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Original Paper

Human Antigen R (HuR) Facilitates miR-19 Synthesis and Affects Cellular Kinetics in Papillary Thyroid Cancer

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Key Words
HuR • ELAVL1 • *miR-17-92* cluster • Splicing • Cancer

Abstract
Background/Aims: Pre-mRNA splicing is an essential step in eukaryotic gene expression regulation. Genes are composed of exons that remain in the mature mRNAs and intervening sequences named introns. Splicing is the removal of introns and ligation of exons in a mature transcript. Splice site or spliceosome component mutations can lead to different diseases, including neurodegenerative diseases and several cancer types. HuR is an RNA-binding protein that preferentially binds to U- and AU-rich elements, usually found at the 3′ UTRs of some mRNAs. We previously observed HuR specifically associated with spliceosomes assembled on introns containing *miR-18a* and *miR-19a*. *miR-18a* and *miR-19a* are components of the intronic *miR-17-92* cluster, along with other five miRNAs. This cluster has been reported to regulate proliferation, migration, and angiogenesis in cells. In this context, we reasoned HuR could be controlling the splicing and processing of these miRNAs, leading to altered cellular phenotypes. ***Methods:*** We induced HuR overexpression in BCPAP and HEK-293T and analyzed the expression of miRNAs using qPCR, as well as the phenotypic effects in those cells. Cell counting to analyze cell growth was performed after trypan blue staining. Migration and invasion assays were performed using transwell filters and cells were counted after staining with crystal violet. We knocked down HuR using a specific siRNA and analyzed expression of miRNAs by qPCR, as well as cellular kinetics. ***Results:*** Our results revealed HuR is associated with *miR-19a* in BCPAP and HEK-293T cells. Conversely, silencing HuR led to reduced *miR-17-5p* and *miR-19a* in BCPAP cells. Our data support that HuR stimulates the expression of *miR-19*, which is further processed and capable of finding its target sequence in a reporter plasmid. Cells overexpressing HuR showed increased cellular proliferation, migration, and invasion rates. Notably, under the presence of antimiR-19a, BCPAP-HuR cells showed reduced cell growth. Taken together, these results indicate the molecular alterations observed are associated with upregulation of *miR-19a*, leading to cellular processes involved in cancer development.

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Conclusion: Our findings propose a connection between HuR, miRNA biogenesis and cellular modifications. HuR stimulates *miR-19a* and *miR-19b* expression, which leads to up-regulation of cell proliferation, migration and invasion, promoting cancer development.

Introduction

Human genes are composed of exons and intervening sequences called introns removed during nuclear processing steps. Pre-mRNA splicing is an essential step in eukaryotic gene expression and consists of removing introns and joining exons, resulting in mature mRNAs transcripts [1]. Splicing regulation is dependent on transcript sequences and regulatory proteins recruited by the spliceosome. Interaction between regulatory sequences found across the pre-mRNAs and regulatory proteins defines splicing fate. Additionally, mutations in spliceosome components and splice sites are among the most significant causes of cancer development [2]. More than 70% of the miRNAs are transcribed from introns in the human genome [3, 4]. MicroRNAs (miRNAs) are small non-coding RNAs (20–25 nucleotides) that directly affect gene expression through mediating transcript stability in eukaryotes. miRNAs were described as part of the oncogenic suppressor's network on tumorigenesis [5, 6]. The oncogenic miRNA cluster *miRNA-17-92* is transcribed from intron 3 of MIR17HG gene, located on chromosome 13q31. Seven miRNAs are transcribed as a polycistron, *miR17-5p, miR17-3p, miR-18a, miR-19a, miR-20a, miR-19b*, and *miR-92a-1*. Changes in these miRNAs' expression patterns have been associated with the development of leukemia, ovarian, lung, and thyroid cancer [7, 8].

The miRNAs transcribed from the *miR-17-92* cluster miRNAs can perform distinct functions. For example, *miR-19a* and *miR-19b* are responsible for the oncogenic and anti-apoptotic effects observed in many lymphomas [8]. Despite being transcribed as a single polycistron, these miRNAs' processing, maturation, and function are independent [9, 10]. Additionally, individual miRNAs might have variable oncogenic features in different cell types [11, 12].

In papillary thyroid cancer, activation of the mutated BRAFV600E oncogene results in the deregulation of *miR-17-92* expression [7]. Among the different targets predicted for *miR-17-92* cluster are the receiver (TGFBR1) and the transducer mRNAs (SMAD2, SMAD3 and SMAD4) present in the TGFβ signaling pathway, an essential track in thyroid cell mitogen activation [13-15]. Regulation of transcription of this cluster might also be related to c-MYC and E2F factors [16]. Indeed, the silencing of Pim-1 and E2F3, controlled by c-MYC and E2F, respectively, can regulate the transcription of this cluster [16, 17]. The relative expression of individual miRNAs from this cluster is important for disease development, yet the mechanism that controls *miR-17-92* biogenesis remains unknown. We previously found Human antigen R (HuR) in spliceosomes assembled from introns containing *miR-18* and *miR-19a* [18]. Although it is not an integral component of the spliceosome, HuR is associated with splicing proteins and the RNA interference machinery, such as the Argonaute proteins [19]. It is also engaged in miRNA processing steps [20].

HuR, also known as embryonic lethal abnormal vision-like 1 (ELAVL1), is a ubiquitously expressed RNA-binding protein (RBP) composed of three RNA recognition motifs (RRMs) [21, 22]. Approximately 90% of endogenous HuR is concentrated in the nucleus, where it has a role in RNA processing, especially in polyadenylation and splicing [21-24]. HuR is overexpressed in many cancer types and has been implicated in regulating the cell cycle, tumorigenesis (cell proliferation, migration, and invasion), immunity, and angiogenesis [24-28]. Silencing HuR using RNAi reduced proliferation, migration, and invasion of ovarian tumor cells [29]. In addition, HuR has been related to the regulation of vascular endothelial growth factor A (VEGFA) expression and angiogenesis [30], also being associated with the inflammatory process in human macrophages [31]. Previous studies reported that HuR could either control the target sites on the 3'UTR of its own mRNA or compete with miRNAs to bind the 3'UTR of other targets [30, 32, 33].

We hypothesized that HuR could associate with miRNAs of this cluster and participate on the processing steps of this intron, therefore controlling miRNA biogenesis and maturation. As a consequence, it would be a key regulator of tumorigenesis processes triggered by these miRNAs. To address that, we investigated the biogenesis and maturation of these miRNAs in papillary thyroid cancer cell line (BCPAP) under altered expression of HuR. Importantly, we observed HuR associates and affects the expression of *miR-19a* and *miR-19b*. We also confirmed phenotypic alterations in cells over-expressing this protein, indicating a possible role in thyroid cancer development.

Materials and Methods

Cell culture

HeLa-Cre, HEK-293T, and papillary thyroid cancer (BCPAP) cell lines were maintained in DMEM/high-glucose (Thermo Fisher Scientific) supplemented with 10% FBS (HyClone), 1 mM sodium pyruvate (Life Technologies), L-glutamine, and 1X penicillin-streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin; Life Technologies) in 60 mm Petri dishes, unless otherwise indicated. Adherent cells were detached using 1X trypsin/EDTA (Life Technologies). Cells were cultured at 37 °C in a humidified, controlled atmosphere incubator (95% air, 5% CO2). According to the manufacturer's instructions, transfections were performed with Lipofectamine 2000 (Life Technologies). HeLa-Cre was kindly provided by E. Makeyev [34], and BCPAP was kindly provided by Massimo Santoro (University "Federico II", Naples, Italy). Transfection selection was performed by gradually increasing geneticin (G418, Sigma) concentration to 1000 µg/mL, generating stably transfected cells. Cells were maintained in geneticin at 200 µg/mL.

microRNA quantification (qRT-PCR) and TaqMan-Based PCR

Whole-cell extracts were prepared using buffer A (10 mM KCl, 1.5 mM MgCl2, 20 mM HEPES [pH 7.5], 0.5 mM DTT) and the Douncer homogenizer (Wheaton, NJ). RNA was extracted using Trizol reagent (Thermo Fisher Scientific) and precipitated with sodium acetate and ethanol. According to the manufacturer's instructions, this material was used for cDNA synthesis using Superscript IV RT enzyme (Life Technologies) and random primers. 100 ng of these cDNAs were used in real-time RT-PCR reactions (qRT-PCR) using SYBR Green reagent (Thermo Fisher Scientific) and specific primers for *miRNAs 17a, 18a, 19a* and *92a*, RNU6B, HuR, and β-actin (Supplementary Table S1 – for all supplementary material see www.cellphysiolbiochem.com). TaqMan analyses were performed using 200 nM of probes for *hsa-miR-19a, hsa-miR-19b*, *hsa-miR-18*, and *hsa-miR-423* (Thermo Fisher Scientific) (Supplementary Table S1). The probes for *hsa-miR-423* were used as endogenous control, as recommended by Thermo. TaqMan universal master mix (ThermoFisher Scientific) was used according to manufacturer's instructions, and ~100 ng DNA, in a total volume of 15 µl were run with the following cycling conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min. Expression of specific miRNAs were normalized with U6 small nuclear RNA (RNU6B) when using SYBR Green or *hsa-miR-423* when using the TaqMan probe system. The fold change calculation was performed using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method [35].

Immunoprecipitation

pFLAG-HuR was transfected into HEK-293T and BCPAP cells. As controls, empty pFLAG and untransfected cells were used. Cells were collected, and extracts were prepared as described above. These extracts were immunoprecipitated by incubation with protein A-Sepharose coupled to anti-FLAG M2 (Sigma) for 16 to 18 h at 4 °C. After incubation, the resin was extensively washed in RIPA buffer (20 mM Tris [pH 8.0], 150 mM of NaCl; 10% glycerol, 2 mM EDTA; 2 mM EGTA, 1% Triton X-100) and the proteins were eluted using 5 µg/µl FLAG peptide for 2 h under rotation. Input and elution fractions were subjected to RNA extraction and qRT-PCR analysis, as described.

Luciferase reporter assay

To develop the luciferase reporter assay, three plasmids were created. We inserted *pri-miR-17-92* sequence into the XhoI/EcoRI sites of the pRD-RIPE intron (kindly provided by Dr. E. Makeyev, Nanyang Technological University, Singapore; [34]), creating pRD-RIPE-1792. This plasmid has a tetracycline-

controlled promoter, which is reversibly turned on or off by the presence of the antibiotic doxycycline. The luciferase reporter plasmid and the "scramble" control were generated using pmir-GLO (Promega). miRbase and TargetScan predicted RAP-IB 3'UTR as a target of *miR-19a* and *miR-19b*. Full length (380 bp) and scrambled (318 bp) sequences of human RAP-IB mRNA 3'-UTR were subcloned downstream into pmir-GLO at XhoI/XbaI sites, generating pmirGLO-RAP-IB-3'-UTR (Luc-RAP-IB-3'-UTR) and pmirGLO-scrambled-3'-UTR (Luc-scrambled-3'-UTR) reporter constructs. The sequence and orientation of the luciferase reporter were verified by NotI cleavage and DNA sequencing. To perform the reporter assay, HeLa-Cre cells were co-transfected with pRD-RIPE-1792 (300 ng) and pCAGGS-Cre (Cre-encoding plasmid) (100 ng) (kindly provided by Dr. E. Makeyev, Nanyang Technological University, Singapore; [34]). Co-transfection was performed using confluent cells on a 24-well plate. These cells were incubated with a mixture containing 2.0 µg of DNA and 1.25 µL Lipofectamine 2000 in 100 µL Opti-MEM I (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. Cells were incubated with the transfection mixtures overnight, the medium was replaced for DMEM, and the incubation continued for another 24 h before adding puromycin. These cells were then used for the transfection of the luciferase reporter plasmid. This was performed with 50 ng/mL of each Luc-RAP-IB-3'-UTR and Luc-scrambled-3'-UTR, separately, using Lipofectamine 2000 as recommended (Life Technologies, Carlsbad, CA). The medium was replaced 6-8 h post-transfection to include the mixture of penicillin and streptomycin, and the incubation continued for another 20 h in DMEM. The two luciferases' activity was measured 48 h post-transfection using the Dual-Glo Luciferase Assay System (Promega).

Small Interfering RNA (siRNA) and antimiR-19a transfection

Silencer selected validated small interfering RNA (siRNA) for HuR (catalogue number 4390824) and negative control siRNA (catalogue number 4390843) were purchased from Life Technologies (Supplementary Table S1). To inhibit HuR expression, three different siRNA concentrations were transfected into BCPAP cells using Lipofectamine 2000 (2.5, 5, and 10 nM) (Life Technologies), following the manufacturer's protocol. Cells were collected after 24 h and subjected to protein preparation and RNA extraction. 25 pM of antimiR-19a (miRVana, Thermo catalogue number 4464084) and the negative control (mirVana, Thermo catalogue number 4464076) (Supplementary Table S1) were transfected into BCPAP-FLAG and BCPAP-HuR cells using Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. Cell growth after 24h and 72h post-transfection was evaluated using trypan blue.

Western Blot

Proteins were extracted using a buffer composed of 10 mM Tris-HCl pH 8, 140 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF and 1 mM DTT. Total protein concentrations were determined using the Bradford reagent (BioRad). Equal amounts of samples prepared from whole-cell extracts were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-HuR rabbit monoclonal (Cell Signaling) and anti-β-actin (Sigma). Following incubation with secondary antibodies IRDye 680/800CW-labeled rabbit or mouse (LI-COR Bioscience), blots were visualized using Odyssey CLx imaging system software (LI-COR Bioscience). Quantification was performed using Image J software and depicted after normalization of optic densitometry.

Cell growth, migration, and invasion

Cell growth was evaluated for 48 h with trypan blue staining. BCPAP cells at an initial concentration of 2.5 x 10^4/mL were cultured in 6-well plates (Corning) at 37 °C in 5% CO_2 for 48 h. After cell suspension, a 0.4% trypan blue solution (Sigma Aldrich) was added in a 1:1 ratio. After 3 min, cells were counted and separated into live cells (no cytoplasmic fluorescence) and dead cells (blue cytoplasmic fluorescence). The trypan blue-positive ratio from 10 random fields was quantified with ImageJ software. Cells were counted using the Countess II FL (Life Technologies). Migration and invasion assays were performed using 8.0 µm pore transwell membranes (Corning). Membranes were incubated with PBS for 1 h at 37 °C, 5 % CO_2 atmosphere for migration assays. For invasion assays, membranes were coated with 25 µg Matrigel® (BD Biosciences) and incubated for 1 h at 37 °C, 5% CO_2 atmosphere. For both assays, about 2.5 x 10^4/mL cells were suspended in a culture medium containing 1 % FBS and plated in the upper chamber, whereas the lower chamber had 10 % FBS supplemented media. Non-migrating cells were removed from the top chamber after 24h using a cotton swab. Migrating cells were fixed with 4 % paraformaldehyde (PFA) in

PBS and stained with 0.5 % Crystal Violet. Cells were photographed using a Nikon Eclipse E600 microscope equipped with optical camera CF160 epifluorescence, and ten representative fields were counted. Total protein concentrations were determined using the Bradford reagent.

Statistical analyses

Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism (GraphPad software, version 9, San Diego). Splicing reporter assay results and immunoprecipitation results were analyzed by two-way ANOVA followed by Tukey's post-test to allow group comparison. Results of qPCR and Taqman experiments were further analyzed using the Mann-Whitney post-test. Differences at *p*-values <0.05 were considered to be significant.

Results

HuR overexpression in BCPAP and HEK-293T cells

We previously observed HuR in spliceosomes assembled upon introns containing *miR-18a* and *miR-19a* [18]. To investigate whether HuR could modulate the expression of *miR-17-92* miRNAs in cancer cells, we induced HuR overexpression *in vitro* in two cell lines: human embryonic kidney (HEK-293T) and papillary thyroid cancer (BCPAP). BCPAP is a papillary thyroid cell line carrying the mutation BRAFV600E [7]. HEK-293T is derived from human embryonic kidney. Previous genomic analysis revealed HEK-293T has 7 copies of MIR17HG [36]. We reasoned that HuR regulatory roles on the expression of the miRNAs could be compared in these two cell lines. HuR overexpression in BCPAP and HEK-293T cells was confirmed by real-time PCR (Supplementary Fig. 1). We first analyzed *miR-17-92* individual levels after HuR overexpression. As a control, empty pFLAG was also transfected into these cells. BCPAP-HuR and HEK293T-HuR showed increased expression of *miR-19a*, suggesting this protein positively regulates *miR-19a* expression independently of the cell line. On the other hand, we observed reduced levels of *miR-92a* in both cell lines (Fig. 1). The evidence for the role of HuR on the regulation of these miRNAs is reinforced by the fact that multiple copies of this cluster are found in HEK-293T cells.

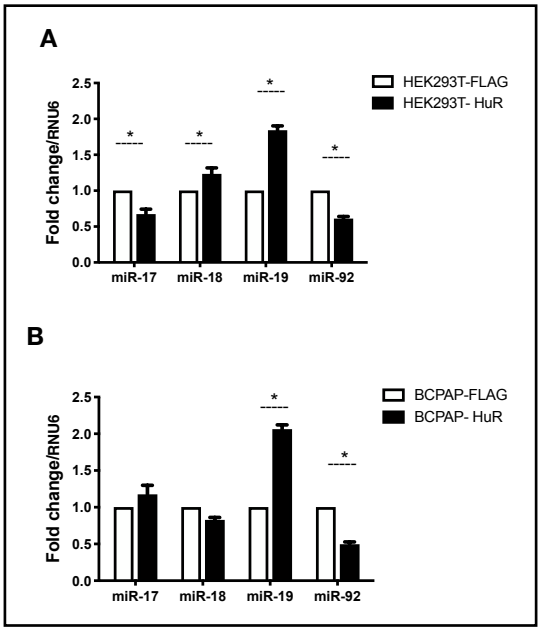

Fig. 1. Graphs representing qPCR analysis for expression of *miR-17-92* cluster miRNAs in (A) HEK293T-HuR and (B) BCPAP-HuR cells. Cells with FLAG epitope (white bars) were used as controls. The y-axis represents the fold change of expression calculated after normalization with RNU6B. Error bars represent standard deviations calculated from three independent measurements. **P<0.05.*

Our qPCR using HuR over-expressing cells and *in silico* analysis retrieved from miRbase and TargetScan databases using query search consistently revealed potential HuR binding sites along pre-*miR-17-92* sequence (GUUU, AUGA, NNUUNNUUU). Specifically, *pre-miR-19a* and *pre-miR-19b* sequences show conserved HuR binding sites (Fig. 2), indicating a possible interaction with these regions of the cluster. To investigate the association of HuR with this region, we performed immunoprecipitation using HuR over-expressing cells. Whole-cell extracts from BCPAP-HuR, HEK293T-HuR, and controls (cells expressing only the epitope FLAG) were subjected to anti-FLAG immunoprecipitation, and input and elution fractions were analyzed using qPCR. With three biological replicates, we observed that levels of *miR-19a* and *miR-92a* increased in BCPAP-HuR elution fractions relative to controls, indicating an association of those miRNAs and HuR (Fig. 3A). HuR associates with *miR-19a* in HEK293T-HuR as well (Fig. 3B). Specifically, HuR is also associated with *miR-18* in this cell line. With the use of Taqman probes, we also confirmed strong HuR association with *hsa-miR-19a* and *hsa-miR19b* in BCPAP (Fig. 3C) and in HEK-293T cells (Fig. 3D). Specific Taqman probes for *hsa-miR18* also confirmed association with HuR in HEK-293T but not in BCPAP cells (Fig. 3C and 3D).

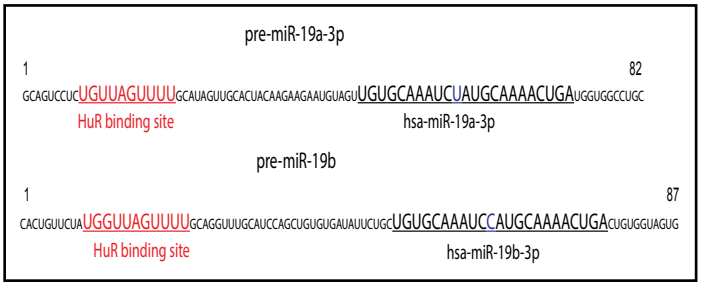

Fig. 2. *In silico* analysis of HuR binding sites on *pre-miR-19a* and *pre-miR-19b* sequences. HuR binding consensus sequences are marked on the pre-miRNA sequences (red). The mature sequences for *miR-19a* and *miR-19b* are underlined in black. miRbase and TargetScan databases were used to search for consensus sequences.

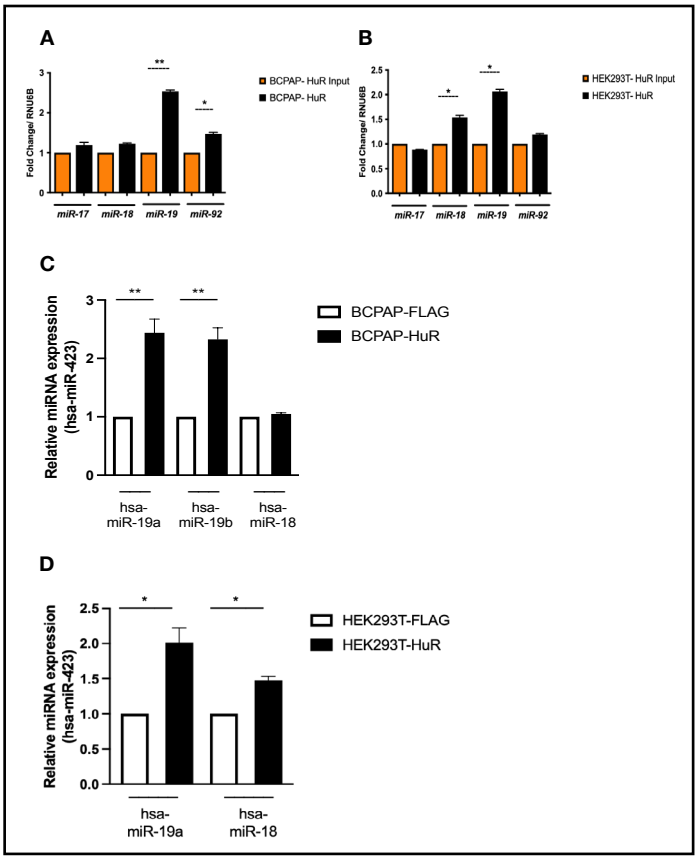

Fig. 3. Analysis of immunoprecipitated RNAs in BCPAP-HuR and HEK293T-HuR. Input and elution fractions from (A) BCPAP-HuR and (B) HEK293T-HuR were analyzed by qRT-PCR. Specific primers for *miR-17a*, *miR-18a*, *miR-19a*, and *miR-92a* designed for qPCR using SYBR-Green were used in these experiments. The snRNA U6 (RNU6B) was used as a normalizer and fold change was calculated using the input samples as controls. Taqman assays were performed using probes for *hsa-miR-19a*, *hsa-miR-19b* and *hsa-miR-18*. Elution fractions of (C) BCPAP-HuR (black bars), BCPAP-FLAG (white bars) and (D) HEK293T-HuR (black bars), HEK293T-FLAG (white bars) were analyzed after normalization with the use of probe for *hsa-miR-423*. Error bars represent standard deviations calculated from three independent measurements, **P<0.05 **P<0.005*.

Knockdown of HuR affects the synthesis of miR-19a and miR-17-5p

Considering HuR was associated with *miR-19a* and *miR-19b*, we asked whether its absence would also interfere with the expression of these miRNAs in BCPAP cells. To reduce the levels of HuR, we transfected BCPAP cells with siRNA against HuR (siHuR). Concentrations as low as 5 nM led to reduced HuR mRNA and protein, as confirmed by qPCR and western blot against HuR (Supplementary Fig. 2). We then analyzed the expression of miRNAs in BCPAP-siHuR cells by qRT-PCR. Our results indicated that *miR-17-5p* and *miR-19a* levels decreased significantly in BCPAP-siHuR cells (Fig. 4). *miR-18* and *miR-92* remained unchanged after using siHuR in BCPAP cells. Therefore, the reduction observed in *miR-19a* might be primarily due to the strong association of HuR with *miR-19a*, as observed with the IP assay (Fig. 3). Additionally, we also observe a reduction in *miR-17-5p* upon knockdown of HuR. Since HuR can bind next to *miR-17-5p* coding region, it is possible that its absence also impacts the processing of this miRNA. This result indicates that HuR not only associates with *miR-19a* but also regulates the expression *miR-19a* and *miR-17-5p*. Thus, it is possible that HuR binding to the pre-miRNA facilitates the processing of the miRNAs transcribed from the 5'-end of the cluster, such as *miR-17-5p, miR-18* and *miR-19a* [37].

HuR induces miR-19 expression and maturation

Our results indicated that HuR binds to and regulates the expression of *miR-19a* and *miR-19b* in BCPAP cells. The connection of HuR expression and *miR-17-92* cluster could point to a new mechanism governing the biogenesis of these miRNAs. To confirm that the induced miRNAs were truly functional, we designed an *in vitro* reporter system using a plasmid containing intronic *pri-miR-17-92*, whose expression is controlled by a tetracycline-inducible promoter (Fig. 5A) [34]. Ras-related protein RAP-IB is a GTPase member of the Ras-associated protein family (RAS). Bioinformatics analysis revealed that RAP-IB 3' UTR has target sequences for both *miR-19a* and *miR-19b*. Therefore, this sequence was inserted into pmiR-GLO to test for *miR-19a* and *miR-19b* activity. If the induced miRNA were correctly processed and functional in our assay, it would hybridize to the sequence cloned next to luciferase. Therefore, it would block luciferase translation, resulting in reduced luciferase activity. Under the absence of doxycycline, and therefore without activation of exogenous *miR-17-92* expression, we observed endogenous *miR-19a* and/or *miR-19b* successfully found the target, resulting in reduced luciferase expression and activity (Luc-RAP-IB-3'UTR). The addition of FLAG-HuR coupled to doxycycline supplementation further reduced luciferase activity, indicating that more *miR-19a* and *miR-19b* were synthesized and able to find their target in pmiR-GLO-RAP-IB (Fig. 5B, Luc-RD-17-92-HuR). Altogether, these results support that induction by HuR generated functional *miR-19a* and *miR-19b*.

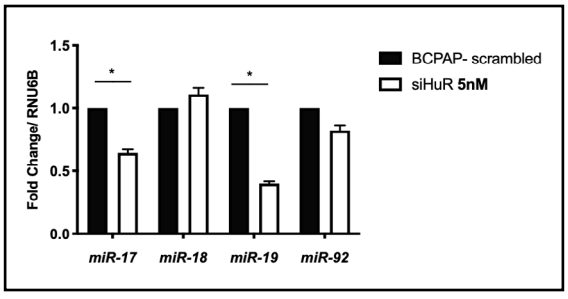

Fig. 4. *miR-17-92* levels were assessed by qRT-PCR after silencing HuR with 5 nM of siHuR (white bars). Transfection with "siRNA scrambled" was used as control (BCPAP-scrambled, black bars). The snRNA U6 was used as the normalizer (RNU6B). The y-axis represents the fold change of expression calculated after the normalization of Cts. **P<0.05.*

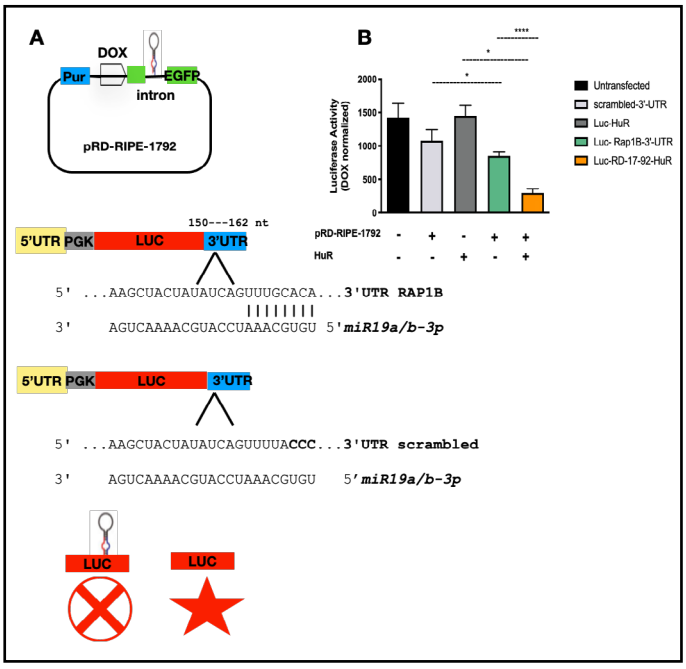

Fig. 5. Splicing reporter system. (A) Schematic representation of pRD-RIPE-1792, with the pri-miRNA-1792 inserted inside the intron and dox-inducible promoter; below, the pmirGLO plasmid with RAP-IB 3'UTR target sequence (Luc-RAP-IB-3'UTR) and the control with the scrambled target sequence (scrambled-3'UTR). (B) HeLa-Cre cells were transiently co-transfected with reporter plasmids Luc-RAP-1B-3'-UTR, scrambled-3'UTR, pRD-RIPE-1792 (Luc-RD-1792-HuR), and pFLAG-HuR (Luc-HuR). Luciferase activity was quantified as described in methods and normalized after doxycycline addition. Induction of pRD-RIPE-1792 or HuR is indicated below the graph. Luciferase activities were analyzed as the relative activity of firefly to Renilla luciferase. Data represent the mean ± SE of six biological replicates. **P<0.05 ****P <0.0005.*

HuR overexpression is associated with tumor progression

Our results indicated that HuR positively controls *miR-19a* and *miR-19b* biogenesis and processing, and these miRNAs were already shown as potent oncogenic miRNAs [8]. We then hypothesized that increased expression of HuR could affect cellular kinetics promoting tumorigenic effects. To evaluate that, we analyzed cell growth, migration and invasion in cells over-expressing HuR. By comparing the phenotypes of BCPAP-HuR and BCPAP-FLAG; and HEK293T-HuR and HEK293T-FLAG, we observed increased growth in BCPAP-HuR and HEK293T-HuR cells (Fig. 6A and 6B), suggesting HuR is stimulating cell growth. We reasoned that HuR was stimulating *miR-19a* expression, which was the responsible for the increased cell growth observed. To address that, we transfected BCPAP-FLAG and BCPAP-HuR cells with antimiR-19a or with a negative control antimiRNA. Following 24h of the transfection, we confirmed *miR-19a* inhibition by qRT-PCR (Supplementary Fig. 3). We then analyzed cell growth for another 48h and compared it with the growth of BCPAP-HuR cells. The results showed inhibition of *miR-19a* has a significant impact on growth of these cells, especially when compared to BCPAP-HuR cells (*P<0.0005*) (Fig. 6C).

Sustained continuous proliferation is one hallmark of cancer [38], and this characteristic indicates that HuR over-expression could affect other tumorigenic aspects. We then sought to investigate migration and invasion rates in cells overexpressing HuR. Transwell migration assays were performed with approximately 2.5 x 10^4 cells/mL after incubation of 24 h in regular culture media containing FBS. Our results showed that BCPAP cells overexpressing HuR migrated faster than control cells. In addition, the number of cells counted on the lower chamber was, on average, 50% higher than the number found in control cells (Fig. 7A).

Similarly, we performed this assay using a matrigel layer over the transwell membrane, simulating the extracellular matrix environment. The number of HuR overexpressing cells that could invade the lower chamber was twice the number found for control cells (Fig. 7B). This result indicated that HuR over-expressing cells also had increased invasive capacity. Altogether, these results suggested that HuR over-expression increases tumorigenic characteristics in thyroid papillary cancer cell line. Indeed, *miR-19a* and *miR-19b* stimulation by HuR over-expression might directly involve these observed effects during tumor progression.

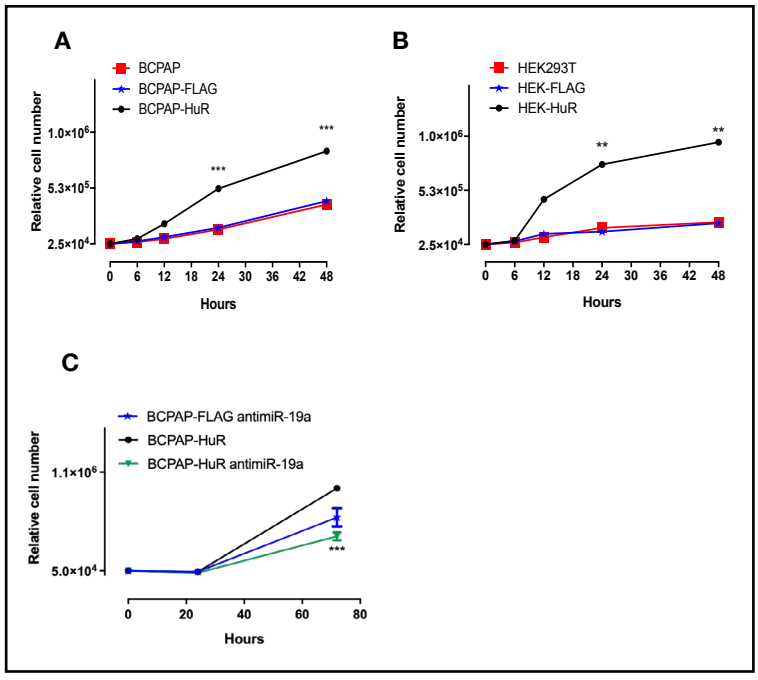

Fig. 6. Growth curves of (A) BCPAP and (B) HEK293T cells overexpressing HuR and the respective controls. Cells over-expressing HuR (black lines) and the controls untransfected cells (red lines) and cells transfected with empty pFLAG plasmid (blue lines) were analyzed for 48h using crystal violet staining. (C) Growth curves of BCPAP-FLAG and BCPAP-HuR after transfection with antimiR-19a. BCPAP-FLAG antimiR-19a (blue line) and BCPAP-HuR antimiR-19a (green line) were analyzed after 24h of transfection throughout 48h. BCPAP-HuR (black line) without anti-miR-19a was used as control. Three biological replicates were performed for each experiment, ***P<0.005; ***P<0.0005.*

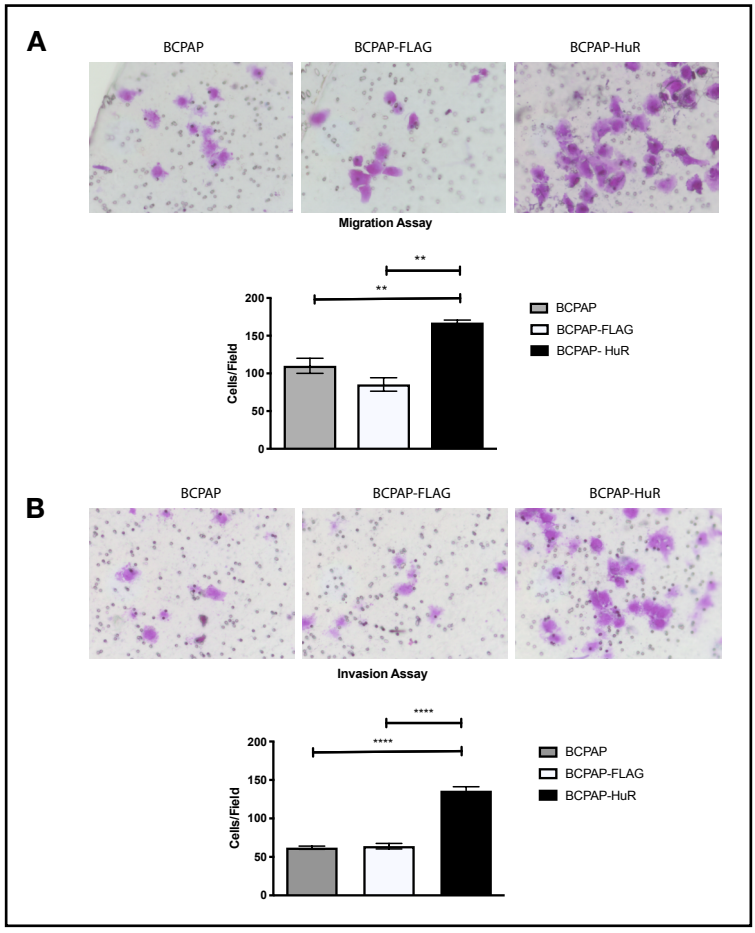

Fig. 7. HuR over-expression affects migration and invasion rates in BCPAP. Cells were fixed and stained with crystal violet and subsequently counted by optical microscopy after assays using the transwell. (A) Migration assays, the upper panel shows representative images of cells stained with crystal violet. The lower panel is a quantification of 10 fields of untransfected BCPAP (gray), BCPAP-FLAG (white), and BCPAP-HuR cells (black). (B) Invasion assays, the upper panel shows representative images of cells stained with crystal violet. Lower panel is a quantification of 10 fields of untransfected BCPAP (gray), BCPAP-FLAG (white), and BCPAP-HuR cells (black). Three biological replicates were performed for each experiment, ***P<0.005 ****P <0.0005.*

Discussion

The ubiquitously expressed HuR protein is an RNA-binding protein already associated with several post-transcriptional regulatory mechanisms [26, 39, 40]. HuR bears RRM motifs, which are important to bind AU- and U-rich sequences on RNAs mediating their stabilization. This protein was found especially upregulated in different cancers, including papillary, follicular, and anaplastic thyroid cancers [39, 41]. We previously observed HuR associated with spliceosomes assembled upon introns containing miRNAs in papillary thyroid cells (BCPAP) [18]. In this paper, we investigated HuR association with intronic miRNAs as well as the molecular and cellular effects of this interaction. Our results contribute to unveil the mechanisms mediated by HuR to cause the cellular transformations that might develop cancer.

Although HuR is not an integral component of spliceosomes, we hypothesized it could be associated with intronic miRNA processing and maturation, therefore affecting the rate of synthesis and expression of these molecules. We first observed BCPAP and HEK-293T cells with increased HuR expression also show increased levels of *miR-19a*. On the other hand, we also observed reduced levels of *miR-92a* in both cells. This expression variation can be due to pri-miRNA secondary structure, which might hide *miR-92a* from processing in both cell lines tested [37, 42]. It is also possible that HuR may regulate the expression of the miRNAs from this cluster in a cell-specific manner. It is known that *miR-19* expression promotes lymphoma, and the co-expression of *miR-92* suppresses this oncogenic activity in mice. The expression ratio between these two miRNAs appears to be dynamically regulated during the lymphoma progression [37, 42].

Next, we confirmed HuR associates to intronic *pre-miR-19a* and *pre-miR-19b* sequences and increased the expression of these miRNAs. Both BCPAP and HEK-293T cells over-expressing HuR showed increased expression of *miR-19a*, suggesting this protein positively regulates *miR-19a* expression independently of the cell line. Additionally, *miR-19a* and *miR-19b* associate with HuR in BCPAP cells. Importantly, by using a reporter system, we confirmed HuR induces expression and proper maturation of *miR-19a* and *miR-19b*. We interpret this result as a strong indicator that HuR is an important regulator of this miRNA cluster expression, specifically stimulating *miR-19a and miR-19b* expression. We then asked whether HuR reduction or absence would interfere with the expression of these miRNAs in BCPAP cells. Our results indicated that knockdown of HuR severely reduces *miR-19a*. Previous studies have shown that HuR is an essential protein for cell survival [43], possibly due to its association with proteins and miRNAs involved in cell cycle regulation and RNA stabilization. In fact, upon cellular perturbation, HuR translocates to the cytoplasm, targeting specific RNAs and with a primary role in RNA stabilization [27, 44]. It binds to the 3'UTR of angiogenic factors such as VEGF-A, COX-2, TGF-β, and TNF-α [45, 46], regulating their stability and affecting cell cycle control. Some cancer-associated stressors, including lipopolysaccharides, chemical compounds, cytokines, and ultraviolet radiation, increase HuR total expression [47, 48], which might lead to the altered expression of oncogenic miRNAs, as confirmed by our results. In bladder cancer, cytoplasmic HuR levels were directly proportional to an increased malignant potential, tumor progression, and poor prognosis [49]. The results observed after HuR knockdown suggest the molecular mechanism related to this phenotype might be associated with the regulation of *miR-19a/b* expression.

Considering the important effects of HuR on mediating *miR-19a* and *miR-19b* expression, we reasoned it could be involved with modifications on the cellular phenotypes. To evaluate that, we analyzed cell growth, migration and invasion in cells over-expressing HuR. Our results suggested that cells over-expressing HuR increase tumorigenic characteristics in the thyroid papillary cancer cell line. In order to investigate the role of *miR-19a* on the altered cell growth, we used antimiR-19a in BCPAP-HuR and BCPAP-FLAG cells. Growth of cells transfected with antimiR-19a was slower than non-transfected cells (BCPAP-HuR) and control transfected cells (BCPAP-FLAG antimiR19). These results support our initial

hypothesis, indicating HuR effect on *miR-19a* is directly responsible for altered cell growth, which can lead to tumorigenic features. Indeed, *miR-19a* and *miR-19b* stimulation by HuR are directly involved with these observed effects during tumor progression. These results are consistent with previous analyses, which showed that HuR silencing in ovarian cancer cells reduced the proliferative profile and invasive migration rates [29]. The same phenotype was observed in lung cancer development, in which knockdown of HuR inhibited migration and invasion [50]. In anaplastic thyroid cancer, HuR silencing increased apoptosis and reduced cell viability [41]. It has been reported that HuR also increases the migration of vascular smooth muscle cells and bone marrow-derived mesenchymal stem cells (BMSCs) [33, 40]. This process can lead tumor cells to invade adjacent tissues, leading to metastasis by growing in a distant organ. Translocation of HuR from the nucleus to the cytoplasm might stabilize target mRNAs essential to promote migration and invasion processes in some types of cancer [51]. The mechanism of action in this process may also be related to the activity of cell cycle regulators mediated by HuR, like VEGF-A in cell proliferation [52], COX-2 and PTEN in cell migration [53, 54], cyclin A in cell invasion [51] and PI3K/AKT/NF-κB or METTL3/ZMYM1/E-cadherin in anti-apoptotic capability [55, 56]. Cell cycle regulators such as PTEN, cyclin A2, cyclin D2, and TGFBR2 mRNAs have target sequences for *miR-19a* and *miR-19b*, suggesting that altered levels of these miRNAs might directly affect the kinetic processes regulated by these molecules. Additionally, HuR increases the stability of cyclin A, c-fos and cyclin E1 by binding to the 3'UTR of the respective mRNAs. HuR increases the mRNA stability of cyclin E1, leading to its overexpression in breast cancer [57, 58], and directly affecting cell proliferation.

HuR might also regulate the cell cycle by controlling HOTAIR long non-coding RNA expression. For example, in bladder cancer, this protein increases the expression of HOTAIR by directly binding to it [59]. On the other hand, knockdown of HuR or HOTAIR can inhibit bladder cancer cell migration, invasion, proliferation, and epithelial-mesenchymal transition (EMT), slowing down bladder cancer progression [59, 60]. Finally, HuR has been reported to bind directly to miRNAs, especially to *miR-21*, apparently acting as a miRNA sponge [61]. Consistent with all these roles, HuR has proved to be a molecular marker of cancer in various tumors [62-64].

Conclusion

Taken together, our results support a role for HuR in papillary thyroid cancer progression. Additionally, we propose that this modulation is related to increased *miR-19a* and *miR-19b* synthesis and maturation. We propose a model in which HuR overexpression triggers an interaction with the pre-miRNA and stimulates *miR-19a* and *miR-19b* biogenesis and maturation, directly affecting cell cycle regulatory pathways like proliferation, migration and invasion in cancer cells (Fig. 8). HuR knockdown led to decreased levels of these miRNAs. The inhibition of *miR-19a* resulted in slower growth of cells overexpressing HuR. Our findings improve the understanding of the functional regulatory role of HuR on *miR-17-92* cluster biogenesis. To conclude, we propose that HuR mediates cancer progression through the upregulation of HuR-targeted miRNAs *miR-19a* and *miR-19b*, which alter the aggressive oncogenic potential of cancer cells. We suggest that targeting *miR-19a/b* and/or HuR might be helpful to therapeutic strategies against tumorigenesis.

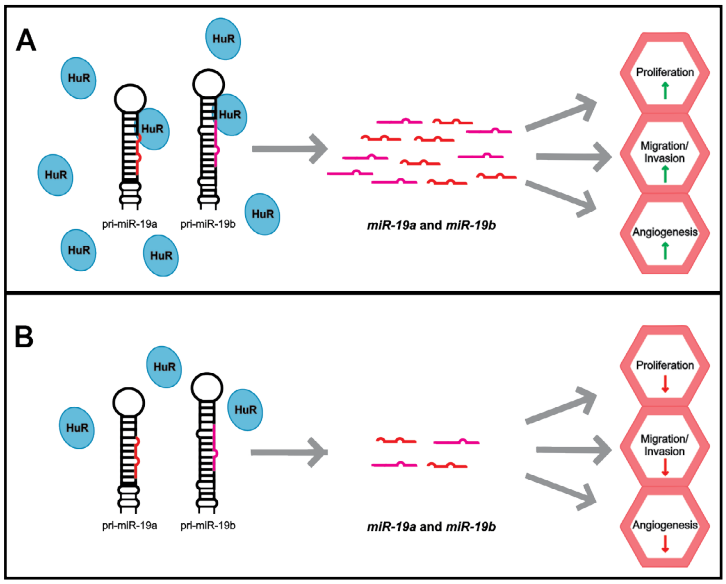

Fig. 8. Schematic diagram showing HuR protein interaction with pri-miR-19a and pri-miR-19b. Based on our results, we propose that (A) over-expression of HuR increases the expression of both *miR-19a* and *miR-19b*, which results in increased cell proliferation, migration, and invasion. (B) Conversely, reduced expression of HuR reduces cell proliferation and other tumoral characteristics, as shown on the right-hand side.

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Author Contributions

Conception of the work: G.H.G.S. and P.P.C.; Acquisition, analysis and interpretation of data: G.H.G.S., M.G.P.S., H.Y.N. and P.P.C.; Manuscript draft: G.H.G.S. and P.P.C.; Manuscript final revision: G.H.G.S., H. Y. N., M.G.P.S. and P.P.C.; Funding: P.P.C.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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