MicroRNA-18a Prevents Estrogen Receptor-α Expression, Promoting Proliferation of Hepatocellular Carcinoma Cells

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Background & Aims: **Men have a higher incidence of hepatocellular carcinoma (HCC) than women, which is believed to partly be because of protective effects of estrogen. We sought to determine whether there were differences in levels of microRNA (miRNA) molecules between male and female HCC samples.** ***Methods:*** **The expression profiles of a panel of candidate miRNAs were compared between male and female HCC tissues using the TaqMan miRNA assay. A luciferase reporter assay was used to identify mRNA targets recognized by specific miRNAs. The levels of pri- and pre-miRNA for each specific miRNA were assayed by quantitative reverse-transcription polymerase chain reaction to delineate the step deregulated in the biogenesis process. Finally, a colorimetric assay was used to determine the effect of specific miRNAs on hepatoma cell proliferation.** ***Results:*** **The miR-18a miRNA increased specifically in samples from female HCC patients (female/male ratio, 4.58; $P = .0023$). The gene ESR1, which encodes the estrogen receptor-α (ERα), was identified as a target of miR-18a. miR-18a can repress ERα translation by binding to its mRNA at the 3′ untranslated region. Increased levels of miR-18a in female HCC tissues correlated with reduced ERα expression; the level of pre-miR-18a changed in concordance with that of mature miR-18a in these tissues. Overexpression of miR-18a decreased ERα levels but stimulated the proliferation of hepatoma cells.** ***Conclusions:*** **This study provides a novel miRNA-mediated regulatory mechanism for controlling ERα expression in hepatocytes. miR-18a prevents translation of ERα, potentially blocking the protective effects of estrogen and promoting the development of HCC in women.**

Hepatocellular carcinoma (HCC) occurs preferentially in males, with a male to female ratio ranging from 2 to 11:1, as described by several cohort reports.[1] Sex hormones, both androgens and estrogens, have long been considered as an explanation for such sex disparity. Evidence from human studies shows that elevated testosterone levels and the presence of genetic polymorphisms linked to increased androgen activity were significantly associated with increased risk of HCC in male hepatitis B surface antigen (HBsAg) carriers.[2,3] In rodent HCC models, castration or treatment with antiandrogen agents can protect male rodents from tumor development.[4] Therefore, up-regulation of the androgen pathway in male patients is considered to accelerate liver carcinogenesis.

Another mechanism that could explain the sex disparity observed in HCC is the increased activity of estrogens in female patients, which might protect them from hepatocarcinogenesis. In fact, our previous study showed that the risk of HCC in females is inversely related to the age of menopause and to the number of full-term pregnancies.[5] In addition, earlier oophorectomy (at age ≤50 years) was identified as a risk factor for HCC in females, whereas postmenopausal hormone replacement therapy was shown to be a protective factor.[5] This is consistent with animal studies in which ovariectomy increased the susceptibility to HCC in female mice.[6,7]

The majority of the supporting evidence for these 2 possibilities comes from epidemiologic studies or animal models, with little understanding of the molecular mechanisms involved. Recently, one study demonstrated that estrogens can protect hepatocytes from malignant transformation via down-regulation of the secretion of interleukin (IL)-6 from Kupffer cells, a critical process in the diethylnitrosamine-induced HCC mouse model.[8] We also showed that the HBV viral protein HBx can enhance the transcriptional activity of the androgen receptor in a ligand-dependent manner and may stimulate HCC in male HBV carriers.[9] Other than these, there are probably other cellular regulatory molecules involved in HCC sex disparity. MicroRNAs are noncoding RNAs 19–25 nucleotides long that can inhibit gene expression by binding to

Abbreviations used in this paper: HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, heptocellular carcinoma; ERα, estrogen receptor α; FNH, focal nodular hyperplasia; miRNA, microRNA.

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complementary sequences at the 3′ untranslated regions (UTRs) of target mRNAs.[10] An increasing list of miRNAs have been identified that function as putative oncogenes or tumor suppressors; these usually show aberrant expression patterns during carcinogenic process.[11] This approach has led to the identification of the *let-7* family of miRNAs in lung cancer, miR-15 and miR-16 in chronic lymphocytic leukemia, miR-17-92 in B-cell lymphoma and lung cancer, and miR-21 in breast cancer and cholangiocarcinoma,[11] and others.

For HCC, the analysis of miRNA expression profiles in paired HCC and adjacent nontumorous tissues has pinpointed several molecules that are deregulated in this type of cancer.[12–17] We propose that a number of these miRNAs may contribute to the unique sex disparity observed in HCC. To test this hypothesis and identify the specific miRNA molecules involved, we compared the expression profiles of miRNAs in HCCs from male and female patients. Interestingly, our analysis led to the identification of 1 miRNA, miR-18a, that showed differential expression between male and female HCCs patients. miR-18a was also found to target the ESR1 gene, which encodes for the estrogen receptor α (ERα) protein. The functional study of the effect of increased levels of miR-18a on both ligand-stimulated transcriptional activation and cell proliferation activity of ERα further supported its involvement in regulating ERα's functions. Our study thus provides a novel miRNA-mediated regulatory mechanism for ERα expression in hepatocytes, which might turn off the protective effect of estrogens in female HCC patients.

Materials and Methods

Study Subjects

From 2002 to 2006, liver tissues from 80 HCC patients were collected from National Taiwan University Hospital. Among them, initial 20 male and 20 female HCC patients were used for screening miRNA showing sex-related different expression pattern. Next, another 20 male and 20 female HCC patients were subsequently recruited for the validation analysis. In addition, 16 focal nodular hyperplasia (FNH) (9 males and 7 females) and 7 adenoma cases (4 males and 3 females) were also included for comparison. For each patient, 1 pair of tumorous and adjacent nontumorous liver tissues was collected. The clinical characteristics of those patients are summarized in supplementary Table 1 (see supplementary Table 1 online at www.gastrojournal.org).

The resected surgical specimens were quickly frozen in liquid nitrogen until RNA and protein extraction. The Institutional Review Board of National Taiwan University Hospital approved the use of these archived tissues. The HBV and hepatitis C virus (HCV) hepatitis viral etiology of these serum samples were determined by assaying both HBsAg and antibody to HCV (anti-HCV) (AxSYM HBsAg version 2 and AxSYM HCV version 3, Abbott Laboratories, Wiesbaden, Germany).

Cell Lines and Cell Culture

Seven hepatoma cell lines (Huh-7, HepG2, Hep3B, HA22T, HCC36, PLC/PRF/5, and SNU-387) were used. SNU-387 was derived from female HCC patients, and the others were derived from male HCC patients. One breast cancer cell line (MCF-7) and another embryonic kidney cancer cell line (293T) were also used. SNU-387 is purchased from ATCC with the accession No. of CRL-2237, and the other cell lines were kindly provided by Dr Hui–Ling Chen at the Hepatitis Research Center at National Taiwan University Hospital, Taipei, Taiwan. SNU-387 was maintained in RPMI 1640 (Gibco, Carlsbad, CA), and the remaining cell lines were cultured in Dulbecco's modified Eagle medium (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 10% fetal bovine serum (Gibco), in a 5% CO_2 incubator at 37°C.

Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis of Mature miRNA

Total RNA was isolated from cell lines and tissues with Trizol reagent (Rezol C & PROtech, Taipei, Taiwan). Quantification of mature miRNAs was performed using the TaqMan miRNA Assay Kit (TaqMan MicroRNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. U6 small nuclear RNA was used as an internal control for determining the relative miRNA expression level.

In relation to the expression of small nuclear U6 RNA, the expression level of specific miRNA for each RNA sample was calculated, reflecting by the value of ΔCt (Ct of miRNA − Ct of U6). The expression level of miR-18a in paired tissues collected from the same individuals were compared, reflected by the value of $-\Delta\Delta$Ct [− (ΔCt of tumor tissues) − (ΔCt of nontumorous tissues)].[18] The value of $-\Delta\Delta$Ct represents the value of $\log_2$ T/NT for each patient.

Luciferase Reporter Constructs

Two reporter plasmids, S-3′UTR and L-3′UTR, were constructed for assaying the effect of miR-18a on ERα expression. Both plasmids were cloned by insertion of DNA fragments derived from the 3′UTR of ESR1 into the *Xba*I site of pGL3-Promoter vector (Promega, Madison, WI), which locates downstream of the luciferase gene. S-3′UTR contained a shorter region covering nucleotides (nt) 1700∼2000 and L-3′UTR contained a longer region covering nt 1∼2000 of 3′UTR (nt 1 of 3′UTR as the first nucleotide after the stop codon of ESR1, at nt position 2151 of ESR1 transcript, NM_000125).

The insert DNA fragments were polymerase chain reaction (PCR)-amplified from the genomic DNA of Huh-7

cells with primers 5′-CCTAGCTAGCGAGCTCCCTGGCTCCCACACGGT-3′ and 5′-CCTAGCTAGCCATTCAATTGTCTGATAAACAAGC-3′ for L-3′UTR and primers 5′-CCTAGCTAGCCAATGACCCAGGTGAGCTGCTCG-3′ and 5′-CCTAGCTAGCCATTCAATTGTCTGATAAACAAGC-3′ for S-3′UTR.

The mutant constructs of S-3′UTR-mut and L-3′UTR-mut were generated using a QuickChange II Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX). The 2 putative miR-18a target sites predicted in 3′UTR of ESR1, with the seed sequence matched with nt 1938∼1945 and the seed sequence matched with nt 1917∼1924, were specifically replaced in the mutant constructs. The oligonucleotides used for site directed mutagenesis contained UTR-mut-forward (5′-GCTGTTTGTTTAAGAAGCACC CG AGTTTGTTTAAGAAGCACC GC ATATAGTATAATAT-3′) and UTR-mut-reverse (5′-ATATTATACTATATGCGGTGCTTCTTAAACAAACTCGGGTGCTTCTTAAACAAACAGC-3′). Another 2 mutant reporters with mutation at either seed sequence of S-3′UTR, S-3′UTR-Seed1-mut and S-3′UTR-Seed2-mut, were constructed using modified UTR-mut-forward and UTR-mut-reverse (at nucleotides underlined), containing mutation for either seed sequence. The 3′UTR(−) ESR1 plasmid (containing full-length human ESR1 complementary DNA (cDNA) cloned in the pCMV5 vector) and the luciferase reporter of pERE-E1b-Luc construct were kindly provided by Prof Shyng–Shiou Yuan at the I-Shou University at Taiwan.

Statistical Analysis

The expression level of each miRNA was expressed as the mean with a 95% confidence interval constructed on the original log scale and then exponentiated back to the ratio scale. The paired *t* test was used to examine the difference of miRNA expression levels between tumor and adjacent nontumor liver tissues. The expression level of specific miRNAs in each subgroup of patients was presented as the mean of T/NT ratios of each paired liver tissues, and the difference between different subgroup of patients (eg, male and female HCC patients) was examined by *t* test. The *t* tests were done on log-transformed data. The *P* values were adjusted for multiple comparisons using Benjamini and Hochberg's method,[19] taking a false discovery approach to correct for multiple comparisons. The number of multiple comparisons is 25, the number of miRNAs analyzed in the current study.

To examine the correlation of T/NT fold change values of miR-18a with pri-18a and pre-miR18a, a regression model with an interaction term for sex by miR-18a was constructed. The log-transformed data were used for analysis. Statistical significance level was defined as $P < .05$ by 2-tailed tests. Stata statistical software (version 8.0; Stata Corp, College Station, TX) was used for all analyses. For other methods, please see the supplementary materials and methods section (see supplementary material online at www.gastrojournal.org).

Results

Increased Expression of miR-18a Preferentially in Female HCCs

The expression pattern of 17 miRNAs previously reported to be deregulated in HCCs (reproducibly documented with abnormal miRNA expression profiles of HCCs in at least 3 reports),[12–17] as well as 4 miRNAs showing no significant expression changes in HCC, and 5 miRNAs enriched in liver (Table 1) were first compared between HCCs and adjacent nontumorous tissues in 20 male and 20 female cases. This screening test aims to identify whether there is any miRNA showing expression pattern with sex-related difference.

Changes in expression levels of specific miRNAs in paired HCC and nontumorous tissues (T/NT) collected from the same individuals were analyzed. Most of the 17 miRNAs previously implicated in HCC also showed significantly deregulated expression patterns in this study (Table 1, T/NT Up and T/NT Down rows in the "No stratification" panel). To identify deregulated miRNAs specifically associated with sex in HCC, these results were further stratified by sex. Only 1 miRNA, miR-18a, was found to be significantly associated with sex, showing a mean T/NT ratio of 1.22 for males and 5.45 for females (fold change of female/male, 4.46; $P = .045$, adjusted by Benjamini and Hochberg's method, Table 1).

To validate this result, we recruited an additional 40 pairs of tumor and nontumor tissues from 20 male and 20 female HCC patients and also 16 pairs of FNH and 7 pairs of adenoma tissues for comparison. The expression patterns of miR-18a in male and female patients were compared, and the results are summarized in Table 2 and also schematically illustrated in Figure 1. When all 80 HCC patients were included in the analysis, a significant increase in miR-18a expression was detected preferentially in female HCC patients, with a mean T/NT ratio of 9.17 for female HCC patients and a ratio of 2.00 for male HCCs patients (Table 2; fold change of female/male, 4.58; $P = .0023$). When the HCC patients were further stratified by the hepatitis viral etiology, the sex-specific significant increase in miR-18a expression still held, both in HBV- and HCV-related HCCs (Table 2; for HBV-related HCC, $P = .0104$; for HCV-related HCCs, $P = .0079$). However, miR-18a expression in tumor tissues was not different from the nontumor tissues in either male or female patient FNHs and adenomas (Table 2 and Figure 1). Moreover, no significant association was identified between the elevation of miR-18a and the other clinical characteristics of HCCs listed in supplementary Table 1 (see supplementary Table 1 online at www.gastrojournal.org).

Table 1. Differential Expression Pattern of 25 miRNAs in 40 HCCs

		No stratification		Stratified by sex factor			
		Fold change (T/NT)	Adjusted	Female	Male	Fold change	Adjusted
Previous studies	miRNA	Mean (95% CI)	*P* value[a,b]	Mean (95% CI)	Mean (95% CI)	Female/male	*P* value[c]
T/NT fUp	hsa-miR-21	3.47 (4.15−2.79)	<.0001[c]	3.38 (4.38−2.37)	3.57 (4.58−2.55)	0.95	.908
	hsa-miR-221	3.79 (4.38−3.20)	<.0001	3.51 (4.39−2.63)	4.07 (4.92−3.22)	0.86	.637
	hsa-miR-222	2.55 (2.88−2.22)	<.0001[c]	2.52 (2.97−2.07)	2.58 (3.10−2.07)	0.97	.927
	hsa-miR-301	3.68 (2.27−5.10)	.0012[d]	5.23 (2.61−7.85)	2.14 (1.22−3.05)	2.45	.278
	hsa-miR-18a	3.33 (2.08−4.59)	.0013[d]	5.45 (3.28−7.61)	1.22 (0.79−1.65)	4.46	.045[e]
	hsa-miR-224	3.80 (2.40−5.19)	.0013[d]	4.72 (2.40−7.05)	2.87 (1.22−4.52)	1.64	.424
	hsa-miR-130b	2.35 (1.66−3.05)	.0086[d]	2.94 (1.61−4.27)	1.76 (1.31−2.22)	1.67	.387
T/NT Down	hsa-miR-199b	0.30 (0.16−0.45)	<.0001[c]	0.25 (0.12−0.37)	0.36 (0.08−0.64)	0.68	.660
	hsa-miR-122a	0.42 (0.27−0.57)	<.0001[c]	0.47 (0.18−0.75)	0.37 (0.24−0.51)	1.26	.747
	hsa-miR-195	0.45 (0.34−0.56)	<.0001[c]	0.40 (0.24−0.56)	0.50 (0.34−0.67)	0.80	.620
	hsa-let-7c	0.44 (0.33−0.55)	<.0001[c]	0.44 (0.29−0.59)	0.44 (0.27−0.60)	1.01	.968
	hsa-miR-199a	0.16 (0.23−0.10)	<.0001[c]	0.11 (0.05−0.16)	0.22 (0.11−0.33)	0.48	.332
	hsa-miR-132	0.84 (0.68−1.00)	.0052[d]	0.94 (0.69−1.19)	0.74 (0.53−0.95)	1.27	.426
	hsa-miR-145	0.57 (0.43−0.70)	.0057[d]	0.51 (0.34−0.68)	0.62 (0.40−0.84)	0.83	.679
	hsa-miR-200a	0.17 (0.25−0.10)	.0000[d]	0.23 (0.09−0.36)	0.11 (0.04−0.19)	2.00	.384
	hsa-miR-223	0.63 (0.39−0.87)	.0314[e]	0.89 (0.43−1.35)	0.37 (0.27−0.47)	2.41	.241
	hsa-let-7g	0.83 (0.63−1.03)	.1021	1.06 (0.71−1.41)	0.60 (0.43−0.78)	1.76	.429
Enriched in liver	hsa-miR-122a[f]	0.42 (0.27−0.57)	<.0001[c]	0.47 (0.18−0.75)	0.37 (0.24−0.51)	1.26	.747
	hsa-miR-192	0.85 (0.64−1.07)	.0071[d]	0.67 (0.50−0.85)	1.03 (0.63−1.43)	0.65	.352
	hsa-miR-148b	1.54 (1.27−1.82)	.0093[d]	1.85 (1.41−2.29)	1.23 (0.93−1.54)	1.50	.288
	hsa-miR-194	0.86 (0.69−1.03)	.0322[e]	0.82 (0.56−1.08)	0.91 (0.67−1.15)	0.91	.766
	hsa-miR-148a	0.86 (0.60−1.12)	.0327[e]	0.88 (0.58−1.18)	0.84 (0.38−1.30)	1.05	.914
T/NT no change	hsa-miR-15b	1.75 (1.33−2.17)	.0283[e]	2.09 (1.28−2.90)	1.41 (1.13−1.70)	1.48	.363
	hsa-miR-320	0.88 (0.70−1.05)	.1006	1.00 (0.69−1.30)	0.76 (0.56−0.95)	1.31	.447
	has-miR-331	1.37 (1.08−1.66)	.1607	1.68 (1.16−2.20)	1.06 (0.83−1.30)	1.58	.208
	hsa-miR-206	1.06 (1.22−0.90)	.5088	1.10 (1.35−0.86)	1.01 (1.23−0.79)	1.09	.742

[a]By paired *t* test.
[b]Adjusted by Benjamini and Hochberg's[19] method (the number of multiple alignments is 25).
[c]$P < .001$.
[d]$P < .01$.
[e]$P < .05$.
[f]Also shown in the panel of T/NT Down.

Inverse Correlation Between the Expression Levels of miR-18a and ERα in Female HCC Patients

Using PicTar and TargetScan algorithms,[20,21] we found that the ESR1 gene, which encodes the ERα protein, seems to be a putative miR-18a target (ranked as the first by PicTar and as the top third by TargetScan). If ESR1 were indeed a putative target for miR-18a, the ERα protein level in primary HCC tissues would be expected to inversely correlate with the level of miR-18a. Using Western blot analysis, ERα was found to be decreased in HCCs showing significantly increased miR-18a expres-

Table 2. Differential Expression Pattern of miR-18a in 80 HCCs, 16 FNHs, and 7 Adenomas

		No stratification		Stratified by sex factor			
		Fold change		Female	Male	Fold change	
Paired liver tissues	n	Mean (95% CI)	*P* value	Mean (95% CI)	Mean (95% CI)	Female/male	*P* value
HCC (all)	80 (40 M, 40 F)	5.59 (3.23−7.94)	<.0001[a]	9.17 (4.77−13.58)	2.00 (1.40−2.61)	4.58	.002[b]
HCC (HBV)	40 (20 M, 20 F)	7.87 (3.37−12.37)	.0065[b]	13.68 (5.45−21.92)	2.06 (1.17−2.95)	6.65	.010[c]
HCC (HCV)	40 (20 M, 20 F)	3.30 (2.26−4.35)	.0003[a]	4.66 (2.94−6.38)	1.95 (1.10−2.79)	2.39	.008[b]
FNH	16 (9 M, 7 F)	1.11 (0.69−1.53)	.8682	1.30 (0.45−2.14)	0.97 (0.58−1.36)	1.34	.450
Adenoma	7 (4 M, 3 F)	1.34 (0.90−1.78)	.2160	1.29 (1.96−0.62)	1.39 (0.52−2.25)	0.93	.828

n, Patient number; M, male; F, female.
[a]$P < .001$.
[b]$P < .01$.
[c]$P < .05$.

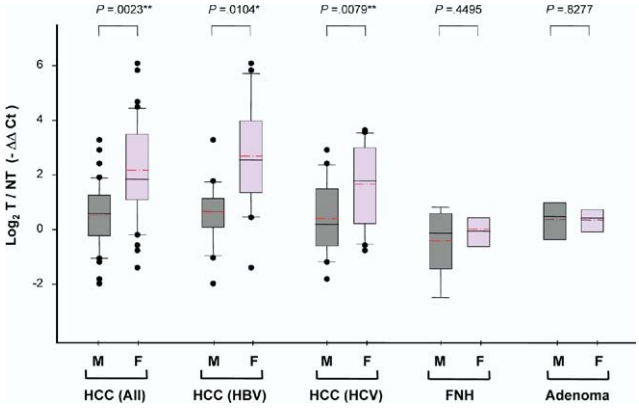

Figure 1. Increase in miR-18a in female HCCs. The changes in miR-18a expression levels in paired liver tissues of each individual collected from 80 HCC, 16 FNH, and 7 adenoma patients were stratified by gender and hepatitis viral etiology. The *box plots* were used to plot the $\log_2$ T/NT ratio of miR-18a expression against the sex and viral factors, with the *dark gray* and *light pink* representing male and female, respectively. The *dashed* and *solid lines* within each box indicate the mean and median, respectively. The *rectangle* indicates the interquartile range (IQR), and the *P* values were calculated by *t* test (*$P < .05$; **$P < .01$; ***$P < .001$).

sion (Figure 2*A*; HCCs with T/NT ratio of miR-18a >5) but was unchanged in HCCs without significant increase in miR-18a expression (Figure 2*B*; HCCs with T/NT ratio of miR-18a <2.5) or in FNHs (Figure 2*C*, *lanes T* vs *lanes N*). Consistently, this inverse correlation between the levels of expression of miR-18a and ERα clearly identified in primary HCC tissues was also detected in hepatoma cell lines (Figure 2*D*, *lanes 3–9*).

miR-18a Regulates ESR1 Gene Expression by Binding to Its Putative 3′UTR Target Sites

The expression results obtained for primary HCC tissues seem to pinpoint ESR1 as a cellular target of miR-18a. To pursue this line of evidence, we used TargetScan to screen for the putative miR-18a target sites in the 3′UTR of ESR1 and found 1 highly conserved site (seed sequence matched with nt 1938–1945, seed 1) and 1 poorly conserved site (with seed sequence matched with nt 1917–1924, seed 2) (Figure 3*A*; ESR1 3′UTR).

The 4 reporter constructs used for evaluation of the capacity of miR-18a to regulate ESR1 by binding to the putative target sites are schematically illustrated in Figure 3*A*. Huh-7 cells expressing the individual reporter constructs were infected with lentiviral vectors of si-GFP, miR-18a, anti-miR-18a, or both in combination and were subsequently assayed for the resulting luciferase activity. No difference was detected when the pGL3-expressing cells were infected with these lentiviruses (Figure 3*B*, *lanes 1–4*), suggesting that the basic luciferase expression vector was not influenced by these mi-RNAs.

The regulatory effect of miR-18a was further verified by infecting the cells with lenti-miR-18a (~5-fold increase of miR-18a expression, revealed by quantitative

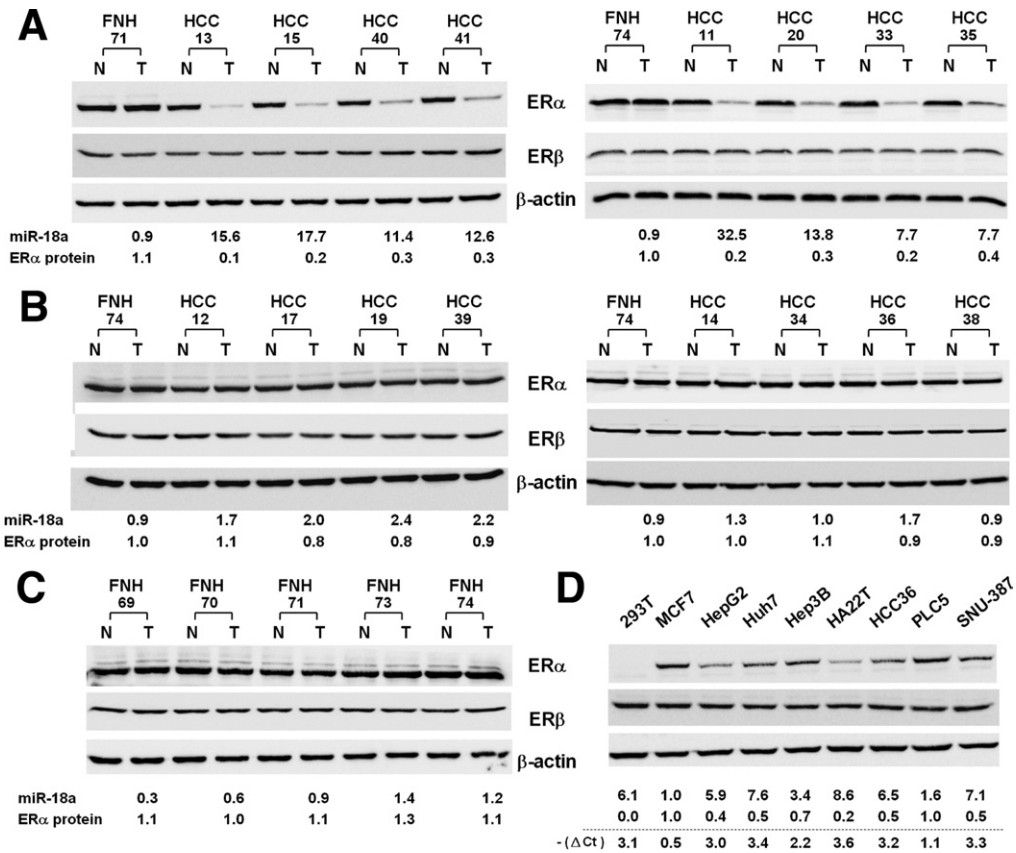

Figure 2. Representative results for the inverse correlation of miR-18a and ERα expression levels in female HCCs and FNHs. Protein lysates from paired female HCC tissues either with (*A*) or without (*B*) significant miR-18a increase, or from paired FNH tissues (*C*), and also from various tumor cell lines (*D*), were processed for Western blot analysis by hybridization with antibodies against ERα, ERβ, and β-actin. After normalization with β-actin, the fold change in ERα expression in tumor tissues vs the corresponding nontumor tissues for each patient were calculated, with the resulting values shown at the bottom of each sample pair. The fold change of miR-18a for each patient was also added at the bottom of each sample pair for comparison. For the cell lines, the value of fold change was derived by comparison with the level of MCF-7, and their −(ΔCt) values for miR-18a (relative to that of U6 RNA) were shown at the bottom of each cell line.

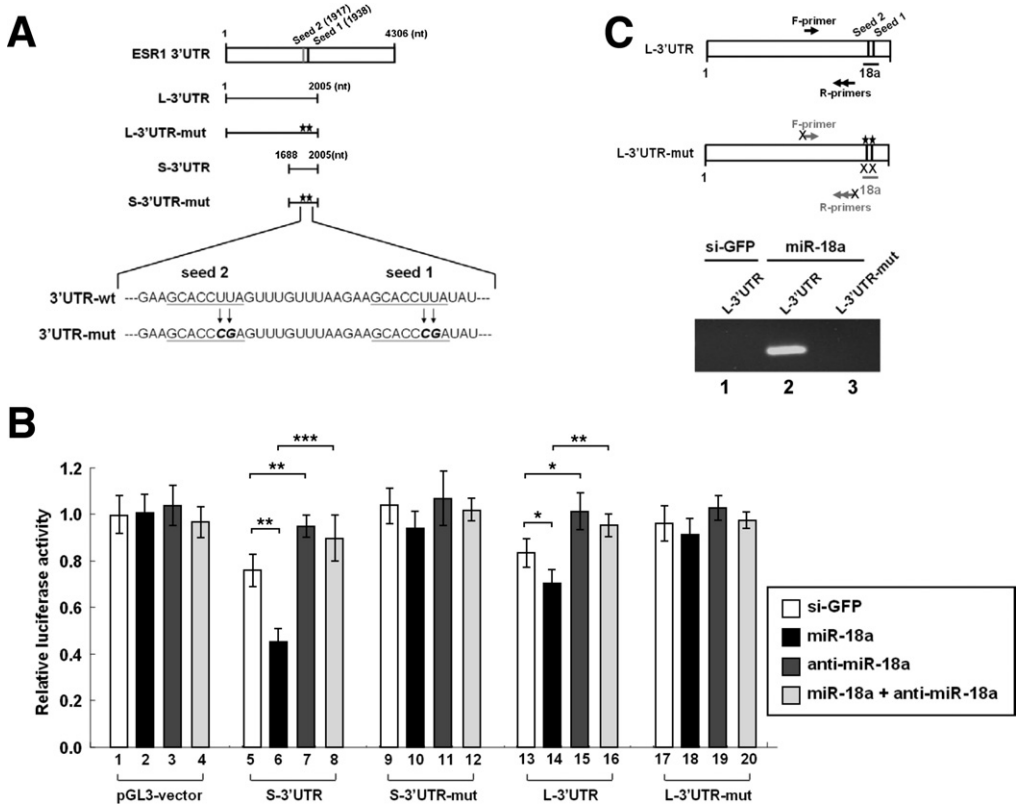

Figure 3. miR-18a represses the activity of luciferase reporter fused with the 3′UTR of the ESR1 gene by binding to its putative target sites. (*A*) The 3′UTR of the ESR1 gene contains 2 putative target sites with the position of seed sequences as indicated. Two reporter constructs containing longer (*L-3′UTR*) and shorter (*S-3′UTR*) 3′UTR regions, as well as constructs carrying mutations corresponding to the seed sequences (L-3′UTR-mut and S-3′UTR-mut), are schematically illustrated. (*B*) Huh-7 cells transfected with pGL3-vector, S-3′UTR, S-3′UTR-mut, L-3′UTR, and L-3′UTR-mut constructs were infected with lentiviral vectors as described. Their effect on reporter activity was illustrated relative to that of pGL3-vector-transfected cells infected with lenti-si-GFP, which was set as 1. All results were the average of 3 experiments (mean ± SD). *$P < .05$; **$P < .01$; ***$P < .001$. The *number at the bottom of each bar* is used for naming each data set. (*C*) RNA extracted from 293T cells transfected with L-3′UTR or L-3′UTR-mut constructs and infected with lenti-miR-18a was processed for reverse transcription reaction and PCR with the primers as indicated (*upper panel*), for analysis of the specific binding of miR-18a to the 3′UTR of ESR1. The PCR products were analyzed by agarose gel electrophoresis (*lower panel*).

reverse-transcription PCR [qRT-PCR]), which led to a decrease in the luciferase activity of S-3′UTR, when compared with that caused by lenti-si-GFP (Figure 3*B*, *lane 6* vs *lane 5*). This effect was diminished in cells expressing S-3′UTR-mut (Figure 3*B*, *lane 10* vs *lane 6*) and was reverted by infection with lenti-anti-miR-18a (Figure 3*B*, *lane 8* vs *lane 6*). By another 2 reporters with mutation at either target site of S-3′UTR, the S-3′UTR-Seed1-mut and 3′UTR-Seed2-mut, we found that the repressive effect of lenti-miR-18a on S-3′UTR reporter can only be partially rescued in either construct (see supplementary Figure 1 online at www.gastrojournal.org), suggesting that both putative sites are targets for miR-18a and are responsible for the miR-18a-mediated repression of S-3′UTR reporter activity. Notably, a similar trend was also identified for the L-3′UTR reporter constructs (Figure 3*B*, *lanes 13–16* for L-3′UTR and *lanes 17–20* for L-3′UTR-mut), suggesting that miR-18a-mediated regulation of ESR1 also occurred in its genomic context containing a longer 3′UTR.

We subsequently tried to assess whether miR-18a could directly bind to the 3′UTR of ESR1 messenger RNA (mRNA), using a newly developed method capable of detecting miRNA-mRNA complexes in cells.[22] 293T cells (with undetectable endogenous ESR1 mRNA expression) transfected with either L-3′UTR or L-3′UTR-mut constructs were infected with lenti-miR-18a. Total RNA was isolated, and RT-PCR was performed using specific primers covering the target sites at the 3′UTR (Figure 3*C*, *upper panel*). The specific PCR product could only be amplified from cDNA prepared from L-3′UTR-transfected cells and not from L-3′UTR-mut-transfected cells (Figure 3*C*, *lower panel*, *lane 2* vs *lane 3*), pinpointing the specific binding sites of miR-18a within the 3′UTR of ESR1 mRNA.

To evaluate the functional role of miR-18a in the regulation of endogenous ERα, 3 hepatoma cell lines (Huh-7, HepG2, and SNU-387) and 1 breast cancer cell line (MCF-7) were infected with either lenti-miR-18a or lenti-anti-miR-18a. Lenti-si-GFP and lenti-si-ESR1 were included as controls. Western blot analysis results revealed that infection with lenti-miR-18a significantly decreased ERα protein expression in all cell lines, an effect that was close to that of infection with lenti-si-ESR1

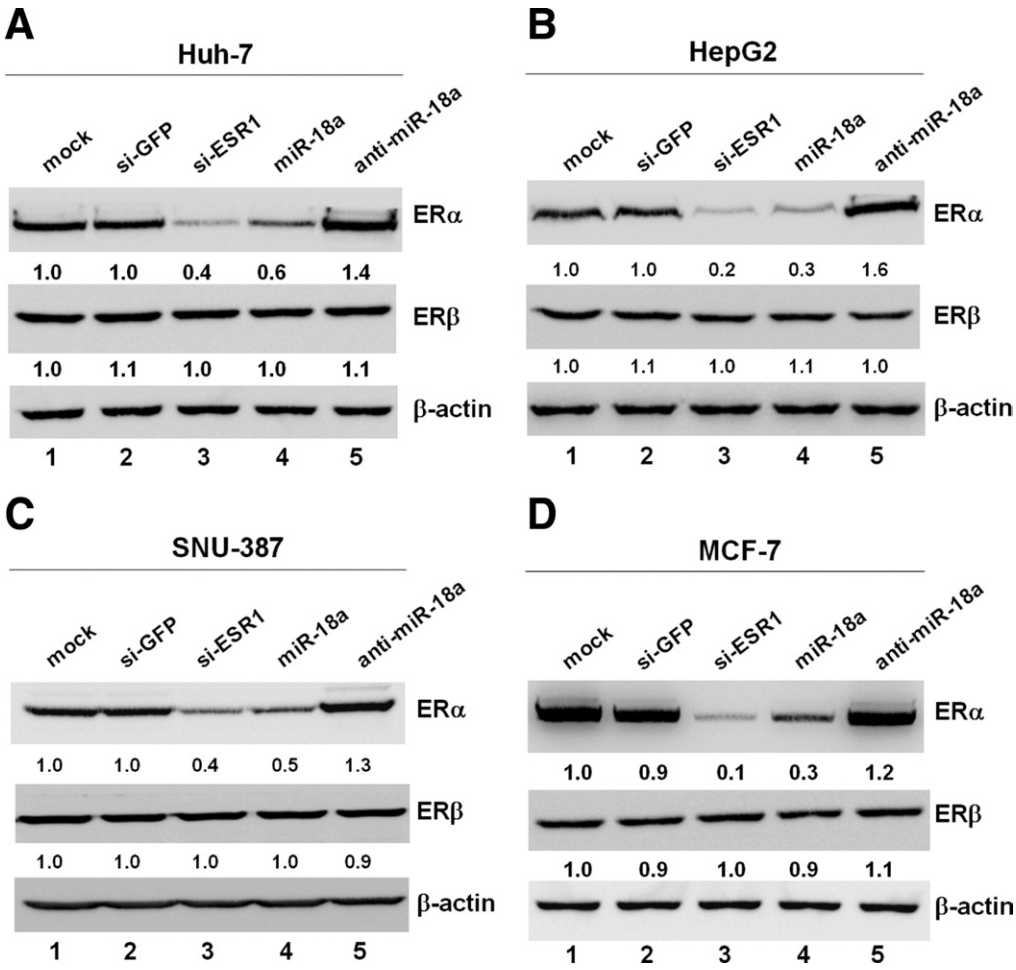

Figure 4. Overexpression of miR-18a decreases the endogenous ERα protein in hepatoma and breast cancer cell lines. Three hepatoma cell lines ([*A*] *Huh-7*, [*B*] *HepG2*, and [*C*] *SNU-387*) and 1 breast cancer cell line ([*D*] *MCF-7*) were infected with various lentiviruses, as described, and were subsequently processed for Western blot analysis by hybridization with antibodies against ERα, ERβ, and β-actin. The expression levels of ERα and ERβ relative to that of β-actin were shown at the bottom of each sample.

(Figure 4*A–D*, *lanes 3* and *4*). In contrast, infection with anti-miR-18a moderately increased the expression level of ERα when compared with infection with lenti-si-GFP (Figure 4*A–D*, *lane 5* vs *lane 1*). Therefore, we have shown that miR-18a down-regulates the expression of endogenous ERα protein in cancer cells.

Level of Expression of pri-miR-18a Correlates With the Increase in miR-18a Expression, Which Down-regulates ERα Posttranscriptionally

To identify the step(s) that is (are) deregulated during the biogenesis of miR-18a and leads to increased expression of miR-18a in female HCC patients, we then compared the T/NT fold change values of miR-18a precursor molecules, pri-miR-18a and pre-miR-18a, to that of mature miR-18a in female HCC patients. The data from 20 female and 20 male HCC patients (first set of samples) are summarized in supplementary Table 2 (see supplementary Table 2 online at www.gastrojournal.org). Correlations were established for the fold change between pri-miR-18a and miR-18a (Figure 5*A*) and also between pre-miR-18a and miR-18a (Figure 5*B*), with the details described in the supplementary Results section (see supplementary material online at www.gastrojournal.org). The results indicated that no significant correlation between pri- and miR-18a was identified, either in male or in female HCC patients. In contrast, there exists a significant correlation between pre- and miR-18a, and the effect seems to be stronger in female than in male HCC patients. The lack of overlap of the male and female data further indicates that the levels of pre- and mature miR-18a are both higher in female than in male HCC patients.

We also investigated whether miR-18a regulated ERα protein expression at the transcriptional or posttranscriptional level. The RNA levels of ESR1 in female HCCs were determined by quantitative PCR and revealed no significant correlation with the levels of ERα in these samples (see supplementary Table 2 online at www.gastrojournal.org values of ESR1 RNA relative to ERα protein, $R^2 = 0.088$). Similar results were also found in cell lines infected with lenti-miR-18a, without showing ESR1 RNA changes correlated with the down-regulation of ERα (data not shown). MiR-18a-mediated decrease of endogenous ERα expression thus seems to mainly occur at the posttranscriptional level.

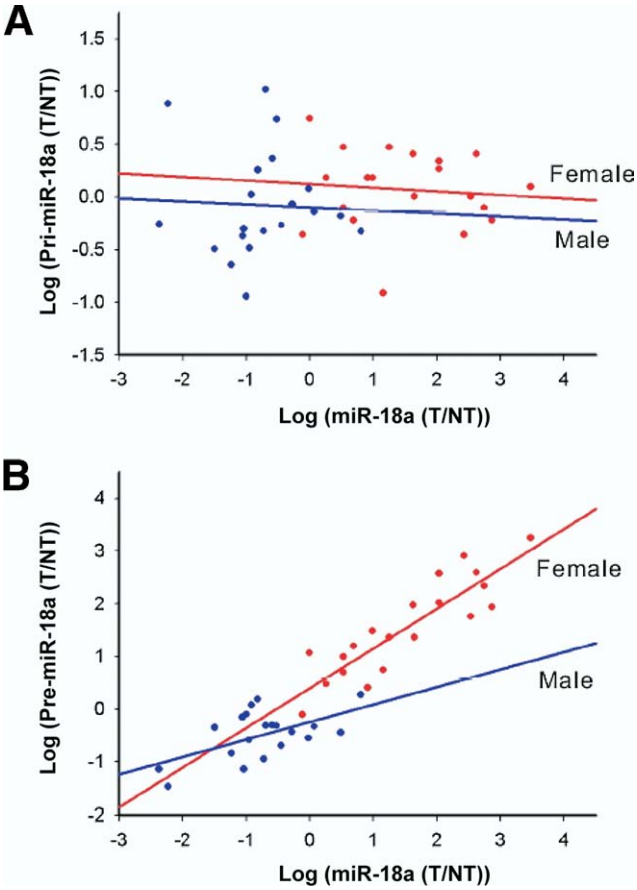

Figure 5. Pre-miR-18a increased expression, but not pri-miR-18a, correlates with the increase in miR-18a expression in HCC tissues. The T/NT ratios of pri-miR-18a, pre-miR-18a, and miR-18a were used for examining the correlation between pri-miR-18a and miR-18a (*A*) and also between pre-miR-18a and miR-18a (*B*). *Red dots* represent females, and *blue circles* represent males. The regression line for females is shown in *red*, and the regression line for males is shown in *blue*.

Increase in miR-18a Expression Down-regulates Estrogen-Stimulated Transcriptional Activity of ERα

The ERα nuclear transcriptional factor can activate the transcription of genes containing estrogen responsive elements (EREs) in their promoter region, usually in a ligand-dependent manner.[23] Aided by the pERE-E1b-Luc reporter, the functional effect of miR-18a increase on the transcriptional activity of ERα was investigated in Huh-7, SNU-387, and MCF-7 cells. The E2-stimulated reporter activity (Figure 6*A–C*, *set 1*) was not affected by the control lenti-si-GFP (Figure 6*A–C*, *set 2*); however, its activity was dramatically decreased by infection with lenti-miR-18a (Figure 6*A–C*, *set 4* vs *2*), close to what was observed following infection with lenti-si-ESR1 (Figure 6*A–C*, *set 4* vs *set 3*). In contrast, lenti-anti-miR-18a significantly increased reporter activity (Figure 6*A–C*, *set 6* vs *set 2*). Notably, the inhibitory effect of miR-18a was rescued by the exogenous expression of 3′UTR(−) ESR1 construct (Figure 6*A–C*, *set 5* vs *set 4*). Thus, miR-18a was shown to regulate the function of ERα protein, in terms of its ligand-stimulated transcriptional activity. Such a conclusion has been further verified by assaying an endogenous target gene of ER, the progesterone receptor gene. In Huh-7 and MCF-7 cells, the mRNA and protein level of progesterone receptor was found decreased by lenti-miR-18a but increased by lenti-anti-miR-18a (see supplementary Figure 2 online at www.gastrojournal.org). The endogenous cellular target gene(s) of ERα can thus be also functionally responsive to the miR-18a changes.

Increase in miR-18a Expression Stimulates the Proliferation Activity of Hepatoma Cell Lines But Represses Proliferation of Breast Cancer Cell Line

To investigate further whether miR-18a increased expression can affect the cell behaviors related with tumorigenesis, we assayed its effect on cell proliferation activity. The proliferation activity of Huh-7, SNU-387, and MCF-7 cells infected with miR-18a, anti-miR-18a, and other control si- or miRNA-expressing lentiviruses were determined by MTT assay.

For Huh-7 and SNU-387 cells, infection with lenti-miR-18a enhanced cell proliferation, an effect that was similar to that caused by si-ESR1 (Figure 7*A* for Huh-7

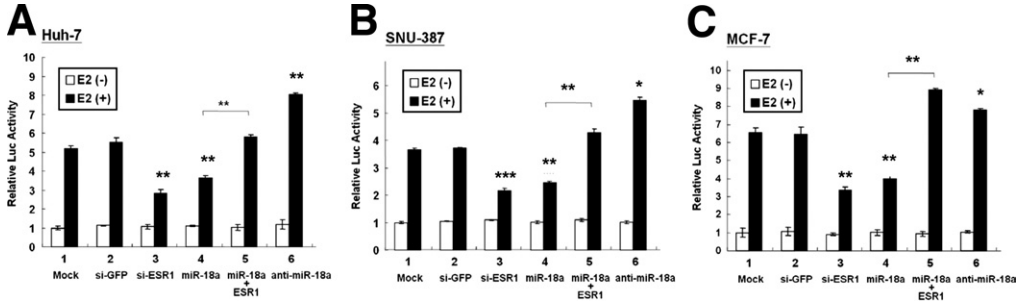

Figure 6. Increased expression of miR-18a down-regulates the estrogen-stimulated transcriptional activity of ERα. Aided by the pERE-E1b-Luc reporter construct, the effect of infection with lentiviruses expressing various si- or miRNAs on the transcriptional activity of ligand-stimulated ERα was evaluated in (*A*) Huh-7, (*B*) SNU-387, and (*C*) MCF-7 cells. The relative luciferase activity was determined relative to that of cells without E2 stimulation. All the results were the average of 3 experiments (mean ± SD). **P* < .05; ***P* < .01; ****P* < .001.

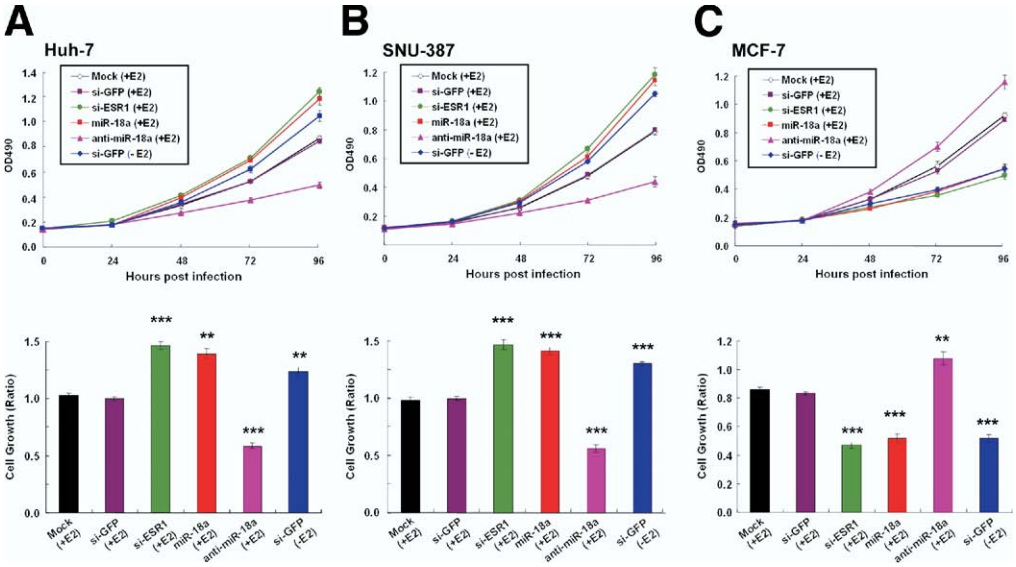

Figure 7. Functional effect of miR-18a increase and ERα down-regulation on the proliferation activity of hepatoma and breast cancer cell lines. The effect of infection with lentiviruses expressing various si- or miRNAs on the proliferation activity of (*A*) Huh-7, (*B*) SNU-387, and (*C*) MCF-7 cells was evaluated using the MTT assay. After puromycine selection, cells infected with lentiviruses were processed for the MTT assay for 4 days. The *upper panel* shows the growth curves reflecting the trend of growth during the follow-up. The *lower panel* shows the results derived at day 4 (end point). All the results were the average of 3 experiments (mean ± SD). $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

and Figure 7*B* for SNU-387, miR-18a and si-ESR1 vs si-GFP). In contrast, for MCF-7 cells, both lenti-miR-18a and the lenti-si-ESR1 repressed cell proliferation (Figure 7*C*, miR-18a and si-ESR1 vs si-GFP). Concordantly, lenti-anti-miR-18a repressed cell proliferation in Huh-7 and SNU-387 cells (Figure 7*A* and *B*, anti-miR-18a vs si-GFP) but in turn enhanced cell proliferation in MCF-7 cells (Figure 7*C*, anti-miR-18a vs si-GFP). miR-18a overexpression displayed intriguing opposite effects on cell proliferation between the hepatoma and breast cancer cell lines.

In support of these results, E2 treatment suppressed the proliferation of Huh-7 and SNU-387 cells but enhanced the proliferation of MCF-7 cells (Figure 7*A* and *B*, si-GFP [+E2] vs si-GFP [−E2]). Furthermore, knockdown of ERα increased cell proliferation in hepatoma cells (close to that of cells without E2 treatment, Figure 7*A* and *B*) but decreased the proliferation of MCF-7 cells (Figure 7*C*). The opposite effect of ligand-stimulated ERα on cell proliferation activity in hepatoma cells or MCF-7 cells was thus confirmed.

Discussion

By comparing the expression pattern of miRNAs between male and female HCCs patients, miR-18a was identified to be preferentially increased in female HCCs. This miRNA was also shown to target the ESR1 mRNA and reduce its protein production, resulting in increased proliferation activity in hepatoma cell lines. These results suggest a new mechanism to suppress the estrogen signaling pathway, which has long been known to protect against HCC development in females.[5–7]

In line to support our finding, ERα was previously reported to be significantly down-regulated in HCCs, both by immunohistochemistry staining or by receptor binding assay (detectable only in 15%–40% of female HCCs patients).[24–26] Notably, one documented mechanism for ERα down-regulation in HCC is the methylation of a CpG island at ESR1's promoter[27]; however, we did not find a decrease in ERα RNA correlated with the decrease of ERα protein in our collection of female HCC patients (see supplementary Table 2 online at www.gastrojournal.org), suggesting that this is not the major mechanism of down-regulation of ERα in these HCCs. Therefore, increased miR-18a expression by binding to the target sites at the 3′UTR of ESR1 is a newly identified major mechanism for down-regulation of ERα at the posttranscriptional level. Meanwhile, we noted a recent publication about the targeting of miR-206 to ERα in breast cancers.[28] However, we did not find any significant change of miR-206 in HCC patients, either in male or female patients (Table 1). Moreover, no correlation between the level of ERα protein and the level of miR-206 in HCC tissues was identified. miR-206 might thus not attribute to the down-regulation of ERα in female HCC patients.

Concerning the contention that long-term use of oral contraceptives could be an inducer of female benign hepatomas, such as adenoma,[29] we included 7 pairs of adenoma tissues for comparison. No increase of miR-18a and down-regulation of ERα were found in these cases, the same as that in FNHs (Table 2), which is consistent with the result from a recent publication by Ladeiro et al.[14] Therefore, although our sample size for adenoma is small, the results did suggest a preferential elevation of miR-18a to be only in the female HCC patients rather than for other benign liver tumors. It further supports that HCC and adenoma/FNH are caused by different carcinogenic mechanisms.

Estrogens are well accepted as cancer-promoting agents in several estrogen-responsive tissues, such as the breasts and the uterus.[30] How they function in a contra-

dictory way to protect females against HCC is intriguing. An opposite effect of miR-18a increase (and concomitant ERα down-regulation) on the cell proliferation activity was noted between hepatoma cells (Huh-7 and SNU-387 cell lines) and breast cancer cells (MCF-7 cell line) because miR-18a increase was shown to activate the proliferation of hepatoma cells but to repress the proliferation of breast cancer cells. These results led us to formulate the possibility that estrogens, through their cell lineage-specific coregulators or tissue-specific selective ER modulators,[31] may exert opposite effects on hepatoma and breast cancer cells, at least in what concerns proliferation activity. This can be addressed in the future by overexpressing miR-18a specifically in the liver or in the breast of transgenic mice.

In addition to the androgen pathway as one major risk factor for male HCC,[2–6] our results further support the protective role of estrogen pathway in female HCC patients. Therefore, the sex disparity characteristic of HCC could be attributed by both sex hormone pathways, with distinct roles in each sex. The higher activity of androgen pathway functions as a tumor-promoting factor in male hepatocarcinogenesis, and the higher activity of estrogen pathway functions as a tumor-suppressing factor in female hepatocarcinogenesis. Because both mechanisms function in a ligand-dependent manner, both the ligand and the receptor of these sex hormones need to be included for assessing the relative risk of HCC patients of each sex. Therefore, although the elevation of miR-18a did not occur in male HCC patients, the lower concentration of estrogens in males will not elicit the estrogen-stimulated tumor-protective pathway in male HCC patients.

Finally, the miR-18a miRNA is expressed as part of a cluster of intronic RNAs (miR-17–92), including miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92.[32] This cluster of miRNAs was reported as potential oncogenes in various tumors,[32] with its elevation either being caused by the genome amplification or by the transcriptional activation by MYC and/or E2F.[32,33] Intriguingly, we did not find other miRNAs in the same cluster of miR-17-92 to be elevated in female HCC patients with increased miR-18a. Our study also found that the pri-miR18a levels were equivalent in all female HCC patients; conversely, pre-miR-18a and miR-18a levels increased concordantly. The elevation of mature miR-18a was thus possibly caused by a deregulated processing of pri-miR-18a to pre-miR-18a or from pre-miR-18a to miR-18a. Guil and Caceres reported a unique mechanism for the accelerated processing of miR-18a, via specific binding of hnRNP A1 to the primary RNA sequence of pri-miR-18a, before Drosha processing.[34] Whether hnRNP A1 or other RNA-binding proteins are involved in accelerating the processing of miR-18a in female HCC patients is currently under investigation. The delineation of the molecular mechanism regulating the increase in miR-18a expression in female HCC patients will be crucial to the future design of strategies specific for blocking female hepatocarcinogenesis.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.10.029.

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