Silencing LRH-1 in colon cancer cell lines impairs proliferation and alters gene expression programs

James R. Bayrer^a, Sridevi Mukkamala^b, Elena P. Sablin^b, Paul Webb^c, and Robert J. Fletterick^{b,1}

^aDivision of Pediatric Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, University of California, San Francisco, CA 94143-0136;

^bDepartment of Biochemistry and Biophysics, University of California, San Francisco, CA 94158-2517; and ^cDepartment of Genomic Medicine, Houston Methodist Research Institute, Houston, TX 77030

Contributed by Robert J. Fletterick, January 16, 2015 (sent for review September 22, 2014)

Colorectal cancers (CRCs) account for nearly 10% of all cancer deaths in industrialized countries. Recent evidence points to a central role for the nuclear receptor liver receptor homolog-1 (LRH-1) in intestinal tumorigenesis. Interaction of LRH-1 with the Wnt/ β -catenin pathway, highly active in a critical subpopulation of CRC cells, underscores the importance of elucidating LRH-1's role in this disease. Reduction of LRH-1 diminishes tumor burden in murine models of CRC; however, it is not known whether LRH-1 is required for tumorigenesis, for proliferation, or for both. In this work, we address this question through shRNA-mediated silencing of LRH-1 in established CRC cell lines. LRH-1 mRNA knockdown results in significantly impaired proliferation in a cell line highly expressing the receptor and more modest impairment in a cell line with moderate LRH-1 expression. Cell-cycle analysis shows prolongation of G0/G1 with LRH-1 silencing, consistent with LRH-1 cell-cycle influences in other tissues. Cluster analysis of microarray gene expression demonstrates significant genome wide alterations with major effects in cell-cycle regulation, signal transduction, bile acid and cholesterol metabolism, and control of apoptosis. This study demonstrates a critical proliferative role for LRH-1 in established colon cancer cell lines. LRH-1 exerts its effects via multiple signaling networks. Our results suggest that selected CRC patients could benefit from LRH-1 inhibitors.

liver receptor homolog 1 | LRH-1 | NR5A2 | nuclear receptor | colorectal cancer

Colorectal cancer (CRC) accounts for nearly 10% of all cancer deaths in the United States. Much is understood about maintenance, but little is known about molecules driving initiation or metastasis of CRCs. Recent discoveries in animal and cellular models of CRC suggest a role for the nuclear receptor liver receptor homolog-1 (LRH-1, also known as NR5A2) (1).

LRH-1, previously thought to be constitutively active, has a functional hormone-binding pocket that binds phosphatidylcholines (2, 3). The structure of the hormone-binding domain of LRH-1 has been determined with four different phospholipids in the binding pocket (4–6).

LRH-1 is vital; knockout mouse embryos fail to undergo gastrulation (7). LRH-1 is expressed in the adult in the gastrointestinal tract, including liver and pancreas, and is also present in ovaries, where it regulates steroidogenesis (8). It is highly expressed in intestinal crypts where it is believed to play a role in intestinal epithelial cell renewal (9). LRH-1 has prominent roles in development, metabolism, stem-cell pluripotency, and tumorigenesis (10–14).

LRH-1 figures prominently in WNT signaling in the intestine (9, 13). The WNT gene family is a large, conserved signaling pathway with roles in development, cellular homeostasis, and tumorigenesis (15). WNT signaling activates β -catenin, an important transcriptional coactivator that is active in over 80% of CRCs (16). LRH-1 physically binds to β -catenin and functions synergistically in the regulation of certain target genes, including cyclin E1 (9).

LRH-1 and β -catenin contribute to tumorigenesis of the gastrointestinal system (17, 18). Investigators studying the effect of LRH-1 in intestinal tumor forming APC^{+/min} heterozygous

mice noted *lrh-1*^{+/-}/*apc*^{+/min} mice developed a lower tumor burden, suggesting that β -catenin activation can be partially offset by decreased LRH-1 activity (17). LRH-1 is overexpressed in many established CRC cell lines (19). LRH-1 increases the expression of key steroidogenic enzymes Cyp11A1 and Cyp11B1, increasing immune-regulating corticosteroid levels and allowing tumor cells to escape the host's natural immune response (20). These findings, combined with in vitro studies of LRH-1 and β -catenin synergistic activation add to the body of evidence linking LRH-1 expression to intestinal tumor formation.

In our prior study, we demonstrated that LRH-1 is up-regulated in pancreatic cancers compared with normal control pancreas (21). Additionally, we showed that siRNA-mediated LRH-1 knockdown results in impaired cellular proliferation via cell-cycle arrest, pointing toward a major role for LRH-1 in pancreatic cancer.

Although tumor burden is attenuated by loss of LRH-1 in genetic models of CRC, it is not known whether continued expression of LRH-1 is required for tumor growth and progression. In the current study, we address this fundamental question in LRH-1 pathophysiology. We created stably transduced CRC cell lines with inducible LRH-1-directed shRNA constructs. We chose two different CRC cell lines for our experiments to examine the effect of LRH-1 suppression in the setting of differential baseline LRH-1 expression on cell growth. Next, we performed a whole-genome microarray expression analysis to better understand the role of LRH-1 in CRC. Finally, we examined the possible role of apoptosis as a mechanism for inhibited CRC growth.

Significance

This work addresses a key question in the field of liver receptor homolog-1 (LRH-1) pathophysiology in colorectal cancer (CRC)—namely, does LRH-1 contribute exclusively to tumorigenesis, or does LRH-1 also drive established CRC tumor growth? These two models have widely different implications for pharmaceutical targeting in CRC. To our knowledge, our work is the first to demonstrate that silencing of LRH-1 in established human CRC cell lines impairs proliferation though G0/G1 phase prolongation. Our microarray gene expression analysis shows that loss of LRH-1 expression yields alterations in diverse cellular pathways consistent with the critical role of LRH-1 in CRC. Taken together, our study suggests that a subset of CRC patients could benefit from selective antagonism of LRH-1.

Author contributions: J.R.B. designed research; J.R.B., S.M., and P.W. performed research; J.R.B. contributed new reagents/analytical tools; J.R.B., S.M., E.P.S., P.W., and R.J.F. analyzed data; and J.R.B. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The microarray data reported in this paper have been deposited into National Center for Biotechnology Information Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE64695).

¹To whom correspondence should be addressed. Email: robert.fletterick@ucsf.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1500978112/-DCSupplemental.

Results

Suppression of LRH-1 Expression. To examine the role of LRH-1 activity in CRC, we chose two well-established cell lines for our studies, Caco2 and HT29. These cell lines were chosen for their prior use in LRH-1 studies as well as for their differential levels of LRH-1 expression (Fig. 1A). To maximize LRH-1 suppression and minimize variability between experiments, we used a lentiviral system to create stably transduced cell lines with tetracycline-inducible shRNA. Two different shRNA constructs were designed (designated sh1 and sh2), each targeting the LRH-1 ligand-binding domain (LBD) to avoid incomplete suppression secondary to LRH-1 RNA splicing isoforms. Doxycycline was used to induce shRNA expression; the concentration was determined for each cell line by expression of a TurboRFP fluorescent marker and the effect on LRH-1 mRNA levels. Suppression of LRH-1 mRNA was confirmed by quantitative PCR (qPCR) (Fig. 1B). Suppression approaching 80% of baseline was achieved for each cell line, with maximal suppression occurring 48–72 h after shRNA induction. After cessation of shRNA expression, LRH-1 mRNA levels began to rebound in the surviving cells by day 6. LRH-1 mRNA levels were not significantly affected by induction of a nonsilencing control RNA (NSC) (Fig. 1B). The effect of shRNA silencing on LRH-1 protein level was measured by Western blotting (Fig. 1B).

Functional Validation of LRH-1 Suppression. To confirm that our shRNA-mediated suppression of LRH-1 resulted in impaired function, we measured the mRNA levels of the known LRH-1 target SHP (Fig. 2). As expected, loss of LRH-1 RNA expression was followed by suppression of *Shp* mRNA by up to 80% compared with uninduced control cells. Upon recovery of LRH-1 activity, *Shp* mRNA rebounded toward baseline. Interestingly, *Shp* mRNA levels showed greater suppression in the Caco2 cell line compared with HT29.

Loss of LRH-1 Impairs CRC Proliferation. Having validated both the absolute and functional suppression of LRH-1 in our CRC shRNA cell lines, we next examined the impact of loss of LRH-1 activity on cell proliferation. To control for both doxycycline and lentiviral effects on cell growth, our inducible nonsilencing RNA was examined in parallel to each of the experimental lines.

Fig. 1. LRH-1 expression levels and knockdown. Expression of LRH-1 in Caco2 is nearly four times higher than HT29; data are normalized to the minimally LRH-1 expressing CRC cell line Colo205 (A). LRH-1 mRNA levels as determined by qPCR are substantially decreased after shRNA induction for both cell lines (B). Data are normalized to NSC for each cell line. Western blot analysis (C) demonstrates corresponding reduction in LRH-1 protein levels. Average density relative to NSC is quantitated (C, Right). * $P < 0.01$, ** $P < 0.005$.

Fig. 2. Expression levels of the LRH-1 target gene SHP after suppression of LRH-1 for Caco2 (A) and HT29 (B) transduced cell lines. Data are normalized to NSC for each cell line. * $P < 0.05$, ** $P < 0.005$.

Suppression of LRH-1 activity in the Caco2 cell lines by both shRNA constructs significantly impaired cell growth to 66% relative to the nonsilencing control (Fig. 3A, Upper). Growth suppression was detectable within 48 h of shRNA induction, with the maximal effect on cell viability occurring by day 6 for both cell lines (data shown for day 6). In contrast, suppression of LRH-1 in the HT29 cell line resulted in a more modest inhibition of growth relative to the nonsilencing control cells to just over 75% (Fig. 3A, Lower). After rebound of LRH-1 expression, surviving cells were able to reestablish proliferation and approached that of controls.

Cell-Cycle Analysis. Our previous work with LRH-1 suppression in pancreatic cancer cell lines by siRNA demonstrated cell-cycle arrest at the G0/G1 checkpoint (21). To examine whether G0/G1 arrest holds true for CRC cells, we assessed the cell-cycle distribution in our CRC cells after silencing of LRH-1. After cell-cycle synchronization by serum starvation, LRH-1-silenced Caco2 cells showed significantly increased G0/G1 phase population relative to the nonsilencing controls. This effect was observed within 24 h of LRH-1 suppression and peaking on day 4 ($P < 0.005$) (Fig. 3B, Upper). Of note, we did not identify a significant population of cells undergoing apoptosis compared with the doxycycline-treated control population. To confirm that the altered distribution was not a cell line-specific artifact, we repeated the experiments using our HT29 shRNA cell lines (Fig. 3B, Lower). Again, there is a significant increase in cells in G0/G1 phase after LRH-1 suppression relative to the doxycycline-treated nonsilencing control starting 48 h after LRH-1 silencing, with maximal effect at day 4 ($P < 0.005$).

Loss of LRH-1 Has Diverse Ramifications for Cellular Function. Previous work has demonstrated that LRH-1 plays important roles in bile-acid homeostasis, lipid metabolism, and steroidogenesis, along with cell proliferation (reviewed in ref. 22). To better define LRH-1 networks in both physiological and pathophysiological contexts, we conducted a whole-genome microarray analysis of mRNA levels in our more LRH-1-sensitive Caco2 CRC cell line. In these experiments, we examined the fold changes of genes from LRH-1-suppressed cells (sh1) compared with untreated controls and the parent Caco2 cell line (with and without doxycycline).

Analysis of the microarray data revealed 463 genes with significant changes in expression levels ($P < 0.05$) attributable to the shEffect, defined as (sh1 Dox – sh1 Control) – (Native Dox – Native Control). The direction of gene alteration was split nearly identically, with 225 genes up-regulated and 238 down-regulated (Fig. 4, Upper Left). Functional Gene Ontology (GO) processes annotation by GeneCodis of up-regulated processes reveals enrichment in apoptosis, transcriptional control, and cell cycle, among others (false discovery rate; FDR < 0.05) (Fig. 4, Upper Right). Down-regulated processes upon LRH-1-suppression show minimal overlap with the exception of coagulatory processes (Fig. 4, lower

Fig. 3. Suppression of LRH-1 results in decreased CRC cell proliferation. Growth inhibition was apparent by day 3 with maximal effect in both cell lines by day 6 (data shown for day 6) (A). Growth inhibition was greatest in Caco2 (A, Upper), and affected by both shRNA constructs similarly. HT29 proliferation is also affected (A, Lower), but to a lesser extent than Caco2. Data are normalized to induced expression of nonsilencing control RNA sequence. Analysis of cell-cycle population after shRNA induction shows an increase in G0/G1 phase in LRH-1-silenced cells compared with nonsilencing control (B), with slightly greater G0/G1-phase expansion for Caco2 (Upper) compared with HT29 (Lower). ** $P < 0.01$; † $P < 0.001$.

right). Annotations include functions previously ascribed to LRH-1, including bile acid metabolism and cholesterol homeostasis along with new areas including metabolic processes (FDR < 0.05). Overall, 90 GO biological process groups containing five or more members and FDR < 0.05 were categorized (Table S1).

To validate our microarray results and to confirm generalizability outside of sh1, we selected representative genes from separate functional categories for individual qPCR experiments using Caco2 sh2 and our nonsilencing control (Fig. 5).

A second analysis of our microarray data comparing our calculated shEffect with pairwise (sh1 Dox) – (Native dox) fold change showed that less than 2% of identified genes had discordant expression changes while also achieving statistical significance ($P < 0.05$) (Table S2). Interestingly, discordant genes all showed an upward change in expression level for the calculated shEffect. This finding may reflect LRH-1's recognized role in effecting positive transcriptional control, implying that negative regulatory activity by the receptor is carried about via secondary targets and is therefore harder to measure via microarray because of the time scale of regulatory activity. Biological processes analysis failed to show enrichment of any categories, suggesting that the discordant results are not the result of systemic bias against a functional group. Interestingly, Wnt5A was uncovered in this analysis and confirmed by individual qPCR (Fig. 5).

Discussion and Conclusion

Impaired CRC Proliferation. In this study, we demonstrate that inhibition of LRH-1 via shRNA leads to decreased CRC proliferation (Fig. 3A). This effect is diminished in a lower LRH-1-expressing cell line, HT29. To our knowledge, our work is the first to directly demonstrate impaired human CRC cell growth with LRH-1 inhibition and is consistent with our prior studies of LRH-1 activity in pancreatic cancer cells highly expressing LRH-1. Importantly, the magnitude of growth inhibition is on par with that reported with β -catenin siRNA knockdown alone, reflecting the synergy of LRH-1 and β -catenin combined activity in gene regulation (9). This finding is consistent with prior studies in

pluripotent stem cells showing that LRH-1 requires the presence of β -catenin to maintain stemness (13).

Growth Inhibition Is Linked to LRH-1 Expression Level. We initially chose to evaluate the effect of LRH-1 suppression on two well-characterized human CRC cell lines, HT29 and Caco2. These lines have been used in prior studies to interrogate LRH-1 function (20). According to our data, LRH-1 expression is nearly fourfold higher in Caco2 compared with HT29 (Fig. 1A), which is consistent with a cell line gene expression repository (23). Although literature suggests that both CRC cell lines show similarly regulated LRH-1-dependent genes (e.g., *Cyp11A1* and *Cyp11B1*) (20), the loss of LRH-1 expression results in a significantly different growth phenotype. Cells with higher LRH-1 baseline expression are more susceptible to LRH-1 inhibition, as evidenced by the greater impairment of Caco2 growth compared with HT29 (Fig. 3A). This finding reinforces our understanding for multiple roles of LRH-1 in CRC cells, from nonpathologic activity through oncogenic gene regulation.

The differential growth effects further suggest that other neoplasm-associated changes influence the effect of LRH-1 activity. For example, in some scenarios, LRH-1 activity may be critical early in tumorigenesis, but then later becomes dispensable due to other acquired genetic and epigenetic changes. In contrast, acquired overexpression of LRH-1 may obviate the need for these additional oncogenic changes, with the consequence that such overexpressing CRC cells are now dependent upon LRH-1 activity to maintain their growth. The two theories are compatible with prior work showing that LRH-1-haploinsufficient mice harboring the *apc*^{min} mutation develop fewer tumors compared with their LRH-1 WT littermates (17). That is, reduction of LRH-1 activity may have led to fewer tumors forming in setting of a separate protumor mutation rather than the abolition of tumors as a whole. The first mechanism is supported by the similar size of tumors in LRH-1 WT and heterozygous mice. Our Caco2 data lend support to the second mechanism where CRC growth remains at least in part LRH-1-dependent. It remains to be seen what effect overexpression of LRH-1 will have on the development of intestinal tumors in an animal system. That we see differential antiproliferative effects in CRC cell lines with LRH-1 suppression suggests that LRH-1-targeted drugs may have a therapeutic effect in a subset of CRC patients. It will be important to identify the cofactors that allow for LRH-1-driven tumor progression to target therapy appropriately.

Fig. 4. Altered gene expression in Caco2 sh1 as determined by microarray analysis. Up- and down-regulated totals were nearly identical (Upper Left). Up-regulated genes were enriched in apoptosis and cell cycling (Upper Right) whereas down-regulated genes were more enriched in metabolic processes including bile acid and cholesterol metabolism (Lower Right). Sorting of altered genes with signaling pathway notation showed significant enrichment in several pathways, including WNT and Rho (Lower Left). Biological process enrichment was determined using GeneCodis (genecodis.cnb.csic.es). An FDR < 0.05 was considered significant.

Fig. 5. Confirmation of microarray targets from select functional groups. Target mRNA levels were determined by individual qPCR reactions. Levels were assessed in both nonsilencing as well as sh2 LRH-1-silenced Caco2 cells to demonstrate consistent regulation between silencing constructs. Data are normalized to the nonsilencing construct. * $P < 0.0005$, ** $P < 0.008$, † $P < 0.05$.

Antiproliferative Mechanism of LRH-1 Inhibition Is Conserved Between Pancreas and Colon. Cell-cycle analysis of LRH-1-suppressed cells demonstrated an increase in the proportion of cells in G0/G1 phase (Fig. 3B). This increase was found in both cell types with sh1- and sh2-mediated LRH-1 suppression. The fractionally larger difference in Caco2 mirrors the alterations found in cell growth. These findings are consistent with our prior studies in a pancreatic cell line where G0/G1 arrest was the primary phenotype (21). It is remarkable that LRH-1 suppression leads to a similar cell-cycle phenotype, given the different underlying proliferative signaling pathways between the tissues (e.g., KRAS in pancreas and WNT/ β -catenin in colon), and suggests that targeting LRH-1 with an antagonist compound could prove clinically important in diverse oncological settings.

Our qPCR data indicate that LRH-1 mRNA levels remain depressed at day 3 in both cell lines (Fig. 1B). Maximal SHP suppression, serving as a marker of LRH-1 transcriptional activity, occurs on day 4 for Caco2 and as early as day 2 for HT29 (Fig. 2). Therefore, the cell-cycle effects may result from downstream regulation of cell-cycle control proteins and are unlikely to be secondary to derepression of LRH-1 expression. Contributing to our finding may be the known synergistic regulation of LRH-1 with β -catenin at the cyclin E1 promoter. Cyclin E1 is necessary for entry into and completion of S phase (24, 25). Cyclin E1 levels are more suppressed in the LRH-1-sensitive Caco2 compared with the less sensitive HT29 cell lines (Fig. S1).

LRH-1 Interacts with Diverse Cellular Processes. To better understand the regulatory activity of LRH-1 in a sensitive CRC cell line, we performed a whole-genome microarray analysis of gene expression (Fig. 4). Our data are consistent in the number of genes identified with altered expression levels with prior LRH-1 studies in breast and liver (14, 26). Over 460 genes were differentially expressed in our shEffect group, encompassing a host of cellular processes from transcriptional control to metabolic activity. Strikingly, altered expression of gene families was identified with consistent fold changes throughout the individual gene members (Table S3). This result adds to our understanding of

LRH-1 as a potent regulator of diverse processes. We highlight some of the more interesting findings below.

Signal Transduction and Cell Proliferation. A major functional group of altered gene regulation is signal transduction, consistent with the known involvement of LRH-1 in multiple signaling pathways. A total of 10 separate pathways with two or more genes were differentially regulated upon LRH-1 suppression (Fig. 4, Lower Left). Here, we discuss the two most represented pathways.

As expected, WNT signaling figures most prominently, accounting for nearly 12% of genes with signal transduction GO annotations. The WNT/ β -catenin pathway is known to be active in the vast majority of colon cancers, and LRH-1 interacts directly with β -catenin to effect synergistic gene regulation (9, 16, 27). This finding helps validate our silencing dataset. An interesting potential target from the WNT dataset is WNT5A, a factor with noncanonical WNT activity, and prominent in intestinal stem cells and colorectal carcinoma development (28, 29). In keeping with nontraditional WNT ligands is Norrin (NDP), which activates through the frizzled class receptor 4 (Fz4) receptors, and is down-regulated with LRH-1 suppression. A prior investigation showed that Fz4 expression is absent in normal colon tissue but reemerges in a number of CRCs (30). Taken together, these results suggest that LRH-1 may contribute to both canonical and noncanonical WNT signaling.

Interestingly, the next two most representative signal transduction pathways are Rho and G-protein-coupled receptor (GPCR), representing ~10% of the signaling total. These two annotations show considerable overlap and will be discussed together. The importance of Rho signaling in CRC is emerging (reviewed in ref. 31) and, to our knowledge, has not previously been associated with LRH-1. Among altered genes in this pathway are two Rho-activating proteins (rho GTPase activating protein 11A and rho GTPase activating protein 28) and two other factors implicated in cancer metastasis. An interesting Rho-signaling protein not previously associated with CRC is neurotrophin 3 (NTF3). NTF3 is a secreted factor important during embryogenesis in gastrointestinal smooth muscle development and central nervous system function. NTF3 was recently shown to be important in breast-cancer metastases to brain where it encourages epithelial transition of breast-cancer cells, facilitating their integration to the CNS microenvironment as well as serving to reduce immunogenicity (32). Interestingly, NTF3 has also been shown to increase resistance to anoikis (33). LRH-1 as well has recently been implicated in pancreatic-cancer metastasis and breast-cancer development; these findings may demonstrate similar molecular underpinnings between cancer metastases (14, 34, 35). Another Rho member in our dataset that promotes metastasis in gastroenterological cancers is semaphorin 5A. Interestingly, this semaphorin gene member was also identified in a genome-wide association study (GWAS) analysis of azoxymethane (AOM)-induced CRC in mice (36), a model also used to demonstrate decreases in aberrant crypt foci in LRH-1-haploinsufficient mice. The functional similarities in this group suggest a previously unappreciated role for LRH-1 in tumor metastasis and Rho signaling and, along with alteration in endopeptidase expression, mark an important area for future study.

Our observations draw intriguing similarities with a recent study of LRH-1 in breast cancer (14). In breast cancer, LRH-1 is highly associated with TGF β signaling, evidenced through microarray analysis of both silencing and overexpression of LRH-1. Remarkably, we see an overlap in several TGF β -associated factor families, including annexin, integrin alpha, and keratin family members. These results further suggest conserved roles for LRH-1 in cancer.

Reverse Cholesterol Transport and Bile Acid Metabolism.

Apolipoproteins. LRH-1 has been shown previously to regulate apolipoprotein A1 (ApoA1) expression (37). In our experiment, we also saw significant alterations in the expression levels of APO family members ApoA4, ApoC3, and ApoH. ApoD was also slightly down-regulated but failed to achieve significance. ApoA4 and ApoC3 are present in the same gene cluster as

ApoA1 (11q23.3), which may have contributed to the overall concerted regulation. ApoH, however, is chromosomally distinct from this cluster (17q24.2) and raises the possibility of coregulation of the Apo family more globally either directly by LRH-1 or indirectly through alteration of other subordinate regulators.

Apolipoproteins have long been associated with cholesterol and triglyceride metabolism. ApoA4 is implicated in diet- and fiber-responsive lipidemia. Population studies have identified several SNPs associated with dyslipidemia and HDL levels. In the same study, SNPs within the ApoC3 locus have been linked with patients with metabolic syndrome (38). Likewise, elevated circulating ApoH levels have been associated with metabolic syndrome and type II diabetes mellitus (39). Taken together, these findings extend the potential importance of LRH-1 as a key factor in metabolic syndrome.

Cholesterol transporters. Recovery of cholesterol from peripheral tissues for hepatic processing and excretion involves mechanisms of cholesterol efflux from lipid-laden sources and uptake by hepatocytes. Prior work demonstrated that LRH-1 is involved in the latter process via expression of the HDL receptor Scavenger Receptor Class B, Member 1 in the liver (40). Remarkably, our data add to this prior work the cholesterol efflux pump ATP-binding cassette, sub-family A (ABC1), member 5 (ABCA5), which is highly expressed in the intestine as well as in macrophages, where it is important in the prevention of atherosclerosis in a murine model (41).

Bile acid transport and metabolism. LRH-1-dependent cholesterol efflux is complemented by bile acid reabsorption and metabolism. Our data demonstrate down-regulation of the bile acid transporter solute carrier family 10 member 1 (SLC10A1) upon LRH-1 knockdown. This finding complements a prior report of SLC10A2 [also known as apical sodium-dependent bile acid transporter (ASBT)] in intestinal cells (42). Interestingly, SLC10A2 absorbs bile acids from the intestinal lumen as part of enterohepatic bile acid circulation. SLC10A1, however, is more known physiologically for its expression in the basolateral membrane of hepatocytes, where it takes up bile acids.

In addition to bile acid transport, a potential role for LRH-1 in bile acid metabolism is suggested by the down-regulation of the aldo-keto reductase family 1 (AKR1) family of enzymes. Key members of this family, AKR1C2 and AKR1D1, are known to bind and metabolize bile acids (43). AKR1D1 in particular catalyzes a key step in bile acid synthesis; deficiency can lead to cholestasis in humans (44).

Drug and Xenobiotic Metabolism. In addition to genes associated with cholesterol metabolism, our study identifies gene families involved in the detoxification pathways of glucuronidation and glutathione metabolism. Three members of the glutathione S-transferase (GST) family and five members of the glucuronosyltransferase (UGT) superfamily of enzymes were all significantly down-regulated in the absence of LRH-1. Members of these families are associated with drug and xenobiotic metabolism, implying that LRH-1 up-regulation may increase cancer-cell resistance to chemotherapy and oxidative injury. GSTs in particular are important for metabolism and detoxification of drugs and have been found to be overexpressed in a variety of tumors, including CRC, where they are thought to contribute to multidrug resistance (44).

Loss of LRH-1 Does Not Induce Apoptosis in CRC. An enigmatic functional cluster identified in the genome analysis is altered levels of genes involved in apoptosis (Fig. 4, *Upper Right*). Prior studies in hepatic cells pointed to a role for LRH-1 in apoptotic death (45). Additionally, a study of pancreatic islet cells that overexpress LRH-1 demonstrated resistance to induction of apoptosis by chemical or cytokine mixtures (46). Our prior studies of pancreatic cancer cells, however, showed cell-cycle arrest at G0/G1 upon suppression of LRH-1 expression but did not demonstrate induction of apoptosis (21). Our flow-cytometry analysis failed to demonstrate induction of apoptosis in LRH-1-silenced

CRC cells relative to doxycycline-treated controls. The altered regulation of apoptotic processes remains enigmatic and likely needs further study in either animal or complete culture systems to explore fully.

It remains possible that these findings are fortuitous or that the major location of expression and activity is extraintestinal (e.g., hepatic for metabolic enzymes). Furthermore, it is not known whether gene-expression alterations are the result of direct regulation by LRH-1 or secondary effects from LRH-1 regulation of key regulatory targets. In either case, our results extend the reach of LRH-1 in signaling, metabolism, and cell growth.

Conclusion. In this study, we have shown that LRH-1 suppression impairs CRC cell proliferation in a context-dependent manner. Impaired proliferation is mediated by effects on cell cycle with G0/G1 arrest rather than apoptosis. Our RNA expression analysis demonstrates that LRH-1 directly or indirectly impacts many distinct pathways. Our work deepens the appreciation for LRH-1's roles in cholesterol trafficking and regulation in nonpathological homeostasis. Additionally, we identify previously unrecognized pathways in drug and xenobiotic metabolism that may provide LRH-1⁺ CRCs a selective advantage against chemotherapy and highlight the importance of future study.

Materials and Methods

Cells and Culture Conditions. The colon cancer cell lines HT29 and Caco2 were obtained from the University of California, San Francisco (UCSF) cell culture facility. HT29 was maintained in McCoy 5A media supplemented with 10% (vol/vol) FBS and antibiotics. Caco2 was maintained in MEM supplemented with 20% (vol/vol) FBS, 1% nonessential amino acids, and antibiotics. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were harvested and passaged when they reached 80% confluence.

Lentiviral Transduction. Two separate DNA sequences targeting the LRH-1 LBD were cloned into parent vector pTripZ RHS4696 (Open Biosystems) packaged by the UCSF viral core facility. Mature antisense sequences were TGTCGGTAAATGTGGTCGA and ATACAAGAACCCTTCTGTC. Cells were transduced with a multiplicity of infection of 2 in the presence of 4 µg/mL or 7 µg/mL polybrene (Millipore) for HT29 and Caco2 cells, respectively. Cells were selected for 10 d using 3 µg/mL or 5 µg/mL puromycin (Corning) for HT29 and Caco2 cells, respectively. Incorporation of silencing vector was confirmed by fluorescent assay for TurboRFP. Nonsilencing control cell lines were produced similarly using the manufacturer's vector (RHS4743; Open Biosystems).

qPCR. Cells were plated at 1×10^5 cells per ml in 12-well plates and allowed to attach overnight. shRNA expression was induced by the addition of 1–5 µg/mL doxycycline when the cells approached 75% confluence and harvested at 24, 48, 72, and 96 h postinduction using TRIzol reagent (Life Technologies). Total RNA was harvested using a PureLink RNA Mini Kit (Ambion) according to the manufacturer's protocol. cDNA was generated from 500 ng of RNA using random primers (Invitrogen). qPCR was performed using Brilliant SYBR Green Master Mix (Agilent) and specific primers, with RS9 as a control housekeeping gene using either Stratagene MXP3005 or Life Technologies ViiA 7 Real-Time PCR machines. The amplification curves were analyzed with either the Mx3005P or ViiA 7 software using the comparative Ct method. A minimum of three individual experiments were run in triplicate for each target.

Western Blot. Cell lysates from control and knockdown cell lines were separated by SDS/PAGE and transferred to nitrocellulose membranes. Western blot analyses were performed using mouse anti-LRH-1 antibody (PP-H2325; R&D Systems) and goat anti-mouse horseradish peroxidase conjugated Igs (Santa Cruz Biotechnology). The membranes were developed using enhanced chemiluminescence according to a standard protocol. Experiments were performed in triplicate. LRH-1 levels were quantitated by density analysis in Photoshop (Adobe) and normalized to β -Actin (Ambion).

Cell Proliferation Assays. Cells were plated at a concentration of 1×10^4 per ml in 96-well plates. Induction of shRNA was performed with 1–5 µg/mL doxycycline following attachment, typically by three hours after seeding. After 72, 96, 120, and 144 h induction, the relative increase in cell population between induced and uninduced wells was quantified using Cell Titer-Glo (Promega). Relative LRH-1-silenced cell quantifications were normalized to nonsilencing controls at

each time point after induction with equal amounts doxycycline. Experiments were performed in replicates of six and repeated a minimum of three times.

Flow Cytometry and Cell-Cycle Analysis. Cells were serum started for 24 h to synchronize populations into G0. On day 0, serum starvation medium was replaced with rich media, and shRNA and nonsilencing control were induced by doxycycline. Cells were harvested and fixed overnight in 70% ethanol. Cells were stained with FxCycle PI/RNase staining solution (Molecular Probes) and analyzed on an LSR II flow cytometer. Cell-cycle analysis and model fitting was performed with FlowJo (FlowJo LLC).

Microarray. Human HT-12_v4 whole-genome expression arrays were purchased from Illumina. cRNA synthesis and labeling were performed using an Illumina TotalPrepTM-96 RNA Amplification Kit (Ambion). Labeling in vitro transcription reaction was performed at 37 °C for 14 h. Biotinylated cRNA samples were hybridized to arrays at 58 °C for 18 h according to the manufacturer's protocol. Arrays were scanned using iScan. Unmodified microarray data obtained from GenomeStudio were background-subtracted and quantile-normalized using the lumi package and analyzed with the limma package

within R (Bioconductor). The effect of LRH-1 knockdown was determined through comparison between native, nonsilencing control, and sh1 cell lines with and without doxycycline. All analysis was corrected for multiple hypothesis testing, and effects were determined to be significant when greater than or equal to twofold with an adjusted *P* value ≤ 0.05 . Microarray data have been deposited into the National Center for Biotechnology Information Gene Expression Omnibus (accession no. GSE64695).

Gene Ontology Assignment and Cooccurrence. Microarray data were examined using the GeneCodis website (47). Data were analyzed as a whole and individually based on the direction of the fold change. An FDR of <0.05 was considered significant.

ACKNOWLEDGMENTS. We thank D. Sieglaff (Department of Genomic Medicine, Houston Methodist Research Institute) for assistance with microarray experiments and data processing and P. Nguyen, Dr. B. Hann, and R. Uthayaruban (University of California, San Francisco) for technical assistance. This work was supported by National Institutes of Health Grants T32 DK007762, F32 CA163092, and K12 HD07222.

- Lazarus KA, Wijayakumara D, Chand AL, Simpson ER, Clyne CD (2012) Therapeutic potential of Liver Receptor Homolog-1 modulators. *J Steroid Biochem Mol Biol* 130(3-5):138–146.
- Sablin EP, Krylova IN, Fletterick RJ, Ingraham HA (2003) Structural basis for ligand-independent activation of the orphan nuclear receptor LRH-1. *Mol Cell* 11(6):1575–1585.
- Lee JM, et al. (2011) A nuclear-receptor-dependent phosphatidylcholine pathway with antidiabetic effects. *Nature* 474(7352):506–510.
- Krylova IN, et al. (2005) Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. *Cell* 120(3):343–355.
- Whitby RJ, et al. (2011) Small molecule agonists of the orphan nuclear receptors steroidogenic factor-1 (SF-1, NR5A1) and liver receptor homologue-1 (LRH-1, NR5A2). *J Med Chem* 54(7):2266–2281.
- Musille PM, et al. (2012) Antidiabetic phospholipid-nuclear receptor complex reveals the mechanism for phospholipid-driven gene regulation. *Nat Struct Mol Biol* 19(5):532–537, S1–S2.
- Gu P, et al. (2005) Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. *Mol Cell Biol* 25(9):3492–3505.
- Mendelson CR, Jiang B, Shelton JM, Richardson JA, Hinshelwood MM (2005) Transcriptional regulation of aromatase in placenta and ovary. *J Steroid Biochem Mol Biol* 95(1-5):25–33.
- Botrugno OA, et al. (2004) Synergy between LRH-1 and beta-catenin induces G1 cyclin-mediated cell proliferation. *Mol Cell* 15(4):499–509.
- Petersen GM, et al. (2010) A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat Genet* 42(3):224–228.
- Annicotte JS, et al. (2003) Pancreatic-duodenal homeobox 1 regulates expression of liver receptor homolog 1 during pancreas development. *Mol Cell Biol* 23(19):6713–6724.
- Lee YK, et al. (2008) Liver receptor homolog-1 regulates bile acid homeostasis but is not essential for feedback regulation of bile acid synthesis. *Mol Endocrinol* 22(6):1345–1356.
- Wagner RT, Xu X, Yi F, Merrill BJ, Cooney AJ (2010) Canonical Wnt/ β -catenin regulation of liver receptor homolog-1 mediates pluripotency gene expression. *Stem Cells* 28(10):1794–1804.
- Lazarus KA, et al. (2014) Conditional overexpression of liver receptor homolog-1 in female mouse mammary epithelium results in altered mammary morphogenesis via the induction of TGF- β . *Endocrinology* 155(5):1606–1617.
- Rao TP, Kühl M (2010) An updated overview on Wnt signaling pathways: A prelude for more. *Circ Res* 106(12):1798–1806.
- Rowan AJ, et al. (2000) APC mutations in sporadic colorectal tumors: A mutational “hotspot” and interdependence of the “two hits”. *Proc Natl Acad Sci USA* 97(7):3352–3357.
- Schoonjans K, et al. (2005) Liver receptor homolog 1 contributes to intestinal tumor formation through effects on cell cycle and inflammation. *Proc Natl Acad Sci USA* 102(6):2058–2062.
- Wang SL, et al. (2008) Increased expression of hLRH-1 in human gastric cancer and its implication in tumorigenesis. *Mol Cell Biochem* 308(1-2):93–100.
- Holbeck S, et al. (2010) Expression profiling of nuclear receptors in the NCI60 cancer cell panel reveals receptor-drug and receptor-gene interactions. *Mol Endocrinol* 24(6):1287–1296.
- Sidler D, et al. (2011) Colon cancer cells produce immunoregulatory glucocorticoids. *Oncogene* 30(21):2411–2419.
- Benod C, et al. (2011) Nuclear receptor liver receptor homologue 1 (LRH-1) regulates pancreatic cancer cell growth and proliferation. *Proc Natl Acad Sci USA* 108(41):16927–16931.
- Fayard E, Auwerx J, Schoonjans K (2004) LRH-1: An orphan nuclear receptor involved in development, metabolism and steroidogenesis. *Trends Cell Biol* 14(5):250–260.
- Kupersmidt I, et al. (2010) Ontology-based meta-analysis of global collections of high-throughput public data. *PLoS ONE* 5(9):e13066.
- Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M (1995) Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* 15(5):2612–2624.
- Eklholm-Reed S, et al. (2004) Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *J Cell Biol* 165(6):789–800.
- Wang SL, et al. (2006) Microarray analysis of gene-expression profile in hepatocellular carcinoma cell, BEL-7402, with stable suppression of hLRH-1 via a DNA vector-based RNA interference. *Yi Chuan Xue Bao* 33(10):881–891.
- Yumoto F, et al. (2012) Structural basis of coactivation of liver receptor homolog-1 by β -catenin. *Proc Natl Acad Sci USA* 109(1):143–148.
- Nishita M, Enomoto M, Yamagata K, Minami Y (2010) Cell/tissue-tropic functions of Wnt5a signaling in normal and cancer cells. *Trends Cell Biol* 20(6):346–354.
- Miyoshi H, Ajima R, Luo CT, Yamaguchi TP, Stappenbeck TS (2012) Wnt5a potentiates TGF- β signaling to promote colonic crypt regeneration after tissue injury. *Science* 338(6103):108–113.
- Planutis K, et al. (2007) Regulation of norrin receptor frizzled-4 by Wnt2 in colon-derived cells. *BMC Cell Biol* 8:12.
- Leve F, Morgado-Díaz JA (2012) Rho GTPase signaling in the development of colorectal cancer. *J Cell Biochem* 113(8):2549–2559.
- Louie E, et al. (2013) Neurotrophin-3 modulates breast cancer cells and the micro-environment to promote the growth of breast cancer brain metastasis. *Oncogene* 32(35):4064–4077.
- Howe EN, Cochrane DR, Cittelly DM, Richer JK (2012) miR-200c targets a NF- κ B up-regulated TrkB/NTF3 autocrine signaling loop to enhance anoikis sensitivity in triple negative breast cancer. *PLoS ONE* 7(11):e49987.
- Chand AL, Herridge KA, Thompson EW, Clyne CD (2010) The orphan nuclear receptor LRH-1 promotes breast cancer motility and invasion. *Endocr Relat Cancer* 17(4):965–975.
- Lin Q, et al. (2014) LRH1 promotes pancreatic cancer metastasis. *Cancer Lett* 350(1-2):15–24.
- Liu P, et al. (2012) Genome-wide association and fine mapping of genetic loci predisposing to colon carcinogenesis in mice. *Mol Cancer Res* 10(1):66–74.
- Delerive P, Galardi CM, Bisi JE, Nicodeme E, Goodwin B (2004) Identification of liver receptor homolog-1 as a novel regulator of apolipoprotein AI gene transcription. *Mol Endocrinol* 18(10):2378–2387.
- Lopez-Miranda J, Williams C, Lairon D (2007) Dietary, physiological, genetic and pathological influences on postprandial lipid metabolism. *Br J Nutr* 98(3):458–473.
- Castro A, et al. (2010) APOH is increased in the plasma and liver of type 2 diabetic patients with metabolic syndrome. *Atherosclerosis* 209(1):201–205.
- Schoonjans K, et al. (2002) Liver receptor homolog 1 controls the expression of the scavenger receptor class B type I. *EMBO Rep* 3(12):1181–1187.
- Ye D, et al. (2010) Macrophage ABCA5 deficiency influences cellular cholesterol efflux and increases susceptibility to atherosclerosis in female LDLr knockout mice. *Biochem Biophys Res Commun* 395(3):387–394.
- Chen F, et al. (2003) Liver receptor homologue-1 mediates species- and cell line-specific bile acid-dependent negative feedback regulation of the apical sodium-dependent bile acid transporter. *J Biol Chem* 278(22):19909–19916.
- Rizner TL, Penning TM (2014) Role of aldo-keto reductase family 1 (AKR1) enzymes in human steroid metabolism. *Steroids* 79:49–63.
- Seki Y, et al. (2013) Two neonatal cholestasis patients with mutations in the SRD5B1 (AKR1D1) gene: Diagnosis and bile acid profiles during chenodeoxycholic acid treatment. *J Inher Metab Dis* 36(3):565–573.
- Wang S, et al. (2005) Suppression of hLRH-1 mediated by a DNA vector-based RNA interference results in cell cycle arrest and induction of apoptosis in hepatocellular carcinoma cell BEL-7402. *Biochem Biophys Res Commun* 333(3):917–924.
- Baquié M, et al. (2011) The liver receptor homolog-1 (LRH-1) is expressed in human islets and protects beta-cells against stress-induced apoptosis. *Hum Mol Genet* 20(14):2823–2833.
- Tabas-Madrid D, Nogales-Cadenas R, Pascual-Montano A (2012) GeneCodis3: A non-redundant and modular enrichment analysis tool for functional genomics. *Nucleic Acids Res* 40(Web Server issue):W478–W483.