

miR-490-3p Modulates Cell Growth and Epithelial to Mesenchymal Transition of Hepatocellular Carcinoma Cells by Targeting Endoplasmic Reticulum-Golgi Intermediate Compartment Protein 3 (ERGIC3)*

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Background: The role of *miR-490-3p* in hepatocellular carcinoma (HCC) has not been well clarified.

Results: *miR-490-3p* is up-regulated in HCC and enhances the cell proliferation, migration, invasion abilities, and stimulates the epithelial to mesenchymal transition (EMT) through targeting *ERGIC3*.

Conclusion: *miR-490-3p* may play an important role in promoting tumor growth and metastasis in HCC.

Significance: *miR-490-3p* may provide beneficial therapeutic strategy for HCC patients.

MicroRNAs (miRNAs) are considered to be regulators of various biological processes in cancers, including the epithelial to mesenchymal transition (EMT), which is a key factor in cancer metastasis. In this study, we aimed to clarify the potential roles of *miR-490-3p* in hepatocellular carcinoma (HCC) cells. Using real-time quantitative RT-PCR, we discovered that *miR-490-3p* was up-regulated in HCC tissues and cells compared with the adjacent non-tumor tissues and normal cells. We also found that overexpression of *miR-490-3p* led to an increase in cell proliferation, migration, and invasion abilities and that it contributed to EMT. The inhibition of *miR-490-3p* had the opposite effect on the cells. We identified *ERGIC3* (endoplasmic reticulum-Golgi intermediate compartment protein 3) as a direct target gene for *miR-490-3p*. Unlike most miRNA-mRNA interactions, *miR-490-3p* increased *ERGIC3* mRNA and protein levels as well as the intensity of expression of the EGFP reporter gene controlled by the 3'-UTR of *ERGIC3* mRNA. The up-regulation by *miR-490-3p* also required the participation of Ago2. The inhibition of *miR-490-3p* reduced the expression of *ERGIC3*. Overexpression of *ERGIC3* led to the same effect on HCC cells as *miR-490-3p* overexpression, including EMT. Importantly, silencing *ERGIC3* reversed the cellular responses mediated by *miR-490-3p* overexpression. In conclusion, our study indicated for the first time that *miR-490-3p* functioned like an oncogenic miRNA in HCC cells and that the inhibition of *miR-490-3p* might provide a potential treatment approach for HCC patients.

Hepatocellular carcinoma (HCC)³ is the sixth most commonly malignant tumor and third leading cause of cancer-related death worldwide (1, 2). It occurs more often in men than in women and has a high mortality (3). Hepatocarcinogenesis is a complicated process involving many gene alterations, such as mutations of *TP53* and β -catenin, the chromosomal amplification of *VEGFA* (vascular endothelial growth factor A), the deletion of cyclin D1 (1, 4), and other forms of gene dysregulation; therefore, some therapeutic strategies for HCC treatment focus on the development of drugs against these targets to augment the traditional treatments (surgery, transplantation, and percutaneous ablation). Currently, the only drug that enhances survival is sorafenib (5), which is a multikinase inhibitor that can block *Raf*, *VEGFR*, and *PDGFR*. Additionally, the 5-year survival rate for HCC is poor, especially for metastatic HCC. One of the common forms of metastasis and invasion is primarily attributed to the process known as epithelial to mesenchymal transition (6) (EMT), during which the cells lose adhesion and show increased mobility, concomitant with increases in mesenchymal components and initiation of the migratory character (7). Therefore, understanding the molecular mechanism of HCC cell growth and metastasis is crucial for HCC therapeutic intervention and management. Currently, miRNA offers a novel molecular approach, and it has been reported to be involved in HCC pathogenesis (8).

miRNAs are a class of short, non-coding RNAs ~22 nucleotides in length and are involved in the regulation of gene expression at the post-transcriptional level through complete or incomplete complementarity with the binding sites in the 3'-untranslated region (3'-UTR) of target genes (9). miRNAs are usually transcribed by RNA polymerase II into primary transcripts (10) that are then recognized and cleaved by the RNase III enzyme, *Drosha* (11), to produce a stem-loop precursor of ~70 nucleotides (12), which is exported from the nucleus

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³ The abbreviations used are: HCC, hepatocellular carcinoma; miR and miRNA, microRNA; EMT, epithelial to mesenchymal transition; RISC, RNA-induced silencing complex; ASO, antisense oligonucleotide.

Regulation of *ERGIC3* by *miR-490-3p* in HCC

TABLE 1
Primer sequences

Primers	Sequences
Plasmid construct primers	
miR-490-3p sense	5'-CGTGGATCCTTCTTCAACCAACGGTGGTG-3'
miR-490-3p antisense	5'-CCAGAAATCAAAGCAGGAAGAGTAAGACTTCC-3'
<i>ERGIC3</i> 3'-UTR sense	5'-GATGGATCCTAACCGAGAAGCACAGGTC-3'
<i>ERGIC3</i> 3'-UTR antisense	5'-GCGGAATTCACATATCACAAATCAATCAATAG-3'
Mut <i>ERGIC3</i> 3'-UTR sense	5'-GCCCCAGCCCGACAGTGATAAATC-3'
Mut <i>ERGIC3</i> 3'-UTR antisense	5'-GATTTATCACTGTGCGGGCTGGGGC-3'
<i>ERGIC3</i> shRNA top	5'-GATCCGTCAATAAGGTGGCCGGAATTCAGAGATCCCGCCACCTTATGACTTTTGGAAA-3'
<i>ERGIC3</i> shRNA bottom	5'-AGCTTTTCCAAAAAAGTCAATAAGGTGGCCGGAATCTCTTGAATCCCGCCACCTTATGAGC-3'
<i>ERGIC3</i> sense	5'-AGGAATTCACCATGGAGGCGCTGGGGAAG-3'
<i>ERGIC3</i> antisense	5'-CTGAGTCGAGTACGTTGTCTTCCCTAGATC-3'
Real-time quantitative RT-PCR primers	
miR-490-3p RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACCTGGATACGACCAGCATG-3'
U6 RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACCTGGATACGACAAAATATGGAAC-3'
miR-490-3p forward	5'-TGCGGTTCAGTAATTCAGGA-3'
U6 forward	5'-TGCGGTGCTCGCTTCGGCAGC-3'
Reverse	5'-CCAGTGCAGGGTCCGAGGT-3'
β -Actin sense	5'-CGTGACATTAAGGAGAAGCTG-3'
β -Actin antisense	5'-CTAGAAGCATTTCGGTGGAC-3'
Twist2 sense	5'-GCAAGAAGTCGAGCGAAGAT-3'
Twist2 antisense	5'-GCTCTGCAGCTCCTCGAA-3'
Snail sense	5'-TTCTCTAGGCCCTGGCTGC-3'
Snail antisense	5'-TACTTCTTGACATCTGAGTGGGTCTG-3'
FN1 sense	5'-CAGTGGGAGACCTCGAGAAG-3'
FN1 antisense	5'-TCCCTCGAACATCAGAAAC-3'

to the cytoplasm by exportin-5 and finally processed by *Dicer-1* into the mature miRNA (13). The mature miRNA is involved in the RNA-induced silencing complex (RISC), leading to mRNA cleavage or translational repression (14). Thus, miRNAs regulate most genes negatively (15). However, previous studies revealed that miRNAs can regulate gene expression positively, but the mechanism is unclear. For example, *miR-346* up-regulates the RIP140 level by targeting the 5'-UTR without requiring Ago2 (16), and *miR-20a* up-regulates *TNKS2* in human cervical cancer cells (17). It has been reported that miRNAs regulate ~60% of the protein-coding genes (18) and participate in several biological processes, such as developmental timing, cell death, cell proliferation, and apoptosis (9, 19) as well as EMT (20, 21). Therefore, miRNAs are considered to be either tumor suppressors or oncogenes in cancers, depending on the genes or pathway they regulate. For instance, *miR-125b* inhibits breast cancer cell proliferation and tumorigenesis *in vivo* by down-regulating *ETS1* (22), whereas *miR-30b/d* enhance invasion by regulation of GalNAc (23). Previous data showed that *miR-449* (24), *miR-214* (25), *miR-221* (26), and *miR-125b* (27) are dysregulated in HCC and have different roles.

In the present study, the aim is to explore the role of *miR-490-3p* in HCC cell growth and the EMT. We discovered that *miR-490-3p* was up-regulated in HCC tissues and cells and that *miR-490-3p* played an important role in the cell proliferation, migration, and invasion and in the EMT. To our knowledge, our study is the first to document the role of *miR-490-3p* in HCC.

EXPERIMENTAL PROCEDURES

Tissue Specimens and Cell Lines—Twenty pairs of HCC tissues and the adjacent non-tumor tissues were acquired from the Sun Yat-sen University Cancer Center. All tissues confirmed by pathology and immunohistochemistry were collected and frozen in liquid nitrogen and stored at -80°C . To detect *miR-490-3p* and *ERGIC3* expression levels, RNA was

extracted from the tissues according to the manufacturer's protocol.

The QGY-7703, PLC/PRF/5, and LO2 cells were cultured in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum FBS and 1% PS (100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin), whereas SK-Hep-1 cells were cultured in DMEM α , and HepG2 cells were incubated in MEM α with 10% dialyzed (FBS) and 1% PS (100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin). All of the cells were maintained in a humidified incubator with 5% carbon dioxide (CO_2) at 37°C . The cells were transfected with pcDNA3/pri-miR-490-3p or the pcDNA3 control, antisense oligonucleotide (ASO)-miR-490-3p or ASO-ctrl, pSilencer/shR-*ERGIC3* or the pSilencer control, or pA3M1/*ERGIC3* or the pA3M1 control using LipofectamineTM 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

Plasmid Construction—The primary *miR-490-3p* was amplified from genomic DNA and cloned into the pcDNA3 vector sites (BamHI and EcoRI). The gene coding *ERGIC3* was amplified from the cDNA of the QGY-7703 cells. The product was ~1000 bp, and it was cloned into the pA3M1 vector sites (EcoRI and XhoI). The shRNA of *ERGIC3* was annealed and cloned into the pSilencer vector sites (BamHI and HindIII). The 3'-UTR of *ERGIC3* (containing the binding sites for *miR-490-3p*) was amplified from cDNA of QGY-7703 cells. The product was ~300 bp and cloned into the pcDNA3-EGFP control vector (downstream of EGFP). The mutant 3'-UTR of *ERGIC3* (4 nucleotides were mutated in the binding sites) was amplified from the construct (pcDNA3-EGFP/*ERGIC3* 3'-UTR). All of the primers for PCR amplification are illustrated in Table 1.

EGFP Fluorescent Reporter Assay—The QGY-7703 cells were co-transfected with pcDNA3/pri-miR-490-3p or ASO of *miR-490-3p* and the 3'-UTR of *ERGIC3* or the mutant 3'-UTR of *ERGIC3* or with the control vectors in 48-well plates. At 48 h after transfection, the fluorescent intensity was measured with

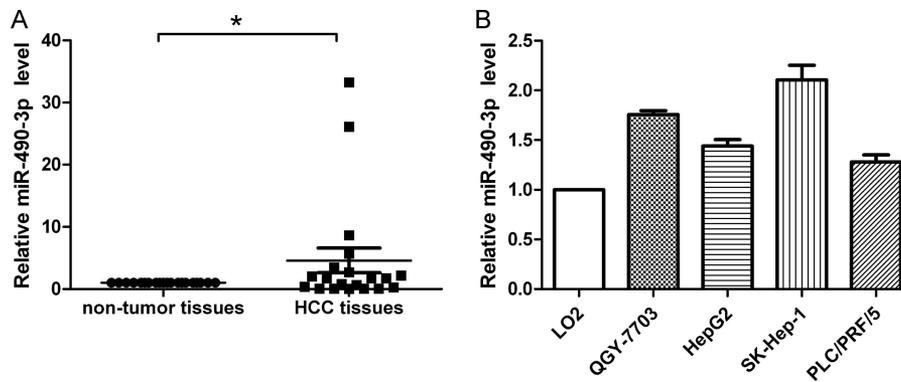


FIGURE 1. **miR-490-3p is up-regulated in HCC.** *A*, miR-490-3p expression was analyzed in HCC tissues and adjacent non-tumor tissues. *B*, miR-490-3p expression was analyzed in LO2, QGY-7703, HepG2, SK-Hep-1, and PLC/PRF/5 cells. U6 was used as the internal control to normalize the miR-490-3p levels. The control was normalized to 1. *, $p < 0.05$. Error bars, S.D.

an F-4500 fluorescence spectrophotometer (Hitachi). The vector pDsRed2-N1 (Clontech) expressing RFP was transfected together with the above vectors and was used as the spiked-in control.

RNA Isolation and Real-time Quantitative RT-PCR Assay—Total RNA was isolated with TRIzol reagent (Sigma) according to the manufacturer's protocol. During the miRNA reverse transcription (RT) reaction, special primers were used, whereas during the RT reaction of the cDNA for the genes, the total RNA was transcribed by the oligo(dT) primer. The PCR assay was performed by the SYBR Premix Ex Taq system (TaKaRa, Madison, WI), and the reaction entailed 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The primers for RT and PCR are shown in Table 1. The U6 small nuclear B non-coding RNA (RNU6B) was used as the endogenous control to normalize the level of *miR-490-3p*, whereas β -actin was used as the endogenous control to normalize the level of *ERGIC3*.

Western Blotting Analysis—The transfected cells were lysed with radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1 mM MgCl₂, 0.1% SDS, 10 mM Tris-HCl, pH 7.4) for 30 min at 48 or 72 h after transfection. The *ERGIC3* levels were analyzed by Western blotting using the rabbit polyclonal anti-*ERGIC3* antibody (1:200; Saier Co.), the E-cadherin levels were detected using the rabbit polyclonal anti-E-cadherin antibody (1:200; Saier Co.), and the vimentin levels were detected using the rabbit polyclonal anti-vimentin antibody (1:100; Saier Co.). The second antibody was goat anti-rabbit antibody (1:1000; Saier Co.). The protein electrophoresis was performed in 12 and 8% SDS-polyacrylamide gels, respectively. *GAPDH* was used as the endogenous control to normalize the expression level of *ERGIC3*, E-cadherin, and vimentin.

Cell Viability Assay (WST-1 Assay)—Cell viability was performed by the WST-1 assay. The transfected cells were seeded into 96-well plates at a density of 2000 cells/well (QGY-7703) or 8000 cells/well (HepG2). At 48, 72, and 96 h after transfection, the cells were incubated with WST-1 reagent for ~1 h at 37 °C, and then the absorbance at $A_{450\text{ nm}}$ was measured with a spectrophotometer.

Colony Formation Assay—The cells were seeded into 12-well plates at a density of 200 cells/well (QGY-7703) or 1000 cells/well (HepG2) at 24 h after transfection. Medium was changed

every 3 days. When most of the colonies contained at least 50 cells, they were stained with crystal violet and counted. The colony formation rate = (colony number)/(seeded cell number).

Migration and Invasion Assays—Migration and invasion assays were performed by Transwell chamber inserts (Millipore) without or with Matrigel (for invasion) according to the manufacturer's protocol. Transfected cells were seeded into the upper chamber of the insert at a density of 5×10^4 cells (QGY-7703) or 10^5 cells (HepG2) and covered with 250 μ l of serum-free medium. The bottom of the insert was incubated in medium containing 20% FBS. The QGY-7703 cells were allowed to migrate for 12 h and invade for 24 h, whereas the HepG2 cells were allowed to migrate and invade for 40 h. The cells in the upper chamber were then removed, and cells that had migrated or invaded were stained with crystal violet and counted using a microscope.

Immunofluorescent Microscopy—Cells were seeded into 14-well chambers at a density of 2000 cells/well after transfection. After 24 h, the cells were washed with $1 \times$ PBS, fixed with 4% paraformaldehyde, and permeabilized in 0.5% Triton X-100. Then the cells were blocked in 10% donkey serum for 1 h. Next, the cells were incubated overnight at 4 °C with rabbit anti-E-cadherin antibody (1:50) in 1% donkey serum. The cells were then stained for 1.5 h with fluorescein isothiocyanate-conjugated secondary antibody (1:300). Finally, the cells were stained with DAPI for 5 min at room temperature. The slides were viewed with the fluorescent microscopy.

Statistical Analysis—Each experiment was repeated at least twice, and all of the data are shown as the means \pm S.D. The statistical analysis between two groups was performed by two-tailed Student's *t* test. For the statistical analysis of the rescue experiment that had four groups, a one-way analysis of variance test and the least significant difference *t* test were performed. A value of $p < 0.05$ was considered significant.

RESULTS

miR-490-3p Is Up-regulated in HCC Tissues and Cells—To evaluate the potential role of *miR-490-3p* in HCC, the *miR-490-3p* expression levels were first evaluated in 20 pairs of HCC tissues and adjacent non-tumor tissues. The results of the real-time PCR analysis illustrated in Fig. 1*A* showed that *miR*-

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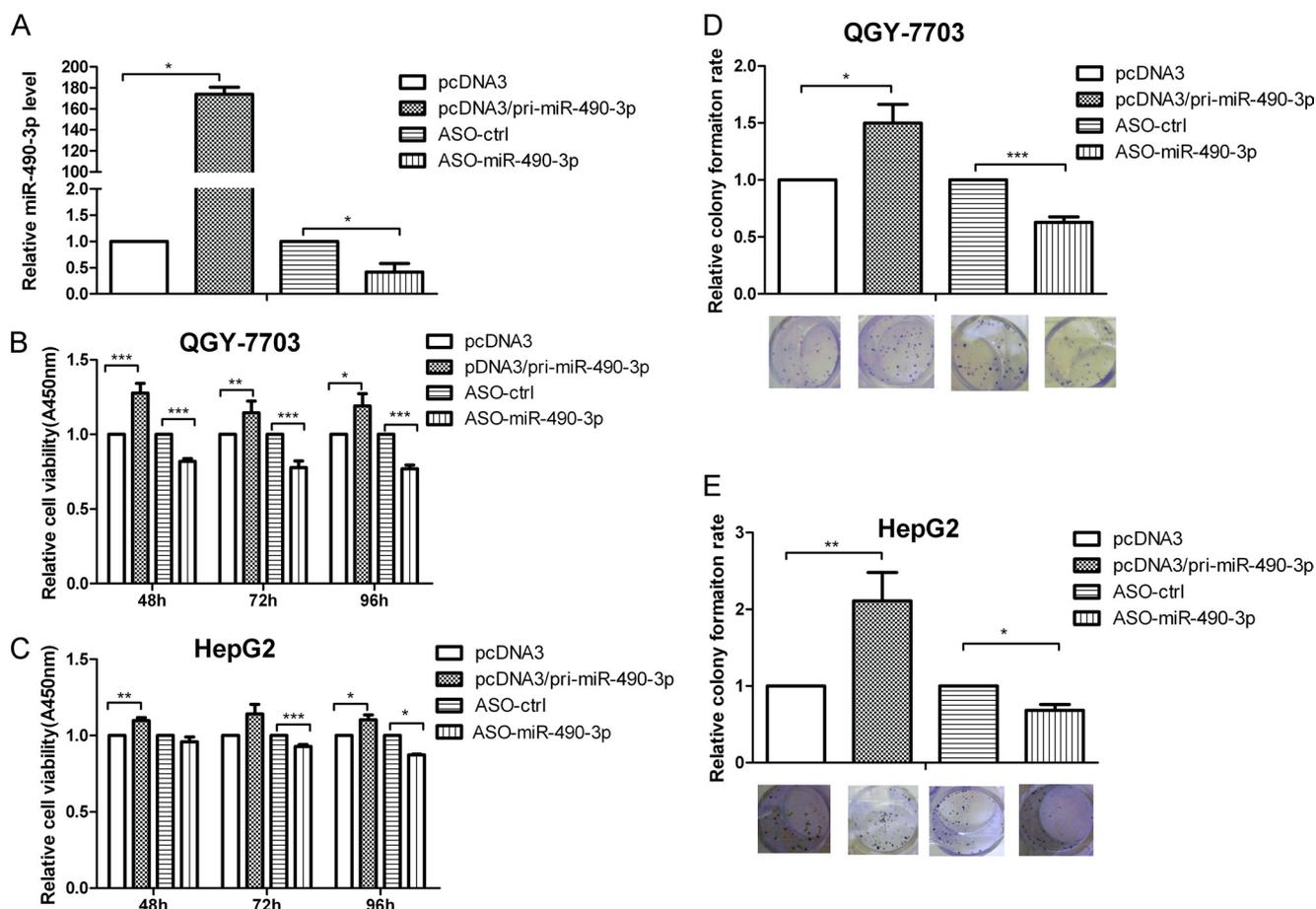


FIGURE 2. miR-490-3p enhances the cell viability and colony formation ability in HCC and stimulates the cell metastasis-related traits. *A*, the expression of miR-490-3p was analyzed by real-time PCR in the cells transfected with pcDNA3/pri-miR-490-3p or ASO-miR-490-3p. *B* and *C*, cell viability was measured in both QGY-7703 (*B*) and HepG2 (*C*) cells at 48, 72, and 96 h after transfection by the WST-1 assay. *D* and *E*, the colonies formed in transfected QGY-7703 (*D*) and HepG2 cells (*E*) were stained with crystal violet at ~8 days after transfection. *F* and *G*, miR-490-3p promotes the migration and invasion abilities of HCC cells. The migrated and invasive QGY-7703 (*F*) and HepG2 cells (*G*) were photographed using a microscope, and the number of migrated and invasive cells in every field was counted. The control was normalized to 1. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *H*, miR-490-3p promotes EMT. *Top*, Western blotting analysis for the expression of E-cadherin and vimentin in the cells transfected with miR-490-3p or the control construct. *Bottom*, real-time PCR analysis for the expression of EMT transcription factor, TWIST2, Snai1, and the mesenchymal marker, FN1, in cells transfected with miR-490-3p or the control construct. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *I*, E-cadherin immunostaining (green) and DAPI staining (blue) in QGY-7703 cells after transfection. Error bars, S.D.

490-3p was up-regulated in HCC tissues compared with the adjacent non-tumor tissues. Next, real-time PCR was performed in the normal liver cell line LO2 and four HCC cell lines, QGY-7703, HepG2, SK-Hep-1, and PLC/PRF/5. Consistently, miR-490-3p was up-regulated in all HCC cell lines compared with LO2 cells (Fig. 1B).

miR-490-3p Promotes HCC Cell Viability and Colony Formation Ability—To determine whether miR-490-3p affects cell growth in HCC, cell viability and colony formation assays were carried out with the QGY-7703 and HepG2 cell lines. First, the overexpression of miR-490-3p was achieved by transfecting the cells with pcDNA3/pri-miR-490-3p, whereas miR-490-3p was inhibited by ASO of miR-490-3p. The miR-490-3p expression level was ~130-fold higher in cells with pcDNA3/miR-490-3p than in the cells with the pcDNA3 control and was ~70% lower in the cells with ASO compared with the cells with the ASO control (Fig. 2A). Second, as shown in Fig. 2B, the overexpression of miR-490-3p increased the cell viability by ~28, 14, and 20% at 48, 72, and 96 h post-transfection, respectively, compared with the control. In contrast, inhibition of miR-490-3p

led to cell viability being reduced by 18, 22, and 23% at 48, 72, and 96 h post-transfection, respectively, in the QGY-7703 cell line. Consistent with the results in QGY-7703, miR-490-3p played a similar role in HepG2 cells (Fig. 2C).

Finally, we performed a colony formation assay with the QGY-7703 and HepG2 cell lines (Fig. 2, D and E). The overexpression of miR-490-3p increased the colony forming rate by ~50% in QGY-7703 and 100% in HepG2 compared with the controls. Conversely, inhibition of miR-490-3p reduced the colony formation rate by ~40 and 35% in QGY-7703 and HepG2, respectively. In combination, these data indicate that miR-490-3p may contribute to HCC tumor growth.

miR-490-3p Promotes HCC Metastasis-related Traits, Including Cell Migration, Invasion, and EMT—Metastasis is one of the most dangerous properties of most cancers, and migration and invasion are two factors essential for metastasis. In this study, migration and invasion assays were carried out in the QGY-7703 and HepG2 cell lines (Fig. 2, F and G). The overexpression of miR-490-3p increased cell migration by ~100% in QGY-7703 and 45% in HepG2 cells compared with the cells

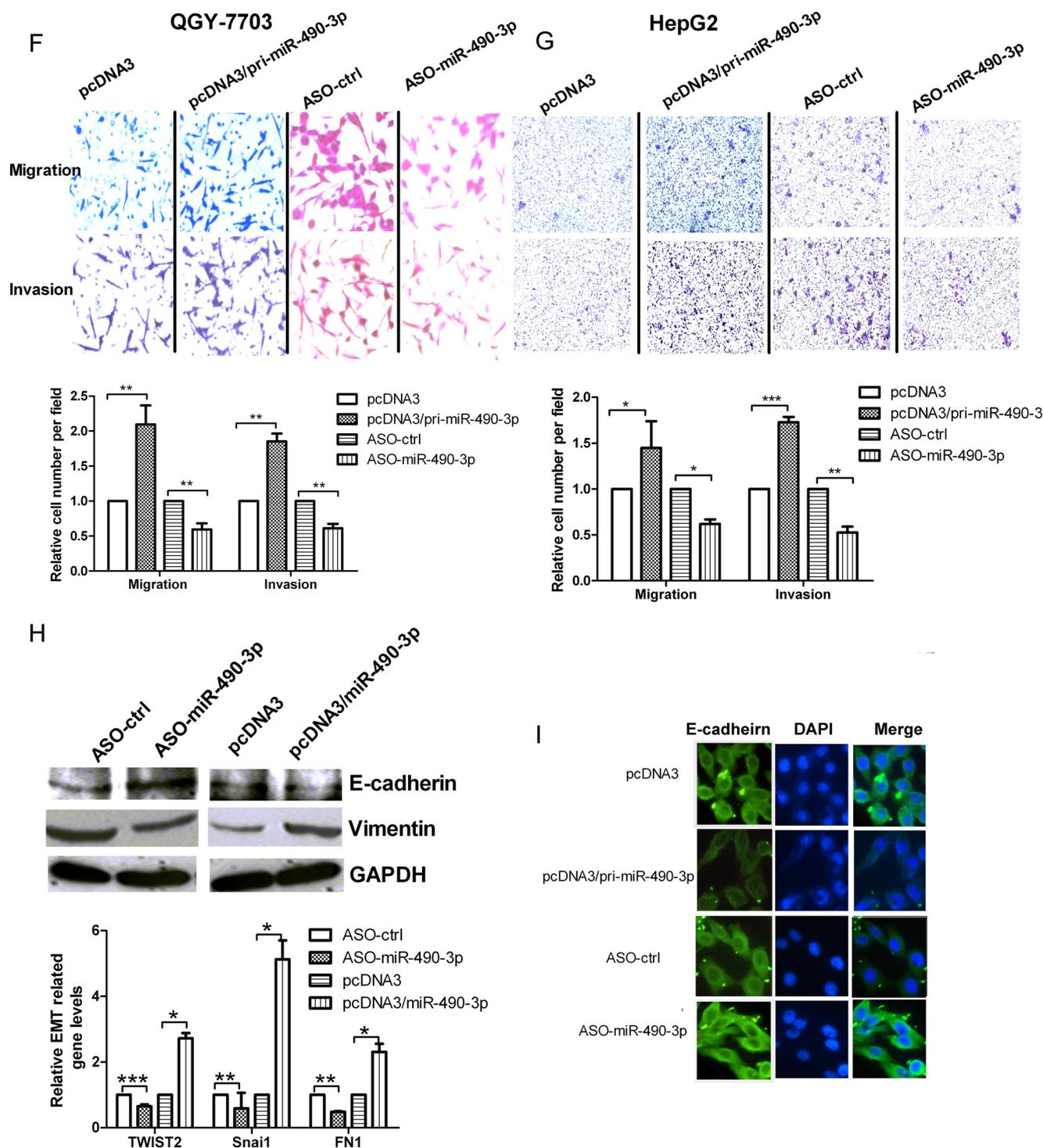


FIGURE 2—continued

with the control constructs. Inhibition of *miR-490-3p* reduced cell migration by ~40% in both cell lines. Similarly, the overexpression of *miR-490-3p* increased cell invasion through the Matrigel-coated membrane by ~85% in QGY-7703 and 70% in HepG2 cells, whereas inhibition of *miR-490-3p* led to cell invasion being reduced by ~40 and 50% in the two cell lines compared with the controls.

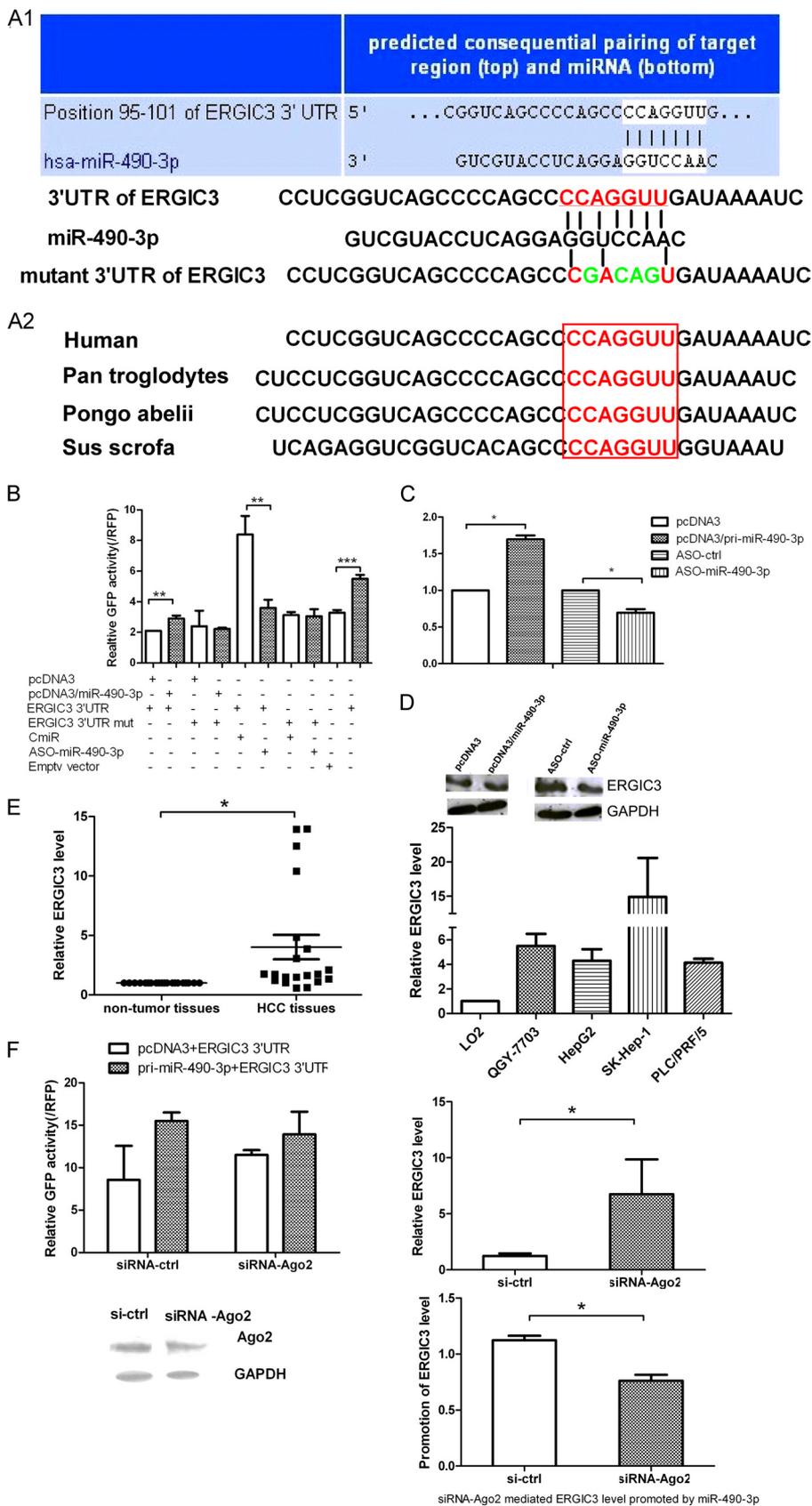
The previous results confirmed that the overexpression of *miR-490-3p* induced some common functions, such as cell pro-

liferation and invasion, required by tumors for metastatic progression. Therefore, we evaluated the relation between *miR-490-3p* and the EMT, which is the basic cause of invasion and a characteristic feature of cells undergoing proliferation. As shown in Fig. 2H, Western blotting analysis indicated that the overexpression of *miR-490-3p* caused a decrease in the expression of the epithelial marker, E-cadherin, and a corresponding increase in the expression of the mesenchymal marker, vimentin. Inhibition of *miR-490-3p* led to the opposite effect. In addi-

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tion, real-time PCR analysis was used to detect other mesenchymal marker, *FN1* (fibronectin) and EMT transcription factors, *Snail* and *TWIST2*. The results shown in Fig. 2H indi-

cated that inhibition of *miR-490-3p* led to the reduction of *TWIST2*, *Snail*, and *FN1* expression by about 35, 42, and 50%, respectively. In contrast, overexpression of *miR-490-3p* had the



opposite role. Consistent with these results, Fig. 2I showed that immunofluorescence analysis confirmed the role of *miR-490-3p* in E-cadherin expression. Therefore, these results suggest that *miR-490-3p* may play an important role in HCC metastasis.

ERGIC3 Is a Direct Target for miR-490-3p and Is Up-regulated by miR-490-3p—The ability of *miR-490-3p* to enhance HCC cell growth and EMT might derive from its regulation of genes involved in these processes. To explore the mechanism of *miR-490-3p* regulation in HCC, we used two algorithms to predict the targets for *miR-490-3p*, TargetScan and microCosm. Among these predicted genes, we selected *ERGIC3* as our candidate target. The binding sites for *miR-490-3p* on the 3'-UTR of *ERGIC3* were conserved among species (Fig. 3A). Next, the EGFP fluorescent reporter assays demonstrated that the overexpression of *miR-490-3p* increased the fluorescent intensity controlled by the *ERGIC3* 3'-UTR by ~40% compared with the empty vector; in contrast, the inhibition of *miR-490-3p* reduced the *ERGIC3* 3'-UTR fluorescent intensity by ~50% (Fig. 3B). However, neither the overexpression nor inhibition of *miR-490-3p* had an effect on the fluorescent intensity of the mutant *ERGIC3* 3'-UTR, in which several nucleotides within the binding sites were mutated (Fig. 3B). The EGFP reporter assay showed that *ERGIC3* was a direct target for *miR-490-3p*. To verify the regulation role of *miR-490-3p* on *ERGIC3*, real-time PCR and Western blotting were performed to detect the effect of *miR-490-3p* on *ERGIC3* mRNA and protein levels. As shown in Fig. 3C, the overexpression of *miR-490-3p* increased *ERGIC3* mRNA levels by ~70% compared with the cells transfected with the control construct, and the inhibition of *miR-490-3p* resulted in the *ERGIC3* mRNA level being reduced by 30%. In accord with these results, the overexpression of *miR-490-3p* increased the *ERGIC3* protein level, and the inhibition of *miR-490-3p* reduced the *ERGIC3* protein level (Fig. 3D).

In addition, we found that *ERGIC3* was up-regulated in HCC tissues and cells compared with the adjacent non-tumor tissues and normal cells (Fig. 3E), similar to the *miR-490-3p* expression trend in both HCC tissues and cell lines. On the basis of these data, we conclude that *ERGIC3* is a direct target gene for *miR-490-3p* and that *miR-490-3p* up-regulates *ERGIC3* expression.

To determine whether this unusual mechanism of mRNA up-regulation by miRNA required the RISC, we used siRNA targeting the critical component of RISC, Ago2, together with the EGFP reporter assay using the *ERGIC3* 3'-UTR. Fig. 3F showed that a knockdown of Ago2 increased the basic activity of the *ERGIC3* 3'-UTR reporter and the *ERGIC3* mRNA levels. Importantly, silencing Ago2 can abolish the *miR-490-3p*-induced up-regulation of *ERGIC3*. Therefore, these results showed that *ERGIC3* up-regulation mediated by *miR-490-3p*

required the involvement of the Ago2 protein and may depend on RISC.

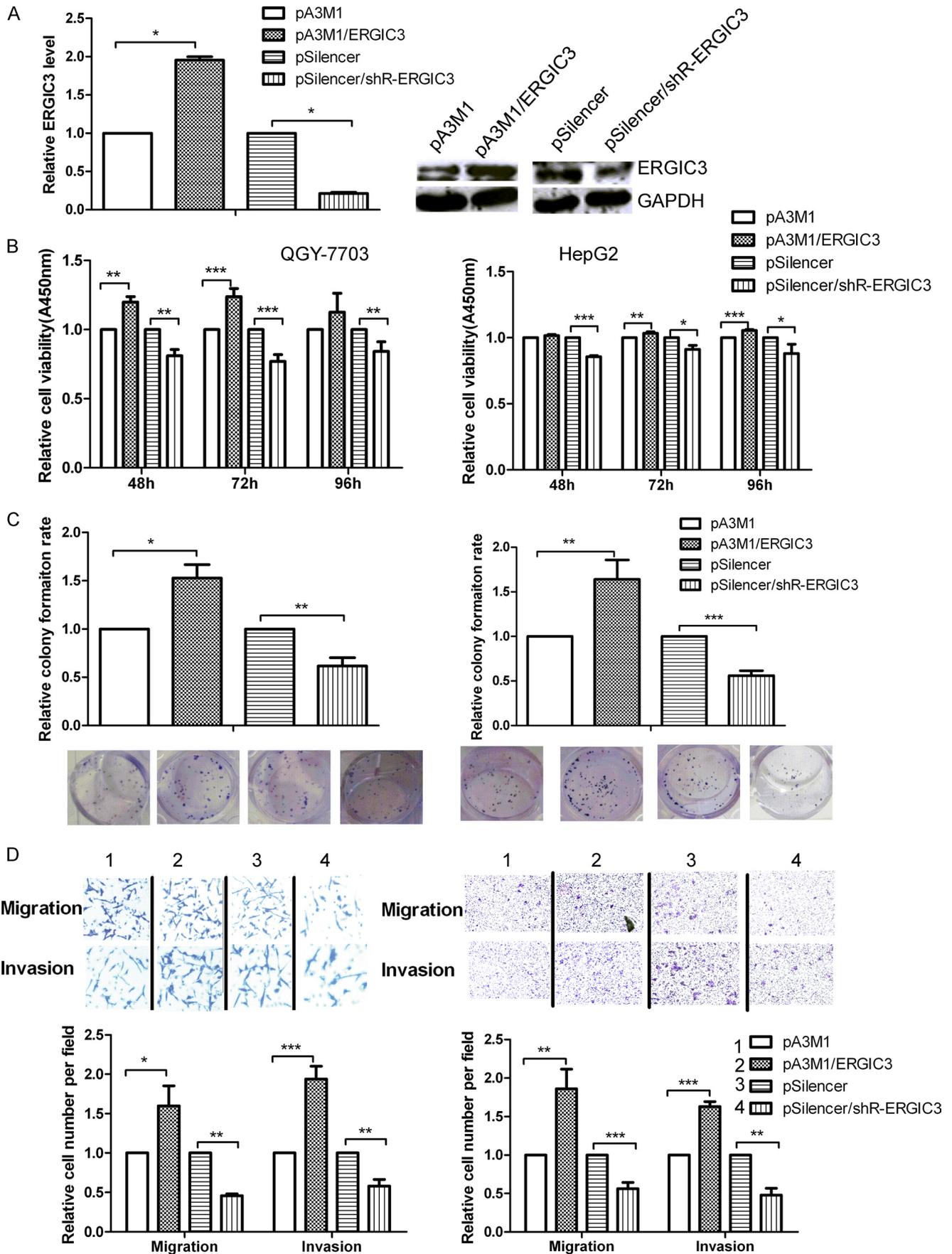
ERGIC3 Promotes HCC Cell Viability, Colony Formation, Migration and Invasion Abilities, and the EMT as Effectively as miR-490-3p—To determine whether *ERGIC3* can phenocopy the role of *miR-490-3p*, we evaluated the effects of *ERGIC3* on the cellular phenotype. As shown in Fig. 4A, compared with the control, *ERGIC3* expression was increased ~4-fold in cells with pA3M1/*ERGIC3* and decreased by ~70% in cells with shRNA for *ERGIC3*. The overexpression of *ERGIC3* increased the cell viability by 20, 24, and 13% in QGY-7703 cells at 48, 72, and 96 h post-transfection, respectively. In HepG2 cells, cell viability increased about 6% at 72 and 96 h post-transfection, respectively (Fig. 4B). The colony formation rate was increased by 50 and 60% in QGY-7703 and HepG2 cells, respectively (Fig. 4C), the migration ability was increased by 60 and 85% in QGY-7703 and HepG2 cells, respectively, and the invasion ability was increased by 90 and 63% in QGY-7703 and HepG2 cell lines, respectively, compared with the cells transfected with the control constructs (Fig. 4D). In contrast, silencing *ERGIC3* inhibited all of the above biological functions (Fig. 4, B–D). In addition, to confirm that the induction of the biological functions by *miR-490-3p* was mediated by *ERGIC3* rather than by other genes, we carried out rescue experiments by co-transfecting the cells with primary *miR-490-3p* and *ERGIC3* shRNA or the control constructs. Fig. 4, E–G, showed that silencing *ERGIC3* can reverse the increases in cell viability, colony formation, and migration and invasion ability induced by *miR-490-3p* overexpression.

Importantly, Western blotting and immunofluorescence staining showed that *ERGIC3* can induce the EMT by repressing the expression of epithelial marker E-cadherin and increasing the expression of mesenchymal marker vimentin (Fig. 4, H and I), consistent with the effect of *miR-490-3p*. In addition, overexpression of *ERGIC3* increased the expression of *TWIST2*, *Snai1*, and *FN1* by 90%, 3.6-fold, and 18-fold, respectively, and silencing *ERGIC3* had the opposite effect (Fig. 4H). In conclusion, these data indicate that *ERGIC3* is a key factor in the regulation of *miR-490-3p* in HCC.

miR-490-3p Plays a Similar Role in the Normal Hepatocytes, LO2 Cells, as in the HCC Cells—Because we previously observed that *miR-490-3p* stimulated the cell growth, migration, and invasion abilities in HCC cells, we tried to test whether *miR-490-3p* played similar roles in the normal hepatocytes, LO2 cells. Fig. 5, A and B, demonstrated that *miR-490-3p* overexpression increased the LO2 cell viability by about 20 and 10% at 48, 72, and 96 h post-transfection, respectively. The colony formation ability was increased by about 50% compared with the cells with controls. Moreover, we discovered that the cells

FIGURE 3. ***ERGIC3* is a direct target gene and regulated by *miR-490-3p* positively.** A, TargetScan was performed to predict the potential binding sites for *miR-490-3p* in the *ERGIC3* 3'-UTR, and several mutated nucleotides within the binding sites are shown. The binding sites were conserved among species. B, cells were co-transfected with *miR-490-3p* and the 3'-UTR of *ERGIC3* or the mutant 3'-UTR and the control construct. The fluorescent intensity was measured at 48 h after transfection. C and D, expression of *ERGIC3* was analyzed by real-time PCR (C) and Western blotting (D) in the cells transfected with *miR-490-3p* or the control construct. E, the expression of *ERGIC3* was analyzed in HCC tissues, adjacent non-tumor tissues, and also in LO2, QGY-7703, HepG2, SK-Hep-1, and PLC/PRF/5 cells by real-time PCR. F, the up-regulation of *ERGIC3* by *miR-490-3p* required Ago2 participation. *Left graph*, EGFP reporter analysis of the effect of Ago2 on the interaction between *miR-490-3p* and *ERGIC3*; *bottom*, Western blotting analysis of Ago2 expression after the cells were treated with siRNA or a control construct. *Right graph*, effect of Ago2 on the interaction of *miR-490-3p* and *ERGIC3* using real-time PCR. The control was normalized to 1. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars, S.D.

Regulation of ERGIC3 by miR-490-3p in HCC



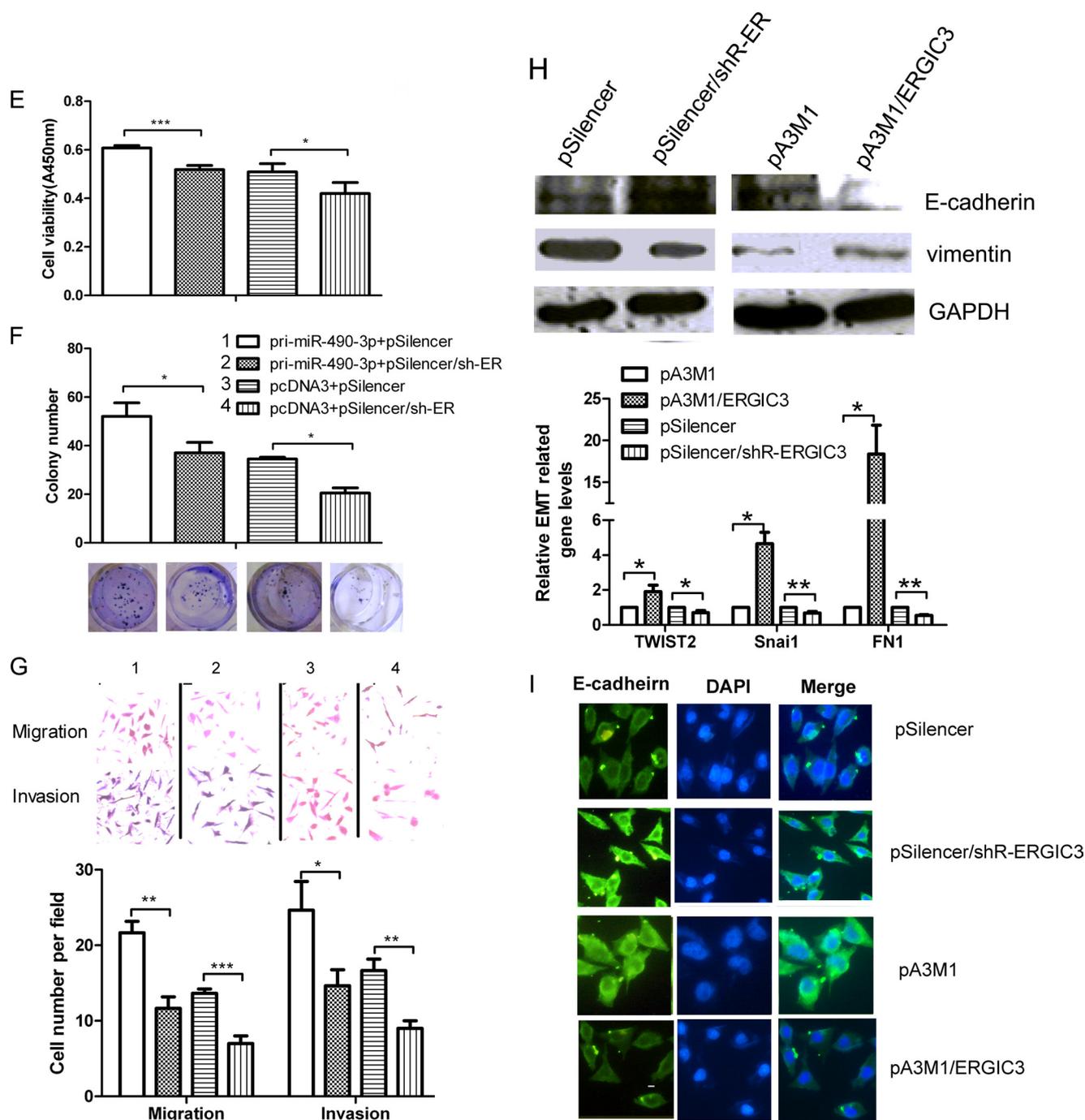


FIGURE 4—continued

with *miR-490-3p* had a relatively high migration and invasion ability, which was 50 and 75% more than that of the controls, respectively (Fig. 5C).

In addition, a rescue experiment was also performed in the cells co-transfected with *miR-490-3p* and shRNA of *ERGIC3* or with the controls. Fig. 5, D–F, indicated that silencing *ERGIC3*

FIGURE 4. ERGIC3 promotes the growth and metastasis-related traits of HCC cells. A, the expression of *ERGIC3* was analyzed by real-time PCR and Western blotting in the cells transfected with pA3M1/*ERGIC3* or pSilencer/shR-*ERGIC3*. B, *ERGIC3* promotes HCC cell viability. Cell viability was measured at 48, 72, and 96 h after transfection by the WST-1 assay. C, *ERGIC3* promotes colony formation ability of HCC cells. The colonies formed in transfected QGY-7703 and HepG2 cells were stained with crystal violet and counted at ~8 days after transfection. D, *ERGIC3* promotes HCC cell migration and invasion abilities. The migrated and invasive QGY-7703 and HepG2 cells were stained with crystal violet and imaged using a microscope, and the number of the migrated and invasive cells was counted. The control was normalized to 1. E–G, rescue experiment. Cells were co-transfected with *miR-490-3p* and shRNA for *ERGIC3*, as well as the control construct and tested for cell viability (E), colony formation (F), and migration and invasion abilities (G). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. H, *ERGIC3* contributes to EMT. Top, Western blotting analysis for the expression of E-cadherin and vimentin in the cells transfected with *ERGIC3* or the control. Bottom, real-time PCR analysis for the expression of EMT transcription factor, TWIST2, Snai1, and the mesenchymal marker, FN1, in the cells transfected with *ERGIC3* or the control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. I, immunostaining of E-cadherin (green) and DAPI (blue) after transfection. Error bars, S.D.

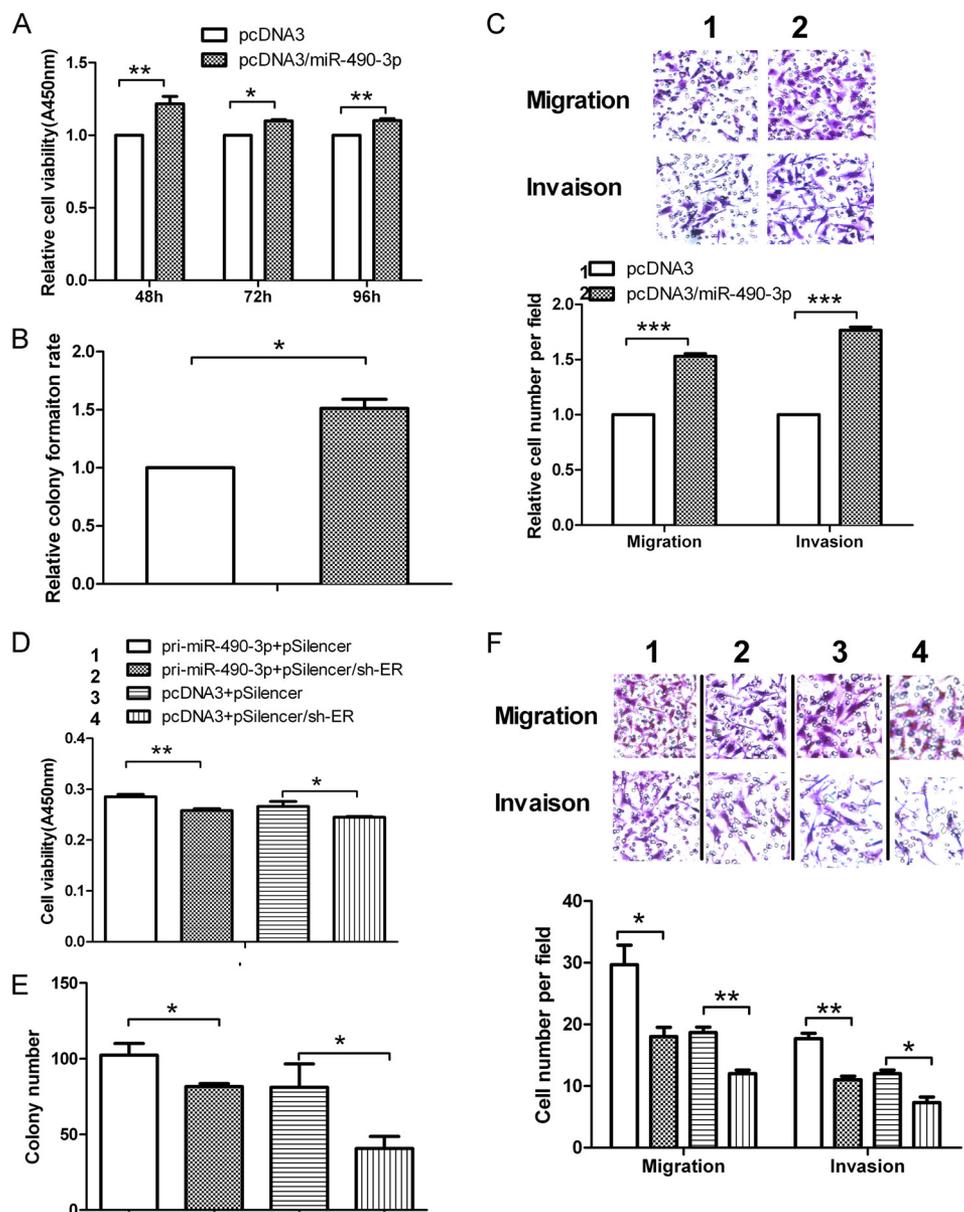


FIGURE 5. **miR-490-3p promotes the cell viability, colony formation, and migration and invasion abilities in LO2 cells.** A, the cell viability was measured in LO2 cells at 48, 72, and 96 h after transfection by the WST-1 assay. B, colony formation ability was measured when the colony involved more than 50 cells, and then the colonies were stained by crystal violet at ~8 days after transfection. C, miR-490-3p enhances LO2 cell migration and invasion abilities. The migrated and invasive cells were photographed using a microscope, and the number of the migrated and invasive cells in every field was counted. The control was normalized to 1. D–F, rescue experiment. LO2 cells were co-transfected with miR-490-3p and shRNA for *ERGIC3* as well as the control construct and tested for cell viability (D), colony formation (E), and migration and invasion abilities (F). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars, S.D.

can restore the LO2 cell growth and the migration and invasion ability increased by *miR-490-3p* overexpression. Overall, these data illustrate that *miR-490-3p* also contributes to LO2 cell growth, migration, and invasion via regulation of *ERGIC3*, further validating that the roles of *miR-490-3p* like those of an oncogene.

DISCUSSION

miRNAs are known to be regulators of many genes at the post-transcriptional level, and their aberrant expression is related to cancer initiation, development, and prognosis; however, most of the details of the mechanism have not been elucidated. Prior reports have suggested that *miR-490-3p* is down-regulated in colon cancer and Ewing's sarcoma (28, 29). It was demonstrated that *miR-490-3p* was associated with the overall

survival of the EWS patients and that the overexpression of *miR-490-3p* represented a high survival rate, acting like a tumor suppressor gene, but the potential mechanism of *miR-490-3p* regulation was not described in both cancers.

The current study indicated for the first time that *miR-490-3p* is highly expressed in HCC tissues and cells, enhancing HCC cell viability, colony formation, migration and invasion abilities, and the EMT. However, the inconsistency of *miR-490-3p* expression levels and roles among colon cancer, Ewing's sarcoma, and HCC may be due to differential regulation of miRNA molecules or cell type specificity. Recent studies have shown that several RNA-binding proteins, such as KSRP (KH-type splicing regulatory protein) and TRBP (TAR RNA-binding protein), can interact with *Drosha* and *Dicer* complex, leading

to the regulation of miRNA (30, 31). In addition, *miR-125b* is known as a tumor suppressor in liver cancer (32), breast cancer (22), and osteosarcoma (33), but it also acts as an oncogene in type II endometrial carcinoma (34) and in breast cancer (35), which further implies that miRNAs may have opposite functions in different cancers and even in the same cancer.

Some miRNAs have been reported to be involved in the process of cancer cell growth. For example, *miR-423* promotes the growth of hepatocellular carcinoma cells by regulating *p21^{Cip1/Waf1}* (36), and *miR-301* inhibits the proliferation and clonogenicity of breast cancer cells through *PTEN* and *FOXF2* (37). Another important characteristic of cancers is metastasis, a complicated, multistep process by which cells detach from the original tissues or organs, migrate into the vascular or lymph circulation, and finally invade another organ, forming a new tumor. It is known that miRNAs are involved in metastasis. For instance, *miR-203* functions as an anti-metastasis miRNA by regulating metastasis-related genes, including *ZEB2*, *Bmi*, *survivin*, and bone-specific effectors, including *Runx2* in prostate cancer (7); *miR-125b* suppresses the metastasis of liver cancer cells by down-regulating *LIN28B2* (32). Accordingly, our study showed that *miR-490-3p* not only promoted the HCC cell viability and colony formation rate but also the cell migration and invasion abilities. Furthermore, the results from the normal hepatocytes, LO2 cells, demonstrated that *miR-490-3p* exerted a similar impact on LO2 cells as on HCC cells, suggesting that the potential roles of *miR-490-3p* are like those of an oncogene.

Importantly, the phenotype resulting from EMT is required for the invasive behavior of cancer cells (6). In addition, the dysregulation of miRNAs is involved in EMT modulation (38). For instance, *miR-194* inhibits EMT by repressing the *BMI-1* in endometrial cancer (21), and the *miR-200* family and *miR-205* inhibit EMT by regulating the E-cadherin transcription repressors, *ZEB1* and *ZEB2*, resulting in an increase in E-cadherin expression (39), a novel biomarker of the epithelial cells. Previous studies indicated that vimentin and fibronectin are key factors for EMT and serve as important biomarkers for the prognosis of cancers (6, 40, 41). In addition, *Snai1* and *Twist2* are key transcriptional factors of EMT, which can contribute to the expression of vimentin and inhibit the E-cadherin expression directly or indirectly (42, 43). Accordingly, our data indicated that *miR-490-3p* enhances the EMT by a reduction in the levels of E-cadherin and an increase in the levels of the mesenchymal markers vimentin and *FNI*, even more, stimulating the expression of *Snai1* and *Twist2*.

As is known, miRNAs regulate gene expression by binding to the 3'-UTR of target genes. In this study, bioinformatics was used to predict target genes, and *ERGIC3* was chosen as the candidate target. The EGFP fluorescence reporter assay, a direct method for target validation, verified that *ERGIC3* was a direct target gene of *miR-490-3p*, but surprisingly, *miR-490-3p* regulated *ERGIC3* positively, enhancing both *ERGIC3* mRNA and protein levels, in contrast to previous reports that a large number of miRNAs regulated gene expression negatively. However, previous studies have revealed several important ways by which miRNA can up-regulate gene expression, but the precise mechanisms involved are still elusive. For example, *miR-10a* enhances ribosomal protein translation by binding to

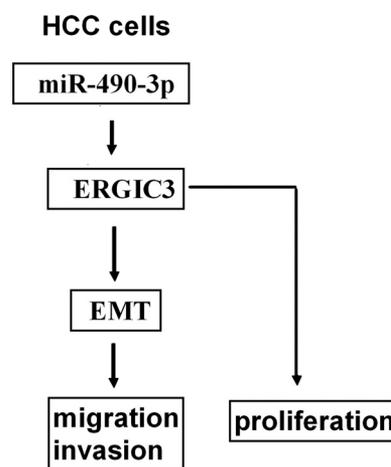


FIGURE 6. A simplified model for the regulation of *ERGIC3* by *miR-490-3p* in HCC cells. *miR-490-3p* up-regulated *ERGIC3* expression and stimulated cell proliferation; *ERGIC3* promoted the EMT, resulting in migration and invasion.

the 5'-UTR of ribosomal protein, interacting with the 5'-TOP motif (44); *miR-122* stimulates hepatitis C virus RNA translation by binding to the 5'-UTR of the virus genome, and *miR-122* functions as the nonmethylated 5'-cap, acting coordinately with Ago2 (45); *miR-346* up-regulates RIP140 (receptor-interacting protein 140) without requiring Ago2 (16); *miR-744* up-regulates cyclin B1 by binding to the promoter (46); and finally, there is also the mechanism related to the A-U-enriched elements during cell starvation (47). In our study, we showed that the up-regulation of *ERGIC3* by *miR-490-3p* required Ago2 participation, but the mechanism needs to be explored further.

ERGIC3, also known as endoplasmic reticulum-localized protein (*ERp43*), is located on chromosome 20 and is a component of ERGIC, which mediates the transport from the endoplasmic reticulum to the Golgi. A previous study (48) indicated that *ERp43* (*ERGIC3*) promoted HEK-293 cell growth. Consistently, our data demonstrated that *ERGIC3* not only enhanced HCC cell viability and colony formation abilities but also stimulated cell migration and invasion ability. Then our data also indicated that *ERGIC3* stimulated EMT by increasing the expression of *TWIST2*, *Snai1*, *FNI*, and vimentin and reducing the expression of E-cadherin. Furthermore, silencing *ERGIC3* can reverse the cell functions induced by *miR-490-3p* overexpression, indicating that *miR-490-3p* stimulated these cell functions, at least partially, by regulating *ERGIC3*.

In conclusion, our study demonstrated a novel regulatory mechanism by which *miR-490-3p* stimulated HCC cell growth and the metastasis-related traits, migration and invasion, by up-regulating *ERGIC3*. We also discovered that *ERGIC3* and *miR-490-3p* contributed to cell invasion via induction of EMT (Fig. 6). Therefore, inhibition of *miR-490-3p* may be considered to be a potentially important molecular treatment strategy for patients with HCC.

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