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REPRESSION OF MULTIPLE CYP2D GENES IN MOUSE PRIMARY HEPATOCYTES WITH A SINGLE siRNA CONSTRUCT

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Abstract

The CYP2D subfamily is the second most abundant subfamily of hepatic drug metabolizing CYPs. In mice, there are nine Cyp2d members that are believed to have redundant catalytic activity. We are testing and optimizing the ability of one siRNA construct to knockdown the expression of multiple mouse Cyp2ds in primary hepatocytes. Expression of Cyp2d10, Cyp2d11, Cyp2d22, and Cyp2d26 was observed in the primary male mouse hepatocytes. Cyp2d9, which is male-specific and growth hormone-dependent, was not expressed in male primary hepatocytes, potentially because of its dependence on pulsatile growth hormone release from the anterior pituitary. Several different siRNAs at different concentrations and with different reagents were used to knockdown Cyp2d expression. siRNA constructs designed to repress only one construct often mildly repressed several Cyp2d isoforms. A construct designed to knockdown every Cyp2d isoform provided the best results, especially when incubated with transfection reagents designed specifically for primary cell culture. Interestingly, a construct designed to knockdown all Cyp2d isoforms, except Cyp2d10 caused a 2.5X increase in Cyp2d10 expression, presumably because of a compensatory response. However, while RNA expression is repressed 24 hours after siRNA treatment, associated changes in Cyp2d-mediated metabolism are tenuous. Overall, this study provides data on the expression of murine Cyp2d's in primary cell lines, valuable information on designing siRNAs for silencing multiple murine Cyps, and potential pros and cons of using siRNA as a tool for repressing Cyp2d and estimating Cyp2d's role in murine xenobiotic metabolism.

Keywords

primary hepatocytes; in vitro; RNAi; P450; Cyp2d

INTRODUCTION

The CYP2D subfamily has one functional isoform in humans, CYP2D6 (Nelson et al. 2004), the second most important CYP in drug metabolism (Ingelman-Sundberg 2001, Zanger and Schwab 2013). CYP2D6, which is highly polymorphic, is involved in the metabolism of over 80 drugs in clinical use, including the selective serotonin reuptake inhibitors (SSRI), tricyclic antidepressants, beta-blockers, opiates, neuroleptics, and antiarrhythmics (Zhou

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2009). In contrast to humans, mice have nine different mouse Cyp2d genes; Cyp2d9, Cyp2d10, Cyp2d11, Cyp2d12, Cyp2d13, Cyp2d22, Cyp2d26, Cyp2d34 and Cyp2d40 (Nelson, et al. 2004). Most of the murine Cyp2d isoforms are expressed in the liver (Miksys et al. 2005) with Cyp2d9 showing strong male specificity because of pulsatile growth hormone release (Park et al. 1999, Wong et al. 1987).

Although mice are used to assess drug and toxicant metabolism, considerable differences exist between the expression and catalytic activity of human and mouse CYP2D isoforms (Ai-Ming and Haining 2006). Understanding these differences will help in properly estimating human risk. Because there are nine murine Cyp2d members, repressing them or knocking them out in cell systems by typical gene targeting is not trivial. RNA interference (RNAi) is one potential method to inhibit multiple Cyp2d isoforms (Elbashir et al. 2001, Fire et al. 1998). Using RNAi to repress multiple CYPs has been done recently, but with Cyp2b (Damiri et al. 2012) and selected Cyp3a (Wang et al. 2012) subfamily members that are significantly more conserved.

We plan to use RNAi to knockdown murine Cyp2d subfamily members with a siRNA homologous to all or most murine Cyp2ds. The objectives of this study are to find and characterize an *in vitro* model system that expresses mouse Cyp2d enzymes, test whether multiple Cyp2d constructs could be repressed using an siRNA approach, and determine the ongoing feasibility of an RNAi approach for repressing multiple Cyp2d enzymes.

MATERIALS AND METHODS

Cell Culture

RHII/Pr1 cells that express Cyp2d22 (Blume et al. 2000) were obtained from Strang Cancer Research Laboratory (New York, NY, USA). *HEPA1C1C7* cells (Hankinson et al. 1985) were purchased from ATCC (Manassas, VA, USA). All cells were incubated and maintained as described previously (Baldwin and Roling 2009). CD-1 mouse primary hepatocytes were purchased from Celsis In Vitro Technologies (Chicago, IL, USA).

siRNA design and transfections

ClustalW was used to align the Cyp2d subfamily members (Suppl File 1). The siRNAs were designed to areas of high homology so that they would knockdown the expression of all or most Cyp2ds (Damiri, et al. 2012). Two siRNA constructs homologous to Cyp2d10 (Cyp2d10-design A, Cyp2d10-design B) and a negative control scrambled construct (SC) were designed by SABiosciences (Frederick, MD, USA). The sequence of each siRNA construct and estimated knockdown efficiency using siRNA scales (http://gesteland.genetics.utah.edu/siRNA_scales/) (Mateeva et al. 2007) is in Suppl File 2.

siRNA Transfections and Sample Preparation

Primary mouse hepatocytes were transfected with 2–12 μ M siRNA using SureFECT® (SABiosciences), TransIT-TKO® or TransIT-siQUEST® (Mirus Bio, Madison, WI, USA) transfection reagents according to the manufacturer's protocol. RNA was extracted 24 hours later and cDNA prepared (Mota et al. 2011). Transfection efficiency was determined with

siRNA labeled with the Label IT® siRNA Tracker Kit® (Mirus Bio). Cells were visualized with a Zeiss LSM510 Laser Confocal Microscope with optics for red and green fluorescence.

RNA and protein expression

PCR and quantitative real-time PCR (qPCR) was performed as described previously (Hernandez et al. 2006, Muller et al. 2002) using gene specific primers (Suppl File 3). Western blots were conducted with a Rabbit anti-Human CYP2D6 polyclonal primary antibody (Chemicon International, Billerica, MA, USA), and goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA) (Mota et al. 2010).

Metabolism

Testosterone hydroxylation was measured using [¹⁴C]testosterone as previously described (Hernandez et al. 2009), but with primary hepatocytes (Kamiguchi et al. 2010). P450-Glo™ CYP2D6 Assay kits were used as described by the manufacturer (Promega, Madison, WI, USA) Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) metabolism was determined in murine hepatocytes following a 2h incubation. Deconjugation was performed by adding bovine β-glucuronidase and *Helix pomatia* sulfatase (Baldwin et al. 1997). Extraction of the metabolites was performed as described by (Lee et al. 2003). Extraction efficiency was determined by using internal standards of tamoxifen, 4-hydroxy-tamoxifen, and endoxifen. Metabolite concentrations were measured using a Waters 1525 binary Reverse Phase-HPLC (HyperSelect Gold C18, 5 μm, 120 Å particle size, 250 mm × 4.6 mm with guard column; ES Industries Chromega Columns, West Berlin, NJ). The mobile phase consisted of 35% acetonitrile and 65% 20 mM potassium phosphate buffer (pH=3) over 70 minutes at a flow rate of 1 ml/min. Chemical detection was determined at an excitation wavelength of 256 nm and an emission wavelength of 380nm.

Computational analysis

Cyp2d10 promoter regions (1500bp upstream of the transcriptional start site through the first intron) with a score greater than 95.0 on TRSearch (Heinemeyer et al. 1998) (<http://www.cbrc.jp/research/db/TFSEARCH.html>) are reported. Statistical significance of the qPCR data was determined by Student's t-tests or ANOVA followed by Tukey's multiple comparison test with GraphPad Prism 4.3 (La Jolla, CA).

RESULTS and DISCUSSION

Expression of Cyp2d isoforms in cell culture

The immortal cell lines tested (*RIIPrI* and *HEPA1C1C7*) do not express enough Cyp2d for subsequent siRNA knockdown as determined by Western blotting and PCR (Suppl File 4AB). Male mouse primary hepatocytes express Cyp2d10/11/22/26 (Suppl File 4CD). Cyp2d9, a male specific CYP, was poorly expressed in the male mouse primary hepatocytes. cDNA from the livers of wild-type male (WT-M) mice expressed Cyp2d9, but wild-type female (WT-F) mice did not show expression of this Cyp2d subfamily member. Cyp2d9 expression is controlled by the pulsatile release of growth hormone (Krebs et al. 2003, Park, et al. 1999). Therefore, we hypothesize that the lack of pulsatile growth hormone (GH)

release from the anterior pituitary in vitro causes the loss of Cyp2d9 expression from the male hepatocytes within 48 hours. This suggests that GH-dependent male sexual dimorphism is difficult to sustain in primary cultures.

Knockdown of Cyp2d isoforms by different siRNAs

ClustalW indicates there are two areas of the Cyp2d subfamily that are highly homologous, and therefore it would be possible that one siRNA could repress the expression of 8 – 9 isoforms (Suppl File 1). siRNA Cyp2d-KD1 is expected to repress the expression of all Cyp2d isoforms; siRNA Cyp2d-KD2 is expected to repress the expression of all but Cyp2d10 because of a single nucleotide mismatch in the middle of the sequence.

Primary hepatocytes were transfected with one of four siRNAs or a scrambled construct as the negative control using SureFECT transfection reagent. qPCR was performed to evaluate the expression of Cyp2d10/11/22/26 (Table 1; Suppl File 5a–d). Cyp2d10 expression was significantly down regulated by siRNA Cyp2d10-design A and Cyp2d-KD1 in a concentration-dependent manner. Cyp2d10-design B only reduced Cyp2d10 expression at 6 μ M. Cyp2d11, 22, 26 all showed dose-dependent trends suggestive of expression repression by the siRNA constructs, but the results were not statistically significant (Table 1; Suppl File 5).

In contrast, Cyp2d10 was up-regulated when Cyp2d-KD2 was transfected into hepatocytes (Table 1). Cyp2d-KD2 was designed to knockdown the expression of all of the Cyp2d members except Cyp2d10, indicating that the loss of the other Cyp2d members caused a compensatory response. The transcriptional regulation of murine Cyp2d isozymes is not well studied. In general, Cyp2d group members are not considered inducible through typical toxicant-sensitive mechanisms (Matsunaga et al. 1989, Oka et al. 2000). However, several liver-enriched transcription factors are responsible for the constitutive expression of select Cyp2d subfamily members in humans and rodents, including HNF4 α , GABP, and C/EBP beta (Cairns et al. 1996, Lee et al. 1994, Yokomori et al. 1995). The expression of murine male-specific Cyp2d9 (Park, et al. 1999), drops precipitously in HNF4 α knockout mice (Wiwi et al. 2004), and siRNA-targeted to HNF4 α represses CYP2D6 expression about 40–45% in human hepatocytes (Jover et al. 2001). Interestingly, Cyp2d11, 22, and 26 are altered in pregnant mice (Topletz et al. 2013), but Cyp2d10 is not. A TSEARCH promoter search suggests several potential promoter elements including 25 HSF sites, 10 ADR1 sites, and a glucose regulated transcription factor (Ratnakumar and Young 2010, Young et al. 2009). Other putative promoter elements include (number of sites in parenthesis) NIT-2 (8), AP-1 (3), SRY (3), HSF2 (3), HSF1 (2), CdxA (2), Sp1 (1), S8 (1), AML-1a (1), GATA-1 (1), GATA-X (1), NF-E2 (1), C/EBP- β (1), and NKX-2 (1).

The two designs specific for Cyp2d10 worked based on statistically analysis; however, subjectively there appears to be down-regulation of other isoforms such as Cyp2d11 and Cyp2d22. Given the high homology between Cyp2d10 and Cyp2d11 this was expected. Overall, we determined that the best siRNA construct for repressing multiple Cyp2d isoforms was Cyp2d-KD1. However, expression was not repressed as much as desired and therefore we attempted to maximize knockdown efficiency by increasing siRNA content (12 μ M) or using multiple siRNA constructs concomitantly. Neither of these approaches worked

because of either enhanced toxicity or potentially competition by a weak acting siRNA that detracts from expression repression (Damiri, et al. 2012).

Therefore, we attempted to maximize transfection efficiency using transfection reagents specifically for use with primary cell culture. Transfection efficiency was greatest using TransIT-siQUEST (Suppl File 6); however, there was greater toxicity at 6 μ M. Subsequent studies were performed with TransIT-TKO, which had a transfection efficiency of about 90%. The first set of quantified transfections performed with TransIT-TKO were done to serve the dual purpose of testing whether this transfection reagent worked, and to test whether the scrambled siRNA construct interfered with Cyp2d expression. The mRNA expression of Cyp2d10/11/22/26 did not vary between mock transfected and scramble-transfected hepatocytes indicating that the scrambled siRNA has no interfering actions. Cyp2d-KD1 significantly repressed the expression of Cyp2d11 and Cyp2d22, and if not for the high variability of Cyp2d10 expression in the mock transfected cells, Cyp2d10 would have been significantly repressed (Fig. 1). Overall, Cyp2d-KD1 in conjunction with TransIT-TKO typically showed increased consistency between wells and an improved knockdown efficiency of 50–70%; a number as good as can be expected based on siRNA Scales (Suppl File 2).

Metabolism

We examined Cyp2d-mediated metabolism following siRNA transfection using testosterone 16 α -hydroxylation (Harada and Negishi 1984, Park, et al. 1999), tamoxifen metabolism (Zanger and Schwab 2013), and a human CYP2D6 P450-Glo substrate luciferin ME-EGE to determine if the increased breakdown of mRNA was translated to reduced CYP metabolic activity. Concomitant 6-well plates were used in these studies; one set for the metabolism studies and one set for qPCR. Cyp2d10/11/22/26 were all repressed significantly (40 – 60%) by 6 μ M Cyp2d-KD1 compared to SC (Fig. 2a).

Testosterone hydroxylation was not significantly reduced by Cyp2d-KD1 siRNA (Fig. 2b). Testosterone is metabolized in the 16 α -position by Cyp2b, Cyp2c, and Cyp2d members (Hernandez, et al. 2009, Waxman 1988). In male mice, Cyp2ds dominate the metabolism of testosterone at the 16 α -position, primarily because of Cyp2d9. However, this CYP appears to drop in expression dramatically in this mouse model because of the loss of pulsatile growth hormone release. Therefore, Cyp2d's role in 16 α -hydroxylation may have been minimized. This in turn may have allowed the Cyp2bs or Cyp2cs to play the primary role in the 16 α -hydroxylation of testosterone, especially since almost all CYP expression decreases approximately 90% within 24 hours in primary culture with the exception of Cyp2b9 and Cyp2b10, which increase (Mathijs et al. 2009).

Furthermore, neither tamoxifen nor the CYP2D6 P450-Glo substrate showed significant changes in metabolism after Cyp2d-KD1 exposure (Fig. 2cd). The P450-Glo assay is specifically designed for human CYP2D6 and has not been tested with mouse Cyp2d members. The murine and human CYP2Ds vary greatly in their substrate specificity (Ai-Ming and Haining 2006, Smith et al.), so using this assay was exploratory. Tamoxifen is in part metabolized by CYP2D6 to 4-hydroxytamoxifen that can be further metabolized by CYP3A4 to Endoxifen (Dezentjé et al. 2009). Tamoxifen is also metabolized by CYP3A4 as

well as other CYPs to N-desmethyldamoxifen (NDMT), which in turn is metabolized primarily by CYP2D6 to endoxifen. After siRNA incubation, 4-hydroxydamoxifen levels were low, endoxifen levels were not perturbed, but N-desmethyldamoxifen levels increased 3-fold in Cyp2d-KD1 cells; albeit not in a statistically significant manner (Fig. 2d). It is interesting to hypothesize that a drop in functional Cyp2d may have repressed the ability of the cells to utilize the NDMT to endoxifen pathway and ultimately lead to the buildup of NDMT. Furthermore, there may also be compensation that caused the increased expression of other CYP subfamilies during siRNA treatment. For example, the Cyp3a-knockout mouse shows increased Cyp2cs (Van Waterschoot et al. 2008). It is also possible that while Cyp2d mRNA levels are lower after 24 hours, a significant drop in protein or functional effects may take 48–72 hours (Paduano et al. 2006).

CONCLUSIONS

A single siRNA construct can be used to repress the expression of number of homologous Cyp2d subfamily members similar to previous studies with Cyp2b and Cyp3a members (Damiri, et al. 2012, Wang, et al. 2012). However, repressing the expression of several Cyps may have unexpected compensatory consequences as Cyp2d10 expression was significantly increased following the knockdown of other Cyp2d members. Metabolic changes following siRNA transfection were weak and their association with perturbed Cyp2d expression tenuous. Taken together, this suggests that the use of siRNA to knockdown multiple Cyp2d isoforms in vitro may need novel, GH-infused, or 3D systems. Overall, this research continues to build a foundation that furthers our toolset for the inhibition of CYPs and understanding their role in the metabolism of endogenous and exogenous chemicals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Repression of CYP2D isoforms in primary mouse hepatocytes by 6 μ M KD1 siRNA following transfection with TransIT-TKO transfection reagent. Cells were either mock-transfected (transfection reagent only; UT), transfected with the scrambled construct (SC), or transfected with the Cyp2d-KD-1 construct (KD-1) and then qPCR performed. Experiments were performed in 6-well plates in order to demonstrate that the SC construct does not repress transcription and KD-1 represses Cyp2d levels when the TransIT-TKO reagent is used. An asterisk indicates statistical significance ($p < 0.05$) by ANOVA followed by Tukey's multiple comparison test ($n = 2$).

Fig. 2. Chemical metabolism following CYP2D expression repression. Repression of CYP2D expression in primary hepatocytes is repeatable using the TransIT-TKO transfection reagent and the KD1 construct (n = 3) (A). Autoradiograph of a Thin-Layer Chromatography (TLC) plate following a testosterone hydroxylase assay (B). Human CYP2D P450-Glo assay from murine primary hepatocytes treated with SC or Cyp2d-KD-1 siRNA (C). The formation of endoxifen and N-desmethyldamoxifen (NDMT) following siRNA treatment in murine primary hepatocytes (D). An asterisk indicates statistical significance ($p < 0.05$) by Student's t-test (n = 3).

Table 1

CYP2D isozyme expression in murine primary hepatocytes treated with different siRNA constructs^a.

siRNA	Cyp2d10	Cyp2d11	Cyp2d22	Cyp2d26
Cyp2d10-A				
2 μ M	0.74 *	0.97	0.76	nd
6 μ M	0.52 **	0.40	0.56	1.13
Cyp2d10-B				
2 μ M	0.84	1.37	1.07	nd
6 μ M	0.68 *	0.65	0.60	0.61
Cyp2d-KD1				
2 μ M	0.73	0.58	0.64	nd
6 μ M	0.57 *	0.40	0.34	0.87
Cyp2d-KD2				
2 μ M	3.68 **	1.45	0.82	nd
6 μ M	2.68 **	0.53	0.41	0.52

nd = not done

^aData presented as relative to 2 or 6 μ M siRNA scrambled construct (SC) (n = 3)

* Indicates a significant difference from the corresponding SC by Student's t-test ($p < 0.05$).

** Indicates a significant difference from the corresponding SC by Student's t-test ($p < 0.01$).