as *Target genes/mouse Cph* and are described as average \pm SEM (n=3). Data represent multiple independent experiments with similar results.

Figure 2. Gene expression analysis of NRs and their function in mature crypt cells.

(A) Shown are nuclear receptor expression levels from mature crypt cells conducted by RT-qPCR analysis, average \pm SEM (n=3). (B-F) Crypts in exponential growth period were treated with different nuclear receptor agonists, including TCDD (50 nM), βNF (20 μM), TCPOBOP (10 μM), PCN (30 μM), T0901317 (100 μM), and WY14643 (100 μM). Twenty four hours after agonist exposure, crypt cells were collected for RT-qPCR analysis (average \pm SEM, experiments carried out in triplicate).

Figure 3. RT-qPCR results of mature crypt cells from both wildtype and genetically modified mice.

(A) Crypt cells were isolated from *Ppara*^{+/-} (wild type) and *Ppara*^{-/-} (*Ppara* deletion) mice. After several passages, crypt cells were treated with vehicle control (Ctrl, DMSO at 0.1%), breast milk (BMK) at 1:100 (v/v) dilution into the culture medium, DHA (5 μM), and WY14643 (100 μM) for 24

h. Cells were collected for RT-qPCR analysis to measure *Cyp4a10* gene expression. Results were normalized with vehicle treated wild-type crypts and described as fold change (average \pm SEM, n=3). (B) Crypt cells were isolated from wild type *Pxr^{+/-}* and *Pxr^{-/-}* knockout mice. Cells were treated with PCN at 30 μ M and TCPOBOP at 10 μ M for 24 h followed by *Cyp3a11* gene expression analyzed by RT-qPCR (n=3). ns: nonsignificant, * p<0.05, **p<0.01, student's t-test.

Figure 4. Irinotecan metabolism and toxicity studies by utilizing crypt organoids from both *Ugt1^{FF}* and *Ugt1 ^{Δ IEC}* mice.

(A-D) Crypts were isolated from *Ugt1^{FF}* mice. Cells were treated with CPT-11 at 0, 0.1, 1, 10, and 100 μ M (in triplicate). After 24 h, both media and cells were collected for HPLC-MS analysis. SN-38 and SN-38G were determined in both (A) extracellular compartment, and (B) intracellular compartment (cells from three wells were combined and then subjected to the bioanalysis), (C) the ratio of SN-38G/SN-38. (D) Apoptosis was analyzed by Western blot of cleaved caspase 3, which was further quantified by using the ChemiDoc Touch Imaging System and Image Lab software (BioRad), and the data was calculated based on independent Western blots (average \pm SEM, n=3). (E-F) Crypts were isolated from both *Ugt1^{FF}* and *Ugt1 ^{Δ IEC}* mice. After three days, cells were treated with control or CPT-11 at 1 μ M. (E) Twenty four hours later, whole cell extracts were prepared for Western blot analysis using an anti-UGT1A, anti-active caspase 3, and anti α -Tubulin antibodies. Western blots of cleaved caspase 3 were further quantified. (F) Cell culture media was collected for bioanalysis of SN-38G (average \pm SEM, n=3). bld, below limit of detection.

Figure 1

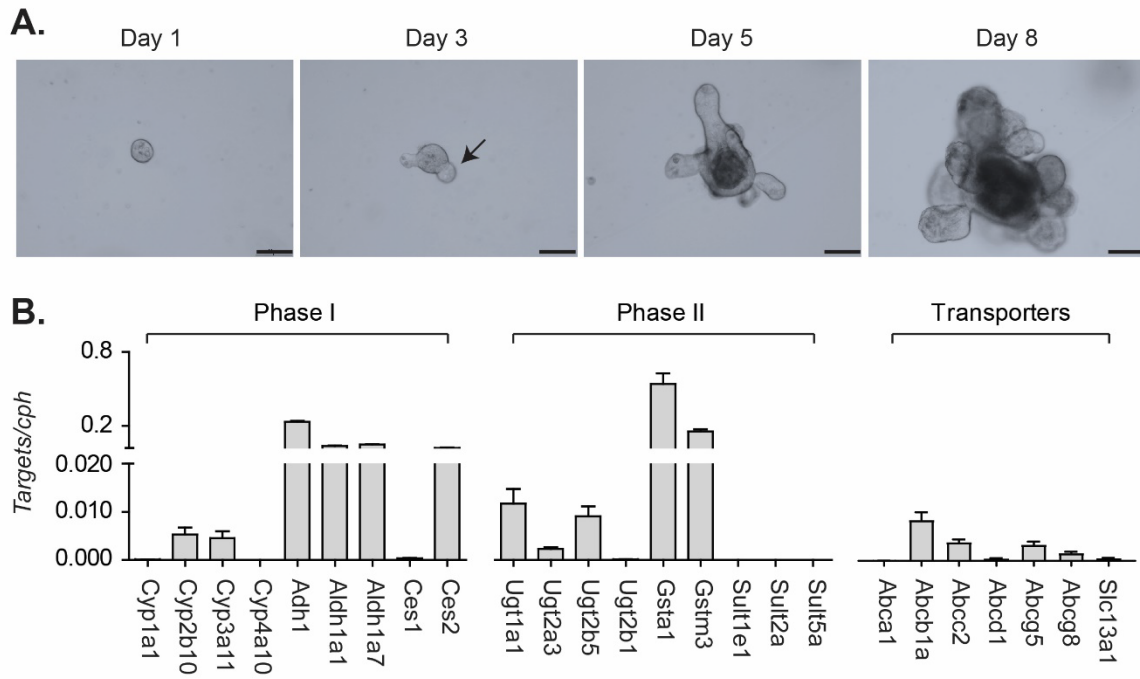


Figure 2

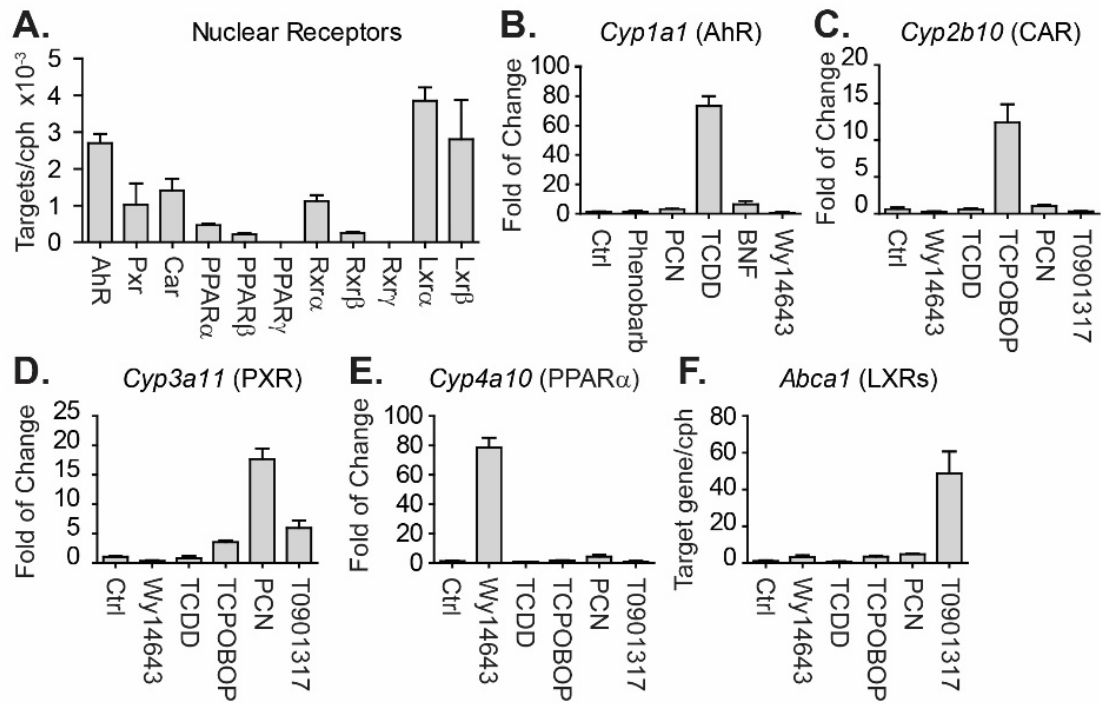


Figure 3

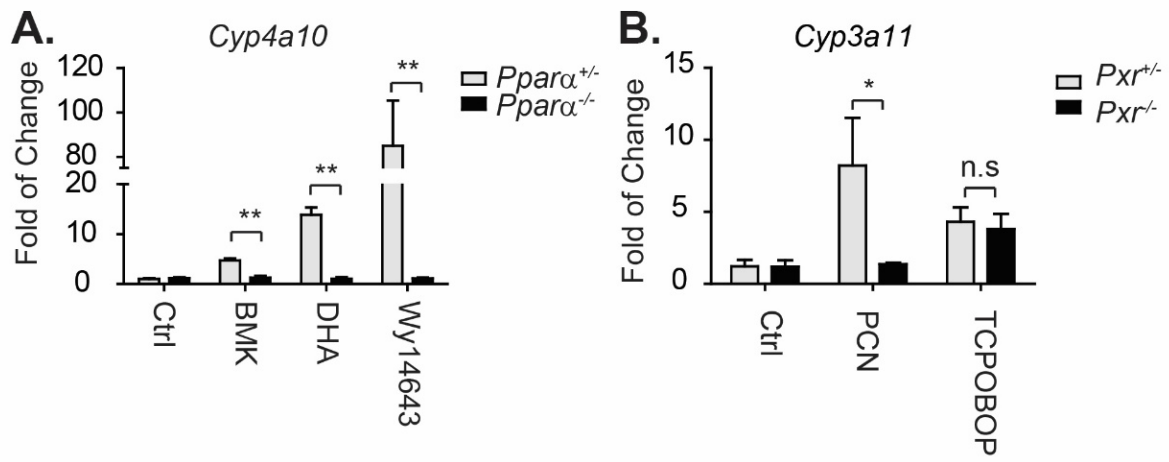


Fig 4

