Published in final edited form as:

Liver Int. 2019 April ; 39(4): 727–739. doi:10.1111/liv.14068.

The concomitant loss of APC and HNF4 α in adult hepatocytes does not contribute to hepatocarcinogenesis driven by β -catenin activation

Chiara Sartor^{1,2,3,4}, Laura Bachelot^{1,2,3,4}, Cécile Godard^{1,2,3,4}, Franck Lager^{1,2,3,5}, Gilles Renault^{1,2,3,5}, Frank J. Gonzalez⁶, Christine Perret^{1,2,3,4}, Angélique Gougelet^{#1,2,3,4}, Sabine Colnot^{#1,2,3,4}

¹Inserm, U1016, Institut Cochin, Paris, France ²CNRS, UMR8104, Paris, France ³Université Paris Descartes, Sorbonne Paris Cité, Paris, France ⁴Equipe labellisée LNCC, Paris, France

⁵Plateforme Imageries du Vivant – PIV, Université Paris Descartes, Sorbonne Paris Cité, Paris, France ⁶Laboratory of Metabolism, National Cancer Institute, Bethesda, Maryland

These authors contributed equally to this work.

Abstract

Background & Aims: Loss of hepatocyte nuclear factor-4 α (HNF4 α), a critical factor driving liver development and differentiation, is frequently associated with hepatocellular carcinoma (HCC). Our recent data revealed that HNF4 α level was decreased in mouse and human HCCs with activated β -catenin signalling. In addition, increasing HNF4 α level by miR-34a inhibition slowed tumour progression of β -catenin-activated HCC in mice.

Methods: We generated a *Hnf4a*^{flox/flox}/*Apc*^{flox/flox}/TTR-Cre^{ERT2} (*Hnf4a/Apc*^{Hep}) mouse line and evaluated the impact of *Hnf4a* disruption on HCC development and liver homeostasis.

Results: There was no significant impact of *Hnf4a* disruption on tumour onset and progression in *Apc*^{Hep} model. However, we observed an unexpected phenotype in 28% of *Hnf4a*^{Hep} mice maintained in a conventional animal facility, which presented disorganized portal triads, characterized by stenosis of the portal vein and increased number and size of hepatic arteries and bile ducts. These abnormal portal structures resemble the human idiopathic non-cirrhotic portal hypertension syndrome. We correlated the presence of portal remodelling with a higher expression of protein and mRNA levels of TGF β and BMP7, a key regulator of the TGF β -dependent endothelial-to-mesenchymal transition.

Conclusion: These data demonstrate that HNF4 α does not play a major role during β -catenin-driven HCC, thus revealing that the tumour suppressor role of HNF4 α is far more complex and

Correspondence Angélique Gougelet, Institut Cochin, INSERM U1016, CNRS UMR8104, Université Paris Descartes, Paris, France. Angelique.gougelet@inserm.fr.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

dependent probably on its temporal expression and tumour context. However, HNF4 α loss in adult hepatocytes could induce abnormal portal structures resembling the human idiopathic non-cirrhotic portal hypertension syndrome, which may result from endothelial- and epithelial-to-mesenchymal transitions.

Keywords

β -catenin; hepatocellular carcinoma; HNF4 α ; portal triad disorganization

1 | INTRODUCTION

Hepatocyte nuclear factor-4 α (HNF4 α , NR2A1) belongs to the nuclear receptor superfamily of ligand-dependent transcription factors¹ and is most highly expressed in the liver,^{2,3} where it binds to around 20 000–30 000 loci.⁴ HNF4 α plays a central role in liver development during gastrulation,⁵ and orchestrates the formation of the adult liver parenchyma.⁶ It also guides hepatoblasts to hepatocyte differentiation,⁷ and regulates a number of genes involved in lipid, glucose, amino acid and xenobiotic metabolisms.³ Our recent findings revealed that HNF4 α governs the expression of the main enzymes involved in the metabolic pathways occurring in portal spaces, antagonizing the gene repertoire regulated by the β -catenin, only active in central areas – a phenomenon defined as the metabolic zonation.⁸ In particular, HNF4 α regulates lipid metabolism and its disruption promotes lipid accumulation.⁹ In addition, studies using knockdown mouse models of hepatic HNF4 α demonstrated a major inhibitory role for HNF4 α in proliferation of postnatal and adult hepatocytes.^{9,10} Considering its antiproliferative role, we and others have reported that HNF4 α expression decreased in human hepatocellular carcinoma (HCC)^{11–13} and more recently loss-of-function mutations in the Zn fingers of HNF4 α have been identified in HCC.¹⁴ Nevertheless, this contrasts with a study suggesting that HNF4 α expression was increased in human HCC.¹⁵ Using mouse models of hepatocarcinogenesis driven by the alkylating carcinogen diethylnitrosamine (DEN),¹⁶ the genetic disruption of the *Hnf4a* gene promoted DEN-driven HCCs while forced HNF4 α expression blocked DEN-induced tumour development through NF- κ B inhibition.^{12,17} More recently, it has been shown that HNF4 α also orchestrates inflammatory feedback loops in liver cancer,^{11,18} and regulates epithelial-mesenchymal transition by repressing mesenchymal genes.^{12,17,19–21} Therefore, HNF4 α appears as a promising therapeutic target for liver diseases, and particularly for liver cancer, a cancer for which mortality remains high because of a lack of efficient treatments.^{11,13.}

Recently, we proposed that a locked nucleic acid (LNA) approach against the microRNA miR-34a could be a potent strategy to treat HCC with activating mutations in the β -catenin gene (*CTNNB1*), which constitute one-third of HCC patients.¹³ In particular, the LNA-34a successfully slowed tumour progression in a mouse model developing β -catenin-activated HCC, owing to the hepatospecific deletion in hepatocytes of *Apc* (*Apc*^{Hep}), the main brake of the Wnt/ β -catenin pathway. This antitumoural effect was in part associated with cyclin D1 repression because of HNF4 α increase in *Apc*^{Hep} tumours. These findings support the protective role of HNF4 α in tumours with Wnt/ β -catenin pathway activation. In the present study, our goal was thus to evaluate the role of HNF4 α in the initiation and progression of HCC driven by the activation of the Wnt/ β -catenin pathway using the *Apc*^{Hep} model. This

tumour model is relevant for modelling human HCC,^{13,22} and distinct from that driven by DEN induction, in which tumours are preferentially mutated in the *Ha-Ras* gene.²³ We thus created a mouse model for liver cancer with a concomitant hepatospecific disruption of *Hnf4a* and *Apc*. Using these mice, we showed that *Hnf4a* disruption had no impact on liver tumourigenesis driven by β -catenin activation if *Hnf4a* and *Apc* were concomitantly lost. Interestingly, it appears that the panlobular *Hnf4a* loss could affect the portal triad architecture, a phenomenon reminiscent of a human disorder, called the idiopathic non-cirrhotic portal hypertension syndrome (IPH).

2 | MATERIALS AND METHODS

See supporting Materials and Methods for additional details.

2.1 | Animals and reagents

Our laboratory engineered few years ago a mouse model with β -catenin overactivation exclusively in the liver, by use of the tamoxifen-inducible and Cre-Lox-mediated inactivation of the Wnt/ β -catenin pathway inhibitor APC (*Apc*^{Hep}).^{22,24} Using this mouse model, we could obtain a panlobular activation of the β -catenin pathway in 90%-100% hepatocytes following one intraperitoneal injection of 1.5 mg tamoxifen (MP Biomedicals, Illkirch, France). This model is so-called the ‘pretumoral’ *Apc*^{Hep} model, since we showed that *Apc*^{Hep} hepatocytes express a similar gene programme than HCC in patients.^{8,13} Additionally, if we invalidate *Apc* in 10%-50% hepatocytes, we generate a tumoural model with mice developing tumours with β -catenin activation in 6 months. To obtain this low percentage of invalidation with precision, we inject an adenovirus coding the Cre recombinase (0.5×10^9 particles), which is more robust and accurate.²²

Apc^{lox/lox}/*Hnf4a*^{lox/lox}/TTR-Cre^{ERT2} mice were generated using *Hnf4a*^{flxed} floxed mice created as described earlier.³ One tamoxifen injection was not sufficient to delete *Hnf4a* gene around 100% for the panlobular model in *Hnf4a*^{lox/lox}/TTR-Cre^{ERT2} mice. To avoid alcoholic stress because of repeated tamoxifen injections, mice received a single injection of 1.5 mg tamoxifen, followed by a tamoxifen-containing diet for 5 days (TD135859, 400 mg/kg, Harlan Laboratories, Indianapolis, IN, USA) and either killed 9 days or 10 months later for tumour follow-up. For all tumoural models, a retro-orbital injection of a low dose of Cre-recombinase adenovirus (0.5×10^9 particles) was sufficient to obtain a focal inactivation of *Apc* and *Hnf4a* in 10%-50% hepatocytes with the same efficiency and to generate tumours. The tumour development was followed twice monthly by 2D-ultrasound (Vevo 770), as previously described.¹³ Only male mice were used throughout this study. All animal procedures were carried out in accordance with French government regulations, with the approval of the Paris-Descartes Ethics Committee for animal experimentation, under protocol 17-082. Animal experimentation permit number B 75-14 02 was obtained from the French “Ministère de L’enseignement Supérieur et de la Recherche”.

2.2 | Statistical analysis

We assessed the significance of differences between two groups of samples using Wilcoxon test or Student *t* test if $n \geq 30$. ANOVA was used to compare three groups of samples. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | HNF4 α expression was impaired during liver carcinogenesis driven by β -catenin overactivation

We previously observed that tumours developed in *Apc*^{Hep} mice expressed a lower level of HNF4 α mRNA as compared to normal liver (Figure 1A and ¹³). Decrease in HNF4 α expression occurred in tumour cells, since a normal level of HNF4 α remained still detectable in *Apc*^{Hep} liver biopsies 90 days after β -catenin activation, as shown by an immunostaining of its direct positive target, the glutamine synthetase (GLUL) (Figure 1B) but also in non-tumoural *Apc*^{Hep} tissue as revealed by recent RNA-seq data (data not shown). Importantly, we have previously published that HNF4 α expression was also downregulated at the mRNA level and more significantly at the protein level in human HCCs with activating mutations of β -catenin consequently to miR-34a induction (figure S4 published in from ¹³). When *Apc*^{Hep} HCCs were divided into two subgroups accordingly to the mean value of *Hnf4a* mRNA level, a high HNF4 α expression in *Apc*^{Hep} tumours was correlated with a lower level of *Snai1* and *Ccnd1* (Figure 1C-E), the two HNF4 α -negative targets that are known to be involved in hepatocarcinogenesis.^{9,19,20} Interestingly, a higher level of HNF4 α was also associated with a lower level of *Axin2*, a positive target of β -catenin (Figure 1F). This corroborates previous studies performed by our laboratory and others suggesting that HNF4 α could impair β -catenin signalling.^{8,12,21,25} Altogether, these data suggest that loss of HNF4 α in pretumoural hepatocytes with β -catenin activation could play a part during hepatocarcinogenesis.

3.2 | *Hnf4a* disruption had no impact on tumour onset and progression of HCCs exhibiting activation in the Wnt/ β -catenin pathway

To elucidate the role of HNF4 α in the particular subtype of HCCs activated for β -catenin, we then created a double mutant *Hnf4a/Apc*^{Hep} model of hepatocarcinogenesis. 6 months after injection of a low dose of Cre adenovirus in 2-month-old mice, we succeeded in focally invalidating both *Apc* and *Hnf4a* in single hepatocytes, as revealed by the presence of an immunostaining for GLUL in HNF4 α -negative hepatocytes (Figure 2A). Ultrasonography follow-up revealed that no mice developed tumours after a single *Hnf4a* invalidation. Tumours emerging from hepatocytes invalidated for *Hnf4a* and *Apc* were GLUL-positive and HNF4 α negative (Figure 2B), and appeared after 5.5 months, as soon as tumours only invalidated for *Apc* (Figure 2C). The number of tumours and their growth were not distinguishable between *Hnf4a/Apc*^{Hep} mice and *Apc*^{Hep} mice (Figure 2C). Tumour differentiation grades were also similar: in both cases, tumours were quite well differentiated with frequent glandular structures, or moderately differentiated (Figure S1). No increase in progenitor-like cells or mesenchymal cells could be detected, and accordingly *Snai1* mRNA amount was not modified (Figure 2D). Their similar proliferation rate was associated with identical *Ccnd1* mRNA level and Ki-67 labelling (Figure 2D). In conclusion, the loss of

HNF4 α concomitantly with β -catenin overactivation did not exacerbate HCC development as compared to β -catenin overactivation only.

3.3 | Hepatospecific inactivation of *Hnf4a* promotes hepatocyte proliferation and lipid accumulation

Regarding the role of HNF4 α in regulating many hepatocyte-specific genes, and particularly metabolic actors,^{8,10} and in view of its controlling on hepatocyte proliferation,^{9,10} we then assessed the impact of *Hnf4a* disruption in the entire liver parenchyma. Using the TTR-Cre^{ERT2} system in *Hnf4a*^{lox/lox} mice, we specifically disrupted around 90% adult hepatocytes the *Hnf4a* gene in *Hnf4a*^{Hep} panlobular model, as shown at the mRNA and protein level (Figure 3A). Loss of HNF4 α was also confirmed by a significant mRNA reduction of the three HNF4 α target genes *Apoc3*, *Ppara* and miR-122 (Figure 3B).

Hnf4a^{Hep} mice exhibited a net increase in liver to body weight ratio, associated with an induction of *Ccnd1* mRNA and Ki-67 immunostaining (Figure 3C). Finally, *Hnf4a*^{Hep} hepatocytes were also characterized by a lipid accumulation in the cytoplasm, as revealed by triglyceride enrichment (Figure 3D). These data add to the body of evidence supporting that HNF4 α downregulation increased cell proliferation^{9,10,16} and lipid metabolism in postnatal as well as in adult hepatocytes.^{8,10} As previously published,^{3,9} despite hepatomegaly and lipid accumulation, the *Hnf4a*^{Hep} liver function was not significantly altered, as shown by the serum level of aspartate and alanine transaminases (Figure 3D). Concerning liver metabolic zonation, we noticed that *Hnf4a*^{Hep} livers exhibited a global staining for GLUL, not restricted to pericentral areas (Figure S2), further supporting the antagonistic link that we and others have previously showed between HNF4 α and the Wnt/ β -catenin pathway in the liver.^{8,26}

Similarly, we created the panlobular model of invalidation in *Apc*^{lox/lox}/*Hnf4a*^{lox/lox} mice. We achieved *Hnf4a* disruption in all *Apc*^{Hep} cells in 2-month-old mice (Figure 4A). No changes neither in the liver to body weight ratio (Figure 4B), in *Ccnd1* mRNA level (Figure 4C), nor in Ki-67 staining (Figure 4D), were observed in these mice as compared to *Apc*^{Hep} ones. Taken together, our data performed in the pretumoural and tumoural models suggest that the loss of hepatic HNF4 α did not alter the proliferation rate of precancerous and cancerous hepatocytes with an overactivation of the Wnt/ β -catenin pathway.

3.4 | Hepatospecific *Hnf4a* disruption disorganized liver zonal architecture

In addition to the impact on liver size and zonation, we observed another intriguing consequence of a panlobular *Hnf4a* disruption, which occurred in 28% of *Hnf4a*^{Hep} mice (8 mice out of 28), only when they were maintained in a conventional animal facility, but never in a specific pathogen-free facility. This was characterized by a disorganization of the totality of portal triads in affected *Hnf4a*^{Hep} livers (Figure 5A), characterized by stenosis of the portal vein, accompanied by an increase in the number and size of hepatic arteries (ha) and bile ducts (bd) within the portal triad (Figure 5B). Populations of small cells were more prominent around the portal vein and also mostly proliferative (Figure 5B). Although HNF4 α deficiency was described to cause epithelial-mesenchymal transition (EMT),^{20,21} E-cadherin was normally membrane localized in hepatocytes lining the disorganized portal triads (Figure 5B). There was also no impact of *Hnf4a* disruption on the zonal pattern of E-

cadherin in the liver lobule (Figure 5B-C), or on *Cdh1* mRNA level (Figure 5D). However, we found that *Hnf4a*^{Hep} livers with triad disorganization overexpressed mRNAs coding for vimentin and snail (Figure 5D), two actors involved in hepatic EMT.¹⁹ Importantly, we also observed that *Hnf4a*^{Hep} livers exhibiting abnormal portal spaces concomitantly expressed higher mRNA level of *Tgfb* and *Bmp7*, a direct negative target gene of HNF4α¹⁰ (Figure 5D). Activation of the TGFβ and BMP7 pathways were also confirmed by immunostaining of TGFβ, SMAD4 and BMP7 exclusively in *Hnf4a*^{Hep} livers exhibiting abnormal portal spaces (Figure 5E). BMP7 is a secreted ligand of the TGFβ family, which preserves the endothelial phenotype,²⁷ and inhibits the endothelial-to-mesenchymal transition (EndMT).²⁸ Therefore, in our study, one can speculate that BMP7 was upregulated in disorganized *Hnf4a*^{Hep} livers to inhibit the TGFβ-driven EndMT and thus to limit fibrosis, as revealed by Sirius red staining (Figure S3). This disorganized liver presented interesting similarities with a human syndrome called idiopathic non-cirrhotic portal hypertension, characterized by an obliterative portal venopathy in the absence of cirrhosis and fibrosis but frequently associated with chronic infections.²⁹

Lastly, we examined the long-term evolution of *Hnf4a*^{Hep} mice with panlobular invalidation. Only one mouse with portal space disorganization among the 15 studied developed one benign and small tumour after 10 months. This mouse exhibited aggravated portal triad disorganization as compared to short-term studies (Figure 6A), together with plenty of hyperplastic and non-dysplastic areas showing a strong accumulation of GLUL, revealing β-catenin activation. All other long-term *Hnf4a*^{Hep} livers possessed portal triads moderately or even are not disorganized (Figure 6B). This less pronounced phenotype was associated with no BMP7 induction at the protein and mRNA levels (Figure 6C,D). In conclusion, portal space remodelling in *Hnf4a*^{Hep} liver was a transient event as a result of *Hnf4a* deletion, which could be reversed by BMP7 secretion in the early steps of this process. This phenomenon did not seem to be sufficient for liver carcinogenesis, as the β-catenin hyperplastic nodules observed were rare and not neoplastic.

4 | DISCUSSION

Although a tumour suppressor role in liver cancer is usually assigned to HNF4α by its frequent downregulation in HCC, the present study reveals that the loss of HNF4α in adult hepatocytes is not sufficient to promote liver tumourigenesis. Firstly, only one mouse with abnormal portal structures because of a panlobular HNF4α loss developed a benign tumour after 10 months. Secondly, in the tumour model of focal inactivation of *Apc*, a concomitant *Hnf4a* genetic deletion did not accelerate tumour onset and progression. Thus, our study contrasts with previous works supporting that *Hnf4a* deletion promotes hepatocarcinogenesis.^{16,30} The work of Yin et al. using HCC cell lines showed that HNF4α attenuated HCC progression *via* miR-134 overexpression, a microRNA expressed from the imprinted DLK1/DIO3 locus.³⁰ This locus, containing the long non-coding RNAs MEG3 and 54 microRNAs, is frequently lost in undifferentiated HCC.³¹ However, our recent data generated by deep-sequencing revealed that this locus was a direct positive target of β-catenin in case of over-activation of this signalling pathway – the DLK1/DIO3 locus including miR-134 being overexpressed in *Apc*^{Hep} tumours.^{8,13} In case of concomitant loss of HNF4α and APC, the β-catenin-mediated induction of miR-134 could counteract the

decrease of miR-134 related to *Hnf4a* disruption and thus prevent the exacerbation of tumour onset and progression. In addition, a study performed in a DEN-induced model of liver cancer using the albumin-Cre^{ERT2} system showed that *Hnf4a* disruption promoted HCC and mixed HCC-cholangiocarcinoma in response to promitogenic signals owing to c-Myc and Cyclin D1 overexpression in the non-tumour tissue.¹⁶ Our laboratory has previously demonstrated that cyclin D1 was a positive β -catenin target, induced not only in pretumoural *Apc*^{Hep} hepatocytes but also in *Apc*^{Hep} tumours. Thus, β -catenin activation in *Apc*^{Hep} liver could constitute a sufficient mitogenic signal for *Apc*^{Hep} tumour via cyclin D1, which could explain the lack of effect of *Hnf4a* disruption. Altogether, our present data support that the activation of Wnt/ β -catenin signalling suppresses the impact of HNF4 α on liver tumorigenesis. Earlier data have shown that HNF4 α -mediated suppression of colony formation was significantly impaired in the SMMC-7721 hepatoma cell line infected with adenovirus expressing a constitutive form of β -catenin (S37A).¹² This demonstrates that inhibition of β -catenin is involved in HNF4 α -mediated suppression of tumorigenesis. In summary, in *Apc*^{Hep} liver, in which β -catenin is constitutively active, HNF4 α does not play an inhibitory role on cell proliferation and EMT.

Since we have shown that HNF4 α expression is maintained in *Apc*^{Hep} non-tumoural tissue but decreased in *Apc*^{Hep} tumours, HNF4 α downregulation appears as a secondary event occurring in tumour cells after β -catenin-induced oncogenic signals to favour HCC progression. In agreement with the importance of the sequence of events responsible for tumorigenesis, there was no difference in tumour growth and onset between tumours emerging from *Apc*^{Hep} cells and those deriving from *Apc*^{Hep} cells in which *Hnf4a* was genetically and thus concomitantly disrupted. However, *Apc*^{Hep} tumour growth could be attenuated with an inhibitor against miR-34a,¹³ which increased HNF4 α level. It thus appears that HNF4 α does not play a major role in the early steps of the tumorigenic process driven by β -catenin but an enforced HNF4 α expression later in tumour clones could be of therapeutic benefit. Accordingly, HNF4 α enforced expression 10 weeks after DEN administration to rats slowed tumour growth,¹² similarly to what we observed in LNA-34a-treated *Apc*^{Hep} tumours.¹³ The use of in vivo Crispr/cas9 strategies to kinetically deplete *Hnf4a* in pretumoural hepatocytes would be determinants to definitely identify the optimal schedule for the tumour suppressive activity of HNF4 α .³²

In *Apc*^{Hep} tumours, we inversely correlated HNF4 α expression with *Snai1* and *Axin2* levels. This confirms earlier works, which high-lighted that HNF4 α inhibited β -catenin-activity to suppress EMT¹² and impaired snail expression.^{17,19} However, the concomitant loss of *Hnf4a* in *Apc*^{Hep} tumours did not affect *Snai1* mRNA levels in the tumours, and their differentiation. This contradicts data from others showing that *Hnf4a* disruption in perinatal hepatocytes using a constitutive albumin-Cre recombinase induced the expression of mesenchymal genes in hepatocytes²⁰. The discrepancies between the later study and ours could be a consequence of the distinct degree of differentiation of the targeted hepatocytes, which are adult hepatocytes in our case. However, we found that a panlobular *Hnf4a* disruption in adult liver could significantly induce the expression of TGF β , snail and vimentin, in association with a global disorganization of portal spaces (Figure 7). This abnormal liver was reminiscent of the human IPH pathology characterized by portal vein stenosis and deregulation of the TGF β signalling.²⁷ This may suggest that TGF β could drive

EMT and EndMT in portal regions following *Hnf4a* deletion. Another argument for an EndMT process occurring after a panlobular *Hnf4a* disruption is the reduction of miR-122 found in *Hnf4a*^{Hep} liver, since a loss of this microRNA is associated with EndMT in the heart.³³ This phenomenon was presented at an incomplete penetrance of 28%, and was not related to the efficiency of *Hnf4a* disruption (around 90% for all mice), suggesting that, apart from disease prevalence, factors other than *Hnf4a* loss are required. The portal remodelling in the double mutant *Hnf4a/Apc*^{Hep} was similar to that of the single mutant *Hnf4a*^{Hep}, although it has been suggested that β -catenin and TGF β could synergize in EndMT process.³⁴ Importantly, livers with abnormal portal architecture were only noticeable in adult mice maintained in a conventional animal facility and never in a specific pathogen-free animal facility. One hypothesis is that susceptibility to abnormal portal structures in *Hnf4a*^{Hep} mice could be because of chronic infections, which could participate in the establishment of IPH in humans.²⁹ In particular, dysbiosis of the gut microbiota is an interesting underlying mechanism, since healthy microbiota transplanted into a diet-induced mouse model of non-alcoholic steatohepatitis could ameliorate endothelial dysfunction and normalize portal pressure.³⁵

Lastly, in long-term experiments, only 1 out of 15 *Hnf4a*^{Hep} mice exhibited an increase in portal disorganization, while the triad disorganization was less pronounced in most *Hnf4a*^{Hep} livers at latter points as compared to day 9. In the long-term *Hnf4a*^{Hep} livers, the level of BMP7, a secreted factor preserving the endothelial phenotype in IPH,²⁷ was not significantly induced as compared to control livers. This implies that the disorganized structures are transient, and portal triads are possibly restored in response to BMP7 secretion during the early phase of EndMT. In addition, BMP7 was described to exert an anti-inflammatory effect in colon.³⁶ Since HNF4 α depletion is usually associated with pro-inflammatory signals,^{11,18} an anti-inflammatory programme in response to BMP7 induction in *Hnf4a*^{Hep} livers balanced, which favour immune cell action to restore portal triad architecture. Kupffer cells activated during portal hypertension, as revealed by CD163 expression, might be preferentially attracted in the neighbourhood of these remodelled structures.³⁷ In this context, even if portal hypertension and BMP7 have been associated with a significant risk of HCC,³⁸ we did not notice a predisposition to HCC in mice exhibiting portal disorganization. In conclusion, HNF4 α plays a complex tumour suppressor role depending on the mutational event responsible for HCC onset. Regarding our present study, HNF4 α does not play a major role during β -catenin-driven HCC. Thus, a precise diagnosis of the mutational status of *CTNNB1*, encoding β -catenin, appears crucial before investigating HNF4 α delivery-based strategies to prevent HCC development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Pascale Bossard for her help with thin layer chromatography experiments and the CRI-Biochemistry Platform of the University Paris Diderot for ASAT and ALAT measurement. We warmly thank Dr Agnès Ribeiro for the gift of *Hnf4a*^{flox/flox} mice and for helpful discussion and Dr Christèle Desbois-Mouthon for her expert advice.

Funding information This work was supported by the *Ligue Nationale Contre le Cancer* Labellisation Program 2011-2019 and the *Agence Nationale de la Recherche* (WNT-METABOLIV 2010-2014).

Abbreviations:

<i>Apc</i>^{Hep}	specific inactivation of <i>Apc</i> in hepatocytes with a liver-specific and tamoxifen-inducible Cre-loxP
Apc	Adenomatous polyposis coli
bd	bile duct
Ctrl	control
cv	central vein
DEN	diethylnitrosamine
Disorg	disorganized
EMT	epithelial-mesenchymal transition
EndMT	endothelial-to-mesenchymal transition
ha	hepatic artery
HCC	hepatocellular carcinoma
<i>Hnf4a</i>^{Hep}	specific inactivation of <i>Hnf4a</i> in hepatocytes
HNF4α	hepatocyte nuclear factor 4α
IPH	idiopathic non-cirrhotic portal hypertension syndrome
LNA	locked nucleic acid
ns	non-significant
pV	portal vein
TG	triglycerides

REFERENCES

- Sladek R, Giguere V. Orphan nuclear receptors: an emerging family of metabolic regulators. *Adv Pharmacol.* 2000;47:23–87. [PubMed: 10582084]
- Garrison WD, Battle MA, Yang C, et al. Hepatocyte nuclear factor 4α is essential for embryonic development of the mouse colon. *Gastroenterology.* 2006;130:1207–1220. [PubMed: 16618389]
- Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte nuclear factor 4α (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol.* 2001;21:1393–1403. [PubMed: 11158324]
- Schmidt D, Wilson MD, Ballester B, et al. Five-vertebrate ChIP-seq reveals the evolutionary dynamics of transcription factor binding. *Science.* 2010;328:1036–1040. [PubMed: 20378774]
- Chen WS, Manova K, Weinstein DC, et al. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Dev.* 1994;8:2466–2477. [PubMed: 7958910]

6. Parviz F, Matullo C, Garrison WD, et al. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nat Genet.* 2003;34:292–296. [PubMed: 12808453]
7. Kuo CJ, Conley PB, Chen L, et al. A transcriptional hierarchy involved in mammalian cell-type specification. *Nature.* 1992;355:457–461. [PubMed: 1734282]
8. Gougelet A, Torre C, Veber P, et al. T-cell factor 4 and beta-catenin chromatin occupancies pattern zonal liver metabolism in mice. *Hepatology.* 2014;59:2344–2357. [PubMed: 24214913]
9. Walesky C, Gunewardena S, Terwilliger EF, et al. Hepatocyte-specific deletion of hepatocyte nuclear factor-4alpha in adult mice results in increased hepatocyte proliferation. *Am J Physiol Gastrointest Liver Physiol.* 2013a;304:G26–G37. [PubMed: 23104559]
10. Bonzo JA, Ferry CH, Matsubara T, Kim JH, Gonzalez FJ. Suppression of hepatocyte proliferation by hepatocyte nuclear factor 4alpha in adult mice. *J Biol Chem.* 2012;287:7345–7356. [PubMed: 22241473]
11. Hatzia Apostolou M, Polyarchou C, Aggelidou E, et al. An HNF4alpha-miRNA inflammatory feedback circuit regulates hepatocellular on-cogenesis. *Cell.* 2011;147:1233–1247. [PubMed: 22153071]
12. Ning BF, Ding J, Yin C, et al. Hepatocyte nuclear factor 4 alpha suppresses the development of hepatocellular carcinoma. *Cancer Res.* 2010;70:7640–7651. [PubMed: 20876809]
13. Gougelet A, Sartor C, Bachelot L, et al. Antitumour activity of an inhibitor of miR-34a in liver cancer with beta-catenin-mutations. *Gut.* 2016;65:1024–1034. [PubMed: 25792709]
14. Taniguchi H, Fujimoto A, Kono H, et al. Loss-of-function mutations in Zn-finger DNA-binding domain of HNF4A cause aberrant transcriptional regulation in liver cancer. *Oncotarget.* 2018;9:26144–26156. [PubMed: 29899848]
15. Xu L, Hui L, Wang S, et al. Expression profiling suggested a regulatory role of liver-enriched transcription factors in human hepatocellular carcinoma. *Cancer Res.* 2001;61:3176–3181. [PubMed: 11306505]
16. Walesky C, Edwards G, Borude P, et al. Hepatocyte nuclear factor 4 alpha deletion promotes diethylnitrosamine-induced hepatocellular carcinoma in rodents. *Hepatology.* 2013b;57:2480–2490. [PubMed: 23315968]
17. Ning BF, Ding J, Liu J, et al. Hepatocyte nuclear factor 4alpha-nuclear factor-kappaB feedback circuit modulates liver cancer progression. *Hepatology.* 2014;60:1607–1619. [PubMed: 24752868]
18. Wang Z, Bishop EP, Burke PA. Expression profile analysis of the inflammatory response regulated by hepatocyte nuclear factor 4alpha. *BMC Genom.* 2011;12:128.
19. Garibaldi F, Cicchini C, Conigliaro A, et al. An epistatic mini-circuitry between the transcription factors Snail and HNF4alpha controls liver stem cell and hepatocyte features exhorting opposite regulation on stemness-inhibiting microRNAs. *Cell Death Differ.* 2012;19:937–946. [PubMed: 22139130]
20. Santangelo L, Marchetti A, Cicchini C, et al. The stable repression of mesenchymal program is required for hepatocyte identity: a novel role for hepatocyte nuclear factor 4alpha. *Hepatology.* 2011;53:2063–2074. [PubMed: 21384409]
21. Yang M, Li SN, Anjum KM, et al. A double-negative feedback loop between Wnt-beta-catenin signaling and HNF4alpha regulates epithelial-mesenchymal transition in hepatocellular carcinoma. *J Cell Sci.* 2013;126:5692–5703. [PubMed: 24101726]
22. Colnot S, Decaens T, Niwa-Kawakita M, et al. Liver-targeted disruption of Apc in mice activates beta-catenin signaling and leads to hepatocellular carcinomas. *Proc Natl Acad Sci U S A.* 2004;101:17216–17221. [PubMed: 15563600]
23. Tolba R, Kraus T, Liedtke C, Schwarz M, Weiskirchen R. Diethylnitrosamine (DEN)-induced carcinogenic liver injury in mice. *Lab Anim.* 2015;49:59–69. [PubMed: 25835739]
24. Benhamouche S, Decaens T, Godard C, et al. Apc tumor suppressor gene is the “zonation-keeper” of mouse liver. *Dev Cell.* 2006;10:759–770. [PubMed: 16740478]
25. Vuong LM, Chellappa K, Dhahbi JM, et al. Differential Effects of Hepatocyte Nuclear Factor 4alpha Isoforms on Tumor Growth and T-Cell Factor 4/AP-1 Interactions in Human Colorectal Cancer Cells. *Mol Cell Biol.* 2015;35:3471–3490. [PubMed: 26240283]

26. Stanulovic VS, Kyrnizi I, Kruithof-de JM, et al. Hepatic HNF4alpha deficiency induces periportal expression of glutamine synthetase and other pericentral enzymes. *Hepatology*. 2007;45:433–444. [PubMed: 17256722]
27. Kitao A, Sato Y, Sawada-Kitamura S, et al. Endothelial to mesenchymal transition via transforming growth factor-beta1/Smad activation is associated with portal venous stenosis in idiopathic portal hypertension. *Am J Pathol*. 2009;175:616–626. [PubMed: 19608867]
28. Zeisberg EM, Tarnavski O, Zeisberg M, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med*. 2007;13:952–961. [PubMed: 17660828]
29. Khanna R, Sarin SK. Non-cirrhotic portal hypertension - diagnosis and management. *J Hepatol*. 2014;60:421–441. [PubMed: 23978714]
30. Yin C, Wang PQ, Xu WP, et al. Hepatocyte nuclear factor-4alpha reverses malignancy of hepatocellular carcinoma through regulating miR-134 in the DLK1-DI03 region. *Hepatology*. 2013;58:1964–1976. [PubMed: 23775631]
31. Anwar SL, Krech T, Hasemeier B, et al. Loss of imprinting and allelic switching at the DLK1-MEG3 locus in human hepatocellular carcinoma. *PLoS ONE*. 2012;7:e49462. [PubMed: 23145177]
32. Loesch R, Desbois-Mouthon C, Colnot S Potentials of CRISPR in liver research and therapy. *Clin Res Hepatol Gastroenterol*. 2018;pii:S2210-7401(18)30085–8
33. Ghosh AK, Nagpal V, Covington JW, Michaels MA, Vaughan DE. Molecular basis of cardiac endothelial-to-mesenchymal transition (EndMT): differential expression of microRNAs during EndMT. *Cell Signal*. 2012;24:1031–1036. [PubMed: 22245495]
34. Liebner S, Cattelino A, Gallini R, et al. Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. *J Cell Biol*. 2004;166:359–367. [PubMed: 15289495]
35. Garcia-Lezana T, Raurell I, Bravo M, et al. Restoration of a healthy intestinal microbiota normalizes portal hypertension in a rat model of nonalcoholic steatohepatitis. *Hepatology*. 2018;67:1485–1498. [PubMed: 29113028]
36. Maric I, Poljak L, Zoricic S, et al. Bone morphogenetic protein-7 reduces the severity of colon tissue damage and accelerates the healing of inflammatory bowel disease in rats. *J Cell Physiol*. 2003;196:258–264. [PubMed: 12811818]
37. Holland-Fischer P, Gronbaek H, Sandahl TD, et al. Kupffer cells are activated in cirrhotic portal hypertension and not normalised by TIPS. *Gut*. 2011;60:1389–1393. [PubMed: 21572121]
38. Ripoll C, Groszmann RJ, Garcia-Tsao G, et al. Hepatic venous pressure gradient predicts development of hepatocellular carcinoma independently of severity of cirrhosis. *J Hepatol*. 2009;50:923–928. [PubMed: 19303163]

Key points

- Genetic loss of HNF4 α in adult hepatocytes did not promote liver tumourigenesis dependent on β -catenin activation.
- HNF4 α loss in adult hepatocytes caused a global portal disorganization in 28% of mice, which was reminiscent of the idiopathic non-cirrhotic portal hypertension syndrome.
- The molecular signatures suggest that TGF β -dependent endothelial- and epithelial-to-mesenchymal transitions could occur in liver after HNF4 α loss.

FIGURE 1.

HNF4α is reduced in tumours from *Apc*^{Hep} mice. A, HNF4α levels, relative to 18S, are determined by qPCR. Results are shown as the mean RQ (relative quantity) values for *Hnf4a* for 30 tumours ± SEM, as compared with normal livers (n = 4). B, HNF4α expression is maintained in precancerous *Apc*^{Hep} liver biopsies 90 days after *Apc* disruption. C,D and F, *Apc*^{Hep} are divided in two groups with either low or high expression of *Hnf4a* (threshold value = 0.5, mean value of *Hnf4a* in the cohort). *Snai1* (C), *Ccnd1* (D), and *Axin2* (F) mRNA levels, relative to 18S, were determined by qPCR, and represented as previously, as compared with non-tumour tissues. E, Inverse correlation between Cyclin D1 and HNF4α by immunohistochemistry in *Apc*^{Hep} tumours. ****P* < 0.001, ***P* < 0.01

FIGURE 2.

Hnf4a disruption does not accelerate the development of tumours from *Apc*^{Hep} mice. A and B, Characterization of *Hnf4a/Apc*^{Hep} mice by HNF4α (left panel) and GLUL immunostainings (right) 6 months after Cre adenovirus injection in *Hnf4a/Apc*^{Hep} liver (A) and tumours (B). C, left panel: Number of months for tumour apparition detected by ultrasounds following adenovirus injection. The results are expressed as the mean of months ± SEM normalized with respect to *t0*, for *Apc*^{Hep} mice (n = 8) and for *Hnf4a/Apc*^{Hep} mice (n = 4). Middle panel: Number of tumours detected during ultrasound following. Right panel: Tumour development was followed by 2D ultrasound bimonthly. The results are expressed as the fold-change in volume over 13 days ± SEM normalized with respect to *t0*, for mice with *Apc* disruption and with (n = 8) or without HNF4α (n = 4). D, *Ccnd1* and *Snai1* mRNA levels determined by qPCR and represented as previously, as compared with non-tumour tissues. Ki-67 quantification represents the mean number of positive cells in the entire tumour. All the results were non-significant (ns)

FIGURE 3.

Characterization of *Hnf4a*^{Hep} mice. A, HNF4α is lost following tamoxifen diet, as revealed by qPCR, western-blot and immunostaining, as compared to control mice without Cre recombinase. *Hnf4a* mRNA levels, relative to 18S, are shown as the mean RQ (relative quantity) values for six mice $\pm$ SEM, as compared to control mice. B, *Apoc3*, *Ppara* and *miR-122* mRNA levels were determined by qPCR, and represented as previously, as compared to control mice (Ctrl). C, Left panel: Liver to body weight ratio of *Hnf4a*^{Hep} mice (n = 9) as compared to control (Ctrl) mice (n = 7). Middle panel: *Ccnd1* mRNA level, relative to 18S, are determined by qPCR, and represented as previously, as compared to control mice (Ctrl). Right Panel: Comparison of Ki-67 immunostaining between control (Ctrl) and *Hnf4a*^{Hep} mice. D, Comparison of triglyceride (TG) content in liver following *Hnf4a* disruption (*Hnf4a*^{Hep}) as compared to control mice (Ctrl) by thin layer chromatography, relative quantity normalized to loading (left) and oil red O staining (middle), together with measuring alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) concentrations in serum (right). ** $P < 0.01$, * $P < 0.05$. ns, non-significant

FIGURE 4.

Hnf4a disruption in hepatocytes with β -catenin activation does not exacerbate hepatocyte proliferation. A, HNF4 α and APC are efficiently lost following tamoxifen diet, as revealed by *Hnf4a* and *Glul* mRNA levels measured by qPCR in five different samples and immunostaining. B, Liver to body weight ratio of *Hnf4a/Apc^{Hep}* mice (n = 9) as compared to control (Ctrl) mice (n = 7). C and D, Comparison of *Ccnd1* mRNA level (C), and Ki-67 immunostaining (D) between *Apc^{Hep}* and *Hnf4a/Apc^{Hep}* mice. Ki-67 quantification represents the mean number of positive cells in the four different liver zones. ns, non-significant

FIGURE 5.

Hnf4a disruption alters liver zonal structure. A-C, Portal triad disorganization following *Hnf4a* disruption is shown by haematoxylin/eosin (A), α-SMA, Cytokeratin-19 (CK19), Ki-67 staining (B) and E-cadherin immunostaining (B-C). Ki-67 quantification represents the mean number of positive cells near four different portal triads. D, Comparison of *Tgfb*, *Vim*, *Snai1*, *Cdh1* and *Bmp7* mRNA levels, relative to 18S, as determined by qPCR and represented as previously, between control mice (Ctrl), *Hnf4a*^{Hep} mice with normal portal triad (norm) and those with disorganized triad (disorg). E, Immunostaining of TGFβ,

SMAD4 and BMP7 in *Hnf4a*^{Hep} mice with normal portal triad (norm) and those with disorganized triad (disorg). bd, bile duct, cv, central vein; ha, hepatic artery, pv, portal vein.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 6.

Portal triad disorganization in *Hnf4a*^{Hep} is a transient event. A, One *Hnf4a*^{Hep} mouse with disorganized portal triad develops a GLUL-positive nodule 10 months after tamoxifen diet. cv : central vein, pv: portal vein, HA : hepatic artery, bd : bile duct, ns : non-significant. ****** $P < 0.01$, ***** $P < 0.05$. B, haematoxylin/eosin staining of long-term *Hnf4a*^{Hep} livers. C and D, Expression of BMP7 in long-term *Hnf4a*^{Hep} livers detected by immunostaining (C) and qPCR (D)

FIGURE 7.

Hnf4a^{Hep} livers were characterized by portal disorganization characterized by stenosis of the portal vein, accompanied by an increase in the number and size of hepatic arteries and bile ducts within the portal triads. These structures are associated with overexpression in hepatocytes of TGFβ and BMP7, a negative target of HNF4α and proliferation/infiltration of non-parenchymal cells (NPC) near the disorganized portal triads. The others features of *Hnf4a*^{Hep} livers are (a) steatosis owing to decrease in apolipoprotein content and thus impaired secretion of very low-density lipoprotein (VLDL); (b) hepatocyte proliferation; and (c) loss of zonal expression of GLUL as a result of the absence of inhibitory activity of HNF4α on β-catenin-driven programme