RNA-binding protein HuR and the members of miR-200 family play an unconventional role in the regulation of *c-Jun* mRNA

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ABSTRACT

Post-transcriptional gene regulation is a fundamental step for coordinating cellular response in a variety of processes. RNA-binding protein (RBPs) and microRNAs (miRNAs) are the most important factors responsible for this regulation. Here we report that different components of the miR-200 family are involved in *c-Jun* mRNA regulation with the opposite effect. While miR-200b inhibits *c-Jun* protein production, miR-200a tends to increase the JUN amount through a stabilization of its mRNA. This action is dependent on the presence of the RBP HuR that binds the 3′UTR of *c-Jun* mRNA in a region including the mir-200a binding site. The position of the binding site is fundamental; by mutating this site, we demonstrate that the effect is not micro-RNA specific. These results indicate that miR-200a triggers a microRNA-mediated stabilization of *c-Jun* mRNA, promoting the binding of HuR with *c-Jun* mRNA. This is the first example of a positive regulation exerted by a microRNA on an important oncogene in proliferating cells.

Keywords: miRNA; HuR; mRNA stability; *c-Jun*

INTRODUCTION

Post-transcriptional gene regulation is crucial to maintain a fine and coordinated production of proteins necessary to the cells in a variety of processes like development, homeostasis, and disease.

To achieve this result, post-transcriptional gene expression is controlled at multiple levels: pre-mRNA splicing and maturation, mRNA stability in the nucleus, mRNA transport, editing, mRNA stability in the cytoplasm, storage, and translation (Bousquet-Antonelli et al. 2000; Mitchell and Tollervey 2000; Orphanides and Reinberg 2000). mRNA turnover and translation are very well-suited steps of control to accomplish quick changes in the pattern of expressed proteins following environmental perturbations. The most important cellular factors that modulate RNA stability and translation are noncoding RNAs, in particular microRNAs (miRNAs), and turnover and translation regulatory RNA-binding proteins (TTR-RBPs). These factors associate with specific *cis* elements in mRNAs to perform their activity. miRNAs are short noncoding RNAs that strongly influence gene expression, according to the most recent data, 2588 mature human miRNAs have been identified and sequenced (Kozomara and Griffiths-Jones 2014). They are produced from long primary transcripts synthesized by RNA Pol II (pri-miRNA) and processed in the nucleus by "microprocessor," a complex of factors containing Drosha and DiGeorge Critical Region8 (DGCR8) ribonucleases to generate precursor miRNA (pre-miRNA). After its translocation to the cytoplasm by Exportin5, the pre-miRNA is cleaved by another ribonuclease, Dicer, to form a duplex RNA of ∼22 nucleotides (nt). One strand is then loaded into miRNA-loaded RNA-induced silencing complex (miRISC), which comprises, among others, Argonaute proteins (for review, see Ha and Kim 2014). The activated RISC can target specific mRNAs containing, generally at the 3′ untranslated region, sequences complementary to miRNA. mRNA and miRNA form a partial hybrid characterized by the presence of a "seed" region (nucleotides 2–7 of the miRNA) perfectly paired between the two. The interaction of miRISC with an mRNA usually inhibits its translation and this effect is often accompanied by a decrease in the stability

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of the mRNA. However, it has been reported that under specific conditions, the activated RISC can also promote translation (Vasudevan et al. 2007).

There are hundreds of RBPs in the human genome; the role of most of them is still poorly understood. However, it is now clear that RBPs together with miRNAs and probably other noncoding RNAs (ncRNAs), target mRNAs in an orchestrated way to regulate their localization, stability, and finally the amount of protein synthesized. The combination of all these effects on mRNAs is known as "post-transcriptional regulatory code" (Keene 2007). Indeed, functionally related groups of mRNAs are tagged in their coding and noncoding regions early in their lives; in this way, their subsequent destinies are organized and coordinated at the various steps of processing and expression. This complex network of interaction is beginning to be addressed in eukaryotic cells where specific techniques and procedures have been devised to examine the coordinated changes in mRNAs expression.

Among the RBPs, the highly conserved ELAV/HU family consists of four family members, including three that are predominantly cytoplasmic and neurospecific (HuB/Hel-N1, Huc, and HuD) and one that can shuttle between nucleus and cytoplasm of all human cells, HuR/HuA/ELAVL1, (HuR from now on) (Keene 1999; Hinman and Lou 2008). HuR is involved in the regulation of cell cycle, cell migration, tumorigenesis and apoptosis; consequently the HuR expression changes in many types of cancers like breast, ovary, colon, and brain, its increase is often associated with poor prognosis (Wang et al. 2013). HuR is also implicated in gametogenesis, cell differentiation, and stem/progenitor cell survival (Levadoux-Martin et al. 2003; Ghosh et al. 2009). In macrophages, HuR is involved in the regulation of inflammatory and angiogenic processes (Zhang et al. 2012; Lu et al. 2014)

HuR binds to mRNAs containing AU-rich element (ARE) and U-rich element (URE) (Abdelmohsen and Gorospe 2010). While these sequences are known to be destabilizing for the RNAs that contain them, the binding of HuR and HuD increases the stability of the target mRNAs and sometimes activates their translation (Jain et al. 1997). Thus, HuR protein appears to be one of the few RBPs found to stabilize ARE and URE containing mRNAs under most conditions (Simone and Keene 2013).

The exact mechanisms of stabilization have not been elucidated, but the binding of HuR to a target mRNA is believed to block the interaction of other RBPs capable of driving the mRNA to sites of decay like processing bodies or to facilitate the entering of the exosome. However, HuR also modulates the translation of several target mRNAs. In some cases, this effect is carried out through the association of HuR with 5′UTR internal ribosome entry site (IRES) while in some others, the effect is due to a competition between HuR and miRNAs.

To this extent there are some important studies analyzing transcriptome-wide HuR-mRNA interactions by PAR-CLIP technique. These results have been compared to analogous known data about miRNA–mRNA interactions to elucidate the resultant regulation (Lebedeva et al. 2011; Mukherjee et al. 2011). Preliminary results and interpretations of these data seem to indicate that when microRNA and HuR binding sites overlap or are in close proximity, the transcripts are preferentially regulated by HuR while when the sites are nonoverlapping the transcripts could be mainly regulated by miRNAs. However, these conclusions are very limited and strongly related to specific mRNAs examined.

Although HuR, as stated before, tends to increase stability/translation of the target mRNAs, there are a few examples of HuR's repressive effect on some mRNAs. In these cases HuR could cooperate with microRNAs to repress target mRNAs through destabilization and/or inhibition of translation (Kullmann et al. 2002; Yeh et al. 2008). So the combined action of HuR and miRNAs on target mRNAs seems to be very complicated and sometimes variable on different mRNAs.

In this article, we present the complex regulation accomplished by miR-200 family microRNAs on the *c-Jun* mRNA and the involvement of HuR in this process. JUN protein is the main component of transcription factor AP1 that is an essential regulator of many different cellular processes. JUN is often deregulated in tumors and its coding gene, *c-Jun*, is considered one of the most important proto-oncogenes of the cell (Lopez-Bergami et al. 2010). We show that *c-Jun* mRNA is regulated in an opposite way by miR-200b with respect to miR-200a: while the first has a classic inhibitory effect on the production of JUN protein, the latter appears to enhance the stability of *c-Jun* mRNA and to increase the protein amount. To our knowledge this is one of the very few examples in which a microRNA is able to up-regulate both the production of the protein as well as the stability of the target mRNA. The opposite effect of miR-200a and miR-200b on the expression of such an important protein like JUN raises interesting questions about the coordinated control that miR-200 family members can carry out on the same mRNA when expressed differently (Chu et al. 2015) and about the importance of this action in tumors development and progression.

RESULTS

miR-200 family affects the expression of *c-Jun* proto-oncogene by opposite way

JUN is the main component of the dimeric AP-1 transcription factor; this complex is involved in almost all areas of eukaryotic cell behavior from cell proliferation and differentiation to stress response and apoptosis. Indeed, AP1 is activated in response to a lot of extracellular signals from cytokines and growth factors to stress and inflammation. Because of its central role in the cells, AP-1 is often involved in tumor progression and malignant transformation during which AP1 proteins are often deregulated by oncogenic signals (Lopez-Bergami et al. 2010).

Despite the fact that *c-Jun* is a central player in a variety of biological processes, little is known about its post-transcriptional regulation, then according to three independent target prediction algorithms, (TargetSCAN, www.targetscan.org; miRanda, www.microrna.org and PicTar pictar.mdc-berlin.de), we found that *c-Jun* mRNA has predicted seed matches in its 3′ untranslated region (UTR), one for miR-200a/141 and one for miR-200b/200c/429 (Fig. 1A).

In our experiments we utilized miR-200a and miR-200b as representative for the two miR-200 family functional groups. To determine whether miR-200s could target the 3′UTR of *c-Jun* mRNA, we cotransfected a reporter construct, in which

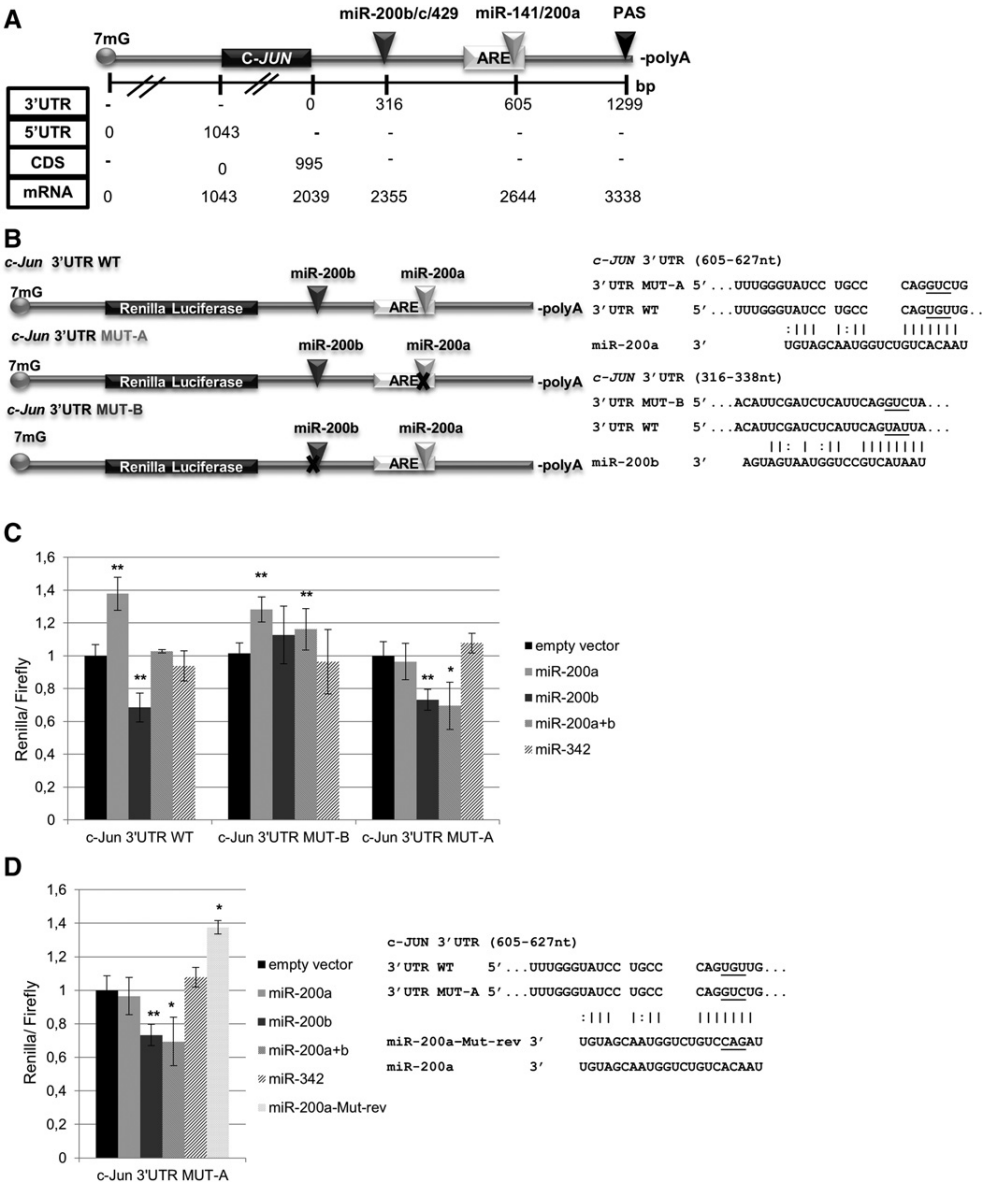

FIGURE 1. (*A*) Schematic of the *c-Jun* 3′UTR with the location of the predicted miR-200a/14 and miR-200b/c/429 target sites. (*B*) Schematic of the Luciferase constructs with *c-Jun* 3′UTR WT, mutants A or B (*left* panel) and alignment of *c-Jun* 3′UTR WT or mutated (MUT-B or MUT-A) with seed region of miR-200b or miR-200a (*right* panel); the mutated nucleotides are underlined. (*C*) Luciferase assay with HEK 293T cotransfected with reporter construct (*c-Jun* 3′UTR WT, *c-Jun* 3′UTR MUT-A, *c-Jun* 3′UTR MUT-B) and a microRNA overexpressing plasmid (miR-200a, miR-200b, miR-342) revealed an antithetical effect of miR-200a and miR-200b on *c-Jun* 3′UTR. The mean values of the corresponding empty vector were set to one. (*D*) Luciferase assay on HEK 293T, cotransfected with reporter construct *c-Jun* 3′UTR MUT-A and microRNA overexpressing plasmids (miR-200a, miR-200b, miR-342, miR-200a-comp-mut), indicated that, in the presence of the compensatory mutation in the seed sequence of miR-200a, the inductive effect of the miRNA on the *c-Jun* 3′UTR is recovered. The mean value of the corresponding empty vector was set to one. Data represented the mean ± SD and asterisks (*) indicate statistically significant modulations with respect to empty vector according to paired Student's test. (*) $P < 0.05$; (**) $P < 0.01$.

the human *c-Jun* 3′UTR WT was fused to Renilla Luciferase (RLuc) and a miR-200a or miR-200b expression plasmid, into HEK 293T cell line; these cells express miR-200s at a very low level. The Rluc activity was strongly repressed in cells transfected with the miR-200b overexpressing plasmid (Fig. 1C; Bracken et al. 2014; Jadhav et al. 2014), as expected from a targeting microRNA. Surprisingly, the overexpression of miR-200a increased the RLuc activity. When both miRNAs were overexpressed together the luciferase activity did not change compared to control. In order to verify that the observed effect could be ascribed to the direct pairing between the microRNAs and the *c-Jun* 3′UTR, we generated two different mutants, one for miR-200b binding site, named mutant B (MUT-B) and one for miR-200a binding site, named mutant A (MUT-A). The two mutants were obtained by the replacement of three nucleotides of *c-Jun* 3′UTR from the 2nd to the 4th nucleotide of miR-200 seeds, with 5′-GUC-3′ (Fig. 1B). The MUT-B 3′UTR was completely resistant to the suppressing activity of miR-200b while the inductive effect of miR-200a was preserved (Fig. 1C). The opposite happened with the MUT-A 3′UTR, using this construct the inductive effect of miR-200a was lost but we were still able to observe the suppressive action of miR-200b (Fig. 1C). We also generated an overexpressing plasmid carrying the sequence for miR-200a pre-microRNA with a mutation in the seed sequence. We changed three nucleotide in the seed sequence (ACA → GAC) to allow the binding of this mutated miR-200a, named the miR-200a compensatory mutant (miR-200a-comp-mut), to the MUT-A 3′UTR. With this mutation we restored the pairing between the microRNA and the mRNA 3′UTR and recovered the inductive effect of the miRNA (Fig. 1D).

To investigate the effect of miR-200a and miR-200b overexpression also on endogenous *c-Jun*, we performed a quantitative real-time PCR (qRT-PCR) to measure the expression level of *c-Jun* mRNA (Fig. 2A) and a Western blot assay for the protein (Fig. 2B). We noticed that in the presence of miR-200b we obtained a reduction in both mRNA and protein of *c-Jun*, compared to the cells transfected with the empty vector. The opposite happened when we transfected the HEK 293T cells with miR-200a overexpressing plasmid, the level of both *c-Jun* mRNA and JUN protein were increased.

Since a change in the level of *c-Jun* mRNA can be caused by either an increase in the transcription level or an alteration of mRNA stability, we further investigated the variations observed. Actinomycin D, an inhibitor of RNA polymerase II, was added to the cell culture media 24 h after transfection of HEK 293T cells with miR-200a overexpressing plasmid or the empty vector control. qRT-PCR performed on total mRNA, after 2, 4, and 8 h by the addition of actinomycin D, showed substantial differences in *c-Jun* mRNA stability (Fig. 2C). Also in this condition, the overexpression of miR-200a increased the *c-Jun* mRNA half-life up to 8 h with respect to the cells transfected with the empty vector where the *c-Jun* mRNA half-life was around 3 h (Fig. 2C) confirming the role of miR-200a in stabilizing *c-Jun* mRNA. These findings indicated an increase of about twofold of *c-Jun* mRNA steady state levels upon miR-200a overexpression.

Considering the increase of endogenous JUN protein, we wondered whether the AP-1 transcription complex could be affected in its functional role. In order to verify that, we transfected the not metastatic breast cancer cell line, MCF7, that expresses the miR-200 family members, with a locked nucleic acid (LNA) anti-miR-200a or miR-200b, to selectively repress the expression of miR-200a or miR-200b in order to highlight the effect of the two microRNAs individually. After 24 h we measured *c-Jun* mRNA quantity by qRT-PCR, a scramble LNA was used as control. We observed that the mRNA level of *c-Jun* was increased in cells transfected with the anti-miR-200b inhibitor, compared to the scramble LNA and, interestingly, we observed the same effect also on matrix metallopeptidase 9 (*Mmp-9*) and vascular endothelial growth factor A (*Vegf-A*) mRNAs (Fig. 2D), that are genes transcriptionally regulated by the AP-1 complex. When we utilized the anti-miR-200a we observed a decrease in mRNAs level suggesting that miR-200 family members have a role also in the regulation of AP-1 complex activity, in which *c-Jun* is the central component.

Previous works have demonstrated that ZEB1 and ZEB2, which regulate the expression of the important adhesion molecule E-Cadherin, are targeted by miR-200 family members (Korpal et al. 2008; Park et al. 2008) this is the reason why this microRNA family is often associated with the inhibition of epithelial to mesenchymal transition (EMT). Here, we checked the importance of mir-200 family action on *c-Jun* mRNA by monitoring the migration capability of the cells that is generally associated with metastatic competence. We transfected a metastatic breast cancer cells line, MDA-MB-231, that do not express the miR-200 family, with the overexpressing plasmid for miR-200b, miR-200a or with the empty vector.

Twenty-four hours after transfection we measured the cells' migration rate through a scratch test in the cells monolayer. The cells transfected with the empty vector closed the scratch in 24 h, whereas the cells transfected with the miR-200b overexpressing plasmid were still maintaining the scratch after 24 h. No differences were observed for the cells transfected with miR-200a with respect to the empty vector control (Fig. 2E). To better clarify the mechanisms by which miR-200b reduces cancer cell migration, we performed the same experiment with cells transfected with a siRNA against *c-Jun* (siJun) or a control siRNA (Fig. 2E). siJun transfection causes a four times reduction of JUN protein amount, in comparison to cells transfected with control siRNA (Fig. 2E, right panel). After 24 h of monitoring, cells transfected with a control siRNA closed the scratch while cells transfected with siJun were still separated by the scratch (Fig. 2E). These experiments show that the miR-200b effect on the *c-Jun* expression is sufficient to inhibit migration of metastatic cells.

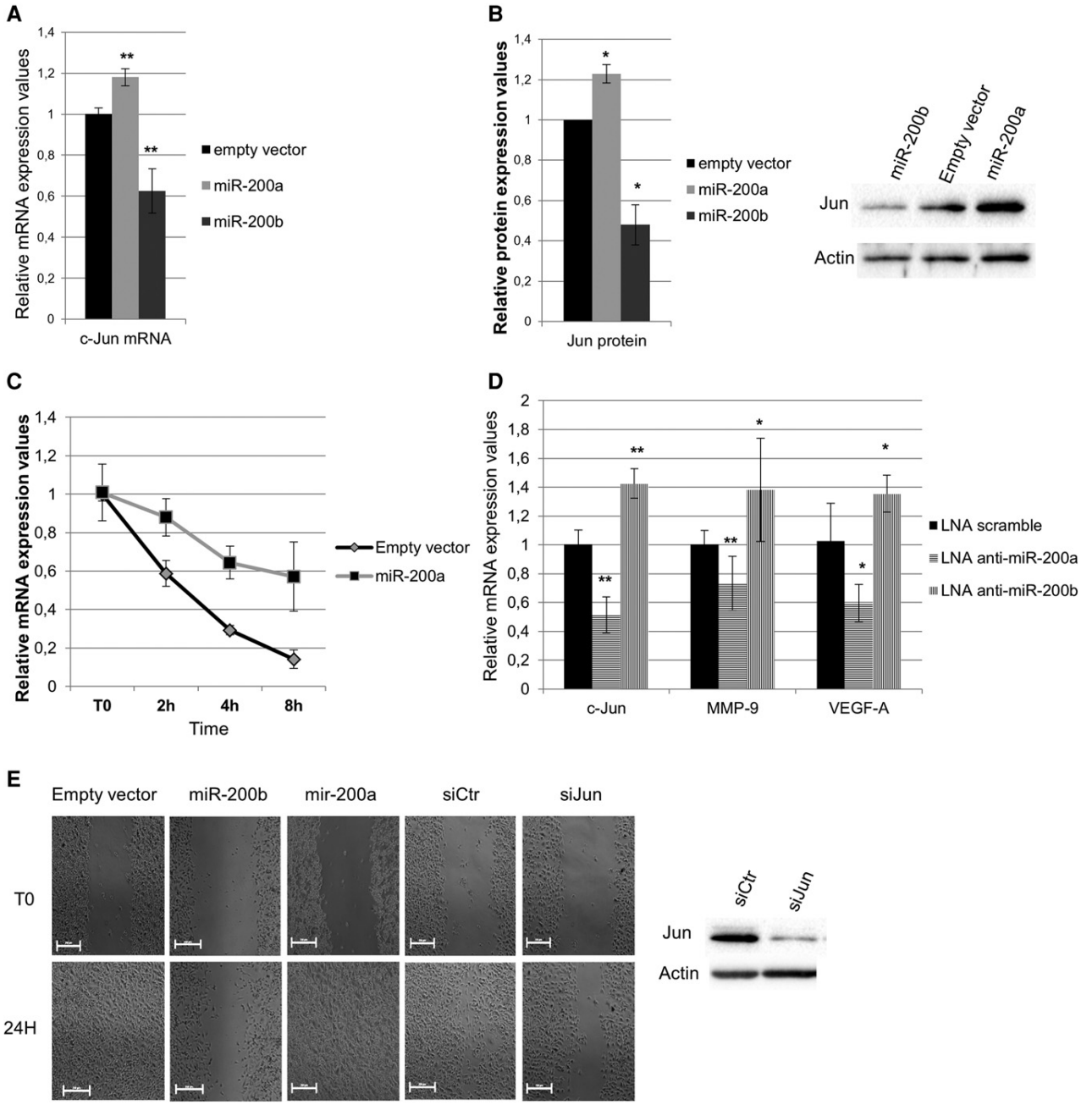

FIGURE 2. (*A*) qRT-PCR of *c-Jun* mRNA showed an opposite effect of the two microRNAs, miR-200a and miR-200b, on the expression level of endogenous *c-Jun* mRNA. The mean value of the corresponding empty vector was set to one, data represented the mean ± SD of two independent experiments. (*B*) JUN protein level measured by Western blotting. The graphic on the *left* represents the densitometry analysis of three independent experiments; on the *right* there is only one representative WB experiment; the empty vector was set to one. (*C*) Inhibition of transcription by actinomycin D. HEK 293T cells were transfected with the miR-200a overexpressing plasmid or with the empty vector. Twenty-four hours after transfection, cells were treated with actinomycin D for the times indicated (*bottom* of the graph). Total mRNA was extracted, quantified, and analyzed by qRT-PCR with primers detecting the endogenous *c-Jun* mRNA. The amount of *c-Jun* mRNA present in the cells, transfected with miR-200a overexpressing plasmid or the empty vector control, before the addition of actinomycin D was utilized as reference point and set to one. (*D*) qRT-PCR of *c-Jun*, matrix metallopeptidase 9 (*Mmp-9*), and vascular endothelial growth factor A (*Vegf-A*) mRNAs performed with RNA from MCF7 cells transfected with LNA microRNA inhibitor anti-miR-200a, miR-200b, or scramble. (*E*) (*Left* panel) Wound healing assay with metastatic breast cancer cells line, MDA-MB-231. Cells were transfected with empty vector or miR-200b and scramble or *c-Jun* siRNA. Twenty-four hours after transfection we generated a scratch in the cells monolayer, and we observe the cells migration rate hourly, for 24 h. The white bar in the panels represents 250 µm. (*Right* panel) Western blotting evaluation of siRNA knockdown. Proteins were isolated after wound healing assay and actin serves as loading control. Data represent the mean ± SD, and asterisks (*) in all the figures indicate statistically significant modulations with respect to empty vector according to paired Student's test. (*) $P < 0.05$; (**) $P < 0.01$.

The noncanonical action of miR-200a is miR-binding site position specific

We tried to understand how miR-200a could enhance the *c-Jun* expression. To explain this unconventional effect, we formulated two hypothesis, (i) miR-200a could compete with a destabilizing factor to bind the *c-Jun* 3′UTR, or (ii) miR-200a could cooperate with a stabilizing factor on *c-Jun* mRNA. We noticed that the *c-Jun* mRNA 3′UTR contains a well-characterized ARE sequence (Peng et al. 1998) and that the miR-200a binding site is located in this sequence, so we assumed that the action of miR-200a on the reporter construct could be related to its localization in the *c-Jun* 3′UTR (Fig. 1A).

The ARE elements are sequences rich in adenines and uracils; they are often bound by specific RNA-binding proteins (RBPs). Systematic study of several RBPs implicated in post-transcriptional gene regulation revealed that HuR, could be a good candidate for our model. In fact, HuR is known as a stabilizer of ARE-bearing mRNAs, it is ubiquitously expressed in the cells and regulates the stability and translation of many ARE-containing mRNAs. It has been reported that HuR could bind the *c-Jun* mRNA 3′UTR, even if in a suboptimal way (Peng et al. 1998). In order to demonstrate that HuR might be involved in the post-transcriptional regulation of *c-Jun*, we performed a luciferase assay with HEK 293T cells transfected with siRNA against HuR mRNA (siHuR) or with a control siRNA. After 24 h the cells were cotransfected with a luciferase construct and a microRNA overexpressing plasmid, as previously described. The luciferase assay reveals that after the depletion of HuR our reporter construct was unaffected by miR-200a overexpression but it was still influenced by the miR-200b overexpression (Fig. 3A). This result suggests an involvement of HuR in the noncanonical action of miR-200a.

In order to analyze the effect of siHuR also on endogenous *c-Jun*, we perform a qRT-PCR and a Western blot assay to measure the mRNA and protein levels of *c-Jun* (Fig. 3B,C). In cells transfected with the control siRNA (siCtr) and miR-200a overexpressing plasmid we are still able to appreciate the inductive effect on *c-Jun* mRNA and protein, mediated by miR-200a. In cells transfected with siCtr and miR-200b overexpressing plasmid, we obtained a reduction in *c-Jun* mRNA (Fig. 3B,C), compared with cells transfected with the empty vector, as observed before (Fig. 2A,B). When we performed the experiment with a siRNA against HuR we confirmed the results obtained in the experiments with reporter construct (3A). Upon HuR depletion (Fig. 3C), the miR-200b mediated reduction of the endogenous *c-Jun* was preserved while the miR-200a mediated increase was abolished (Fig. 3B,C). These results clearly suggest an involvement of HuR in the noncanonical effect mediated by miR-200a on *c-Jun* mRNA.

To determine whether miR-200a could actually enhance the binding affinity of HuR to *c-Jun* 3′UTR, we performed an RNA immunoprecipitation (RIP) to study the association of endogenous HuR with *c-Jun* mRNAs, using cytoplasmic fractions of cells transfected with empty vector or miR-200a overexpressing plasmid. When the lysate from cells overexpressing miR-200a was processed, the qRT-PCR assay revealed the enrichment of *c-Jun* mRNA levels in the IP sample (with anti-HuR antibody), in the presence of miR-200a, relative to the IgG control (Fig. 3D). In the presence of the empty vector, we observed a similar *c-Jun* mRNA level in the IP sample compared to the IgG control, this result confirms that in the absence of stabilizing factors, HuR cannot bind the *c-Jun* ARE sequence with high affinity. As a negative control, we checked the abundance of *c-Jun* mRNA in only beads (OB) samples: it remained unchanged independently of miR-200a. In the input samples, we observed an increase in the endogenous level of *c-Jun* mRNA expression in the cells transfected with a miR-200a overexpressing plasmid compared to the cells transfected with the empty vector (Fig. 3E) as we previously observed (Fig. 2A). Taken together, these observations indicate that HuR is involved in the inductive action of miR-200a and that HuR associates more strongly with the *c-Jun* mRNA after miR-200a overexpression.

Next, we decided to evaluate the importance of the microRNA binding site localization inside the *c-Jun* ARE sequence versus the specificity of the microRNA involved. In order to do that, we generated a mutant with a swap in the binding site of the members of miR-200 family (Fig. 3F). The mutant, named *c-Jun* 3′UTR MUT-INV, presented the position of miR-200a and miR-200b binding sites reciprocally exchanged. We performed a luciferase assay with the *c-Jun* 3′UTR MUT-INV reporter construct and we found that in this new sequence rearrangement, overexpression of miR-200a induced a significant decrease in RLuc activity whereas the overexpression of miR-200b caused an increase in RLuc activity (Fig. 3G). With the 3′UTR MUT-INV we also performed a luciferase assay with a HuR siRNA and we observed that with the depletion of HuR our reporter construct, *c-Jun* 3′UTR MUT-INV, was completely resistant to the inductive effect of miR-200b (Fig. 3H), as we previously observed for the *c-Jun* 3′UTR WT in the presence of miR-200a (Fig. 3A). These results suggest that the noncanonical effect is HuR dependent and it is due to the localization of the microRNA binding site.

Finally, we questioned whether this noncanonical action of a microRNA could encompass other mRNAs, so we looked at mRNAs with a 3′UTR organization similar to *c-Jun*. We found that Dual Specificity Phosphatase 1 (*Dusp1*) mRNA 3′UTR contain an extended ARE sequence (Kuwano et al. 2008) and it has two binding sites for miR-200 family members, one for miR-200a/141 and one for miR-200b/200c/429 and the miR-200a binding site is again into an AU-rich element. We performed a luciferase assay, in HEK 293T cells, with the reporter construct carrying the *Dusp1* 3′UTR, cotransfected with the microRNA overexpressing plasmids and we obtained the same noncanonical event (Supplemental

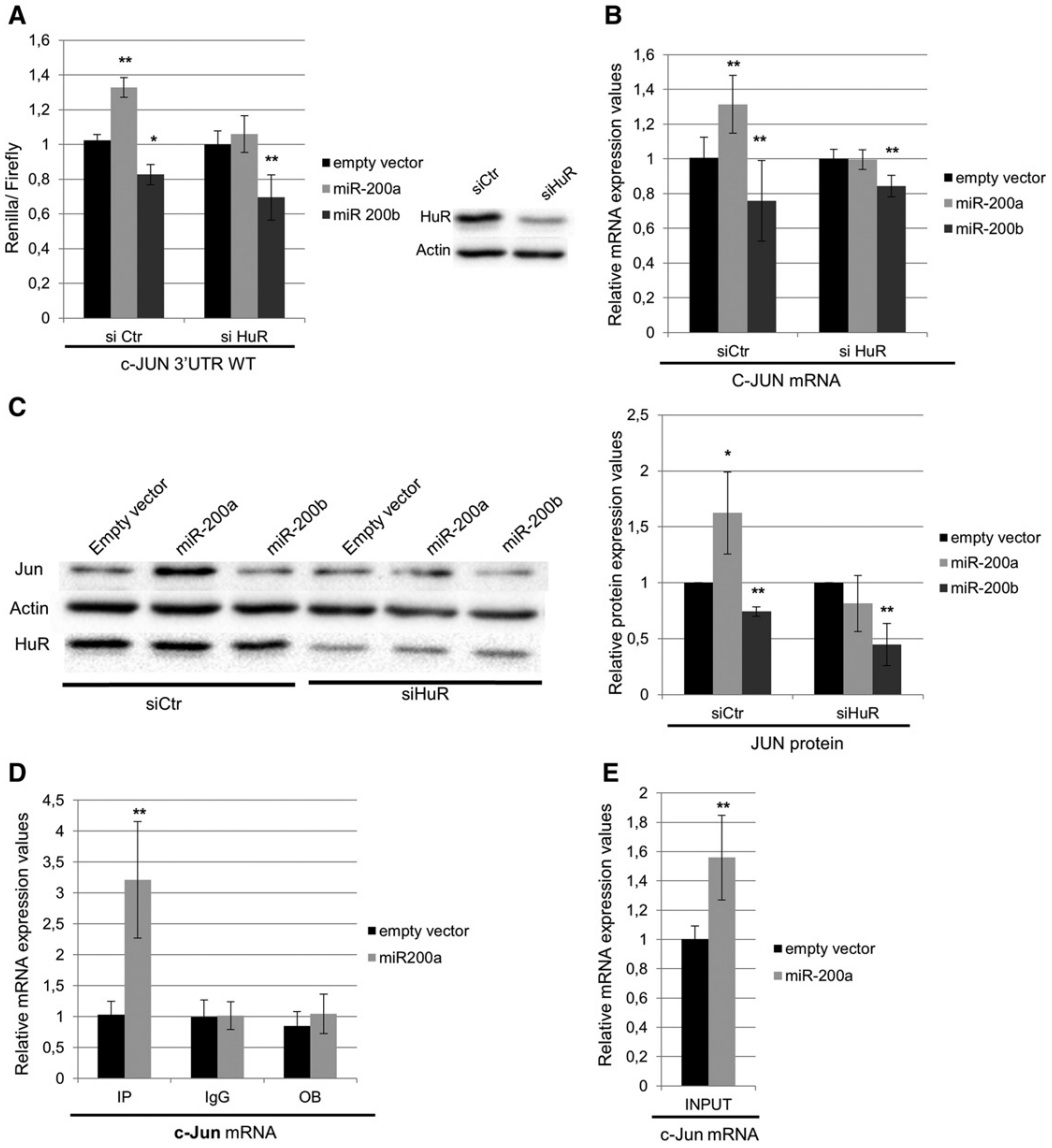

FIGURE 3. (*A*) (*Left* panel) Luciferase assay with HEK 293T cells transfected with a siHuR or a scramble siRNA (siCtr) shows that HuR is involved in the noncanonical regulation mediated by miR-200a on the *c-Jun* 3′UTR WT. The mean value of the corresponding empty vector was set to one. (*Right* panel) Western blotting evaluation of siRNA knockdown. Proteins were isolated after wound healing assay and actin serves as loading control. (*B*) qRT-PCR of *c-Jun* mRNA on cells transfected with the microRNAs overexpressing plasmids (miR-200a, miR-200b, empty vector) and a siRNA control (siCtr) or a siHuR. In the cells transfected with the siHuR, we completely lost the miR-200a inductive effect on *c-Jun* mRNA, suggesting an involvement of the RNA-binding HuR in the noncanonical action mediated by miR-200a. The mean value of the corresponding empty vector was set to one. (*C*) JUN protein level measured by Western blotting. The graphic on the *left* represents the densitometry analysis of three independent experiments; on the *right* there is only one representative WB experiment; the empty vector was set to one. (*D*) qRT-PCR with samples from RNA-immunoprecipitation assay (IP, IgG, OB) show a three times enrichment of HuR/*c-Jun* mRNA interaction in the presence of miR-200a. The mean value of the corresponding IgG was set to one; OB (only beads) was the negative control. (*E*) qRT-PCRs on input samples from the RIP assay in HEK 293T cells confirmed that *c-Jun* mRNA was more abundant in the cells transfected with the miR-200a overexpression plasmid compared to the cells transfected with the empty vector. The mean value of the corresponding empty vector was set to one. (*F*) Schematic of the Luciferase construct with *c-Jun* 3′UTR MUT-INV that contains a reciprocal exchange in the sequence bounded by the seed region of miR-200a and miR-200b. The mutated nucleotides are underlined. (*G*) HEK 293T transfection with reporter construct. *c-Jun* 3′UTR MUT-INV showed that in the new sequence rearrangement, miR-200b induced the RLuc whereas the miR-200a repressed it. The mean value of the corresponding empty vector was set to one. (*H*) Luciferase assay performed with HEK 293T cells transfected with a HuR or a scramble siRNA revealed that also with the 3′UTR MUT-INV there was an involvement of HuR in the noncanonical effect. The mean value of the corresponding empty vector was set to one. Data represented the mean ± SD and asterisks (*) (in panels *A–E*,*G*,*H*) indicate statistically significant modulations with respect to empty vector according to paired Student's test. (*) $P < 0.05$; (**) $P < 0.01$.

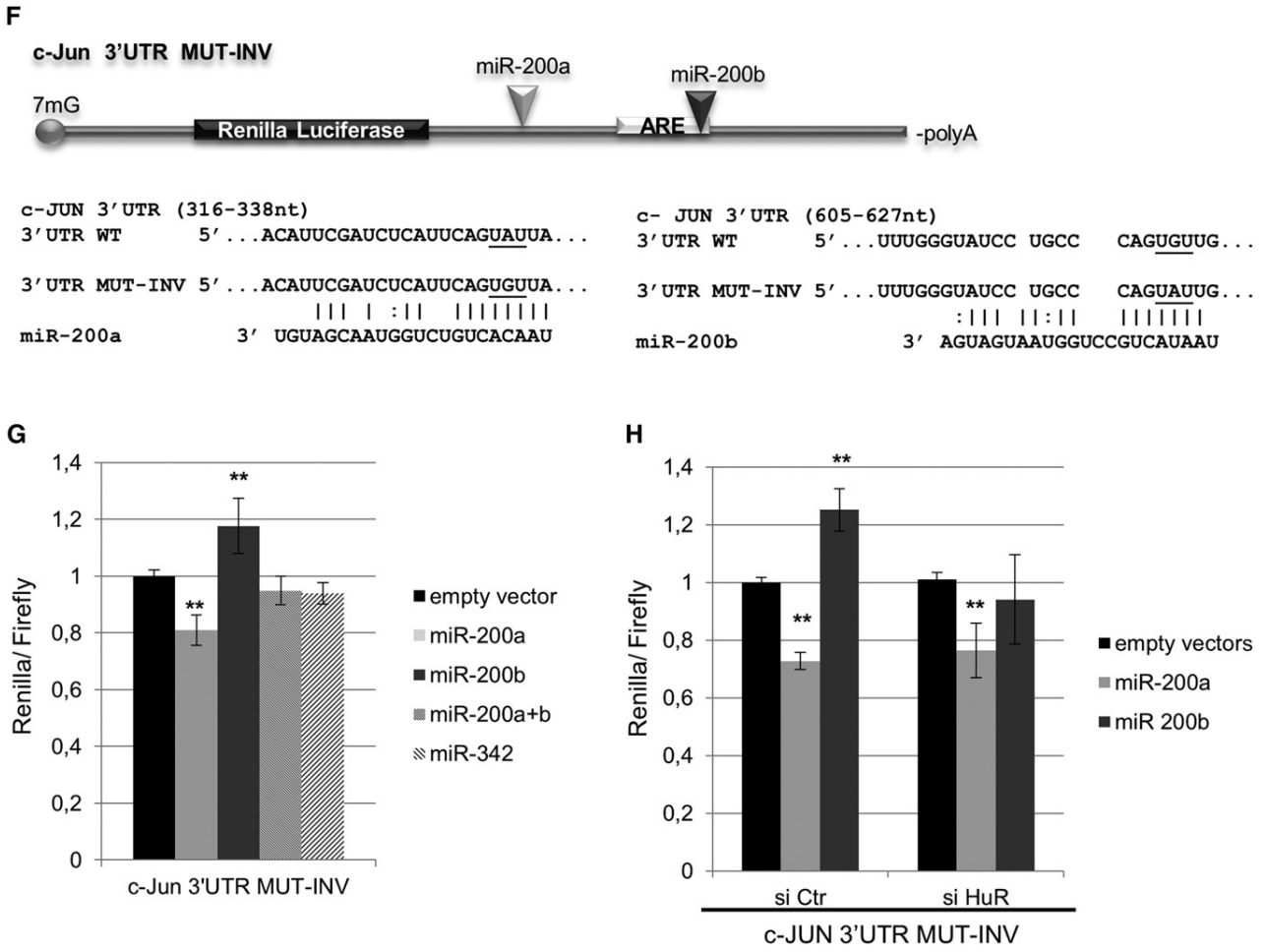

FIGURE 3. *Continued.*

Fig. S1). The overexpression of miR-200a increased the RLuc activity compared with the empty vector whereas miR-200b targeted the *Dusp1* mRNA and the luciferase activity was repressed. With a quantitative real-time PCR we also analyzed the endogenous mRNA level of *Dusp1* and we found that with the overexpression of miR-200a the mRNA level of DUSP1 was enhanced compared to the empty vector but with miR-200b over expression the *Dusp1* mRNA level was decreased (Supplemental Fig. S1).

To exclude the fact that such an unusual feature of regulation could be ascribed to a specific cellular background, we repeated most of the experiments in HeLa cells with identical results (Supplemental Fig. S2).

DISCUSSION

The two most important mechanisms for regulating mRNA stability, turnover and translation are the A/U-rich elements (AREs) with their binding proteins, and microRNAs. Both these pathways are localized on conserved elements located in the 3′UTR of mRNAs, so it is likely the factors involved in the two processes may interact with each other. Among the ARE binding proteins, HuR plays a fundamental role in regulating the stability of mRNAs deeply involved in oncogenesis, inflammatory pathways, and stress response; so there have been many attempts to elucidate how HuR may affect mRNAs function through interactions with microRNAs. The common view is that HuR can globally repress the degradative/inhibitory effect of miRNAs on the target mRNAs, through a direct competition for the binding sites. Indeed, when binding sites for miRNAs and HuR are proximal, HuR (and probably other RBPs) can either block or displace the miRNP complex, allowing mRNA targets to be stabilized (for review, see Simone and Keene 2013). Still, there does not appear to be a single general mechanism by which HuR antagonizes miRNPs: for example, HuR has shown to actually decrease mRNA stability and translation of c-myc mRNA by the recruiting of let-7 miR and RISC complex (Kim et al. 2009). On the other hand, HuR appears to positively regulate c-Myc both directly and indirectly (for review, see

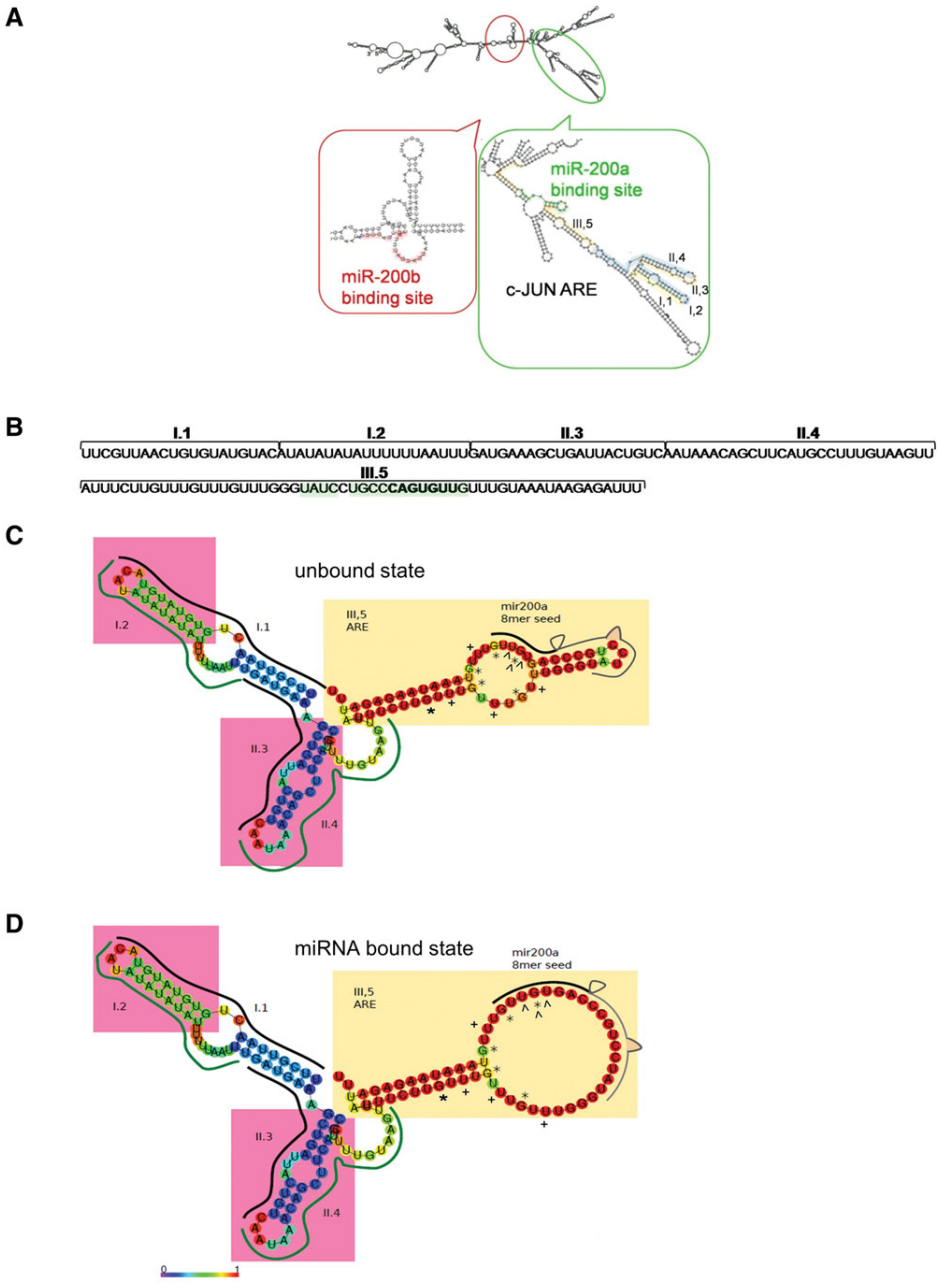

FIGURE 4. miR-200a binding may alter *c-Jun* ARE structure. (*A*) 2D prediction of the entire *c-Jun* 3′UTR available on UCSC (energy = −386.00 kcal/mol). miR-200b binding region is zoomed into the red box. *c-Jun* ARE containing miR-200a binding site is into the green box. ARE domains and subdomains are reported and indicate alternatively in blue and yellow. (*B*) RNA sequence of the *c-Jun* ARE. miR-200a binding site is highlighted in green. (*C,D*) Schematic representation of the conformation of *c-Jun* ARE unbound (*C*) or bound by miR-200a (*D*), predicted using RNAfold algorithm. Base pair probability is reported in a color scale. Modifier letter up arrowheads (^) show UGU triplet we have change for GUC to generate miR-200a binding site mutant (MUT-A); plus signs (+) indicate U → A mutations that do not affect mRNA stability, as reported by Peng et al. (1998); asterisks (*) indicate G → C mutations that increase mRNA stability as Vlasova et al. (2008) described.

van Kouwenhove et al. 2011) so the action of of HuR-miRNP interactions can be different and also divergent, depending on the context such as cell types and cell status. There have been a few examples of miRNAs able to enhance the production of specific proteins. Most of the evidence comes from the work of Vasudevan and colleagues at the end of the past decade indicating a convincing direct relationship between the presence of specific miRNAs and the expression of a definite set of proteins. These noncanonical, positive effect of microRNAs seems to be delimited by a series of conditions,

the most important being the growth condition (Vasudevan et al. 2007) and the stress status of the cells (Prislei et al. 2013).

In the experiments we present here, miR-200a and miR-200b regulate the production of *c-Jun* in an opposite way: while mir-200b has an inhibitory effect on translation of *c-Jun* mRNA and, notably, this effect is sufficient to prevent the migration of MDA-MB-231 cells in a scratch test; miR-200a,in concert with HuR, is able to stabilize *c-Jun* mRNA and, in turn, to increase the amount of JUN protein, AP1 transcription factor and some of the genes regulated by AP1. This noncanonical effect is not dependent on the specific miRNA, instead it seems to be dependent on the location of the binding site on *c-Jun* mRNA 3′UTR. In fact, when mir-200a site on the 3′UTR is mutated to recognize mir-200b, this microRNA is now able to stabilize the mRNA. Remarkably, the binding site of mir-200a is located inside the ARE recognized by HuR, so the action of the microRNA could be due to a direct influence on the structure of *c-Jun* 3′UTR. This possibility seems to be supported by RNA folding predictions obtained through the utilization of "state of the art" algorithms (Fig. 4). With regard to this, it is important to notice that a fundamental feature able to determine the interplay between RBPs and miRNAs on target mRNAs is the structure and the sequence of the AREs present in the 3′UTR. Different AREs containing mRNAs, respond in quite a different extent to changes in HuR activity (Peng et al. 1998). These differences probably reflect a different binding affinity of HuR for the AREs containing mRNAs, the stronger the binding, the stronger the effect on the mRNA. However, this effect can be modulated: a study by Sharma and colleagues in 2013 indicates that the presence of a human microRNA, miR-3134, can affect the binding of HuR on a subset of human ARE bearing transcripts including *Vegf*, *Sox9*, and *Egfr* (Sharma et al. 2013). Our experiments suggest the intriguing possibility that a subclass of ARE containing mRNAs are able to bind HuR with high efficiency only in the presence of miRNAs, (and maybe other cofactors). In the case of the mir-200 family, different expression of members of the family could finely regulate the production of such an important oncogene like *c-Jun*.

Another condition possibly able to influence miRNA-HuR action on the target mRNAs is the structure of the miRNA binding sites. Bracken and colleagues performed a genome-wide analysis of mRNAs targeted by mir-200 family members in different cell lines (Bracken et al. 2014). The complex results obtained seem to indicate that while a 8mer binding site preferentially leads to an inhibition of translation and to a destabilization of the mRNA, the results are less clear when the binding site is a 7mer, 6mer, or a M(is)M(atch) site. In our cases, while the miR-200b has a 8mer binding site, miR-200a interacts through a 7mer binding site; moreover, when the two sites are reversed, miR200-b is able to increase the production of *c-Jun* protein when interacting with the ARE of *c-Jun* 3′UTR through a 7mer site.

In conclusion, our findings elucidate an unexpected complex post-transcriptional regulation of *c-Jun* mRNA, based on the interplay between miR-200 family members and HuR RBP. This coordinated process opens new insights into the contribution of these factors to development, differentiation, and carcinogenesis.

MATERIALS AND METHODS

Cell culture

All cell lines (HEK 293T, HeLa, MCF7, MDA-MB-231) were cultured in D-MEM (PAA) supplemented with 10% fetal bovine serum (FBS, PAA) at 37° C with 5% CO_2.

Plasmid construction

To generate the constructs over expressing miRNAs, the genomic fragments containing the pri-miR-200a or pri-miR-200b were PCR amplified and cloned using BglII and XhoI restriction sites of U1snRNA expression cassette (Denti et al. 2004).

The full-length 3′UTR sequence of *c-Jun* was amplified by PCR and then cloned in XhoI restriction site of the psicheck-2 plasmid (Promega), downstream from the Renilla luciferase (RLuc) gene. The same plasmid contains also the Firefly luciferase (FLuc) to normalize transfection efficiency.

The 3′UTR sequences of *Dusp1* was amplified by PCR and then directionally cloned in NotI and XhoI restriction site of psicheck-2 plasmid.

Mutant derivatives of *c-Jun* 3′UTR were obtained by generating partially complementary PCR fragment that were subsequently used as templates for PCR to generate complete mutated 3′UTRs. They were, finally, cloned as described for the wild type. Specifically, MUT-A and MUT-B 3′UTRs were generated by replacement of three nucleotides of *c-Jun* 3′UTR pairing from the second to the fourth nt of miR-200 seeds with 5′-GUC-3′ (Fig. 1B right panel); MUT-INV 3′UTR presented the position of miR-200a and miR-200b binding sites reciprocally exchanged in the *c-Jun* 3′UTR (Fig. 3A). The compensatory seed mutation (ACA → GAC) in miR-200a overexpressing plasmid was performed to restore the interaction with MUT-A 3′UTR.

All the sequences of the resulting vectors were verified by sequence analysis. Oligonucleotides used for cloning are listed in Supplemental Table I.

Transfection and reporter assays

For reporter assays 1×10^5 HEK 293T cells or 8×10^4 HeLa cells were plated in 24-well plates and cotransfected with 100 ng of *c-Jun* 3′UTR reporter plasmid (*c-Jun* 3′UTR, MUT-A 3′UTR, MUT-B 3′UTR, MUT-INV 3′UTR, or DUSP1 3′UTR) and 900 ng of miR-200a or miR-200b overexpressing plasmid using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were lysed in Passive Lysis Buffer (Promega), and the luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) using the GloMax Multi Detection System Luminometer (Promega). All reporter assays were shown

as relative luciferase activity (averaged ratios of RLuc/FLuc) and combined from at least three separate experiments.

For the experiments on endogenous *c-Jun*, cells were transfected at 80% confluency with microRNA overexpressing plasmids as previously described. Cells were harvested 24 h after the plasmids transfections for downstream processing.

HEK293T cells, 24 h after transfection with microRNA over expressing plasmids were treated with actinomycin D (10 µg/mL, Sigma) for 2, 4, and 8 h to inhibit transcription. Cells were harvested after treatment for downstream processing.

For siRNA experiments, cells were transfected with 30 nM control or HuR (Dharmacon) siRNAs and in some cases after 24 h the cells were also cotransfected with reporter construct and microRNA over expressing plasmids, as previously described. Cells were harvested 48 h after the siRNAs transfections for downstream processing.

For the experiments with LNA microRNA inhibitor, cells were transfected with 30 nM of LNA inhibitor scramble, anti-miR-200a, anti-miR-200b (miRCURY LNA microRNA inhibitor, Exiqon) and 24 h after transfection, the cells were lysed and processed as previously described.

Quantitative real-time PCR

Total RNA was isolated using the miRNeasy Mini Kit according to manufacturer's instructions (QIAGEN). cDNA generation was carried out using the miScript Reverse Transcription Kit (QIAGEN). The real-time PCR detection of mRNAs was performed using miScript SYBR-Green PCR Kit and DNA oligonucleotides by QIAGEN, on a 7300 Real-Time PCR (Applied Biosystems). The values obtained were normalized for *Gapdh* and analyzed by the unpaired Student's *t*-test. Relative quantification was performed using the comparative cycle threshold (ΔΔCt) method. *P*-values were calculated for samples from at least three independent experiments unless otherwise indicated.

Western blotting

Whole-cell protein extracts were prepared from cells lysed in RIPA buffer. Proteins were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membrane (Whatman, Scheleicher & Schuell, Springfield Mill). The immunoblots were incubated with the following antibodies: anti-actin (A2066, Sigma-Aldrich), anti-JUN (ab31415, Abcam). The densitometric analysis was performed using Image Lab software (Bio-Rad).

Wound healing assay

Of note, 5×10^5 MDA-MB-231 cells were plated in the uncoated microslide two well (ibidi) and transfected with 2 ug of miR-200a or miR-200b over expressing plasmid or with 30 nM of *c-Jun* (Dharmacon) siRNAs, using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection the cell monolayer was scraped in a straight line with a p200 pipette tip. The time-lapse imaging of living cells were acquired with a Nikon Eclipse Ti inverted microscope hourly for 24 h.

RNA IP assays

RIP was performed by incubating 20 ug of antibody anti-HuR (sc-5261; Santa Cruz) or isotypic IgG (12–371, Millipore) to 30 mL of Protein A/G salmon sperm agarose beads (Millipore) for 2 h at 4° C. HEK 293T cytoplasmatic lysates were prepared from cells cultured in complete medium for 24 h, after microRNA over expressing plasmid transfection, with 100 mL of passive lysis buffer (PLB). Two hundred milligrams of each lysate was used for each RIP assay. Samples were precleared for 1 h at 4°C with 30 mL of beads, and the supernatant was then resuspended in 600 mL of NT2 buffer and added to antibody-coated beads for 4 h at 4°C. The beads were washed with NT2 buffer five times and split for protein (one-third) and RNA analysis (two-thirds). One-fifth of the input lysate was used as control. PLB and NT2 buffers were prepared according to Tenenbaum et al. (2002).

Secondary structure predictions

The secondary structure prediction of *c-Jun* ARE and the comparison between the structures of the "unbound state" and of the "miR-200a-bound state" were performed by using RNAfold (Vienna RNA package). In addition to the minimum free energy (mfe) (Stiegler et al. 1981) the partition function (pf) base pair probabilities in the thermodynamic ensemble were calculated (McCaskill 1990). Turner energy parameters from 2004 were used. The probability of a base pair sort of calculated by counting the number of structures in the whole ensemble of all possible structures that include that particular base pair and the weight of each structure by its free energy. This sum is then normalized by the ensemble of all possible structures, regardless of whether they include that base pair or not. To get the secondary structure of 152-nt-long *c-Jun* ARE by RNAfold, the sequence was cut out of the 3′-UTR manually and was folded separately. According to Lange et al. (2012), RNAfold is only accurate for short sequences (max 150 nt) and RNAplfold is the preferred method for long sequences twofold folding prediction. Thus, RNAplfold was used to take into account the influence of the context sequence and to check the structure calculated with RNAfold. Both algorithms gave similar results.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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Author contributions: F.D.V. and G.D.V. designed and performed most of the experiments, analyzed data, interpreted results, and participated in manuscript writing; A.R. and C.M. performed clone construction and in silico analysis; S.J.S. performed 2D-RNA folding analysis; I.B. oversaw the project; and C.P. designed experiments, analyzed, and interpreted results and wrote the manuscript.

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