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Coordinate regulation of *Cyp2e1* by β-catenin- and hepatocyte nuclear factor 1α-dependent signaling

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ABSTRACT

Depending on their position within the liver lobule, hepatocytes fulfill different metabolic functions. Cytochrome P450 (CYP) 2E1 is a drug-metabolizing enzyme which is exclusively expressed in hepatocytes surrounding branches of the hepatic central vein. Previous publications have shown that signaling through the Wnt/β-catenin pathway, a major determinant of liver zonation, and the hepatocyte-enriched transcription factor HNF (hepatocyte nuclear factor) 1α participate in the regulation of the gene. This study was aimed to decipher the molecular mechanisms by which the two transcription factors, β-catenin and HNF1α, jointly regulate CYP2E1 at the gene promoter level. Chromatin immunoprecipitation identified a conserved Wnt/β-catenin-responsive site (WRE) in the murine *Cyp2e1* promoter adjacent to a known HNF1α response element (HNF1-RE). *In vitro* analyses demonstrated that both, activated β-catenin and HNF1α, are needed for the full response of the promoter. The WRE was dispensable for β-catenin-mediated effects on the *Cyp2e1* promoter, while activity of β-catenin was integrated into the promoter response *via* the HNF1-RE. Physical interaction of β-catenin and HNF1α was demonstrated by co-immunoprecipitation. In conclusion, present data the first time identify and characterize the interplay of HNF1α and β-catenin and elucidate molecular determinants of CYP2E1 expression in the liver.

1. Introduction

The expression of many drug-metabolizing enzymes in the liver is dynamically controlled by drug-sensing nuclear receptors following their activation by foreign compounds. Well-studied examples of such receptors are, for example, the constitutive androstane receptor or the aryl hydrocarbon receptor AHR (Abel and Haarmann-Stemmann, 2010; Molnar et al., 2013). While the abovementioned concept is valid for many genes encoding proteins involved in phase I and phase II of drug metabolism, the murine *Cyp2e1* gene and its human ortholog *CYP2E1*, which encode the phase I enzyme cytochrome P450 (CYP) 2E1, are rather constitutively expressed in the liver in hepatocytes surrounding the central veins of the individual liver lobules (Buhler et al., 1992; Buhler et al., 1991) and Cyp2e1 expression is barely influenced by the well-studied xeno-sensing receptors which regulate most of the important drug-metabolizing enzymes.

Instead, *Cyp2e1* mRNA levels seem to be controlled mainly by rather internally-regulated signaling pathways, in particular by the hepatocyte-enriched transcription factor HNF1α and by the canonical Wnt/β-catenin pathway. The role of HNF1α in the transcriptional regulation of Cyp2e1 levels is well-established and has been shown by different groups *in vitro* and *in vivo*: transgenic mice with hepatic HNF1α deficiency exhibit markedly reduced levels of Cyp2e1 (Cheung et al., 2003). Molecular analyses have

Abbreviations: AHR, aryl hydrocarbon receptor; ChIP, chromatin immunoprecipitation; CMV, cytomegaly virus; CYP, cytochrome P450; GS, glutamine synthetase; HNF, hepatocyte nuclear factor; HNF1-RE, hepatocyte nuclear factor 1α response element; IP, immunoprecipitation; KO, knockout; TCF, T-cell factor; WB, Western blotting; WRE, wnt response element.

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demonstrated binding of HNF1α to the 5′-upstream regulatory region of the *Cyp2e1* gene and identified a functional HNF1α binding site at a position approximately 100 bp 5′ of the transcription starting point (Liu and Gonzalez, 1995; Ueno and Gonzalez, 1990). HNF1α also participates in the regulation of Cyp2e1 expression by cytokines (Hakkola et al., 2003).

It has been discovered about 10 years ago that signaling through the Wnt/β-catenin signaling pathway, highly important in embryogenesis, adult tissue homeostasis and tumorigenesis, is a master regulator of zonal gene expression in the liver, which especially controls the expression of genes which are physiologically expressed in hepatocytes near the central vein; for example see Braeuning and Schwarz (2010a),Hailfinger et al. (2006), Torre et al. (2011), and Yang et al. (2014). For details about the components of this signaling pathway, please refer to Lustig and Behrens (2003). In brief, activation of membrane-bound Frizzled receptors by Wnt agonists leads to destabilization of a cytosolic multi-protein complex which, in the absence of Wnts, controls β-catenin stability by phosphorylation of several residues near its N-terminus, with the latter post-translational modification being a pre-requisite for ubiquitinylation and proteasomal degradation of β-catenin. When the pathway is activated, β-catenin degradation is inhibited and accumulating β-catenin will translocate to the nucleus, where it functions as transcriptional co-activator. The classic binding partner of β-catenin is the transcription factor TCF4 (T-cell factor), but it has also been reported that β-catenin interacts in hepatocytes with other transcription factors important for the regulation of gene expression in hepatocytes, for example HNF4α (Colletti et al., 2009; Gougelet et al., 2014) and the AHR (Braeuning et al., 2011; Schulthess et al., 2015). Published analyses have shown that the Cyp2e1 mRNA is subject to regulation by β-catenin signaling *in vivo* and *in vitro*, both in liver cells of human and murine origin: for example, mice with hepatocyte-specific knockout (KO) of *Ctnnb1*, the gene encoding β-catenin, lack hepatic Cyp2e1 expression (Ganzenberg et al., 2013; Sekine et al., 2006), whereas activation of β-catenin signaling by expression of mutant, constitutively active β-catenin due to mutational activation of *Ctnnb1* in mouse liver tumors or in transgenic hepatocytes induces Cyp2e1 expression also in liver cells which are located in zones 1 or 2 of the liver acinus and therefore do not physiologically express Cyp2e1 (Schreiber et al., 2011). *In vitro*, modulators of the Wnt/β-catenin pathway are capable of inducing or inhibiting the expression of Cyp2e1 in primary hepatocytes of human and murine origin (Gerbal-Chaloin et al., 2014; Hailfinger et al., 2006), as well as in human HepaRG hepatocarcinoma cells (Thomas et al., 2015). Very recently, it has been discovered that the Wnt signaling agonist Rspondin3 is secreted by venous endothelial cells and determines zonal gene expression in the liver *in vivo* (Rocha et al., 2015).

While, as detailed above, it has been clearly demonstrated that β-catenin has a decisive role in the expression of Cyp2e1 in hepatocytes, the molecular mechanisms of that regulation are still unknown. This study was conducted to elucidate Cyp2e1 regulation by β-catenin and to analyze the convergence of the β-catenin and HNF1α pathways at the *Cyp2e1* promoter.

2. Materials and methods

2.1. Tissue samples

Mouse liver samples from previous studies were used (Braeuning et al., 2016; Braeuning et al., 2009; Braeuning and Schwarz, 2010b), including (i) wildtype mice, (ii) transgenic mice with expression of mutant, constitutively active human β-catenin^{S33Y} in a number of hepatocytes, (iii) mice with Albumin promoter-driven hepatocyte-specific knockout (KO) of *Ctnnb1* (encoding β-catenin) in practically all hepatocytes, as well as (iv) mice with a tamoxifen-inducible hepatocyte-specific KO of *Apc* in a number of hepatocytes. Mouse liver tumors samples from a previous experiment were used, in which mouse liver adenoma with mutationally activated β-catenin had been generated by the use of a N-nitrosodiethylamine/phenobarbital initiation/promotion protocol (Braeuning et al., 2016). Mouse liver adenoma samples with activated mitogen-activated protein kinase signaling due to activating mutations in *Ha-ras*, but without β-catenin activation were taken from another previous study (Jaworski et al., 2005). In addition, livers from mice with a tamoxifen-inducible hepatocyte-specific KO of *Apc* in >70% of hepatocytes were obtained by intraperitoneal injection of 1.5 mg tamoxifen to 8 weeks old mice similar to the protocol described in Braeuning et al. (2016), followed by sacrifice 13 days later. Only samples from adult male mice (C3H/HeN or C57Bl/6 background) were used.

2.2. CYP2E1 protein analysis

Immunohistochemical staining for CYP2E1 and glutamine synthetase (GS) using antibodies and standard methodology was performed as recently described (Braeuning et al., 2010), together with hematoxylin counterstaining. CYP2E1 protein was indirectly quantified by measuring the proteotypic peptide NEFSGR in the presence of an isotopically labeled reference peptide containing a C13/N15- labeled arginine (INTAVIS, Cologne, Germany). Briefly, liver samples were homogenized using a ball mill. Lysis buffer was added in a ratio of 1:10 (w/v) to the tissue sample (Eisen et al., 2013). Total protein was determined using a bicinchinonic assay method (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Enzymatic fragmentation by trypsin was performed using 60 μg protein extract per sample, triethanolamine buffer (50 mM, pH 8.5) and 0.5% *N*-octyl-glycopyranoside. Samples were reduced by adding tris(2-carboxyethyl)phosphine (5 mM) for 5 min at 99 °C and alkylated by incubation with iodoacetamide (10 mM) for 30 min at room temperature. Trypsin was added in a ratio of 1:20 (trypsin:protein). The digestion was performed for 16 h at 37 °C and stopped by heating samples at 99 °C for 5 min and adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM. Then 20 μg digested sample were mixed with stable isotope-labeled peptide and 1 μg antibody recognizing the c-terminal epitope FSGR. The antibody has been generated as described previously (Hoeppe et al., 2011). Peptide-antibody-complexes were precipitated with magnetic protein G microspheres (Thermo Scientific) (Volk et al., 2012). Precipitated peptides were separated by nLC. The CYP2E1 peptide was detected using targeted Single-Ion-Monitoring (tSIM) on a Q Exactive Plus (Thermo Scientific) as described earlier (Weiss et al., 2015). Raw data were evaluated using Pinpoint 1.4 (Thermo Scientific). The CYP2E1 peptide was quantified at parent ion level by calculating the peak area ratio of isotopically labeled and endogenous peptide.

2.3. Chromatin immunoprecipitation and sequencing

ChIP-seq and mRNA-seq experiments have been described elsewhere (Gougelet et al., 2014). Briefly, hepatocytes were isolated from a murine model of hepatic β-catenin loss-of-function (*Ctnnb1*$^{loxP/loxP}$ × *TTR-CreER*^{T} tamoxifen-injected mice, referred to as β-catenin^{ko}) and from a hepatic β-catenin gain-of-function (*Apc*$^{loxP/loxP}$ × *TTR-CreER*^{T} tamoxifen-injected mice, referred to as Apc^{ko}). Thereafter mRNAs were extracted, and chromatin was immunoprecipitated using an antibody against TCF4, the transcriptional partner of hepatic β-catenin, or against β-catenin itself. For further details, please refer to the above-mentioned paper.