



miR-127-5p targets the 3'UTR of human β -F1-ATPase mRNA and inhibits its translation

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ABSTRACT

The mitochondrial H^+ -ATP synthase is a bottleneck component in the provision of metabolic energy by oxidative phosphorylation. The expression of its catalytic subunit (β -F1-ATPase) is stringently controlled at post-transcriptional levels during oncogenesis, the cell cycle and in development. Here we show that miR-127-5p targets the 3'UTR of β -F1-ATPase mRNA (β -mRNA) significantly reducing its translational efficiency without affecting β -mRNA abundance. Despite the reduced expression of β -F1-ATPase in most human carcinomas, we observed no expression of miR-127-5p in different human cancer cell lines, minimizing the potential role of miR-127-5p as a regulator of the bioenergetic activity of mitochondria in cancer. In contrast, miR-127-5p is highly over-expressed in the human fetal liver. Consistent with previous findings in the rat, the expression of β -F1-ATPase in the human liver also seems to be controlled at post-transcriptional levels during development, what might suggest a role for miR-127-5p in controlling β -mRNA translation and thus in defining the bioenergetic activity of human liver mitochondria. Moreover, immunolocalization techniques and subcellular fractionation experiments using different antibodies against β -F1-ATPase reveal that the ectopic expression of β -F1-ATPase at the cell surface of the hepatocytes and HepG2 cells is negligible or stands for scrutiny.

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1. Introduction

Mitochondrial activity provides the energy required for adaptation to distinct environmental and physiological conditions of the organism. The mitochondrial H^+ -ATP synthase is the engine used for ATP production by oxidative phosphorylation [1]. Previous studies in yeast and mammalian tissues have shown that the expression of the catalytic subunit of the H^+ -ATP synthase (β -F1-ATPase), which is encoded in the nuclear genome, is mainly regulated by post-transcriptional mechanisms that affect mRNA localization, stability and translation [2–6]. In particular, during development of rat liver [2,7], in brown adipose tissue [8] and in rat [4] and human [9] carcinomas the expression of β -F1-ATPase is regulated at the level of translation. Mechanistically, both during development and in oncogenesis β -F1-ATPase mRNA (β -mRNA) translation is masked by

specific factors that hinder ribosome recruitment onto the mRNA [2,4,10,11]. In this regard, an important *cis*-acting element required for β -mRNA translation is the 3'UTR of the transcript [2–6]. The 3' UTR of β -mRNA acts as a translational enhancer both *in vitro* [2,9,12] and *in vivo* [13], and is essential for the localization [5,6] and efficient translation of the transcript [2,9].

Post-transcriptional control of the expression of specific mRNAs can be explained by the binding of regulatory proteins and/or miRNAs to *cis*-acting elements of the mRNA. Recently, Ras-GAP SH3 binding protein 1 (G3BP1) has been shown to interact with the 3'UTR of β -mRNA to inhibit the initiation step of translation [11] contributing in this way to the observed down-regulation of β -F1-ATPase expression in cancer. On the other hand, miRNAs are important post-transcriptional regulators of gene expression [14]. miRNAs are short, approximately 22-nucleotides long RNAs, that are transcribed as a long primary transcript that folds into a hairpin structure. After nuclear processing, the ~70 nucleotide precursor miRNA (pre-miRNA) is exported to the cytoplasm and processed by Dicer into miRNA duplexes. One strand of the duplex represents the mature miRNA that has to be incorporated into a miRNA-induced silencing complex (RISC) to exert its biological function [14]. miRNAs regulate various biological processes including developmental timing, cell fate determination, apoptosis and metabolism [15–17]. Changes in the expression of miRNAs are associated with human pathology including cancer [14,18–21]. Animal miRNAs regulate gene expression by

Abbreviations: β - and γ -F1-ATPase, subunits β , α and γ of the mitochondrial H^+ -ATP synthase; β - and γ -mRNA, mRNAs of subunits β - and γ -F1-ATPase; G3BP1, Ras-GAP SH3 binding protein 1; GFP, green fluorescent protein; SDH-B, succinate dehydrogenase subunit B; 3'UTR, 3' untranslated region; ERR γ and ERR α , estrogen receptor gamma and alpha transcription factors; PGC-1 α , Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ATP5B, β -F1-ATPase gene

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imperfectly base pairing to the 3'UTR of target mRNAs inhibiting protein synthesis or inducing the degradation of the targeted mRNA [22]. However, it has also been reported that some miRNAs can induce translation of the target mRNA [23–25].

Due to the relevant implication of β -F1-ATPase in cell biology and in human pathology [10,26] the aim of this study was to identify miRNAs that might be involved in post-transcriptional control of β -F1-ATPase expression. For this purpose we generated stable cell lines expressing GFP fused to the 3'UTR of human β -mRNA and searched for potential miRNA that can modify the fluorescence intensity of the cells. We show that β -mRNA is specifically targeted and translationally silenced by miR-127-5p. We report that miR-127-5p is highly expressed in human fetal liver and show that β -F1-ATPase expression is post-transcriptionally regulated during human liver development. We suggest that miR-127-5p might play a relevant role in controlling the bioenergetic phenotype of the hepatocyte during human development. Moreover, we have estimated that the content of β -F1-ATPase at the plasma membrane of human and rat hepatocytes and HepG2 cells is insignificant as assessed by conventional immunological techniques with validated antibodies.

2. Material and methods

2.1. Plasmids and cloning strategies

The plasmids expressing a hybrid GFP fused in frame with the mitochondrial targeting sequence of β -F1-ATPase ($p\beta$) [27] containing either the 3'UTR of human β -mRNA ($p\beta$ GFP-3' β h, pIW22) or α -F1-ATPase mRNA (α -mRNA) ($p\beta$ GFP-3' α h, pIW23) were cloned as described [9]. The plasmid $p\beta$ -GFP-3' β MUT (pIW29) contains four point mutations in the 3'UTR seed sequence of the hsa-miR-127-5p binding site of β -mRNA. To introduce the mutations, two overlapping PCR products containing the desired mutations were amplified using oligos F-pre β -EGFP-KpnI: 5'-GCGGGTACCCGAATCCAGTCTC-3'/R-3' β hBmut-PCR1a: 5'-CACAGAAAATAAGGGTCTTTGGGTTAGG-3' and F-3' β -h-EcoRI: 5'-GCGGAATTCGGGGTCTTTGTCTCTGTA-3' / F-3' β hBmut-PCR1b: 5'-CCTAACCCAAAGACCTTATTTTCTGTG-3'. The PCR products were mixed 1:1, incubated for 5 min at 94 °C and 30 min at 37 °C to allow annealing of complementary tails. To form one full length mutated PCR fragment, 1.75 mM dNTPs and 1 U/ μ l of Klenow DNA polymerase were added and incubated for 30 min at 37 °C. The product from this reaction was used as a template for a second PCR reaction using the outer primers F-3' β -h-EcoRI: 5'-GCGGAATTCGGGGTCTTTGTCTCTGTA-3' and R-3' β -h-XhoI: 5'-GCGCTCGAGTTTTTTTTTTTGGAGGGGTGTA-3'. The amplified PCR fragment was purified, digested with EcoRI and XhoI and cloned into the $p\beta$ GFP-3' Δ plasmid [9].

2.2. Cell cultures, generation of GFP expressing cells and transfection of plasmids and miRNAs

The human BT549 breast and HepG2 liver cancer cell lines were grown in RPMI and DMEM respectively, supplemented with 10% FBS, 400 μ M non-essential amino acids, 1 mM glutamine, 100 UI/ml penicillin and 0.1 mg/ml streptomycin. Cells were transfected with plasmids $p\beta$ GFP-3' α h or $p\beta$ GFP-3' β h using Lipofectamine and Plus reagent (Invitrogen). After 24 h of transfection cells were split 1:10 and 1:20 into 10 cm plates and grown in the appropriate medium containing 0.3 mg/ml geneticin (G418, GIBCO). After 1–2 weeks isolated colonies began to appear and the drug-resistant GFP-fluorescent clones were isolated with cloning cylinders and sub-cloned into 96-well plates. The clones were expanded and the purity of the cell population analyzed by flow cytometry. miRNAs were transfected at the time of plating using siPORT NeoFx reagent (Ambion/Applied Biosystems). The medium was changed at 24 h post-transfection and the cells were analyzed at 48 h post-transfection. miRNAs were used at a final concentration 30 nM. The pre- and anti-miRNAs used in this study were purchased

from Ambion/Applied Biosystems and were PM11164 (pre-miRNA hsa-miR-423-5p), PM11414 (pre miRNA hsa-miR-101), PM13001 (pre-miRNA hsa-miR-127-5p), PM11646 (pre-miRNA hsa-miR-581), PM10492 (pre-miRNA hsa-miR-200b), PM11753 (pre-miRNA hsa-miR-186), PM10632 (pre-miRNA hsa-miR-103), AM11164 (anti-miRNA hsa-miR-423-5p), AM11646 (anti-miRNA hsa-miR-581), AM10492 (anti-miRNA hsa-miR-200b), AM11753 (anti-miRNA hsa-miR-186), AM10632 (anti-miRNA hsa-miR-103), AM13001 (anti-miRNA hsa-miR-127-5p) and AM17110 (miRNA negative control #1). Co-transfection of miR-127-5p and the plasmids $p\beta$ -GFP-3' β MUT or $p\beta$ -GFP-3' β were performed in a 1:1 molar ratio using Lipofectamine and Plus reagent (Invitrogen). In all cases the cell culture was incubated with the cationic-lipid-DNA complexes for 2 h at 37 °C.

2.3. Flow cytometry

After 48 h of transfection cellular pellets were resuspended in 200–400 μ l FACS solution (1% FBS, 0.1% NaN₃ and 1 \times PBS) and analyzed by flow cytometry. The fluorescence intensity of at least 10,000 events was determined in a FACScan cytometer (Becton–Dickinson) using CellQuest (Becton–Dickinson) acquisition software. The results were analyzed with the program FlowJo (TreeStar).

2.4. Screening of potential miRNAs by fluorescence reporter assays

Over-expression and silencing assays of the selected miRNAs were performed in BT549 clones stably expressing a mitochondrial localized GFP fused to the 3'UTR of β -F1-ATPase mRNA (BT549: $p\beta$ GFP-3' β) using the indicated miRNAs precursors (pre-miRNA), anti-miRNAs and miRNA negative control #1. As an additional control of the screening experiments BT549 clones stably expressing a mitochondrial localized GFP fused to the 3'UTR of α -F1-ATPase mRNA (BT549: $p\beta$ GFP-3' α) were studied in parallel. The assays were carried out in 96-well plates with a black border and a clear flat bottom (Corning). After 48 h of transfection the cells were stained for 10 min at 37 °C with 5 μ g/ml of Hoescht (Sigma) diluted in cell culture medium without phenol red. The fluorescence intensity of Hoescht (excitation: 340–10 nm, emission: 470–10 nm) and of GFP (excitation: 485 nm, emission: 510–10 nm) was determined using a FLUOstar Omega Microplate Reader (BMG Labtech). The fluorescence intensity of GFP was normalized by the number of cells as determined by Hoechst fluorescence intensity and quantified relative to the cells transfected with a non-relevant miRNA (control).

2.5. Cloning, expression and purification of γ -F1-ATPase and antibody production

The cDNA (BC000931) encoding human γ -F1-ATPase (AAH35198) was amplified by PCR using the LGC-Promochem MGC-5380 clone and primers 5'-cgcgagctcatgttctctcgcgcg-3' and 5'-atagtttagcggccgcatccagagctgctgc-3' which add SacI and NotI restriction sites, respectively. The resulting product was purified and first cloned into pGEM-Teasy vector (Promega) and after into pQE-Trisystem [28]. The resulting plasmid, pQE- γ F1, that encodes γ -F1-ATPase with C-terminal 6 \times His and streptavidin tags, was used to transform *Escherichia coli* BL-21 cells. Protein expression was induced by addition of 1 mM IPTG. After overnight induction, the cells were collected and the expressed protein purified using Ni-NTA superflow resin (Qiagen) [28]. To produce monoclonal antibodies against human γ -F1-ATPase, we proceeded as recently described [28]. In brief, BALB/c mice were immunized with various doses of the purified protein (20 μ g) and the hybridomas produced by fusing spleen cells with the SP2 myelomas. Supernatants of the hybridomas were screened by indirect ELISA on γ -F1-ATPase-coated polystyrene plates. Bound antibodies were detected using horseradish-peroxidase-labeled goat anti-mouse antibodies (1:1000 DAKO). The positive colonies were

cloned by limiting dilution. Mouse monoclonal antibodies were purified with Montage antibody purification kit (Millipore) and used at a 1:1000 dilution.

2.6. Quantification of miRNA and mRNA

RNA was extracted from cell cultures using TRIzol Reagent (Invitrogen). Human fetal (20–44 weeks, #636540 from Clontech and A601605 and A604419 from BioChain) and adult (51–64 year-old men, #636531 from Clontech and B510061 and B510092 from BioChain) total liver RNA were purchased. The expression level of mature miRNAs was determined using TaqMan MicroRNA Assay from Applied Biosystems, which includes a unique set of RT-PCR primers and a specific TaqMan probe. RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The TaqMan MicroRNA Assays used in this study were RNU48 (#001006, Applied Biosystems), U6 snRNA (#001973, Applied Biosystems) and hsa-miR-127-5p (#002229, Applied Biosystems). The qPCR reaction was performed as indicated by the supplier using 2X Universal Master Mix without UNG (Applied Biosystems) and an ABI PRISM 7900 SDS thermocycler. Expression of miR-127-5p was determined according to the $\Delta\Delta C_t$ method using RNU48 (cell lines) or U6 (human samples) as internal controls, respectively. Quantification of β -mRNA, γ -F1-ATPase and succinate dehydrogenase subunit B (SDH-B) mRNAs and 18S rRNA expression in human liver samples by RT-PCR was performed as described [9,11].

2.7. Protein extraction and Western blotting

For protein extraction, cellular pellets were resuspended in lysis buffer (25 mM Hepes, 2.5 mM EDTA, 1% Triton X-100, 1 mM PMSF and 5 μ g/ml leupeptin) supplemented with complete protease inhibitor cocktail EDTA-free (Roche). Cell lysates were clarified by centrifugation at 10,000 \times g for 7 min. Adult human liver samples were obtained from the Banco de Tejidos y Tumores, IDIBAPS, Hospital Clinic, Barcelona, Spain, coded for anonymity [29]. The Institutional Review Board approved the project. Fetal human liver lysates were purchased from ProSci Incorporated (#XBL-10409), Abcam (ab29890) and BioChain (A605254). Protein concentrations were determined with the Bradford reagent (Bio-Rad Protein Assay). Protein extracts were fractionated on SDS-PAGE and transferred onto PVDF membranes for immunoblot analysis. The primary monoclonal antibodies used were: anti-Hsp60 (Stressgene SPA-807, 1:5000), anti-GFP (Clontech, 1:10,000), anti- β -actin (Sigma, 1:100,000), anti- γ -F1-ATPase (1:1000), anti-E-cadherin (BD Pharmingen, 1:1000) and anti-SDH-B (Molecular Probes, 1:500), anti- β -F1-ATPase (Ab1) (Molecular Probes, 1:1000) and the polyclonal rabbit anti- β -F1-ATPase (Ab2) (1:15,000) [29] and (Ab3) [30]. Peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Nordic Immunology, 1:5000) were used as secondary antibodies. The blots were developed using the ECL reagent (Invitrogen).

2.8. Immunohistochemistry

Paraffin-embedded human liver 5 μ m tissue sections were stained using a diaminobenzidine-based method using the Envision-Plus-horseradish peroxidase system (DAKO) as detailed [29]. The dilution of the anti- β -F1-ATPase (Ab2) used was 1:3000. Nuclei were counterstained with hematoxylin.

2.9. Electron microscopy

Small pieces (~1 mm³) of rat liver samples were fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1 M Sørensen phosphate buffer, pH 7.2 and supplemented with 6% sucrose, for 2 h at 4 °C. Samples were rinsed in buffer and the free-aldehyde groups

were quenched with 50 mM ammonium chloride in PBS for 60 min at 4 °C. Afterwards, the samples were rinsed in PBS, dehydrated in acetone and finally processed for embedding in Lowicryl K4M (Polysciences Europe, Eppelheim, Germany) according to manufacturer's instructions. Gold interferential color ultrathin sections were collected in collodion/carbon-coated nickel grids. For the immunocytochemical localization of β -F1-ATPase, the grids were incubated with a 1:50 dilution of anti- β -F1-ATPase (Ab3) [30]. After three washes with PBS, grids were incubated for 45 min with Protein A-gold complex (10 nm). Fixation was carried out with 1% glutaraldehyde in PBS. Counterstaining was performed with 2% uranyl acetate (7 min) and 1% lead citrate (45 s). The grids were observed in a Jeol 1010 electron microscope under 80 kV accelerating voltage.

2.10. Immunofluorescence

For double-immunofluorescence microscopy HepG2 cells were grown on coverslips and fixed with 2% paraformaldehyde in PBS for 30 min. Cellular permeabilization was achieved by incubation with 0.1% Triton X-100 for 10 min. Coverslips were first incubated in 1% FCS in PBS for 10 min and washed before incubation with the primary monoclonal anti- β -catenin (BD Pharmingen, 1:100) and the polyclonal anti- β -F1-ATPase (Ab2) antibodies. After, coverslips were incubated for 1 h in the dark with goat-anti rabbit IgGs conjugated to Alexa 594 and goat anti-mouse IgGs conjugated to Alexa 488. Cellular staining was analyzed in a confocal microscope Biorad Radianc 2000 Zeiss Axiovert S100TV.

2.11. Purification of mitochondria and plasma membranes

Rat liver homogenates were prepared in a solution containing 0.25 M sucrose, 1 mM EGTA and 10 mM Tris-HCl pH 7.4 and centrifuged at 800 g for 10 min to remove nuclei and unbroken cells. The resulting post-nuclear supernatants (H) were fractionated on 35 ml sucrose density gradients [24–54% (w/v)]. The gradients were centrifuged at 145,000 g for 70 min at 4 °C. Fractions were collected from the bottom of the tube and the localization of mitochondria (Mi) performed by western blotting using antibodies against β -F1-ATPase and Hsp60. Liver post-nuclear supernatants (H) were centrifuged first at 8500 g for 10 min and the resulting supernatants were further centrifuged at 180,000 g for 1 h. The resulting microsomal pellet was homogenized in 20 ml of 57% sucrose and placed on the bottom of the centrifuge tube. Thirty ml of 34% sucrose was added on top of the microsomal sample. The discontinuous gradient was centrifuged at 54,000 g for 16 h at 4 °C. Fractions were collected from the bottom of the tube and the localization of the plasma membrane (PM) fraction performed by western blotting using antibodies against E-cadherin (BD Pharmingen, 1:1000).

2.12. Statistical analysis

Statistical analyses were performed using a two-tailed Student's t-test. The results shown are means \pm SEM. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Potential miRNAs targeting the 3'UTR of human β -mRNA

In order to identify potential miRNAs that could bind the 3'UTR of human β -mRNA we performed a bioinformatic study using the miRNA target prediction software TargetScan5.1 (<http://www.targetscan.org/>). The TargetScan prediction algorithm is based on few binding rules [31–33]. In brief, the most important requirement seems to be a contiguous and perfect Watson-Crick base pairing of the 5'-miRNAs "seed" region. Matching of the mRNA to the miRNA seed can be divided into 8mer-, 7mer- and 6mer-sites. An A residue

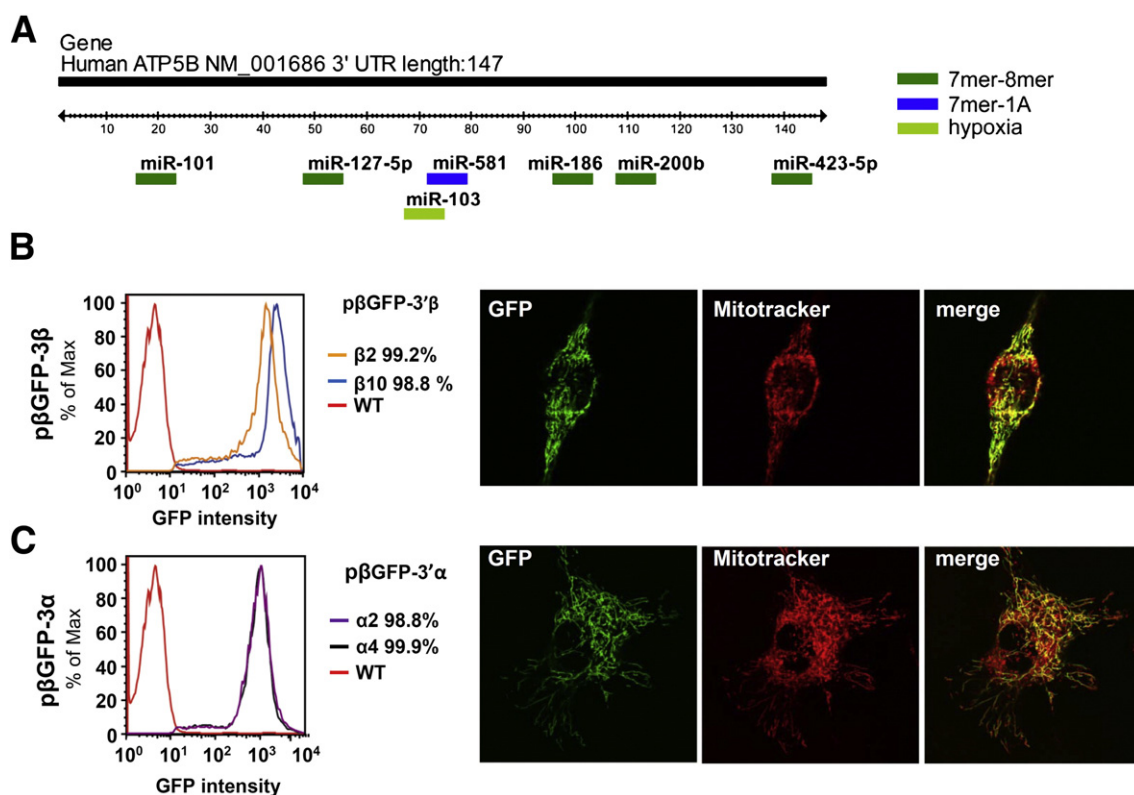


Fig. 1. Tools for the identification of miRNAs targeting the 3'UTR of human β -mRNA. (A) Schematic illustration of the 3'UTR of human β -mRNA and of the miRNAs predicted to bind the 3'UTR with higher affinity (TargetScan). Binding sites and types of the miRNAs selected are indicated in color code. The hypoxia regulated miR-103 (light green) was also included in the study for the putative relevance that hypoxia might play in regulating β -F1-ATPase expression. Generation and analysis of the BT549:p β GFP-3 β (B) and BT549:p β GFP-3 α (C) cell lines used to screen the activity of miRNAs. Graphs in B and C illustrate the GFP fluorescence of BT549 clones that express mitochondria-targeted GFP fused to the 3'UTR of human β -mRNA (clone β 2, orange and β 10, blue) or to the 3'UTR of α -mRNA (clone α 2, magenta and α 4, blue), respectively. The percentage of GFP expressing cells is indicated. The basal cellular fluorescence of the parental wild type (WT) BT549 cells is shown in red trace. Representative immunofluorescence examples of the targeting of GFP (green) to MitotrackerRed stained mitochondria (red) in BT549:p β GFP-3 β (B) and BT549:p β GFP-3 α (C) cells.

at position 1 (7mer-A1) and an A or U at position 9 of the miRNA improves miRNA activity [33]. In addition, miRNAs that are conserved among different species are preferentially selected. Following these binding rules and with the purpose of covering the entire length of the 3'UTR of β -mRNA we selected miR-101, miR-127-5p, miR-186, miR-200b, miR-423-5p and miR-581 as the strongest candidates from the *in silico* results (Fig. 1A). miR-103 was also included in the study because of the putative relevance that hypoxia [34] might have in regulating β -F1-ATPase expression, even though miR-103 does not fit to the above mentioned rules.

3.2. miR-127-5p is a potential regulator of β -F1-ATPase expression

For screening purposes, and in order to analyze the influence of miRNAs on the expression of β -F1-ATPase in breast cancer cells, we generated BT549 cell lines that stably express a mitochondrial targeted GFP reporter bearing the 3'UTR of β -mRNA (Fig. 1B). The pre-sequence of β -F1-ATPase (p β) was fused in frame upstream of the GFP coding region to localize the reporter protein into mitochondria [27] because this *cis*-acting element of β -mRNA is also required for the appropriate subcellular localization of the mRNA [6]. To assess the specificity of the action of the selected miRNAs on the 3'UTR of β -mRNA we also developed BT549 cell lines stably expressing the same reporter but containing the 3'UTR of α -F1-ATPase mRNA (α -mRNA) (Fig. 1C) which is a counterpart subunit of β -F1-ATPase in the mitochondrial H^+ -ATP synthase [1]. After generation of the stable cell lines both the p β GFP-3 α and p β GFP-3 β expressing cells were analyzed by flow cytometry to analyze the purity of the clones as

well as their GFP fluorescence intensity. The analysis showed that the selected p β GFP-3 β (β 2 and β 10 in Fig. 1B) and p β GFP-3 α (α 2 and α 4 in Fig. 1C) clones exhibited a well-defined and single peak of high-intensity GFP fluorescence ($> 10^3$ a.u.), indicating a very high degree of purity ($> 98\%$). The red traces in Fig. 1B and C show the background of the fluorescence signal of wild type cells (10^0 – 10^1 a.u.). Therefore, shifts in the fluorescence intensity of the generated clones can be taken as indicative of interfering GFP expression by the effect of the transfected miRNA precursor molecules (pre-miRNAs), which are designed to directly enter the miRNA processing pathway and mimic endogenous miRNAs in the cells. Moreover, all the clones targeted GFP to mitochondria as revealed by the co-localization of GFP fluorescence with Mitotracker Red by confocal microscopy (Fig. 1B and C).

In order to identify which of the selected miRNAs might be relevant in controlling β -F1-ATPase expression we first carried out miRNA over-expression experiments using miRNA precursors (pre-miRNA) in the GFP-tagged cells. The relative activity of the miRNAs targeting the 3'UTR of β -mRNA was assessed by determining the relative GFP fluorescence intensity of p β GFP-3 β clones when compared to that of p β GFP-3 α clones (Fig. 2A). The study revealed that from all the miRNAs analyzed only miR-127-5p triggered a significant decrease in GFP fluorescence in both p β GFP-3 β clones (β 2 and β 10 in Fig. 2A) when compared to p β GFP-3 α clones (α 4 in Fig. 2A) and cells transfected with a non-relevant miRNA (control in Fig. 2A). Western blot analysis of p β GFP-3 β 2 cells confirmed a reduced expression of the GFP reporter when cells were treated with miR-127-5p (Fig. 2B). Notably, silencing experiments using the corresponding anti-miRNAs, including anti-

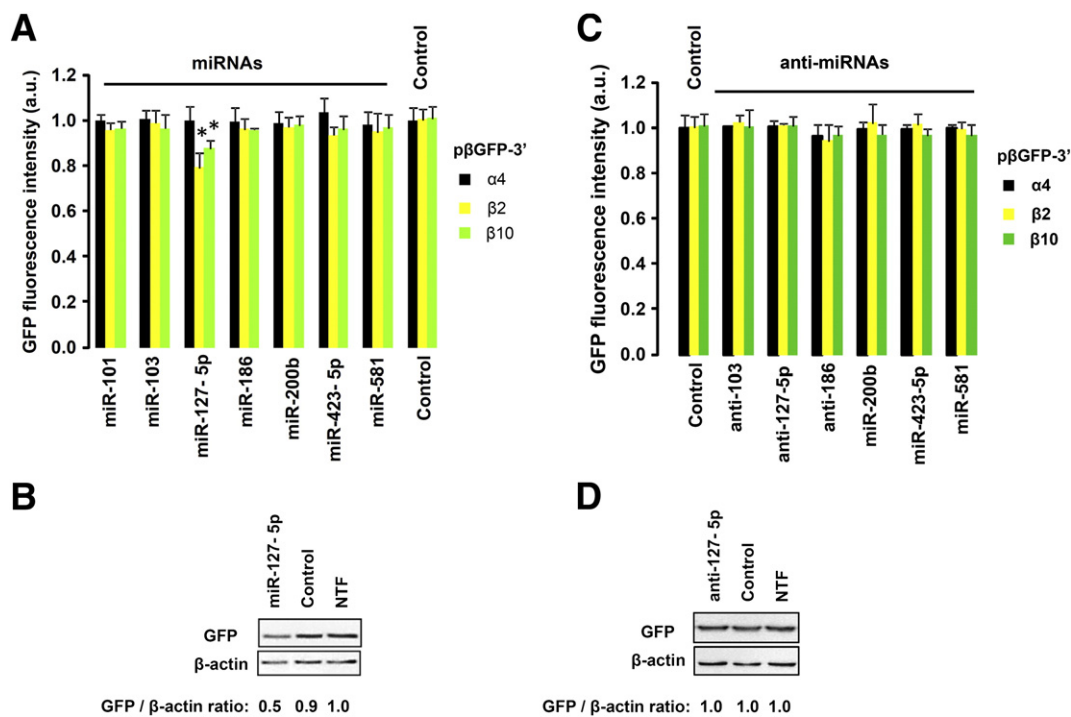


Fig. 2. Fluorescence reporter assays identify miR-127-5p as a potential regulator of β -F1-ATPase expression. **A**, The GFP fluorescence intensity of two BT549 cell clones ($\beta 2$ in yellow and $\beta 10$ in green) expressing p β GFP-3' β was determined after 48 h of transfection with the indicated miRNAs precursors using a fluorescence luminometer. Data were normalized relative to the number of cells as determined by Hoechst fluorescence intensity and quantified relative to the mean GFP fluorescence of control cells transfected with a non-relevant miRNA (control). The fluorescence intensity of BT549 cells expressing p β GFP-3' α ($\alpha 4$ in black) was also analyzed as an additional control of the screening. The results shown are the means \pm SEM of three independent experiments. *, $p < 0.0001$ when compared to p β GFP-3' α by Student's t-test. **B**, Western blot analysis of GFP expression in total cell lysates of BT549:p β GFP-3' $\beta 2$ cells non-transfected (NTF) or transfected with pre-miR-127-5p (miR-127-5p) or a scramble miRNA (control). The GFP/ β -actin ratio is indicated. **C**, Fluorescence reporter assays using anti-miRNAs. The GFP fluorescence intensity of two BT549 cell clones ($\beta 2$ in yellow and $\beta 10$ in green) expressing p β GFP-3' β was determined after 48 h of transfection. Data were normalized as indicated in **A**. The fluorescence intensity of BT549 cells expressing p β GFP-3' α ($\alpha 4$ in black) was also analyzed as an additional control of the screening. The results shown are the means \pm SEM of three independent experiments. **D**, Western blot analysis of GFP expression in total cell lysates of BT549:p β GFP-3' $\beta 2$ cells non-transfected (NTF) or transfected with anti-miR-127-5p or a scramble miRNA (control). The GFP/ β -actin ratio is indicated.

miR-127-5p, revealed that none of them had a relevant effect on the fluorescence intensity (Fig. 2C) and GFP expression (Fig. 2D) of p β GFP-3' β clones when compared to controls.

3.3. β -mRNA is targeted and silenced by miR-127-5p

Next, we questioned about the activity of miR-127-5p on the expression of the endogenous β -F1-ATPase. Transfection of miR-127-5p precursors in parental BT549 cells provoked a significant decrease in the expression of β -F1-ATPase protein (Fig. 3A, histogram to the left). In this situation, the cellular content of β -mRNA was not affected when compared to control cells that were transfected with a non relevant miRNA (Fig. 3A, histogram to the right). Consistent with the above mentioned findings (Fig. 2C and D) the transfection of BT549 cells with anti-miR-127-5p had no effect on β -F1-ATPase expression (Fig. 3B), suggesting that BT549 breast cancer cells do not express significant levels of miR-127-5p. The determination of miR-127-5p expression in BT549 and other cancer cell lines confirmed the lack of expression of this miRNA in these cells (see Fig. 5A), raising the question of what is the physiological context where miR-127-5p might be of relevance in controlling the expression of human β -F1-ATPase. Anyway, the negative effect of miR-127-5p on β -F1-ATPase expression was further underlined by the observation that the expression of β -F1-ATPase was restored to that of control cells when miR-127-5p expression was silenced by co-transfection with anti-miR-127-5p (Fig. 3B).

To verify that the down-regulation of β -F1-ATPase expression is due to the targeting of miR-127-5p to its presumed binding site on the 3'UTR of β -mRNA we generated a mutated version of the

p β GFP-3' β expression plasmid (p β GFP-3' β MUT) that contains four point mutations on the miR-127-5p binding region (Fig. 4A). Preliminary experiments indicated that the fluorescence intensity of p β GFP-3' β MUT expressing cells was not significantly affected by the co-expression of miR-127-5p (1.00 versus 0.96 arbitrary units in the absence or presence of miR-127-5p, respectively). Analysis of GFP fluorescence in BT549 cells co-transfected with the miR-127-5p precursor and p β GFP-3' β or p β GFP-3' β MUT plasmids revealed that whereas miR-127-5p significantly inhibited GFP fluorescence derived from the p β GFP-3' β plasmid the expression of GFP derived from the mutated plasmid was not affected (Fig. 4A). These findings were further confirmed by western blot analysis (Fig. 4B), that illustrated a very sharp reduction of GFP expression derived from the p β GFP-3' β plasmid whereas GFP expression was not affected from the p β GFP-3' β MUT expressing cells. It should be noted that over-expression of miR-127-5p promoted in both p β GFP-3' β and p β GFP-3' β MUT expressing cells a significant reduction of the endogenous β -F1-ATPase (Fig. 4B). Taken together, these results illustrate that miR-127-5p targets the 3'UTR of human β -mRNA and interferes with translation of the transcript.

It appears that western blots are more sensitive than fluorescence analysis to illustrate the activity of a particular miRNA (compare differences in GFP expression between Fig. 4B and A). Since this situation raised the possibility that other of the miRNAs studied in this work (Fig. 2A) could have some effect on β -F1-ATPase expression, presumably with less activity than miR-127-5p, we also verified the activity of the miRNAs by western blotting (Fig. 4C). The results obtained confirmed the data of the fluorescence study (Fig. 2A), i.e., only miR-127-5p has relevant activity as regulator of the expression of β -F1-ATPase (Fig. 4C).

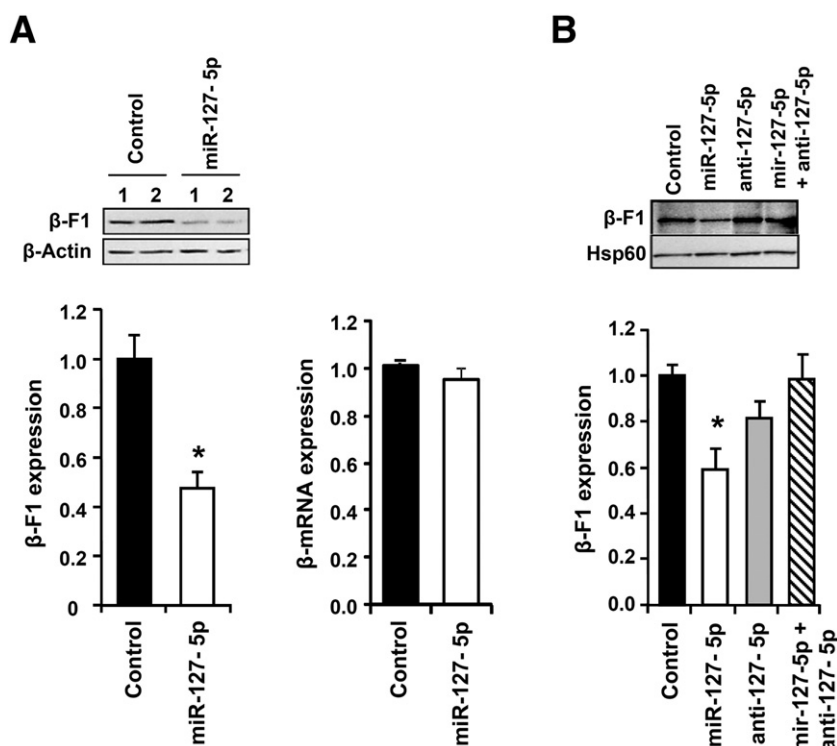


Fig. 3. miR-127-5p inhibits the translation of β -mRNA. **A**, Western blot analysis of the expression of β -F1-ATPase in total cell lysates (histogram to the right) and RT-qPCR quantification of β -mRNA (histogram to the left) of BT549 cells transfected with miR-127-5p precursor (miR-127-5p, open bars) or a scramble miRNA (control, closed bars). Lanes 1 and 2 correspond to two different experiments of the same experimental condition. The results shown are the mean \pm SEM of three independent experiments. The quantification of β -F1-ATPase expression is indicated using β -actin as reference protein. *, $p < 0.001$ when compared to control by Student's t-test. **B**, Analysis of β -F1-ATPase expression in BT549 cells transfected with miR-127-5p precursor (open bar), anti-miR-127-5p (gray bar), both miR-127-5p precursor + anti-miR-127-5p (hatched bar) or a negative control miRNA (control, closed bar). The quantification of β -F1-ATPase expression is indicated using Hsp60 as reference protein. The results shown are the means \pm SEM of four independent experiments. *, $p < 0.05$ when compared to control by Student's t-test.

3.4. miR-127-5p is expressed in human liver but not in cancer cells

To identify in which physiological context miR-127-5p could affect β -F1-ATPase expression we first analyzed by RT-qPCR its expression in several human cancer cell lines. The results obtained revealed that breast (BT549 and T47D), colon (HCT116), lung (Hop62), HeLa and Jurkat cancer cells do not express significant levels of miR-127-5p (Fig. 5A), most likely indicating that miR-127-5p does not play a role in silencing β -mRNA translation in these carcinomas. In contrast, human fetal liver samples expressed highly significant levels of miR-127-5p when compared to adult livers (Fig. 5A), suggesting that it might play a role in controlling β -F1-ATPase expression in the human liver during development. Interestingly, the expression of proteins involved in energy metabolism, such as subunits β -F1-ATPase and γ -F1-ATPase of the mitochondrial H^+ -ATP synthase and of subunit B of succinate dehydrogenase (SDH-B) is barely detectable in fetal liver extracts but show a remarkable increase in their expression in the adult liver (Fig. 5B). In contrast, it should be noted that the expression of the structural mitochondrial protein Hsp60 did not show significant differences between fetal and adult liver extracts (Fig. 5B). Therefore, the β -F1-ATPase/Hsp60 ratio increased significantly during development of the human liver (Fig. 5B), in agreement with previous results in rat liver that illustrated the rapid postnatal biogenesis and functional differentiation of mammalian mitochondria [7,35–37]. Consistent with the relevance of translational control for the biosynthesis of β -F1-ATPase observed in lower mammals [36], we noted that liver mRNA levels for both the β and γ subunits of the H^+ -ATP synthase as well as of SDH-B showed no relevant changes during development (Fig. 5C), i.e., the mRNAs are represented at the time of development when the amount of the proteins is very limited. This was not the situation for the liver content of Hsp60 where both mRNA (Fig. 5C) and protein (Fig. 5B) levels correlated.

3.5. β -F1-ATPase is not expressed at the plasma membrane of the hepatocytes

It has been reported that β -F1-ATPase is a high-density lipoprotein (HDL) receptor for apolipoprotein A-I localized on the plasma membrane of rat and human hepatocytes and HepG2 cells [38]. To find out the fraction that plasma membrane β -F1-ATPase might represent from the total cellular content of the protein we have studied the expression and localization of β -F1-ATPase in rat and human liver as well as in HepG2 cells using different experimental techniques and antibodies (Ab1, Ab2 and Ab3) (Fig. 6). Immunohistochemistry of paraffin-embedded human liver sections using a polyclonal antibody against β -F1-ATPase (Ab2) [29] revealed no immunoreactive material on the plasma membrane of the hepatocytes whereas a strong labeling of cytoplasmic organelles was observed (Fig. 6A). High-resolution immunoelectron microscopy of rat liver thin sections, using a different antibody (Ab3) [30], showed a strong gold decoration of mitochondria of the hepatocytes in the absence of plasma membrane recognition of any immunoreactive material (arrows in Fig. 6B). Double-immunofluorescence microscopy of non-permeabilized HepG2 cells using an antibody against β -catenin, a plasma membrane protein (green, Fig. 6C) and anti- β -F1-ATPase (Ab2) (red, Fig. 6C) showed the almost complete absence of β -F1-ATPase on the plasma membrane. The only red signals appeared decorating the mitochondria of the few cells (<10%) that are inevitably permeabilized as a result of the fixation procedure (arrow in Fig. 6C). Similar findings were obtained using a commercially available antibody against β -F1-ATPase (Ab1) (data not shown). As expected, both Ab2 (Fig. 6D) and Ab1 (data not shown) specifically recognized mitochondria in permeabilized HepG2 cells. Altogether, we show that various cellular fixation procedures and localization techniques using different antibodies against β -F1-ATPase only

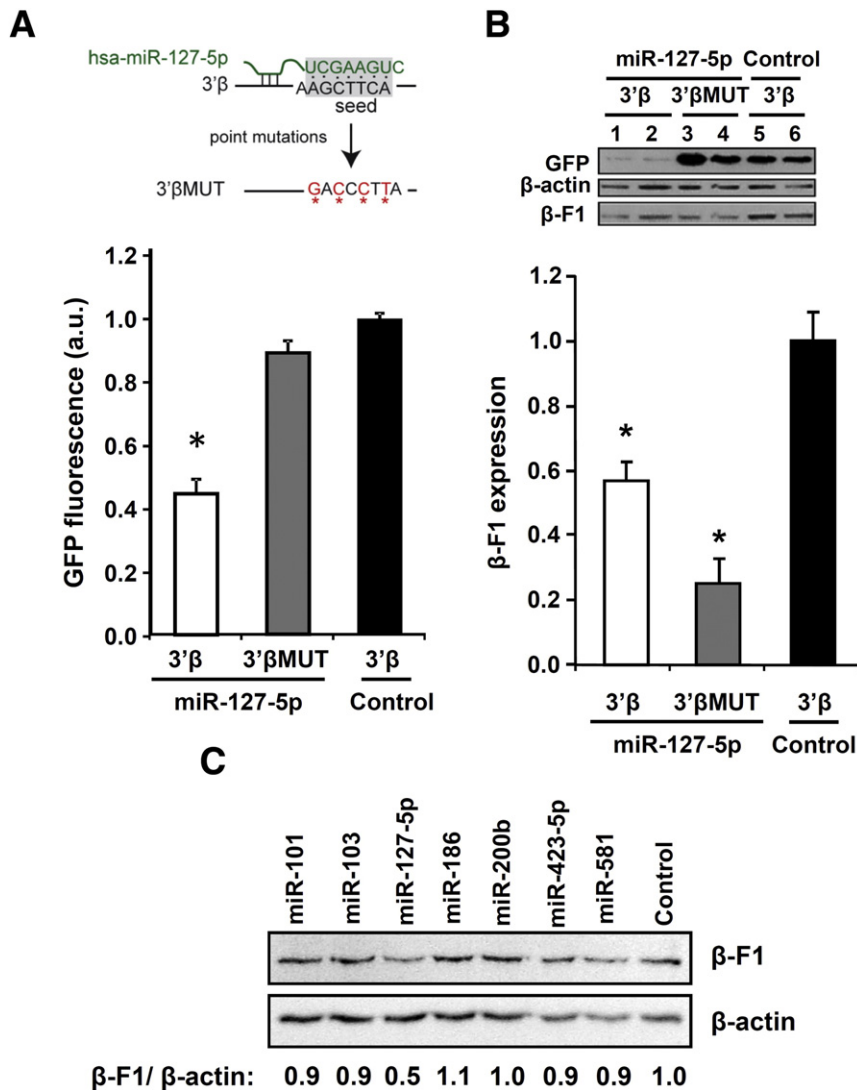


Fig. 4. miR-127-5p specifically targets the 3'UTR of human β -mRNA. **A**, Schematic illustration of the four point mutations (in red, asterisks) introduced in the 3'UTR of β -mRNA to generate the p β -GFP-3 β MUT plasmid (3 β MUT). On top, the miR-127-5p (green) seed matching sequence on the 3'UTR of β -mRNA is indicated (highlighted in gray). The histogram shows the fluorescence intensity of BT549 cells co-transfected with miR-127-5p or a negative control miRNA (control) and reporter plasmids expressing GFP fused either to the 3'UTR of β -mRNA (3'β) (open and closed bars) or to the mutant 3'UTR of β -mRNA (gray bar, 3'βMUT). The results shown are the mean \pm SEM of three independent experiments. *, $p < 0.001$ when compared to control by Student's *t*-test. **B**, Western blot analysis in total cell lysates of BT549 cells co-transfected with miR-127-5p precursor (lanes 1–4) or a negative control miRNA (control, closed bar) (lanes 5 and 6) and the reporter plasmids expressing GFP fused to the wild type 3'β (open bar) (lanes 1 and 2) and control (lanes 5 and 6) or mutant (3'βMUT, gray bar) (lanes 3 and 4) 3'UTR of β -mRNA. Two different experiments assayed in the same gel for each condition are shown. The lower histogram shows β -F1-ATPase (β -F1) expression relative to that of β -actin. The results shown are the mean \pm SEM of four experiments. *, $p < 0.005$ when compared to control by Student's *t*-test. Note the lack of effect of miR-127-5p on GFP expression derived from the 3'βMUT and the silencing of the endogenous β -F1-ATPase. **C**, Western blot analysis of β -F1-ATPase (β -F1) expression in BT549 cells transfected with the indicated miRNAs precursors or a scramble miRNA (control). The β -F1/ β -actin ratio is indicated.

recognized immunoreactive material in mitochondria supporting the marginal presence of β -F1-ATPase at the plasma membrane of mammalian liver cells.

Furthermore, rat liver homogenates were prepared and the mitochondria and plasma membrane fractions purified (Fig. 6E). Western blotting of proteins from these fractions using specific antibodies against mitochondria (Hsp60 and β -F1-ATPase) and plasma membrane (E-cadherin) markers, confirmed the enrichment of mitochondria (compare H versus Mi in Fig. 6E) and plasma membranes (compare H versus PM in Fig. 6E) in their respective fractions. Remarkably, none of the three antibodies used against β -F1-ATPase recognized a band in the plasma membrane fractions (Fig. 6E). Similar negative findings have been obtained using a monoclonal anti- β -F1-ATPase (data not shown) developed recently [39]. These results also support that there is no ectopic expression of β -F1-ATPase on the

cell surface of liver cells or, alternatively, that the content of β -F1-ATPase in the plasma membrane is negligible when considered the total cell content of the protein. Therefore, and for the rest of this work, we will not take into consideration the contribution that the plasma membrane protein could have in this study.

4. Discussion

The control of β -F1-ATPase expression is essential for normal cell function. In fact, down-regulation of its expression is linked to cancer progression [10,29,40] or to the onset of a small group of devastating rare diseases [26]. In the present study we have investigated the role that miRNAs could play in controlling β -F1-ATPase expression in humans and the likely physiological context where they might exert their function. This is part of an ongoing project aimed at the

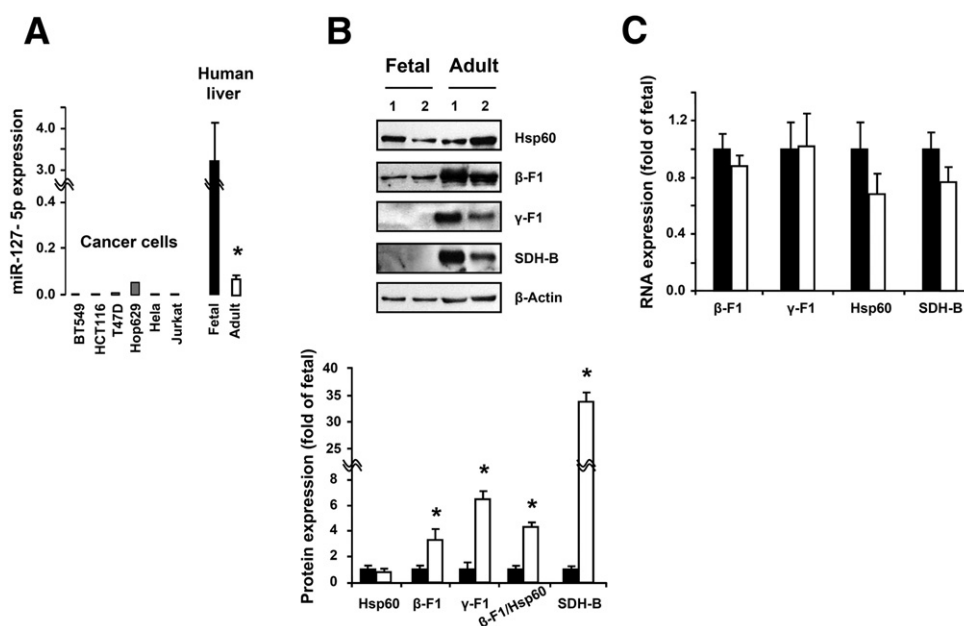


Fig. 5. miR-127-5p is expressed in fetal human liver. **A**, Quantification of miR-127-5p expression by RT-qPCR in human cancer cell lines (BT549, T47D, HCT116, Hop62, Jurkat and HeLa) and in fetal and adult human liver. miR-127-5p expression in the cell lines and during liver development was normalized to RNU48 and U6 RNA, respectively. The results shown are the mean \pm SEM of three experiments. *, $p < 0.005$ when compared to fetal by Student's *t*-test. **B**, Western blot analysis of the expression of the mitochondrial proteins Hsp60, β -F1-ATPase (β -F1), γ -F1-ATPase (γ -F1) and subunit B of succinate dehydrogenase (SDH-B) in human fetal and adult liver extracts. The expression of β -actin was used as reference of protein loading. Lanes 1 and 2 under the same heading correspond to two different fetal or adult liver extracts. The histogram summarizes the relative expression level of each protein in adult liver extracts (open bars) relative to the level observed in fetal extracts (closed bars). The β -F1-ATPase/Hsp60 ratio is also shown. The results shown are the mean \pm SEM of three different extracts. *, $p < 0.01$ when compared to fetal by Student's *t*-test. **C**, RT-qPCR analysis of β -F1-ATPase mRNA, γ -F1-ATPase mRNA, SDH-B mRNA and Hsp60 mRNA in fetal (closed bars) and adult (open bars) human liver samples. Data were normalized relative to 18S rRNA and are shown as fold change relative to the values in fetal liver. The results shown are the mean \pm SEM of three different samples.

characterization of the post-transcriptional mechanisms involved in the regulation of β -F1-ATPase expression in human pathophysiology [4,10,11]. Initially, we intended to characterize the mechanism of repression of β -F1-ATPase expression in breast cancer [9]. Therefore, the BT549 breast cancer cell line was selected for developing the GFP-3' β and GFP-3' α clones because it is a cell line that shows an intermediate expression level of β -F1-ATPase when compared to other breast cancer cells of the NCI panel (T47D, MDA-N > BT5490 = HS578T > MCF7, ADR-RES, MDA-231, MDA-435). Of the seven miRNAs studied we found that only miR-127-5p functionally interferes with β -F1-ATPase expression. Moreover, it was further found that this miRNA is not expressed in many cancer cells. This unexpected finding promoted the search for other physiological contexts where miR-127-5p might play a role in regulating the translation of β -mRNA and found its likely participation in development of the liver. A recent report identified miR-101 as a post-transcriptional regulator of β -F1-ATPase expression in HeLa cells [41]. Our fluorescence and western blot data in BT549 cells does not support such a role for miR-101 perhaps due to differences in the cells studied. In general, miRNAs silence gene expression by induction of mRNA degradation or by inhibition of its translation [22,42–44]. We illustrate that expression of miR-127-5p brings about a profound decrease in the steady state level of the endogenous β -F1-ATPase without affecting β -mRNA levels. Moreover, we show that miR-127-5p specifically targets the 3'UTR of the transcript. These results indicate that the mechanism of action of miR-127-5p on β -F1-ATPase expression is exerted by repressing β -mRNA translation.

In the mouse miR-127 is expressed in embryos, placenta and post-natal brain and muscle [45] as well as in fetal lung [46]. It is expressed as a part of a cluster with miR-136, miR-431, miR-432, and miR-433 both in normal tissues and cultured fibroblasts of mouse and humans [45,47]. The expression of the cluster and specifically of miR-127 is down-regulated in primary tumors and various cancer cell lines [47]. Moreover, the miR-127 promoter is embedded in a CpG island and

remains methylated in most tissues except sperm [47]. Treatment of cancer cells with chromatin-modifying drugs promoted the up-regulation of miR-127 which targets the mRNA of the protooncogene BCL6 to inhibit its expression [47]. Consistent with these findings, miR-127 is over-expressed in diffuse large B-cell lymphomas [48] and Epstein-Bar virus (EBV) positive Burkitt lymphomas [49], supporting that miR-127 acts as a potential tumor suppressor by blocking terminal differentiation of B-cells and hence favoring their proliferation [47–49]. A role for miR-127 has been suggested in the control of cellular differentiation because it is preferentially expressed in the mesenchymal rather than in the epithelial compartment of the lung towards the end of fetal stages of development [46]. In fact, over-expression of miR-127 in fetal lung cultures impedes normal lung differentiation [46]. Consistent with these findings we report that miR-127-5p is highly over-expressed in the fetal liver. Hence, we suggest that miR-127-5p does not play a role in controlling β -F1-ATPase expression in oncogenesis and thus in promoting the down-regulation of the protein that accompanies the bioenergetic switch during cellular transformation [10,40] but rather it represses the differentiation of mitochondria during fetal liver development [35].

Cancer cells and embryonic tissues display large phenotypic similarities regarding the molecular and functional activities of mitochondria [37,50]. In fact, fetal hepatocytes and hepatocarcinomas are highly glycolytic cells because both the number and the bioenergetic activity of their mitochondria are very low [35,37,50]. Consistent with the observations in lower mammals recent reports demonstrated that the activity of oxidative phosphorylation complexes increases significantly in liver, brain, muscle and heart of humans after birth [51,52]. The analogies also affect the mechanisms that control the expression of genes of the H^+ -ATP synthase because β -mRNA is accumulated in a translation repressed state in fetal rat liver [2,7,36], in rat hepatocarcinomas [4] and in human lung, colon and breast carcinomas [9]. Accumulation of β -mRNA in developing liver and in rat

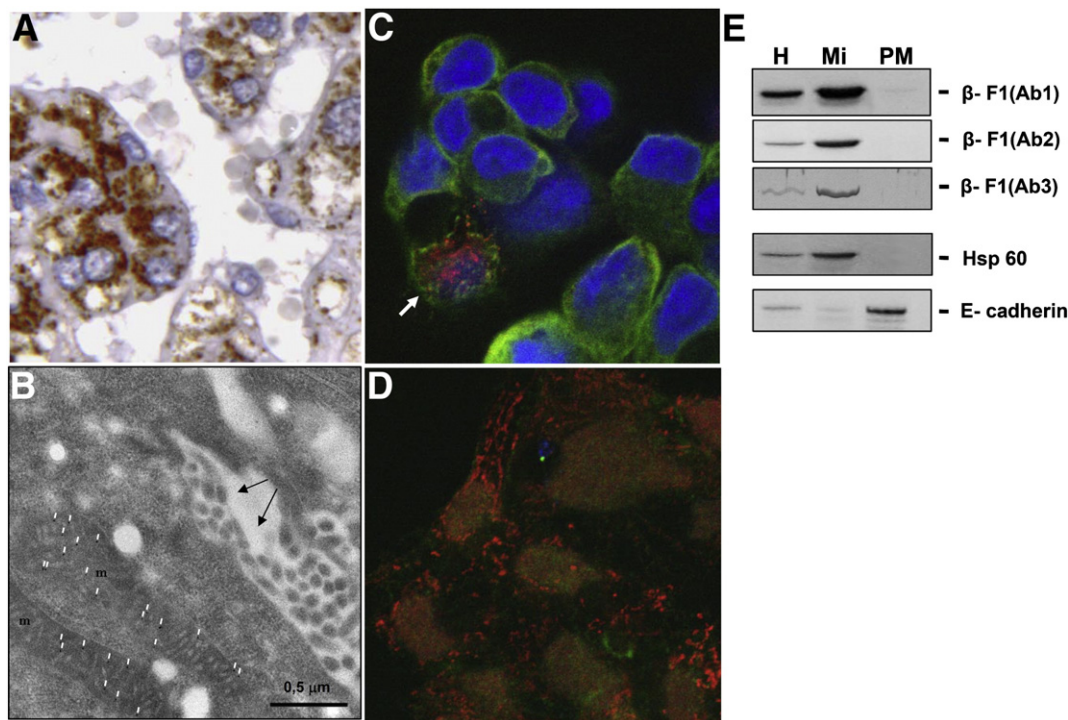


Fig. 6. No evidence for the localization of β-F1-ATPase at the plasma membrane. **A**, Immunohistochemical characterization of the expression of β-F1-ATPase in human liver sections using a polyclonal anti-β-F1-ATPase (Ab2) only reveals the mitochondrial localization of the protein. **B**, High-resolution immunoelectron microscopy of rat liver ultrathin sections processed with a polyclonal anti-β-F1-ATPase (Ab3) reveals the specific labeling (10 nm gold, white stripes) of mitochondria (m) in the absence of gold decoration of the plasma membrane (arrows) of the hepatocyte. **C**, Double immunofluorescence microscopy of non-permeabilized HepG2 cells with anti-β-catenin (green) and anti-β-F1-ATPase (Ab2) (red) reveals non-relevant immunolabeling of β-F1-ATPase on the plasma membrane. The only red signals observed (β-F1-ATPase) are confined to cytoplasmic organelles in few partially permeabilized cells as a result of the fixation procedure (arrow). **D**, Double immunofluorescence microscopy of permeabilized HepG2 cells reveals the almost complete absence of β-catenin signal (green). The strong red signals of the β-F1-ATPase antibody (Ab2) are confined to cytoplasmic organelles. **E**, Western blotting of rat liver postnuclear supernatants (H), purified mitochondria (Mi) and plasma membrane (PM) proteins using three different antibodies against the β-subunit of the H⁺-ATP synthase (Ab1, Ab2 and Ab3). Probing of the membranes with anti-hsp 60 and anti-E-cadherin, markers of mitochondria and plasma membrane, respectively, confirmed the enrichment of mitochondria and plasma membranes in their respective fractions. The blots show no immunoreactivity against β-F1-ATPase at the PM fraction.

carcinomas is accounted for by controlling the rate of turnover of the transcript [4,36]. The storage of masked β-mRNA in the fetal liver and its rapid translational activation immediately after birth [2,7,36] is required to promote the functional differentiation of liver mitochondria [35] allowing adaptation of mammals to the aerobic environment. In agreement with these findings our study illustrates that subunits β and γ of the H⁺-ATP synthase are hardly expressed in human fetal liver boosting their expression in the adult liver. Paradoxically, and consistent with findings in rat liver [36], we observed no differences in steady state β-mRNA levels in the human liver during development strongly suggesting that the low content of β-F1-ATPase in humans also results from translation silencing of the corresponding mRNA. Similar paradoxical findings were obtained for the counterpart γ-F1-ATPase subunit. The mechanisms that control the translation of β-mRNA are very complex [10] and, in addition to miR-127-5p, β-F1-ATPase expression is also regulated by specific RNA-binding proteins such as G3BP1 that also targets the 3'UTR of the transcript [11]. In this scenario, we suggest that protein- and RNA-mediated mechanisms that regulate the interaction of essential *cis*-acting elements of β-mRNA with the translational machinery are of utmost importance to unveil β-F1-ATPase expression in different physiological contexts [10]. Importantly, the β-F1-ATPase/Hsp60 ratio, which illustrates the energetic competence of mitochondria [29], showed a significant increase after birth. This finding indicates that human fetal liver mitochondria are deficient in the molecular components of the H⁺-ATP synthase and perhaps in mitochondrial functionality, resembling very much the findings obtained in lower mammals [37,50]. Within this context, miR-127-5p might be of physiological relevance

to regulate the attenuation of β-mRNA translation during development of the human fetal liver.

Remarkably, the promoter of human miR-127 gene can be activated by estrogen related receptor gamma (ERRγ) and alpha (ERRα) transcription factors [53]. Functional and biochemical studies have shown that ERRα is involved in energy metabolism and mitochondrial biogenesis [54]. Together with PGC-1α, ERRα is involved in the induction of the ATP5B gene that encodes β-F1-ATPase [55,56]. Thus, one can speculate that during fetal stages of human liver development ERRα simultaneously activates the transcription of β-F1-ATPase and of miR-127-5p, the repressor of β-mRNA translation. This could promote the accumulation of masked β-mRNA in the fetal liver ensuring a less functionality of the H⁺-ATP synthase to allow an active glycolysis which is the main pathway of the proliferating hepatocyte [40]. It is likely that the targeting of miR-127-5p to its presumed binding site in the 3'UTR of β-mRNA is an assisted process exerted in cooperation with the RNABPs that built the human β-RNP [11,21]. Post-translational modification of any of these proteins [21] by the cellular signaling events that occur in mammals after birth [57] might induce the release of miR-127-5p from β-mRNA to allow its effective translation and the functional differentiation of human liver mitochondria. Within this context, miR-127-5p could function in human liver development in a similar way to that of miR-378* in cancer, which has been shown to be an important regulator of the bioenergetic switch experienced in breast cancer for adjusting the cell to a changing metabolic demand [58].

Taken together, our results indicate that translational control of β-mRNA is also operative during human liver development. Since

miR-127-5p is highly expressed in the fetal human liver and targets the 3'UTR of β -mRNA repressing its translation we suggest that it offers a potential candidate for controlling the expression of β -F1-ATPase during development and therefore in regulating the bioenergetic differentiation of human liver mitochondria.

5. Conclusion

miR-127-5p targets the 3'UTR of β -F1-ATPase mRNA (β -mRNA) and inhibits its translation without affecting β -mRNA abundance. The expression of β -F1-ATPase is controlled at the level of translation during development of the human liver. Since miR-127-5p is highly expressed in the fetal human liver we suggest that it might be involved in defining the bioenergetic activity of mitochondria in the hepatocyte during development.

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