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MiR-15/16 mediate crosstalk between the MAPK and Wnt/β-catenin pathways during hepatocyte differentiation from amniotic epithelial cells

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ABSTRACT

MiR-15/16 play an important role in liver development and hepatocyte differentiation, but the mechanisms by which these miRNAs regulate their targets and downstream genes to influence cell fate are poorly understood. In this study, we showed up-regulation of miR-15/16 during HGF- and FGF4-induced hepatocyte differentiation from amniotic epithelial cells (AECs). To elucidate the role of miR-15/16 and their targets in hepatocyte differentiation, we investigated the roles of miR-15/16 in both the MAPK and Wnt/β-catenin pathways, which were predicted to be involved in miR-15/16 signaling. Our results demonstrated that the transcription of miR-15/16 was enhanced by c-Fos, c-Jun, and CREB, important elements of the MAPK pathway, and miR-15/16 in turn directly targeted adenomatous polyposis coli (APC) protein, a major member of the β-catenin degradation complex. MiR-15/16 destroyed these degradation complexes to activate β-catenin, and the activated β-catenin combined with LEF/TCF7L1 to form a transcriptional complex that enhanced transcription of hepatocyte nuclear factor 4 alpha (HNF4α). HNF4α also bound the promoter region of miR-15/16 and promoted its transcription, thereby forming a regulatory circuit to promote the differentiation of AECs into hepatocytes. Endogenous miRNAs are, therefore, involved in hepatocyte differentiation from AECs and should be considered during the development of an effective hepatocyte transplant therapy for liver damage.

1. Introduction

Transplantation of organs or tissues is one of the main aims of regenerative medicine. A major barrier to successful implementation is the requirement for an effective and safe source of cells for transplantation. Isolated cells from specific tissues, including stem cells, are increasingly considered as possible donor cells for the treatment of human disease including liver damage. Hepatocyte transplantation to treat liver damage is largely limited by the availability of suitable hepatic cells. Previous reports revealed that the use of stem cells may be the most effective approach to producing functional hepatic cells for cell transplantation [1,2]. Thus, hepatic cells differentiated from stem cells have the potential to overcome the shortage of hepatic cells for clinical application.

Currently, to induce the differentiation of hepatic cells from stem cells in vitro, hepatocyte growth factor (HGF) and fibroblast growth factor-basic are typically used. A study by Chen et al. used HGF and fibroblast growth factor 4 (FGF4) to induce hepatocyte differentiation from embryonic stem cells, which activated the Wnt signaling pathway during differentiation [3,4]. The Wnt signaling pathway plays an important role in regulating the self-renewal and multi-lineage differentiation potential of stem cells. Wnts are separated into two classes: canonical Wnts and non-canonical Wnts. The Wnt/β-catenin signaling pathway comprises canonical Wnts, which associate with LRP5/6–Frizzled (Fzd) receptor complexes to stabilize β-catenin. These complexes then enter the cell nucleus to activate the TCF/LEF family of transcription factors [5–7]. HGF and FGF also activate the MAPK signaling pathway [8,9]. Although the Wnt pathway has been clearly

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demonstrated to determine the differentiation fate of stem cells, the upstream mechanisms by which the p38 MAPK kinase signaling pathway activates the Wnt/β-catenin pathway to promote hepatocyte differentiation remain to be elucidated.

A major group of endogenous, small, noncoding ribonucleotides (18–24 nt) called microRNAs (miRNAs), which are present in animals and plants, has been shown to play important roles in the regulation of gene expression at the post-transcriptional level [10]. Liver-specific miRNAs have been widely reported, and include miR-122, miR-192, miR-194, and miR-106 [11–15]. The miR-15 cluster includes miR-15a and miR-16, which are clustered within 500 base pairs of each other at chromosome position 13q14 in humans. These miRNAs are abundantly expressed in the human embryonic liver [14,16]. Previous research has revealed that miR-15/16 play a key role in hepatocyte differentiation from mesenchymal stem cells, but the transcriptional regulation of this cluster has not been fully investigated.

Amniotic epithelial cells (AECs) derived from the placenta exhibit biological characteristics similar to embryonic stem cells (ESCs) and have the capacity for differentiation into all three germ layers, as well as hepatic cells [17,18]. In the present study, HGF and FGF were used to activate the MAPK and Wnt/β-catenin pathways to induce AEC differentiation into hepatocytes. To better illuminate the roles of miR-15/16 in the transcriptional regulation of hepatocyte differentiation from AECs, we investigated the effect of mutations on the transcription factor-binding sites of the various genes of interest along the MAPK and Wnt/β-catenin pathways. Additionally, we over-expressed miR-15/16 and knocked down its target genes in vitro. Using this approach, we systematically elucidated the molecular mechanisms involved in growth factor-induced hepatocyte differentiation from AECs.

2. Results

2.1. Growth factor-induced hepatocyte differentiation from AECs

HGF, FGF4, and Oncostatin M, classical cocktail factors for hepatocyte differentiation from stem cells, were added to the culture medium of AECs to induce hepatocyte differentiation. Albumin (ALB) and CYP3A4, both mature liver markers, and hepatocyte nuclear factor 4 alpha (HNF4α), a special transcription factor in the liver that is induced during differentiation, were detected in the induced AECs with immunofluorescence staining (Fig. 1A) and western blotting (Fig. 1B). HepG2 cells were used as the positive control, and normal (i.e., uninduced) AECs were used as the negative control. ALB and CYP3A4 were expressed in the cytoplasm of induced AECs and the HepG2 cells, while HNF4α was expressed in the nuclei of the same cells. These markers were not detected in the normal AECs. Glycogen staining showed that glycogen was synthesized and stored in the induced AECs during the three-week induction period, as indicated by the bright red color observed in induced AECs, while the normal AECs exhibited no such staining (Fig. 1C). Based on the results of previous reports, miR-15/16 play a key role in liver development during the fetal period. We, therefore, tested the relative expression levels of miR-15/16 as well as that of adenomatous polyposis coli (APC) protein, a putative target of miR-15/16 predicted in our study, in the normal and induced AECs using real-time quantitative PCR (RT-qPCR; Fig. 1D). MiR-15/16 increased dramatically following hepatocyte differentiation (8-fold increase), while APC was significantly down-regulated (3-fold reduction).

2.2. HGF and FGF4 activate the MAKP and Wnt/β-catenin pathways during hepatocyte differentiation

We hypothesized that for the AECs, MAPK pathway activation begins at the cell membrane, where JNK, p38, ERK, c-Fos, CREB, and c-Jun, the major members of the MAPK pathway, are activated by HGF, FGF-4, and Oncostatin M to induce hepatocyte differentiation. To investigate the role of each of these three factors in the activation of the MAPK pathway, different combinations of the factors were added into the culture medium and their effects on the expression of proteins involved in this pathway were analyzed with western blotting. Our results indicated that while Oncostatin M did not affect activation of the MAPK pathway, HGF and FGF4 play an important role in hepatocyte differentiation (Fig. 2A).

JNKs (c-Jun NH2-terminal kinases) phosphorylate and thereby activate c-Jun. JNK-IN-8 is an irreversible JNK inhibitor that regulates the phosphorylation and expression of JNK1, JNK2, and JNK4, and influences the activation of c-Jun into the nuclear form [19]; therefore, JNK-IN-8 was used to demonstrate the activation of c-Jun by a cocktail of factors via JNK phosphorylation in induced AECs. SB203580 is a p38 MAPK pathway inhibitor that is widely used as a research tool, and it blocks classical p38 pathways by inhibiting the α and β isoforms. The transcription factor CREB is a target of the p38 MAPK pathway via the downstream kinases, MAPKAP kinases and MNK1/2 [20]. Therefore, SB203580 directly inactivates CREB by blocking the p38 MAPK kinase pathway. SCH772984, a selective inhibitor of ERK1/2 that displays behaviors of both type I and type II kinase inhibitors, has nanomolar cellular potency in tumor cells and induces tumor regressions in xenograft models at tolerated doses [21]. In our experiments, JNK-IN-8, SCH772984, and SB203580 were tested separately and in combination for treating induced AECs. As a result, the related transcription factors, c-Jun, c-Fos, and CREB, showed significant changes in nuclear levels, as did HNF4α (Fig. 2B). This indicated that the addition of these inhibitors resulted in the down-regulation of not only their respective target's expression but also that of HNF4α. Crosstalk between the MAPK and Wnt/β-catenin pathways has been widely reported [22,23]. In this study, we investigated for crosstalk by adding the inhibitor XAV-939 or activator LiCl into the medium to evaluate the role of the Wnt/β-catenin pathway in cocktail factor-induced hepatocyte differentiation from AECs. XAV-939 is known to antagonize Wnt/β-catenin-mediated transcription, and the addition of this inhibitor led to significant down-regulation of activated β-catenin, HNF4α, and the mature liver markers, ALB and CYP3A4, with some down-regulation also observed for ERK (Fig. 2B). The results demonstrated that the Wnt/β-catenin pathway acts as a switch in the differentiation of hepatocytes by regulating the transcription of its downstream genes. LiCl, a GSK-3β inhibitor, represses the formation of β-catenin/GSK-3β/APC complexes to release active β-catenin, which, in turn, activates the Wnt/β-catenin pathway. Our data demonstrated that LiCl enhanced the activation of β-catenin to increase expression of ALB, CYP3A4, and HNF4α (Fig. S1). Using immunofluorescence staining, c-Fos, CREB, c-Jun, and nuclear β-catenin were all shown to have clear up-regulation in the nuclei of AECs following growth factor induction relative to normal, uninduced AECs (Fig. 2C). This clearly illustrated the activation of the MAPK and Wnt/β-catenin pathways in these cells. The addition of the four different inhibitors (JNK-IN-8, SCH772984, SB203580, and XAV-939) resulted in the down-regulation of not only their respective target's expression, but also significantly reduced the level of AEC differentiation into hepatocytes, which indicated a positive correlation between the affected transcription factors and hepatocyte formation from AECs (Fig. 2D). These results implied that the Wnt/β-catenin pathway plays an important role in hepatocyte differentiation from AECs, but the mechanisms by which the various cocktail factors regulate MAPK pathway to activate the Wnt/β-catenin pathway are poorly understood.

MiR-15/16 play a key role in liver development during fetal development, and our results indicated a positive correlation between the expression of miR-15/16 and HNF4α in AECs following treatment with the growth factor cocktail (Fig. 3). To illustrate the role of miR-15/16 in hepatocyte differentiation, a miR-15/16 mimic and miR-15/16 sponge were added to induced AECs, and the expression rates of the two liver markers, albumin and CYP3A4, and that of the hepatocyte differentiation marker, HNF4α, were analyzed using flow cytometry (Fig. 3A). Induced AECs showed significant up-regulation of all three markers, and this effect was reversed in the presence of the miR-15/16 sponge.

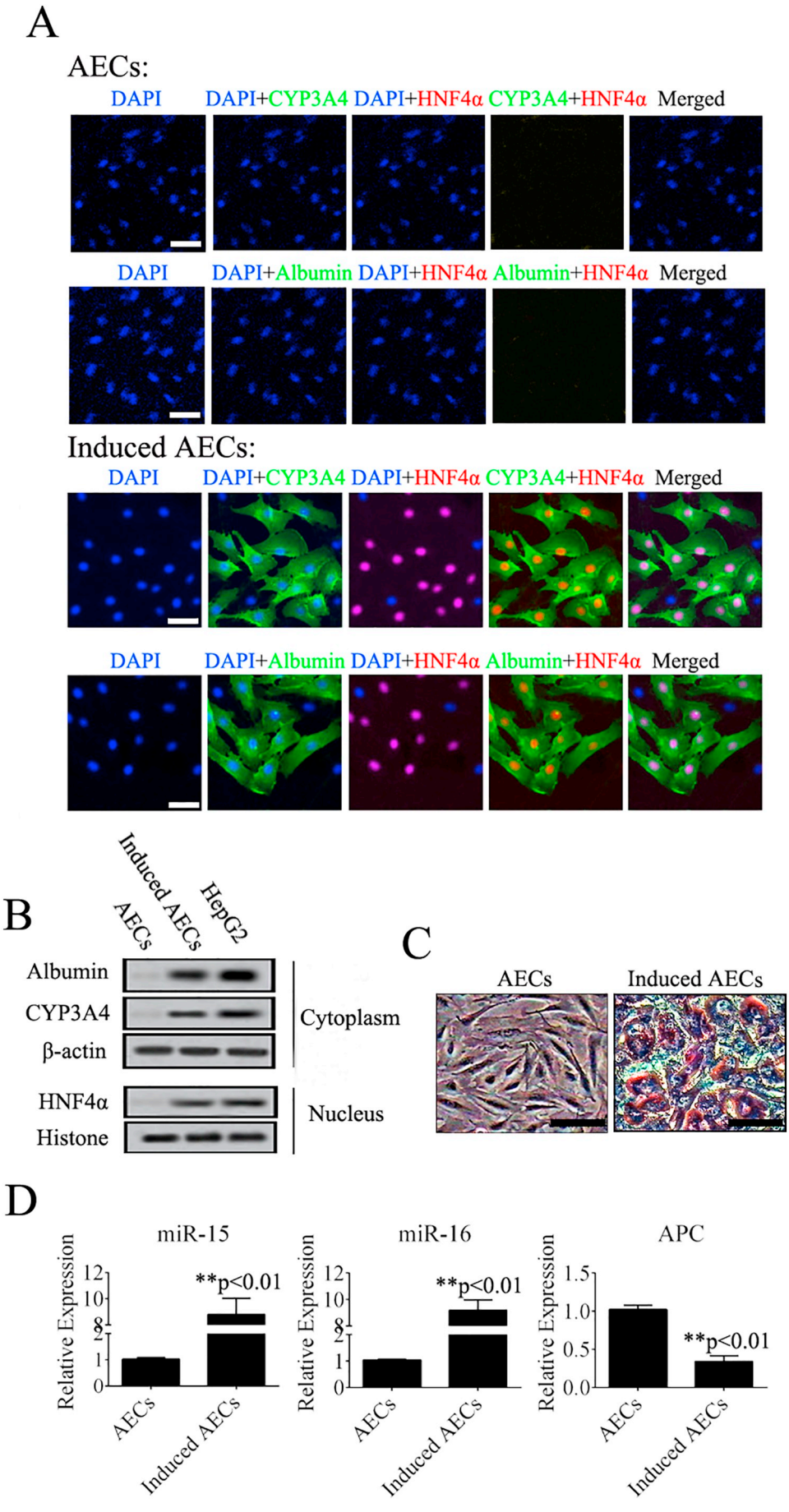

Fig. 1. Hepatocytes can differentiate from amniotic epithelial cells (AECs). The AECs were induced with a cocktail of factors (HGF, FGF4, and Oncostatin M) for three weeks. A. Hepatocyte markers were detected by immunofluorescence staining. Normal AECs were negative for hepatocyte markers (top panels), while the induced AECs were positive (bottom panels) (scale bar = 100 μm). B. Hepatocyte markers were confirmed with western blotting. HNF4α is a special transcription factor expressed in the nuclei of liver cells, while albumin (ALB) and CYP3A4 are mature liver markers expressed in the cytoplasm of induced AECs. HepG2 cells and normal AECs were the positive and negative control, respectively. C. Glycogen staining was positive in the induced AECs, which presented bright red in color, indicating glycogen synthesis and storage in these cells; in contrast, the normal AECs showed no such staining. All images are representative of at least three independent replicates. D. RT-qPCR was used to determine the relative expression of miR-15/16 and that of their putative target, APC (adenomatous polyposis coli), in AECs and in hepatocytes differentiated from AECs. Values represent the mean ± SD of at least three replicates.

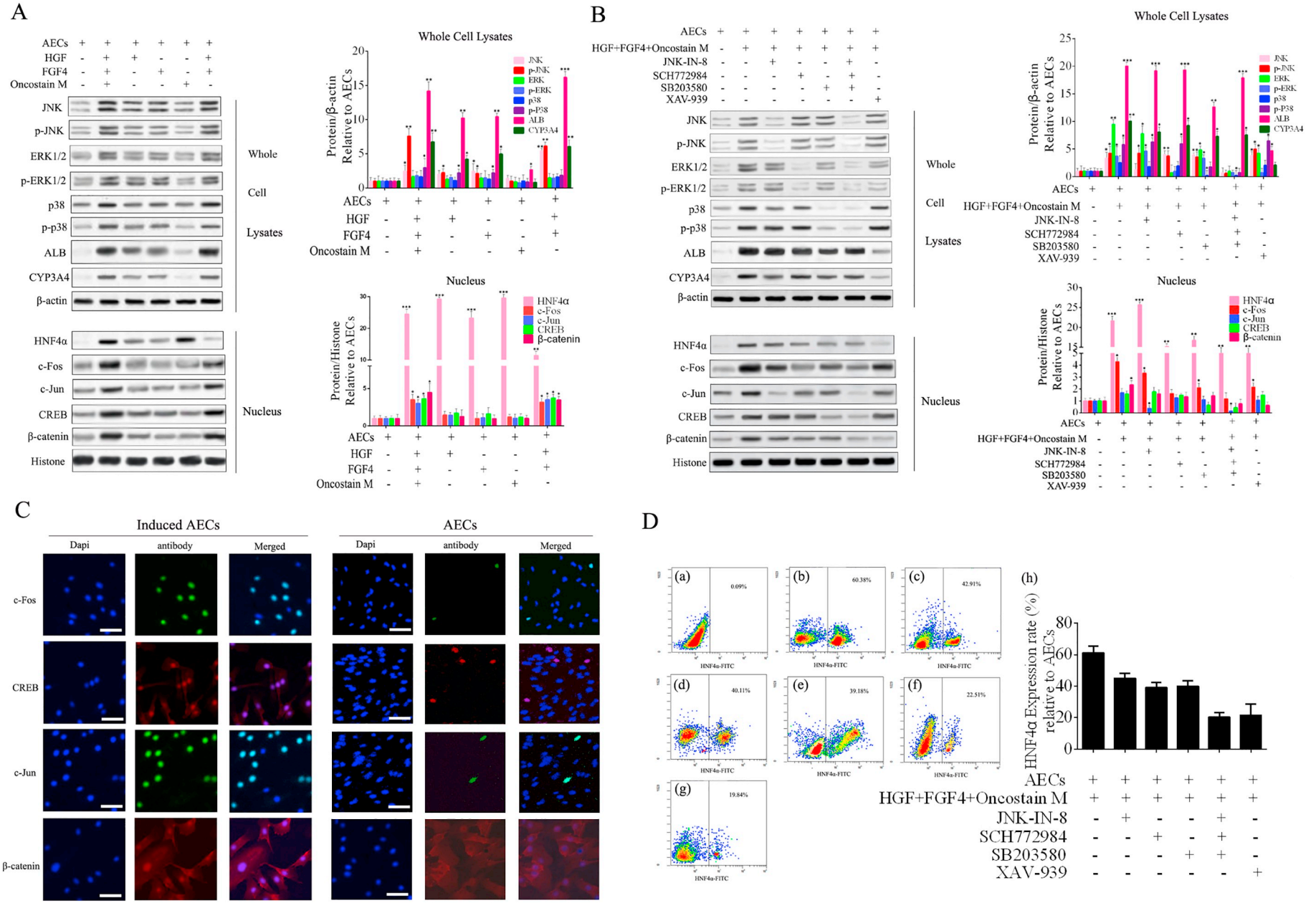

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Fig. 2. Activation of the MAPK and Wnt/β-catenin pathways during hepatocyte differentiation of AECs. A. Hepatocyte markers were tested using western blotting after AECs were exposed to various growth factors. The addition of growth factors to AECs demonstrated the critical roles that HGF and FGF4 play in the activation of both the MAPK and Wnt/β-catenin pathways. B. The role of the MAPK and Wnt/β-catenin pathways in the expression of hepatocyte markers was confirmed by the addition of specific inhibitors of different components of these pathways. The results demonstrated that the Wnt/β-catenin pathway acts as a switch in the differentiation of hepatocytes by regulating the transcription of its downstream genes. JNK-IN-8, JNK pathway inhibitor; SCH772984, ERK pathway inhibitor; SB203580, p38 pathway inhibitor; and XAV-939, Wnt/β-catenin pathway inhibitor. Western blot images are representative of at least three independent replicates. Protein abundance was analyzed using ImageJ tools. C. Immunofluorescence staining illustrated the activation of the MAPK and Wnt/β-catenin pathways based on the nuclear expression of c-Fos, c-Jun, CREB, and β-catenin after growth factor induction. The results showed that c-Fos, c-Jun, CREB, and β-catenin in induced AECs were dramatically up-regulated compared with normal, uninduced AECs (scale bar = 100 μm). D. Role of the MAPK and Wnt/β-catenin pathways in hepatocyte differentiation. The same inhibitors used to investigate hepatocyte differentiation were tested singly and in combination using flow cytometry to determine their effect on the differentiation rate of the hepatocytes. (a) Normal, uninduced AECs; (b) AECs induced with a cocktail of growth factors; (c) induced AECs exposed to the inhibitor, JNK-IN-8; (d) induced AECs exposed to the inhibitor, SCH772984; (e) induced AECs exposed to the inhibitor, SB203580; (f) induced AECs exposed to a combination of the inhibitors, JNK-IN-8, SCH772984, and SB203580; (g) induced AECs exposed to the inhibitor, XAV-939; (h) the percentages of HNF4α-positive cells following the above different treatments. The results showed that not only the MAPK pathway but also the Wnt/β-catenin pathway has a significant effect on the differentiation of AECs into hepatocytes. Values represent the mean ± SD of at least three replicates.

These results imply that HNF4α and miR-15/16 play a critical role in growth factor-induced hepatocyte differentiation from AECs. Therefore, HNF4α was used as a special marker to determine the level of hepatocyte differentiation in the presence or absence of the growth factor cocktail, miR-15/16, and the miR-15/16 sponge (Fig. 3B). The results demonstrated that miR-15/16 are indeed an important molecular node in growth factor-induced hepatocyte differentiation. To further elucidate the role of HNF4α in hepatocyte differentiation, a siRNA against HNF4α was designed, synthesized, and transfected into AECs during differentiation. Based on qPCR, the mature liver markers, ALB and CYP3A4, were significantly reduced after silencing of HNF4α compared with treatment with the scrambled siRNA control (Fig. 3C). To further analyze the expression of miR-15/16 during hepatocyte differentiation, we added the same inhibitors to AECs singly or in different combinations, to test their effect on the expression of miR-15/16 using qPCR (Fig. 3D). The data showed dramatic down-regulation of miR-15/16 after single or combination treatment with inhibitors of the MAPK pathway, but a non-significant blocking effect on the Wnt/β-catenin pathway. Taken together, the results indicate a positive correlation between miR-15/16 expression and activation of the Wnt/β-catenin pathway; however, no reports regarding miR-15/16-activation of the Wnt/β-catenin pathway during hepatocyte differentiation have yet been reported, and the molecular mechanisms underlying this process are unclear.

2.3. MAPK pathway enhances transcription of miR-15/16

It is clear that miR-15/16 play an important role in hepatocyte differentiation from AECs. Despite the expression levels of miR-15/16 showing a significant change after the addition of XAV-939, an inhibitor of the Wnt/β-catenin pathway, compared with normal AECs (Fig. 3D), the data strongly suggest that the MAPK pathway rather than the Wnt/β-catenin pathway is a key regulator of the transcription of miR-15/16. To determine the mechanisms underlying the elevated expression of miR-15/16 in induced AECs, we used bioinformatic algorithms to screen for binding sites of the c-Fos, c-Jun, and CREB transcription factors within the region 3 kbp upstream of pre-miR-15/16. Multiple binding sites were found (Fig. 4A, B). To test the regulation of these sites by their respective transcription factors, the binding sites for each factor were mutated singly or in combination using site-directed mutagenesis of the pGL3.0-miR-15/16 promotor vectors. The different vectors were then transfected into HEK293T cells for analysis using a sensitive luciferase assay system to measure transcription factor-mediated transcriptional activation. HEK293T cells are extensively used for luciferase assays because of their ease of use and high transfection efficiency. We co-transfected the wild-type (WT) promotors of miR-15/16 or their mutated (MUT) counterparts together with c-Fos, c-Jun or CREB over-expression vectors into the HEK293T cells. Luciferase activity was measured as a proxy for miR-15/16 expression. For these assays, either the full-length miR-15/16 promoter (WT) or the promoters with different mutated sites (MUT) were co-transfected with a luciferase reporter vector (pRL-40) into the cells. After 48 h, the cells were harvested and lysed, and luciferase activities were measured using a dual luciferase reporter assay. The pRL-40 vector alone was used as an internal control. The results revealed that c-Fos, c-Jun, and CREB bind to sequences within the miR-15/16 promoters to enhance their transcription (Fig. 4C–E).

To test the physical interactions between the miR-15/16 promoter and these transcription factors, PCR primers spanning the factor-binding sites (Fig. 5A) were used to detect the presence of protein binding within these sites using ChIP-PCR (Fig. 5B–D). For each of the three transcription factors, two or more fragments of the promotor were found to be positive for factor binding after PCR amplification. To accurately compare the amounts of the three transcription factors enriched in the miR-15/16 promoter region, ChIP coupled with quantitative PCR analysis (qPCR) was performed. Nonspecific IgG was used as a negative control, and input sample (whole lysate prior to ChIP being performed) was used as a positive control. As shown in Fig. 5E–G, the amounts of immunoprecipitated chromatin were significantly higher in the hepatocytes than in the AECs, with a fold change in occupancy of 4.76, 5.28, and 4.09 for c-Fos, c-Jun, and CREB, respectively.

Electrophoretic mobility shift assay (EMSA) is a common affinity electrophoresis technique used to study protein-nucleic acid interactions. To confirm the interactions observed with ChIP-PCR, EMSA probes were designed for each transcription factor based on their respective binding sites within the promoter sequence. Only the binding sites predicted by bioinformatic tools to have the highest probability score for each factor were selected for probe design. The EMSA results showed that c-Fos, c-Jun, and CREB did indeed bind to their respective binding sites (Fig. 5H–J). The specificity of protein binding to these sites was further confirmed by competition with 100–300-fold molar excess of unlabeled EMSA probe. Supershift bands were also clearly observed after incubation with specific antibodies against each of the transcription factors.

2.4. MiR-15/16 are important mediators in the crosstalk between the MAPK and Wnt/β-catenin pathways

In agreement with previous reports, we observed that the expression levels of miR-15/16 were significantly increased during hepatocyte differentiation. To determine the role of miR-15/16 in hepatocyte differentiation, a miR-15/16 over-expression vector and a miR-15/16 sponge were designed, synthesized, and added to the hepatic induction medium. Western blotting revealed that HNF4α and the liver-specific genes, CYP3A4 and ALB, as well as dephosphorylated β-catenin, were all down-regulated after sponge treatment (Fig. 6A–C).

The target mRNAs of miR-15/16 were then predicted using starBase V2.0, and gene ontology analysis of the predicted targets was performed with PANTHER. Several signaling pathways were predicted to be targets of miR-15/16, including the Wnt, TGF-β, Notch, and PDGF

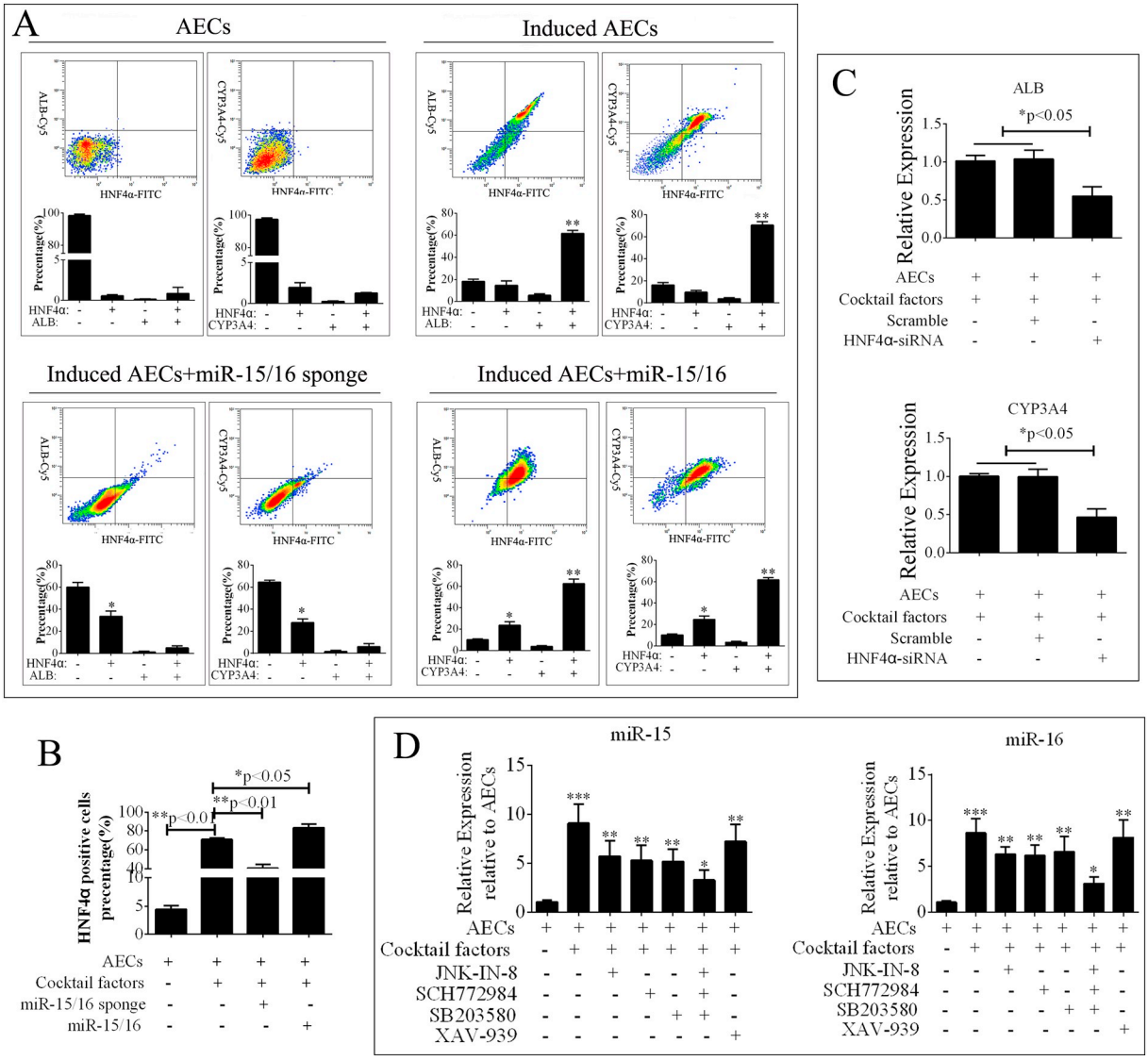
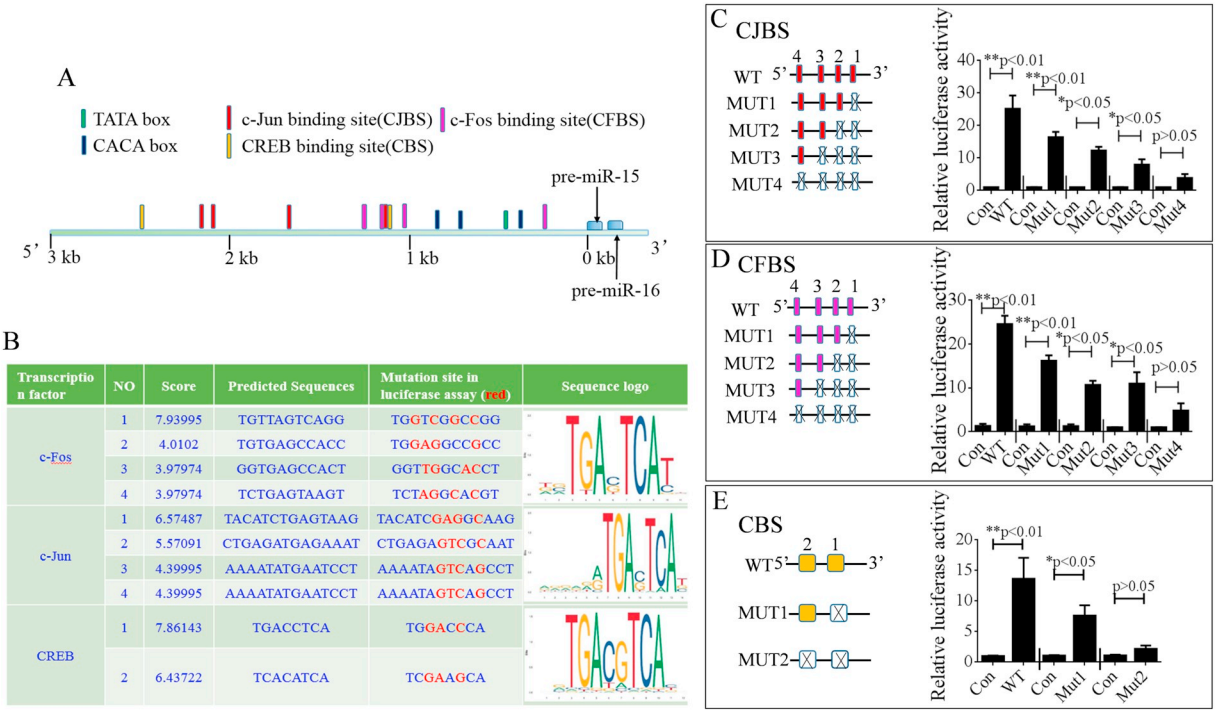


Fig. 3. Role of miR-15/16 in hepatocyte differentiation from AECs. A. miR-15/16 directly affects the expression of the hepatocyte markers, CYP3A4 and albumin, and that of the hepatocyte differentiation marker, HNF4α, in induced AECs based on flow cytometry analyses. MiR-15/16 increased expression of these hepatocyte markers when it was over-expressed in induced AECs, but treatment with a miR-15/16 sponge produced the opposite effect. B. MiR-15/16-mediated hepatocyte differentiation. HNF4α was used as a special marker to calculate the proportion of AECs differentiating into hepatocytes in A. C. HNF4α is an important transcription factor for hepatocyte differentiation from AECs. A siRNA of HNF4α was designed and added during hepatocyte differentiation, and the mature liver markers, ALB and CYP3A4, were tested using RT-qPCR. The results demonstrated that HNF4α influenced the expression of ALB and CYP3A4 during hepatocyte differentiation from AECs. A scrambled siRNA (negative control) had no such effect. D. The effects of the MAPK and Wnt/β-catenin pathways on the expression of miR-15/16. For this analysis, different inhibitors were added singly or in combination during hepatocyte differentiation, and RT-qPCR was used to test the expression level of miR-15/16 relative to that of normal, uninduced AECs. The results illustrated a positive correlation between miR-15/16 expression levels and activity of the MAPK and Wnt/β-catenin pathways. JNK-IN-8, JNK pathway inhibitor; SCH772984, ERK pathway inhibitor; SB203580, p38 pathway inhibitor; and XAV-939, Wnt/β-catenin pathway inhibitor. Values represent the mean ± SD of at least three replicates.

signaling pathways. Adenomatous polyposis coli (APC), a negative regulator of the Wnt/β-catenin pathway, was found to have a conserved miR-15/16-binding site within its 3′UTR region, and western blotting demonstrated that APC underwent dramatic down-regulation after miR-15 or miR-16 agomir treatment, with the opposite results observed after miR-15/16 sponge over-expression (Fig. 6D, E). The Argonaute 2 (AGO2) protein plays a central role in RNA silencing processes, as an essential component of the RNA-induced silencing complex (RISC). To determine whether APC serves as a binding platform for AGO2 and miR-15/16, we performed Myc-AGO2 immunoprecipitation in HEK293T cells containing either an AGO expression vector or an empty vector and transiently co-expressing miR-15/16 or Let-7a (negative control). The resulting APC mRNA levels were analyzed by RTq-PCR of the immunoprecipitation products. Endogenous APC was specifically enriched by > 23–28-fold in the presence of AGO2 in miR-15/16-transfected cells compared with the control (Let-7a-transfected cells) (Fig. 6F). These results suggest that miR-15/16 facilitate AGO2 association with APC.

To determine whether the putative binding site in the 3′UTR of APC was responsible for silencing APC gene expression by miR-15/16, we next cloned the wild-type (WT) and mutated (MUT) version of the APC 3′UTR region downstream of a luciferase reporter gene and co-transfected either of these vectors into HEK293T cells with either pre-miR-15/16 or a control precursor. In HEK293T cells co-transfected with the pre-miRNA and pRL-APC-WT, luciferase activity was significantly decreased relative to that in HEK293T cells co-transfected with control



Transcription factor	NO	Score	Predicted Sequences	Mutation site in luciferase assay (red)	Sequence logo
c-Fos	1	7.93995	TGTTAGTCAGG	TGGTCGGCCGG	
	2	4.0102	TGTGAGCCACC	TGGAGGCCGCC	
	3	3.97974	GGTGAGCCACT	GGTTGGCACCT	
	4	3.97974	TCTGAGTAAGT	TCTAGGCACGT	
c-Jun	1	6.57487	TACATCTGAGTAAG	TACATCGAGGCAAG	
	2	5.57091	CTGAGATGAGAAAT	CTGAGAGTCGCAAT	
	3	4.39995	AAAATATGAATCCT	AAAATAGTCAGCCT	
	4	4.39995	AAAATATGAATCCT	AAAATAGTCAGCCT	
CREB	1	7.86143	TGACCTCA	TGGACCCA	
	2	6.43722	TCACATCA	TCGAAGCA	

Fig. 4. c-Jun, c-Fos, and CREB promote the transcription of miR-15/16 via physical interaction with their promoter. A. Schematic of the predicted binding sites of each of these transcription factors in the promoter region of miR-15/16. B. The promoter-binding sites for c-Fos, c-Jun, and CREB were predicted with bioinformatic algorithms and subsequently mutated to determine the effect on miR-15/16 expression. C–E. The full-length miR-15/16 promoter (WT) and the miR-15/16 promoter containing different mutated variants of the c-Jun/c-Fos/CREB binding sites (MUT) were tested to determine the effect on miR-15/16 expression. For these assays, the WT and MUT vectors were co-transfected with a luciferase reporter vector (pRL-40) into HEK293T cells. After 48 h, cells were harvested and lysed, and luciferase activities were measured using a dual luciferase reporter assay. The pRL-40 vector alone was used as an internal control. Results are expressed as relative luciferase activity and represent the mean ± SD of at least three replicates.

precursor or pRL-APC-MUT (Fig. 6G, H).

2.5. β-Catenin/LEF/TCF7L1 complex promotes transcription of HNF4α

The Wnt/β-catenin pathway also plays an important role in regulating hepatocyte differentiation from stem cells and in regulating the expression of hepatocyte-specific genes. However, there is no direct evidence of the mechanisms of this regulation following the activation of β-catenin. APC is a major member of the β-catenin degradation complexes in the cytoplasm. These APC/β-catenin/GSK3β/AXIN complexes accelerate proteolysis. We used Myc as a tag to construct a Myc-APC plasmid, which was co-transfected with miR-15/16 into AECs to demonstrate the miR-15/16-mediated reduction in the formation of the APC/β-catenin/GSK3β/AXIN complexes via the targeting of APC and the activation of β-catenin. We then performed immunoprecipitation and immunoblotting of transfected AECs using specific antibodies to detect Myc-APC, β-catenin, GSK3β, and AXIN. The results showed that miR-15/16 down-regulates APC, which leads to decreased formation of APC/β-catenin/GSK3β/AXIN complexes and increased levels of activated β-catenin (Fig. 7A, B). The Wnt signaling pathway regulates target gene expression by stabilizing the cytoplasmic component β-catenin, which enters the nucleus and forms complexes with TCF/LEF factors bound to specific promoter sequences. There are four TCF/LEF factors in mammals: TCF7, TCF7L1, TCF7L2, and LEF1 [24]. Our western blotting data revealed that TCF7 and TCF7L1 are both expressed in AECs, and the expression level of TCF7L1 was higher than that of TCF7 (Fig. S2). Therefore, the Duolink PLA fluorescence assay was used to analyze the interaction of endogenous β-catenin and TCF7L1 proteins in the nuclei of cells following different treatments. The results revealed that the number of positive cells was significantly elevated after treatment with a cocktail of growth factors and miR-15/16 compared with normal AECs, while the cells treated with the miR-15/16 sponge or APC exhibited dramatic down-regulation relative to the induced AECs alone (Fig. 7C).

HNF4α is a constitutive activator of the transcription of liver-specific genes, including genes involved in intermediary metabolism as well as in xenobiotic and drug metabolism [25,26]. To determine the molecular mechanisms that cause elevated HNF4α during hepatocyte differentiation, we used the ALGGEN-PROMO (v8.3) and JASPAR (v7) tools to screen for potential LEF/TCF7L1-binding sites in the promoter region of HNF4α. By combining these tools, we found two putative binding motifs within the HNF4α promotor (Fig. 8A). We next constructed the pGL3.0-HNF4α promotor vector and generated mutants of the two binding sites. HEK293T cells were then co-transfected with either the wild-type (WT) promotor of HNF4α or its mutated (MUT) counterparts together with the TCF7L1 over-expression vector, and luciferase activities were measured (Fig. 8B). EMSA, ChIP-PCR, and qPCR were subsequently used to confirm the physical association between this transcription factor and the promoter (Fig. 8C–E). The results clearly revealed that TCF7L1 bound to the two predicted regions within the HNF4α promoter to enhance its transcription.

2.6. HNF4α enhances the transcription of miR-15/16 by forming a regulatory circuit

Activation of the MAPK pathway induced by growth factors in vitro is a finite process, but hepatic differentiation in vivo occurs serially, implying the induction of an endogenous regulatory circuit. Bioinformatic algorithms were used to screen for HNF4α-binding sites in the pre-miR15/16 promotor sequence and three binding sites were predicted (Fig. 9A, B). And the physical association of HNF4α and the miR-15/16 promotor was confirmed with ChIP-PCR and ChIP-coupled qPCR (Fig. 9C, D). HEK293T cells were co-transfected with a luciferase reporter vector and a vector containing the miR-15/16 promoter region

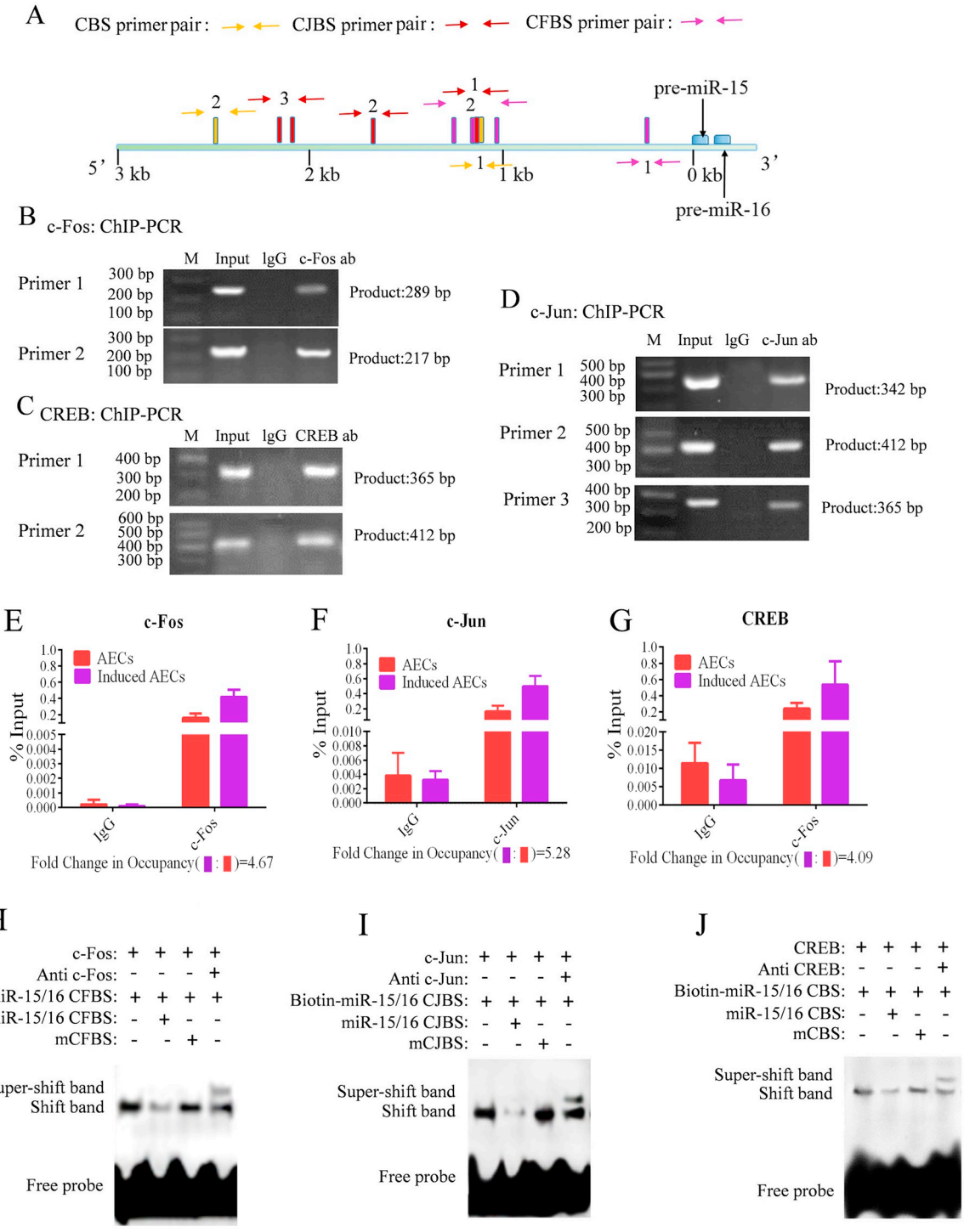


Fig. 5. ChIP-PCR and EMSA assay of the physical binding of c-Fos, c-Jun, and CREB to the miR-15/16 promoter region. A. Diagram indicating the predicted binding sites for these transcription factors within the miR-15/16 promoter and the primer pairs (arrows) used for the ChIP-PCR assays. B–D. The ChIP-PCR results showed the binding of c-Fos, c-Jun, and CREB to their respective binding sites within the miR-15/16 promoter region in amniotic epithelial cells. Protein-DNA complexes in the cells were immunoprecipitated in the positive control ("Input", i.e., whole cell lysates prior to ChIP), non-specific mouse IgG ("IgG") or monoclonal antibodies against each of the transcription factors (lane 4 in each image). ChIP-PCR was then performed with primers flanking the binding sites within the miR-15/16 promoter. CBS, CREB-binding site; CJBS, c-Jun-binding site; CFBS, c-Fos-binding site. E–G. The amounts of c-Fos, c-Jun, and CREB enriched within the miR-15/16 promoter region in normal and induced AECs were quantified by ChIP-coupled real-time PCR. The percentages of input were calculated according to threshold cycle values (CT). Data are presented as the mean ± SD of three independent experiments. H–J. EMSA assay of the physical binding of c-Fos, c-Jun, and CREB to the miR-15/16 promoter region. The promoter-binding site with the highest score for each of these transcription factors based on bioinformatic predictions was selected for EMSA probe design. Following induction, cell nuclear extracts were prepared and incubated with the specific biotinylated EMSA probe for each transcription factor. Non-biotinylated EMSA probes were used as competitor probes. c-Fos, c-Jun, and CREB were all observed to bind their respective predicted binding sites within the miR-15/16 promoter sequence incorporated into the EMSA probes. Furthermore, incubation with specific antibodies against each of the transcription factors clearly resulted in supershifted bands. Probes containing mutated binding sites (mCBS, mCJBS, and mCFBS) were unable to compete for binding. CBS, CREB-binding site; mCJBS, c-Jun-binding site; CFBS, c-Fos-binding site.

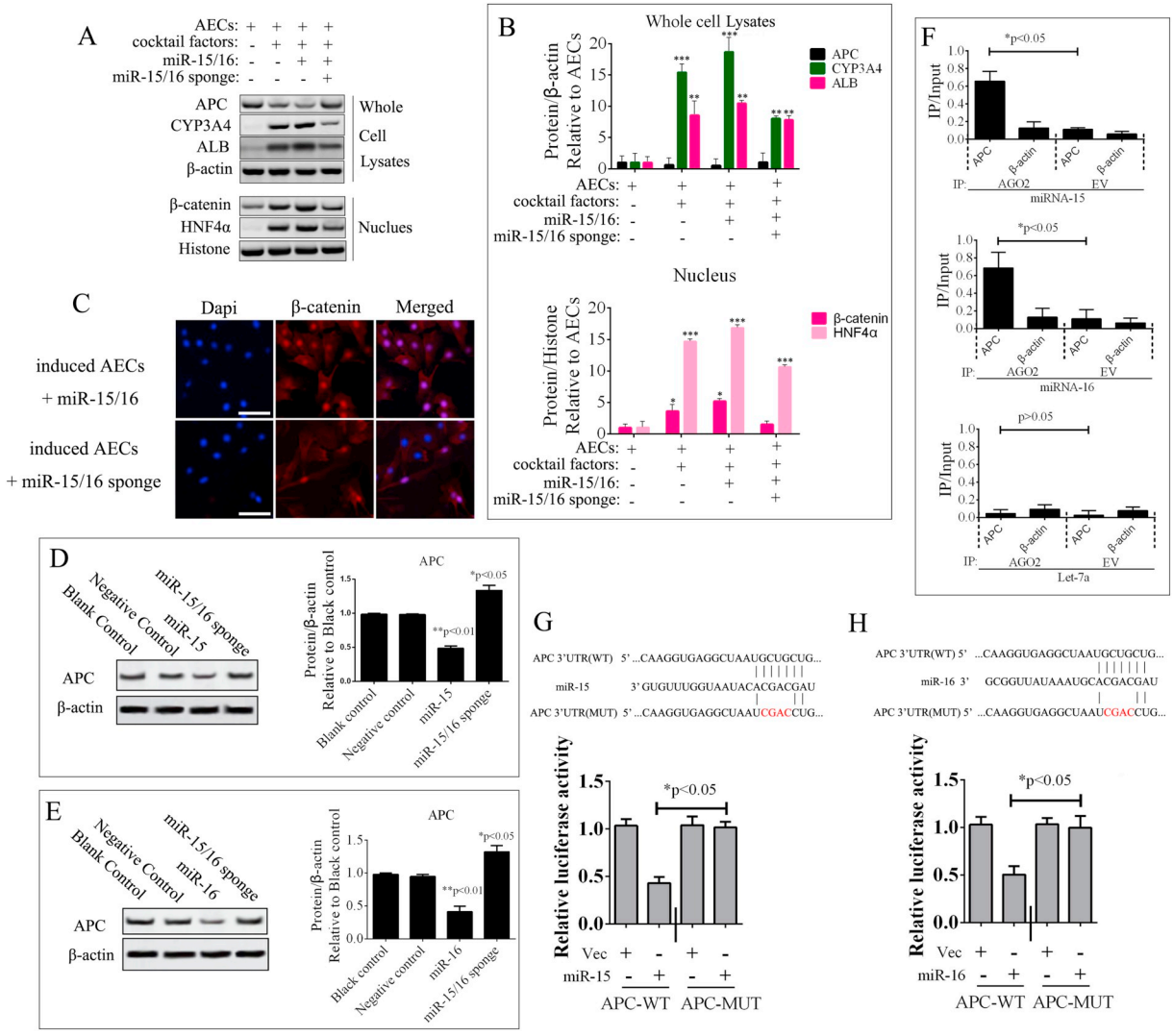


Fig. 6. MiR-15/16 target APC to increase hepatocyte differentiation in AECs. A. MiR-15/16 influence the expression of specific genes during hepatocyte differentiation from AECs. Adenomatous polyposis coli (APC), a putative target of miR-15/16, was down-regulated during hepatocyte differentiation or treatment with a miR-15/16 agomir, but the opposite effect was observed after miR-15/16 sponge treatment. The reverse effect was observed for CYP3A4, ALB, β-catenin, and HNFα, which were up-regulated by miR-15/16 and down-regulated by the sponge. Active β-catenin is a key factor in hepatocyte differentiation, and its expression level was positively correlated with that of the hepatic-specific genes, implying that miR-15/16 regulates the activation of β-catenin by inhibiting APC expression. B. Protein abundance in A was analyzed using ImageJ tools. C. Immunofluorescence images of activated β-catenin in induced AECs following different treatments (scale bar = 100 μm). The results showed that activated β-catenin levels were dramatically elevated after over-expression of miR-15/16, but the opposite results were observed following miR-15/16 sponge treatment. D and E. Western blot analysis of APC expression following the over-expression of miR-15/16 or their sponge in normal AECs. Protein abundance was analyzed using ImageJ tools. Over-expression of mir-15/16 for 72 h inhibited endogenous expression of APC, but the mir-15/16 sponge enhanced its expression. β-Actin was used as an endogenous control. Blank control, normal AECs; negative control, empty vector. Western blot images are representative of at least three independent replicates. F. Immunoprecipitations of Myc-tagged AGO2 from AECs co-transfected with Myc-AGO2 and either miR-15/16 or Let-7a (negative control) were next performed. The empty vector (EV) served as the Myc-AGO2-related negative control. The APC and β-actin mRNA levels were quantified using qPCR, and the relative immunoprecipitate (IP)/input (cell total RNA) values were plotted. The results showed that miR-15/16 facilitates AGO2 association with APC. G and H. The effect of miR-15/16 on APC expression was further validated using luciferase reporter assays. HEK293T cells were used as part of a sensitive luciferase assay system to measure APC transcriptional activation. The cells were co-transfected with a luciferase reporter vector and a vector containing either the wild-type (WT) miR-15/16-binding 3′UTR region of APC or the mutated (MUT) 3′UTR region. Mutating the miR-15/16 target site in the 3′UTR of APC abolished the inhibition of luciferase activity by endogenous miR-15/16. The relative luciferase activity and qPCR values represent the mean ± SD of at least three replicates.

with either the wild-type (WT) or mutated (MUT) HNF4α-binding sites. A luciferase assay was then used to demonstrate the regulation of miR-15/16 by this transcription factor (Fig. 9E), Additionally, EMSA was performed using an EMSA probe designed based on the highest-scoring HNF4α-binding site (Fig. 9F). To test the requirement of HNF4α for miR-15/16 expression, a siRNA of HNF4α was transfected into induced AECs, and based on qPCR, the expression of miR-15/16 was shown to be significantly reduced after silencing of HNF4α compared with treatment with the scrambled control siRNA (Fig. 9G–I). Taken together, the results showed that HNF4α physically interacts with the miR-15/16 promoter to up-regulate their expression. Exogenous activation of the MAPK pathway, therefore, forms a regulatory circuit, thereby increasing the transcription of endogenous miR-15/16 via the miR-15/16-APC/β-catenin/TCF7L1-HNF4α pathway and promoting hepatic differentiation (Fig. 10).

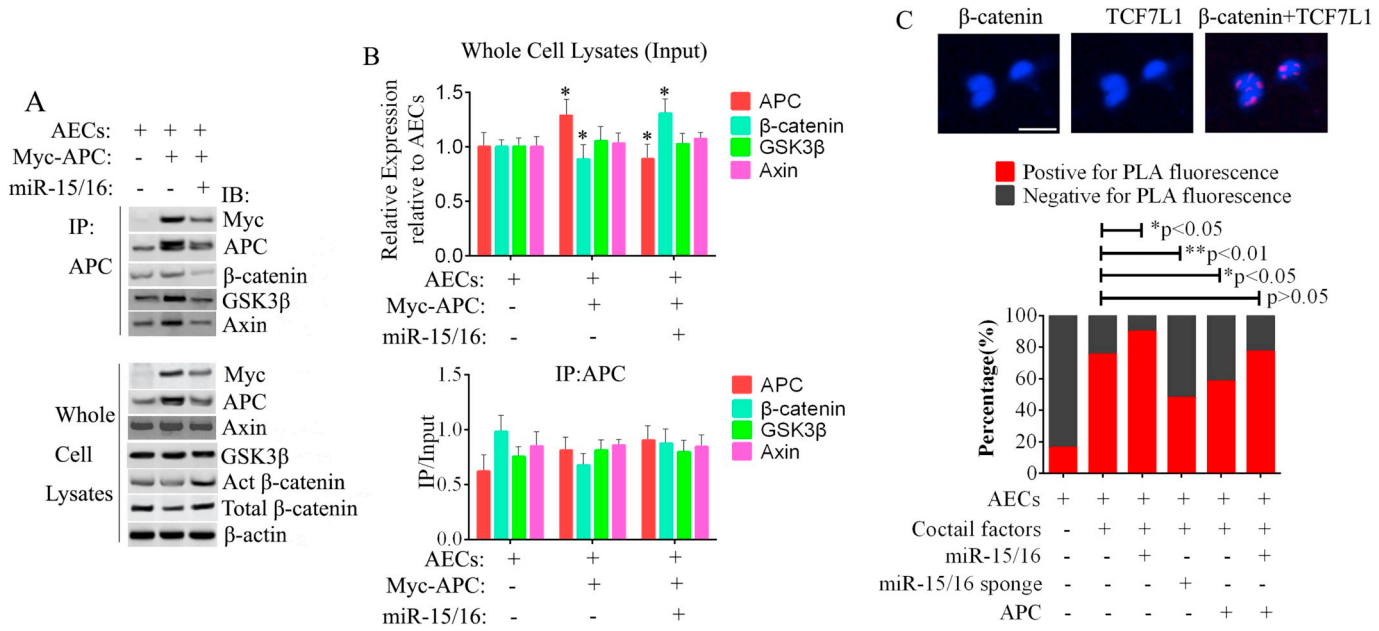


Fig. 7. MiR-15/16 degrade the GSK3β/APC/β-catenin/AXIN complexes to activate the Wnt/β-catenin pathway via the targeting of APC. A. Immunoprecipitation of APC was performed to confirm that endogenous GSK3β/APC/β-catenin/AXIN complex formation is regulated by miR-15/16 and APC in AECs. B. Protein abundance in A was analyzed using ImageJ tools. The whole cell lysates were the input for immunoprecipitation, and the data were normalized using β-actin. The protein levels in the whole cell lysates (input sample) are shown in the upper panel. The abundance of IP products was calculated relative to the protein levels in the input and shown in the bottom panels. The abundance of active β-catenin was increased following over-expression of miR-15/16 and decreased by APC-treatment. These results revealed that miR-15/16 reduced the formation of APC/β-catenin/GSK3β/AXIN complexes and activated the Wnt/β-catenin pathway by targeting APC. C. A Duolink PLA fluorescence assay of the interaction of β-catenin and TCF7L1 in AECs following different treatments. Colocalization of β-catenin and TCF7L1 in nuclear foci is shown in the upper panel, which corroborated the interaction of these proteins in AECs (Scale bar = 20 μm). The percentages of positive cells following the different treatments were calculated and shown in the bottom panel. The number of positive cells was significantly elevated after treatment with the growth factor cocktail and miR-15/16 compared with normal AECs. In contrast, the numbers of positive cells treated with the miR-15/16 sponge or APC were dramatically down-regulated relative to the induced AECs alone.

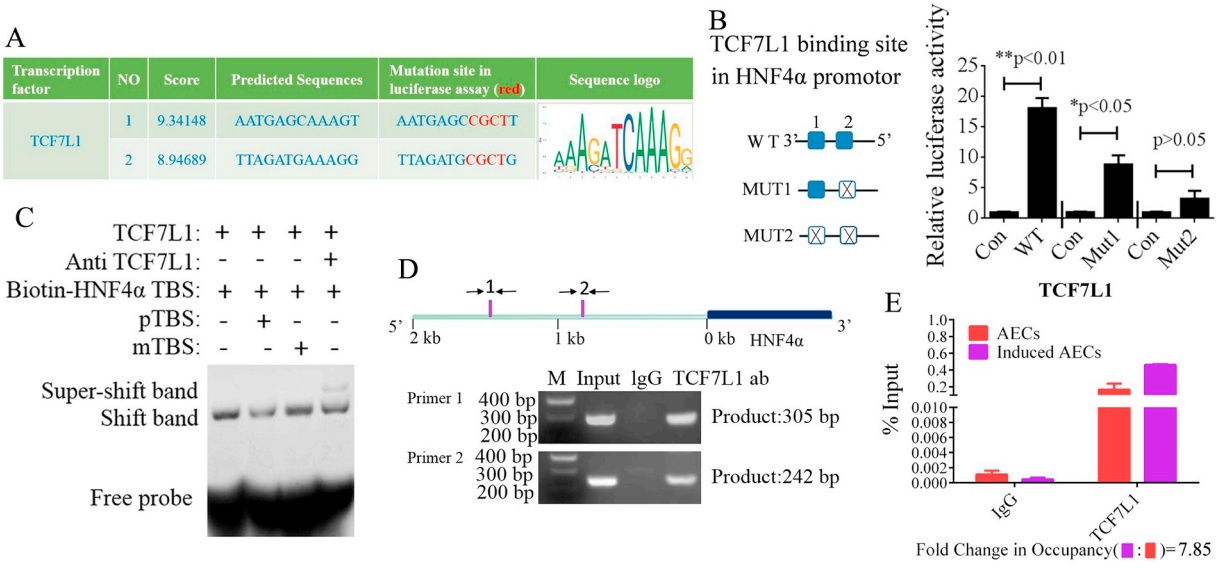


Fig. 8. LEF/TCF7L1 promotes the transcription of HNF4α via physical interaction with its promoter during hepatocyte differentiation. A. Two TCF7L1-binding sites were predicted in the promoter region of HNF4α using bioinformatic algorithms, and mutations of these sites were generated. B. HEK293T cells were co-transfected with a luciferase reporter vector and a vector containing either the wild-type (WT) or mutated (MUT) TCF7L1-binding promoter region of HNF4α. The luciferase activity of these transformants was then analyzed. C. TCF7L1 binding to the specific predicted binding site within the HNF4α promoter region was analyzed in vitro using EMSA. mTBS, unlabeled and mutated EMSA probe. D and E. The physical binding of TCF7L1 to the HNF4α promoter region was validated in vivo using ChIP-PCR, and the amounts of TCF7L1 enriched in the HNF4α promoter regions was quantified by ChIP-coupled real-time PCR. These results suggested that TCF7L1 binds the promoter region of HNF4α and promotes its transcription. All images are representative of at least three independent replicates, and data represent the mean ± SD of at least three replicates.

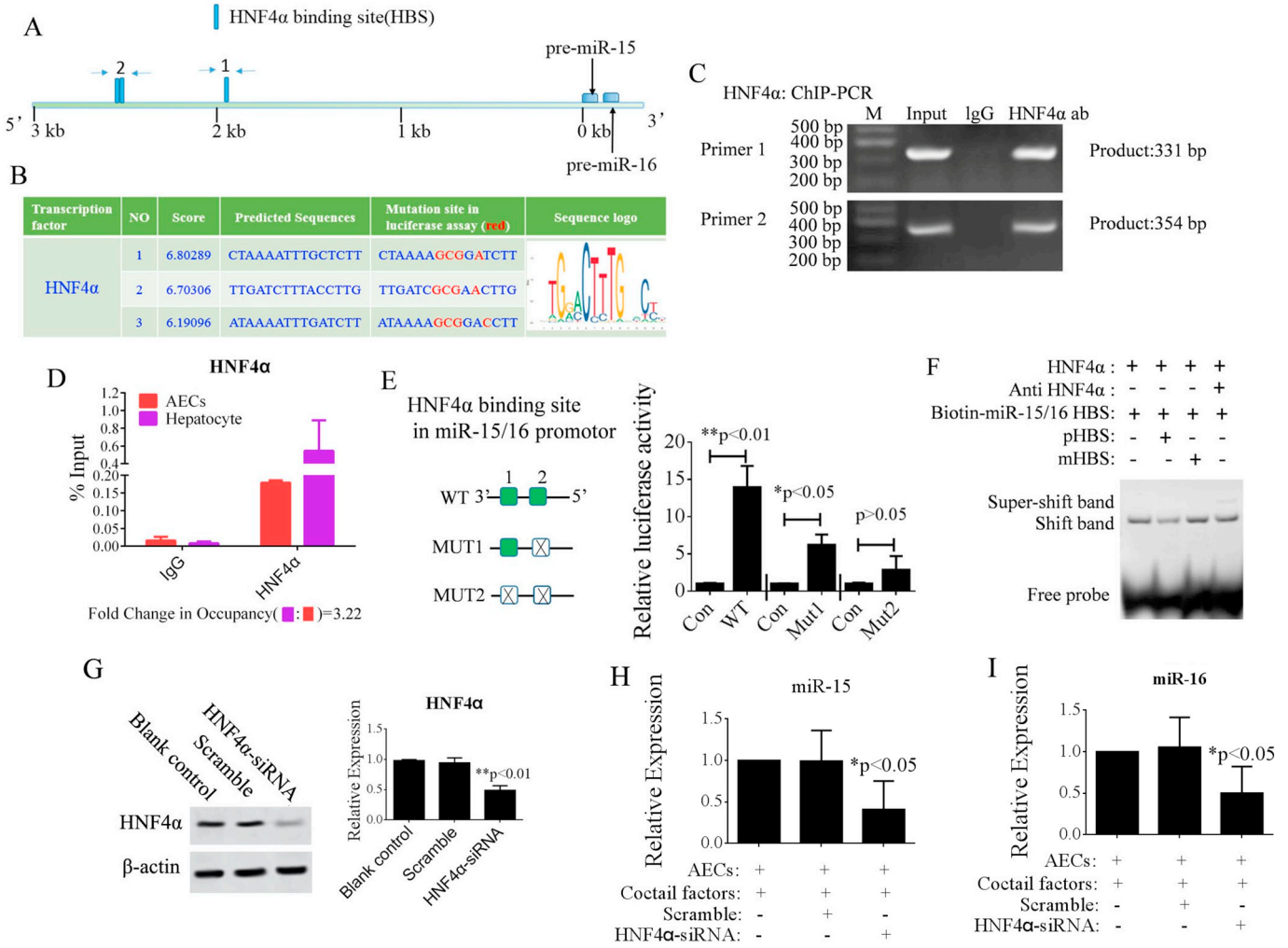


Fig. 9. HNF4α enhances the transcription of miR-15/16. A. Schematic of the predicted binding sites of HNF4α in the promoter region of miR-15/16. B. The binding sites were predicted with bioinformatic tools, and mutations of these sites were generated. C and D. Binding of HNF4α to the promoter region of miR-15/16 was validated in vivo using ChIP-PCR, and the amounts of HNF4α enriched in the miR-15/16 promoter region was quantified by ChIP-coupled real-time PCR. E. HEK293T cells were co-transfected with a luciferase reporter vector and a vector containing either the wild-type (WT) or mutated (MUT) HNF4α-binding promoter region of miR-15/16. The luciferase activity of these transformants was then analyzed. F. HNF4α binding to the specific predicted binding site within the miR-15/16 promoter region was analyzed in vitro using EMSA. The results suggested that HNF4α binds the promoter region of miR-15/16 and promotes its transcription. G. Western blot analysis of HNF4α expression following siRNA targeting. Protein abundance was analyzed using ImageJ tools. Over-expression of siRNA for 72 h inhibited the endogenous expression of HNF4α. β-Actin was used as an endogenous control. Scramble, negative control. Western blot images are representative of at least three independent replicates. H and I. The effects of HNF4α on the expression of miR-15/16. To test the effect of HNF4α on the expression of miR-15/16, a siRNA of HNF4α was transfected during hepatocyte differentiation and real-time PCR was used to test the expression of miR-15/16. The results demonstrated that HNF4α forms a regulatory circuit that increases the expression of endogenous miR-15/16. All images are representative of at least three independent replicates, and data represent the mean ± SD of at least three replicates.

3. Discussion

Liver transplantation is an effective treatment for both metabolic and end-stage liver disease, but the lack of donor livers has hampered its clinical application. Hepatocyte transplantation is an attractive alternative to liver transplantation, and the generation of hepatocytes from stem cells or other cell types could potentially be used to help repair damaged liver tissue [27]. AECs have important properties that contribute to their promising potential for use in regenerative medicine. These include differentiation into all three germ layers, anti-inflammatory properties, and low immunogenicity. In previous research, murine AECs demonstrated the ability to differentiate into cells with characteristics of functional hepatocytes in vitro and in vivo using the microenvironment of the liver [28], but this approach is not yet suitable for hepatocyte production for clinical applications. Cocktails of growth factors have been extensively used to induce the differentiation of various stem cells, including AECs [29].

Combined HGF and FGF induction is currently the most frequently used method for producing hepatic cells from stem cells, which are activated in response to FGF in the hepatic progenitors [30]. Studies have suggested that FGF4 is a major stimulus for activating the ERK cascade in stem cells to exit self-renewal and enter directed differentiation into terminal cells. Meanwhile, JNK and p38, major members of the MAPK pathway, have been shown to play an important role in mediating stem cell proliferation and differentiation. Recent research reports that HGF and FGF4 also promote hepatocyte differentiation via activation of the Wnt/β-catenin pathway in human embryonic stem cells. The Wnt/β-catenin pathway appears to play an important role in regulating the stemness, proliferation, and differentiation of stem cells, especially in liver development [31,32]. However, no direct evidence has shown the role of the MAPK pathway in degrading β-catenin complexes and stabilizing active β-catenin in the cytoplasm. HGF and

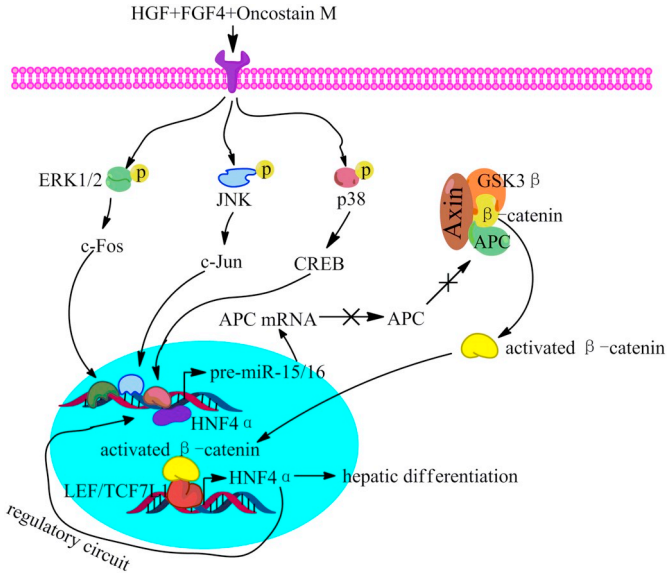


Fig. 10. Schematic diagram of the various factors promoting hepatocyte differentiation from human amniotic epithelial cells. Under culture conditions that include specific growth factors, the major transcription factors of the MAPK pathway, c-Fos, c-Jun, and CREB, are activated. These factors then enter the nucleus to bind the promoter region of miR-15/16 and promote its transcription. APC, a negative regulator of the Wnt/β-catenin pathway, is dramatically down-regulated by miR-15/16, leading to the degradation of APC/β-catenin/GSK3β/AXIN complexes and increased levels of active β-catenin. Free β-catenin after dephosphorylation is translocated into the nucleus where it binds to LEF/TCF7L1 to trigger the expression of HNF4α, thereby promoting hepatocyte differentiation. HNF4α also binds the promoter region of miR-15/16 and promotes its transcription, thereby forming a regulatory circuit.

FGF4, ligands of MAPK, activate the Wnt/β-catenin pathway to enhance hepatocyte differentiation. The study by Gao et al. also demonstrated that the MAPK and Wnt/β-catenin pathways are synergistically involved in effecting early hepatic differentiation from embryonic stem cells [30]. In contrast, Wnt3a, a ligand of the Wnt/β-catenin pathway, combines with a cell surface receptor and induces the rapid and transient activation of the p38 and ERK pathways. Such studies provide evidence of crosstalk between the Wnt and MAPK pathways [33]. However, the molecular mechanisms involved in the activation of the Wnt/β-catenin pathway via the MAPK pathway remained unclear.

MiRNAs have been implicated in the regulation of gene expression in most stages and processes of embryonic development, such as cell differentiation, proliferation, and organ formation. The liver is a multifunctional organ that undergoes rapid changes during the developmental period and relies on tightly regulated gene expression. Previous studies uncovered multiple regulated miRNAs and their targets throughout human liver development, including miR-15/16, which are key miRNAs in the liver that are up-regulated during the early stages of liver formation. Other studies showed that miR-15/16 are up-regulated during hepatic differentiation from mesenchymal stem cells [16,34], and our study similarly showed that miR-15/16 were significantly up-regulated. However, the roles of miR-15/16 and its targets in hepatic differentiation still remained unclear.

The Wnt/β-catenin pathway regulates gene expression by stabilizing cytoplasmic β-catenin, which enters the nucleus and forms transcription complexes with TCFs that bind to target promoter regions, thereby inducing transcription of a variety of target genes including regulators of cell proliferation and differentiation. β-Catenin usually forms a destruction complex with GSK3β, AXIN, and APC in the cytoplasm, which induces β-catenin phosphorylation and degradation and prevents activation of Wnt/β-catenin signaling. APC protein contains a multitude of β-catenin-binding motifs, and it interacts with multiple AXIN molecules to form an inhibitory complex that prevents dephosphorylation of β-catenin. In this study, we demonstrated that miR-15/16 is a mediator of both the MAPK and Wnt/β-catenin pathways in HGF- and FGF4-induced hepatocyte differentiation from AECs. The Wnt signaling pathway has been implicated in the earlier stages of human liver development [35,36], during which it initially inhibits the induction of differentiation but shortly afterward promotes liver bud growth and differentiation [37]. β-Catenin is a component of adherens junctions with E-cadherin, which are ubiquitous in all hepatocytes. The Wnt/β-catenin pathway is therefore constitutively active in hepatic cells. In addition, activation of the Wnt/β-catenin pathway is important for hepatic specification, as it maintains the activity of hepatic-specific transcription factors including hepatocyte nuclear factor (HNF)-1β, forkhead box A1 (FOXA1), FOXA2, and GATA-binding protein 4 (GATA4). Herein, we addressed the role of β-catenin dephosphorylation in hepatocyte differentiation via the miRNA-mediated down-regulation of APC. Our results were in agreement with previous studies where conditional deletion of APC revealed the spatiotemporal role of Wnt/β-catenin signaling in the development of liver zonation in the embryonic mouse [38,39]. HNF4α is an essential transcription factor for promoting the transcription of mature liver-specific genes during hepatoblast differentiation into hepatocytes [40], and our results indicated that the transcription factor, LEF/TCF7L1, binds to the promoter region of HNF4α and enhances its transcription for hepatocyte differentiation.

4. Conclusions

We systematically elucidated the molecular mechanisms involved in growth factor-induced hepatocyte differentiation from AECs. Growth factor induction enhances the transcription of the liver-specific transcription factor, HNF4α, via crosstalk between the MAPK and Wnt/β-catenin pathways. In addition, miR-15/16, a key mediator of the coordinated interactions between these two pathways, is up-regulated by CREB, c-Fos, and c-Jun, and it, in turn, directly down-regulates APC, a member of the β-catenin degradation complex. This results in the activation of β-catenin to enhance the transcription effects of LEF/TCF7L1. Our study, which particularly focused on miR-15/16 involvement in hepatocyte differentiation from stem cells, may assist in the future development of effective cell transplant therapies for the treatment of liver disease.

5. Materials and methods

5.1. Ethics statement

The ethics committee of the Jining Medical University approved the collection process of placentas from voluntary donors through signed informed consent from the mothers (License ID: 2017-JZ-003). The placentas from healthy mothers undergoing elective cesarean deliveries were used. Placentas obtained from patients testing positive for human immunodeficiency virus, hepatitis B virus, hepatitis C virus, tuberculosis, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* or syphilis were excluded from the study, as were any placentas with macroscopic abnormalities.

5.2. Cell culture

Human AECs were isolated from the amniotic membrane using enzyme digestion as described in a previous report [41] and cultured in complete medium (DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, and 55 μM β-mercaptoethanol).

5.3. Hepatocyte differentiation of AECs

For hepatocyte differentiation, AECs were grown to approximately

60% confluence and cultured in hepatic induction medium (DMEM supplemented with 5% FBS, 20 ng/mL HGF, 20 ng/mL FGF4, 20 ng/mL Oncostatin M, 1 μM dexamethasone, and 1 × insulin-transferrin-selenium) for 21 days. Glycogen staining using the Periodic acid–Schiff method was applied to detect biological characteristics of the hepatocytes, and western blotting was used to analyze the expression of HNF4α, a specific transcription factor for hepatocytes, as well as the mature liver markers, albumin, and CYP3A4. We tested potential mechanisms of growth factor-mediated hepatocyte differentiation using an ERK pathway inhibitor (SCH772984), a p38 pathway inhibitor (SB203580), a JNK pathway inhibitor (JNK-IN-8), and a Wnt/β-catenin pathway inhibitor (XAV939).

5.4. miRNA RT-qPCR

Based on previous research, miR-15a and miR-16 transcription levels were analyzed with RT-qPCR after hepatocyte differentiation. In brief, miRNAs were isolated from AECs and hepatocytes with the miRcute miRNA Isolation Kit, and the pure miRNAs were poly(A)-tailed and reverse-transcribed with the miRcute miRNA First-strand cDNA Synthesis Kit. qPCR was performed with the miRcute miRNA qPCR Detection Kit (SYBR Green-based) on an ABI 7500 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. All kits and primers were obtained from Tiangen Biotech (Beijing) Co., Ltd. (Tiangen, Beijing, China).

5.5. MiR-15 cluster target prediction and transfection with miRNA mimics or sponge

MiRNA targets were predicted with starBase v2.0 (http://starbase.sysu.edu.cn/targetSite.php), which searches for intersections among the predictions made by multiple miRNA prediction tools (TargetScan, PicTar, PITA, miRanda/mirSVR, and RNA22). Predicted targets were subjected to gene ontology analysis using Panther online tools (http://www.pantherdb.org/), and the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) database was used to identify predicted target pathways. MiR-15 and miR-16 agomirs (mimics) or sponge were then synthesized or designed, and transfected into AECs using Lipofectamine 3000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The protein expression levels of potential targets were analyzed with western blotting.

5.6. RNA interference

To investigate the role of miRNA targets in hepatocyte differentiation, small interfering RNAs (siRNAs) against specific miRNA targets were designed, synthesized, and transfected into AECs prior to hepatocyte differentiation, and target expression levels were detected with western blotting.

5.7. Co-immunoprecipitation

Hepatocytes cultured in 6-cm culture dishes were removed from the culture medium, washed once with Modified Dulbecco's PBS, harvested, and lysed in 1 mL lysis buffer (CWBio, Beijing, China). The lysates were centrifuged, and 1 μg β-catenin antibodies were added to 500 μL of the supernatant. Immunoprecipitation (IP) was carried out with the Pierce Co-Immunoprecipitation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, US) according to the manufacturer's instructions, and the IP products were analyzed by western blotting.

5.8. Western blotting

The target genes of the miRNAs were detected by western blot analysis following miRNA transfection, the RNA interference assays, and the IP studies. Cells were lysed with the Mammalian Protein Extraction Reagent (M-PER, Thermo Fisher Scientific, Waltham, Massachusetts, US) and supplemented with a protease inhibitor (Thermo Fisher Scientific, Waltham, Massachusetts, US). Protein concentrations were measured with the BCA assay. The extracts were loaded and subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes. Primary antibodies (HNF4α, 1:200; albumin, 1:400; CYP3A4, 1:300; β-catenin, 1:200; APC, 1:300; and β-actin, 1:2000) and HRP-labeled secondary antibodies (1:5000) were purchased from Abcam (Cambridge, MA, USA). Proteins were visualized with the Pierce ECL western blotting substrate (Thermo Fisher Scientific) for HRP. β-Actin was used as the internal control.

5.9. Transcription factor-binding site prediction

Putative transcription factor-binding sites within the promoter sequences of miRNA-15/16 and the other genes of interest were predicted using the TRANSFAC and JASPAR CORE databases [42,43], which construct specific binding site weight matrices for binding site prediction. The promoter sequences of the miRNAs were obtained from the UCSC Genome Browser Gateway using the approach described in a previous report [44] and were defined based on the location of the TATA and GC boxes.

5.10. Site-directed mutagenesis

Site-directed mutagenesis was performed with a PCR-based method using the Fast Site-Directed Mutagenesis Kit (Tiangen Biotech). Primers containing the appropriate base substitutions are listed in Table S1. The PCR reactions comprised 1 μL template plasmid DNA, 0.5 μL Primer STAR DNA polymerase, and 1 μL of the primer pairs in a final volume of 50 μL. PCR products were digested with *Dpn*I at 37 °C for 8 h and then transformed into competent *E. coli* cells. The mutations were confirmed with Sanger sequencing.

5.11. Luciferase reporter assays

To test whether the predicted miR-15/16-binding sites in the 3′UTRs of target mRNAs were responsible for silencing their expression, firefly luciferase reporter vectors were constructed with the pGL3.0-Luc plasmid and the target 3′UTRs. A mutation at nucleotide position 4 of the miRNA seed sequence in each 3′UTR was generated with the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Constructs containing the mutated target (MUT) 3′UTRs were used as the negative controls. Lipofectamine 3000 was used to transfect HEK293T cells with a mixture of firefly luciferase reporter plasmid, miRNA precursor or control precursor, and *Renilla reniformis* luciferase-encoding plasmid (pRL-TK; Promega, Madison, WI, USA). Cells transfected without precursors served as controls for normalization. For examination of promoter activity, serial mutation fragments of promoter region were inserted pGL3.0-Luc. The plasmids were co-transfected with Renilla luciferase expression vector (pRL-40) into HEK293T cells grown in 96-well plates. To study the effect of putative transcriptional factors on promoter activity, the c-Jun, c-Fos, CREB, TCF7L1 and HNF-4α expressing plasmid was respectively mixed with pRL-40 and promoters-luciferase reporter constructs with normal or mutant putative binding sites, and were mixed and co-transfected into HEK293T cells. Luciferase activity was measured at 48 h post-transfection using a dual-luciferase assay system (Promega). All transfections were repeated independently at least three times.

5.12. Chromatin immunoprecipitation assay (ChIP)-PCR

ChIP was performed with the ChIP assay kit (Beyotime Institute of Biotechnology, Beijing, China). The antibodies used in the assay included anti-c-Fos, anti-c-Jun, anti-CREB, anti-HNF4α, and anti-TCF7L1

antibodies (all of rabbit origin; Abcam), and anti-rabbit IgG antibodies (Abcam). The ChIP DNA was extracted with the DNA Purification Kit (Beyotime), and the purified sample was subjected to PCR amplification with different primer pairs spanning the protein-binding sites. PCR products were resolved with 1% agarose–ethidium bromide gel electrophoresis and visualized with ultraviolet light.

5.13. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from human AECs were prepared as described previously using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). Protein concentrations were determined with the Bradford Protein Assay Kit (Beyotime). The EMSA probes used for the gel-shift assays are listed in Table S1. Gel-shift assays were performed with the EMSA/Gel-Shift Kit (Beyotime). The super-shifting antibodies (ChIP Grade) were purchased from Abcam and Santa Cruz biotechnology Co., Ltd.

5.14. In situ PLA

AECs were seeded on glass plates. After miRNA transfection, the cells were fixed with cold 4% paraformaldehyde for 15 min at room temperature followed by cell membrane permeabilization with 0.25% Triton-X-100 in PBS for 10 min. The cells were washed three times with PBS, blocked for 1 h at room temperature with 4% bovine serum albumin in PBS containing 0.1% Tween-20 and then incubated with the indicated antibody pairs at 37 °C. Duolink In Situ PLA (Sigma-Aldrich, St. Louis, MO, USA) was performed according to the manufacturer's instructions. Images were obtained with a TE2000-E confocal microscope (Nikon, Yokohama, Japan).

5.15. Statistical analysis

All studies were performed in three to five separate experiments, each performed in triplicate. All data are represented as the mean ± standard deviation. Differences between experimental groups were assessed with the two-tailed *t*-test. Statistical significance was defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

List of abbreviations

AECs	amniotic epithelial cells
HNF4α	hepatocyte nuclear factor 4α
APC	adenomatous polyposis coli
HGF	hepatocyte growth factor
FGF	fibroblast growth factor
MAPK	mitogen-activated protein kinase
CREB	cAMP-response element binding protein
UTR	Untranslated Region

Declarations

The authors declare that they have no competing interests.

Author contributions

Gao Y. performed cell differentiation, western blotting, and FCM, and drafted the manuscript, Bai C. performed RNAi, cultured cells, and reviewed the manuscript, Li X and Zhang H analyzed data and reviewed the manuscript, Zhang X and Yang W conceived of the study, and participated in its design and coordination.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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