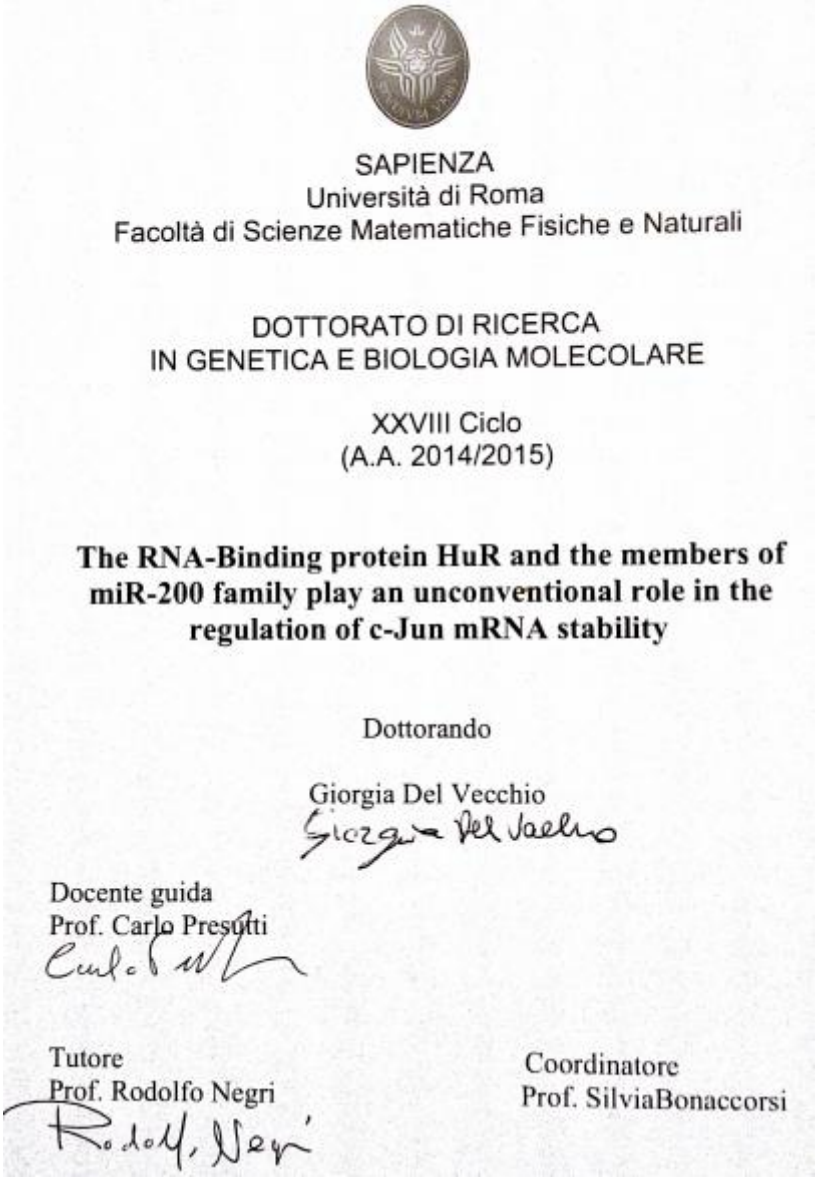

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Università di Roma
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DOTTORATO DI RICERCA
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The RNA-Binding protein HuR and the members of miR-200 family play an unconventional role in the regulation of c-Jun mRNA stability

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GLOSSARY

RBP	RNA- Binding protein
miRNA	microRNA
3'UTR	3' untranslated region
Chr	chromosome
EMT	epithelial-to-mesenchymal transition
MET	mesenchymal-to-epithelial transition
TSS	transcriptional start site
ZEB	zinc-finger enhancer binding
HuR	Hu-antigen R, also known as ELAV-like protein 1
DGCR8	DiGeorge syndrome critical region 8 gene.
LNA	Locked Nucleic Acid
c-Jun	proto-oncogene c-JUN
mRNA	messenger RNA
MMP9	Matrix metalloproteinase 9
PAS	PolyAdenylation Site
pre-miRNA	miRNA precursor
pri-miRNA	miRNA primary transcript
RBP	RNA-Binding protein

SUMMARY

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 present the complex regulation accomplish 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-oncogene of the cell. While miR-200b inhibits c-Jun protein production, miR-200a tends to increase c-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 comprehending mir-200a binding site. By mutating this site, we demonstrate that this non-canonical 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 on c-Jun mRNA. This is the first example of a positive regulation exerted by a microRNA on an important oncogene in proliferating cells. Moreover, the control exerted by 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 and about the importance of this action in tumors.

INTRODUCTION

1. Post-transcriptional gene regulation

Post-transcriptional gene regulation is a fundamental step for coordinating cellular response in a variety of processes like development, homeostasis and disease.
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 (*Orphanides et al, 2002; Mitchell et al, 2000, Bousquet-Antonelli et al, 2000*).

Throughout their lifetimes, mRNAs are escorted by a host of associated factors, some of which remain stably bound while others are subject to dynamic exchange . Together with mRNA, this complement of proteins and small noncoding RNAs constitute the messenger ribonucleoprotein particle (mRNP). It is the unique combination of factors accompanying any particular mRNA, as well as their relative positions along the transcript, that dictates almost everything that happens to that mRNA in the cytoplasm (*Moore et al, 2005*).

Inside the cell, it is possible to identify mRNAs ranging from very stable (many hours) to heavily unstable (a few minutes). Their rates of degradation are controlled by orchestrated interactions between specific cis-acting motifs (present within the sequence of the transcript itself) and trans-acting factors, such as the so-called RNA-binding proteins (RBPs), which bind to the former. Therefore, depending on which RBP is associated to a given mRNA, this interaction may result in a higher/lower stability and/or in an enhanced/reduced translation of the transcript itself (Fig. 1) . Distinct RBPs can interact with the same mRNA in different binding sites or even compete for the same binding site.

Finally, among the several transacting factors binding to the transcript, the involvement of non-coding RNAs that function directly as structural, catalytic, or regulatory RNAs, should also be taken into account. Among these, particular attention has been devoted to microRNAs (miRNAs) (*Fabian et al, 2010*). Obviously, any deregulation within all of these processes may determine an alteration in protein expression and hence metabolic changes leading to disease (*Nguien-Chi et al, 2008*).

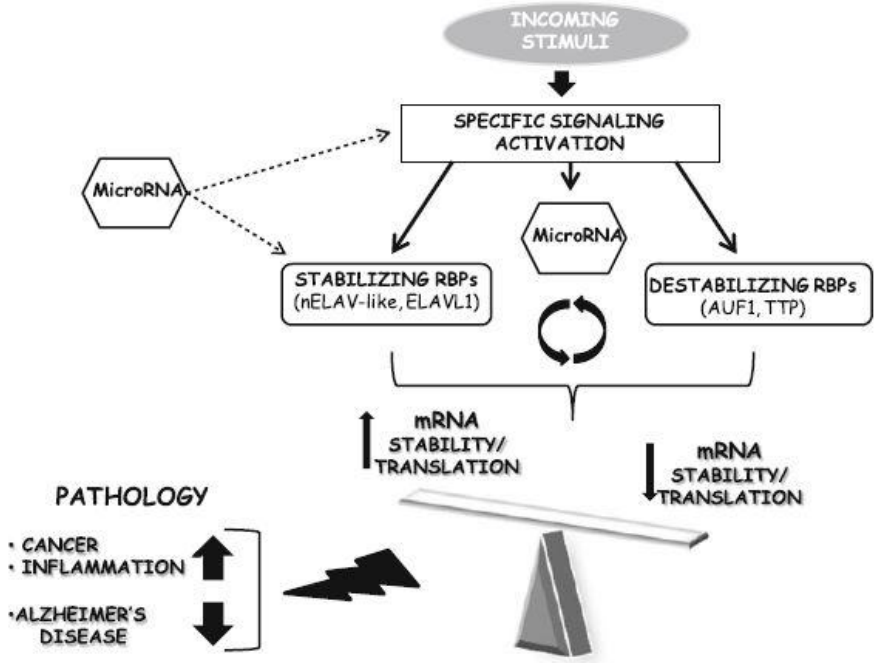


Figure 1 | Scheme showing the influence of post-transcriptional mechanisms on mRNA stability/translation. Different signaling cascades are triggered upon distinct stimuli, and their activation influences the interplay among stabilizing, destabilizing RBPs, and miRNAs. The resulting effect has consequences on the target mRNA half-life and/or its translation, thus determining an increase or a decrease of the correspondent protein. A deregulation of this fine balance may play a role in the development of

several pathologies, such as Alzheimer's disease, cancer, and chronic inflammation. (adapted from *Pascale A and Govoni S, Cell. Mol. Sci. 2012*).

2. Mechanisms of post-transcriptional regulation by microRNA

miRNAs are short noncoding RNAs that strongly influence gene expression, according to most recent data, 2588 mature human miRNAs have been identified and sequenced (*Kozomara et al, 2014).* They are produced from long primary transcripts (Fig 2), synthesized by RNAPolII, (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 about 22nt. One strand of the duplex is then loaded into the miRNA containing ribonucleoprotein inhibitory complex (activated RISC) which contains, among others, Argonaute proteins (*Kim et al, 2009*). 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 miRNA-RISC with an mRNA usually inhibits its translation and this effect is often accompanied by a decrease in the stability of the mRNA.

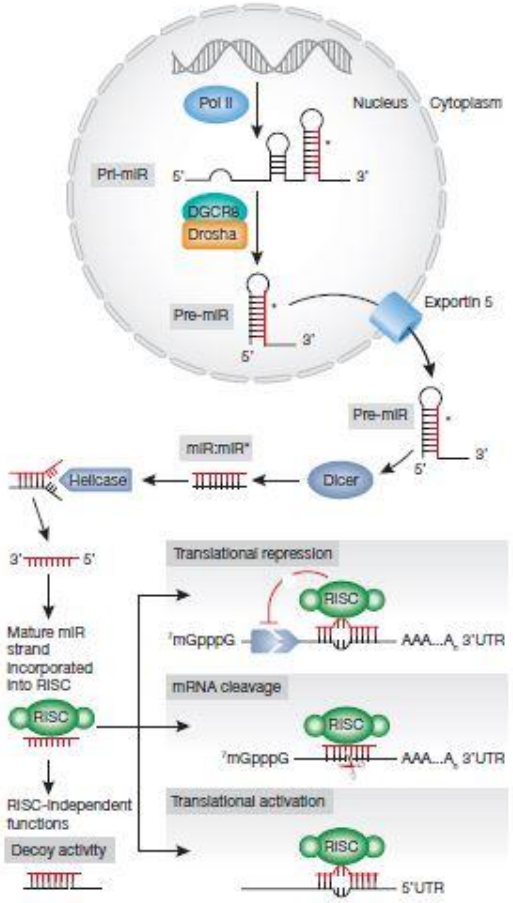


Figure 2 | Biogenesis of miroRNAs and their assembly into microribonucleoproteins. MicroRNAs are transcribed as long primary transcripts and processed into the nucleus by RNAse III Drosha into long pre- microRNAs The originated precursor molecules are exported by an Exportin 5-mediated mechanism to the cytoplasm, where Dicer generates a dsRNA approximately 22 nts long, named miRNA/miRNA , including the mature miRNA guide, and the complementary passenger strand, the miRNA*. The mature single stranded miRNA product is then incorporated in the complex known as miRNA-containing ribonucleoprotein complex (miRNP), a ribonucleoproteic complex containing Argonaute proteins. Now, guided by the base pairing between the non- coding RNA and the target mRNA, miRNA-RISC

could mediate, site-specific cleavage; enhance mRNA degradation; mediate translaional inhibition or activation (adapted from *Iorio M V and Croce C M, EMBO Mol. Med. 2011*).

miRNAs may inhibit protein translation by more than one mechanism (Fig 3). Binding of micro-ribonucleoproteins (miRNPs), possibly complexed with accessory factors, to mRNA 3'UTR can induce deadenylation and decay of target mRNAs (*Wu et al, 2006; Giraldez et al, 2006*) (Fig 3, upper left). Alternatively, miRNPs can repress translation initiation at either the cap-recognition stage (*Mathonnet et al, 2007; Wakiyama et al, 2007*) or the 60S subunit joining stage (*Chendrimada et al, 2007*) (Fig 3, bottom left). mRNAs repressed by deadenylation or at the translation-initiation stage are moved to P-bodies for either degradation or storage. The repression can also occur at post-initiation phases of translation (*Petersen et al, 2006; Maroney et al, 2006*), owing to either slowed elongation or ribosome 'drop-off' (Fig 3, bottom right). Proteolytic cleavage of nascent polypeptides was also proposed as a mechanism of the miRNA-induced repression of protein production (*Nottrott et al, 2006*) (Fig 3, upper-right). However has been reported that under specific condition, the activated RISC can also promote translation (*Vasudevan et al, 2007*). To complicate the already intricate scenario, it has been recently reported that miRNAs can bind to ribonucleoproteins in a seed sequence and a RISC-independent manner and then interfere with their RNA binding functions (*Beitzinger et al, 2010*) (decoy activity Fig 2). Finally, three studies have reported that miRNAs can also regulate gene expression at the transcriptional level by direct binding to the DNA (*Gonzalez et al, 2008; Khraiwesh et al, 2010, Kim et al, 2008*.

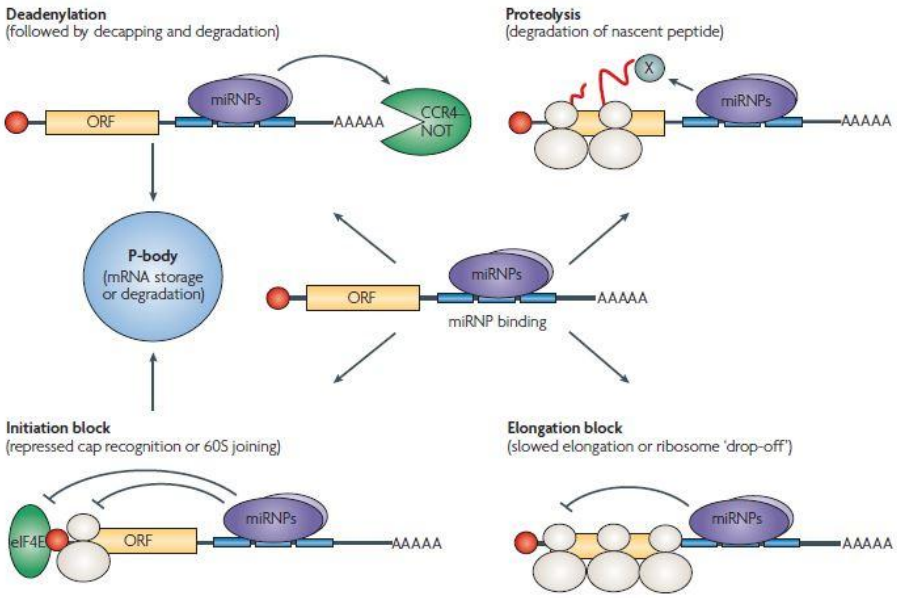


Figure 3 | Possible mechanisms of the microRNA-mediated post-transcriptional gene repression in animal cells. See text for details. (adapted from Filipowicz et al, Nat. Rev. Genet. 2008)

miRNAs can regulate various physiological and pathological processes by modulating the expression of their target mRNAs that play important roles in diverse cellular processes including differentiation, proliferation, growth, migration and survival. Thus, miRNAs constitute one of the most abundant classes of gene-regulatory molecules in animals and given the wide impact of miRNAs on gene expression, it is not surprising that they exert a uniquely important role in disease phenotypes, such as cancer. miRNA expression profling analysis reveals a global down-regulation of mature miRNAs levels in primary human tumors relative to normal tissue (*Lu et al, 2005*). Thus, miRNAs may

function as tumor suppressors or oncogenes and dysregulation in their expression may contribute to tumor cell metastasis.

3. Functional interplay between RNA-Binding protein and microRNA : the ELAV/HU family

There are hundreds of RBPs in the human genome, the role of mostly of them is still poorly understood. However it is now clear that RBPs together with miRNAs and probably other non-coding 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 eukariotic 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 member, including three that are predominantly cytoplasmic and neurospecific (HuB/Hel-N1, Huc and HuD) and one that is expressed primarily in the nucleus of all human cells (HuA/HuR/ELAVL1) (*Keene, 1999; Hingman, 2008*).
Hur is involved in the regulation of cell cycle, cell migration, tumorigenesis and apoptosis; consequently Hur expression changes in many type of cancers like breast, ovary, colon and brain and 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, 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 et al, 2010*). While these sequences are known to be destabilizing for the RNAs that contain them, the binding of Hur and Hub increases the stabilty of the target mRNAs and sometimes activates their translation (*Jain et al, 1997*. Thus, Hur proteins appear to be one of the few RBPs found to stabilize ARE and URE containing mRNAs under most conditions (*Simone et al, 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 to drive 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 of HuR with 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, the transcripts are preferentially regulated by HuR while when the sites are non overlapping 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 example 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 (Fig 4) and is probably under tight spatio-temporal control. In this way, the 3′ UTR can be considered a multi-faceted docking platform for post-transcriptional regulators that either synergistically or antagonistically fine-tune gene expression in time and space. The fact that 3′ UTRs frequently contain multiple evolutionary conserved binding sites for both miRNAs and RBPs suggests that the interplay between RBPs and miRNAs is a crucial component of gene regulation. Novel high-throughput techniques to measure RNA–RNA and RNA–protein interactions as well as to monitor mRNA secondary structure, should enable us to connect networks of post-transcriptional regulation and decipher their relevance for cancer initiation and progression (*Kouwenhove et al, 2011*).

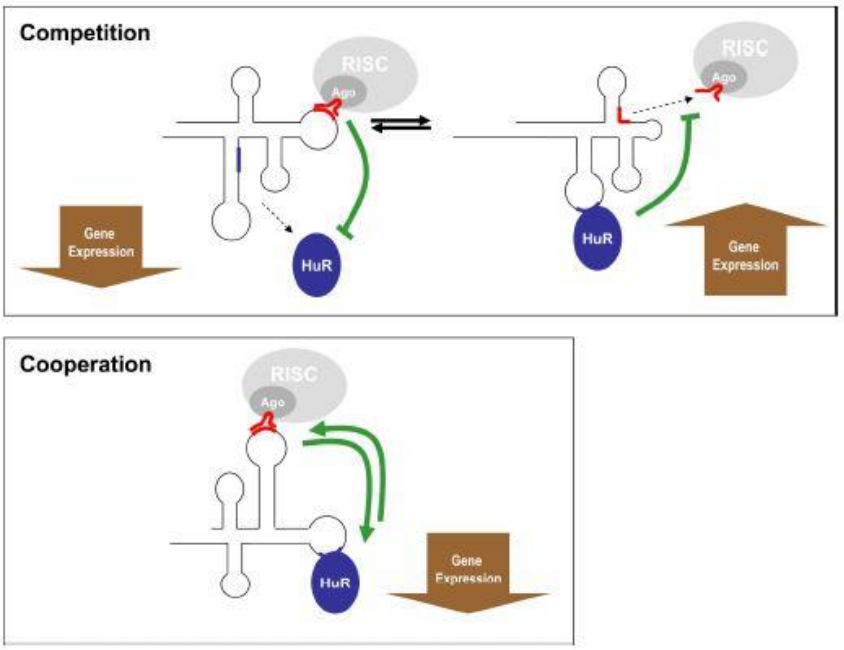


Figure 4 | Schematic of competitive and cooperative interactions of HuR and microRNAs on shared target mRNAs. Top, under the 'competition' model, binding of miRNA-RISC may trigger a conformational change that hides the site of HuR binding to the mRNA, in turn lowering expression of the mRNA (left); conversely, HuR binding may trigger changes in RNA structure that conceal the site of interaction with miRNA-RISC, causing increased expression of the mRNA (right). Bottom, in the 'cooperation' model, binding of HuR may trigger conformational changes that allow binding of miRNA-RISC or vice versa, resulting in repression of the mRNA. (adapted from Srikantan S et al, Curr Protein Pept Sci. 2012).

4. Role of miR-200 family in epithelial- mesenchymal transition and cancer metastasis .

Controlled activation of stemness and cellular motility are of outstanding importance in embryonic development and adult tissue homeostasis. Uncontrolled activation or maintenance of these properties is associated with the pathogenesis of various diseases, in particular cancer. Pure epithelial and mesenchymal phenotypes mark the extreme endpoints of stationary and highly mobile cell types, respectively. Epithelial-to-mesenchymal transition (EMT) is a reversible embryonic programme that allows partial or complete transition between these extreme phenotypes and is essential for embryonic processes such as gastrulation. However, if EMT is aberrantly activated it is a trigger for tumour progression and metastasis (*Thiery et al, 2009*). it is now known that EMT activation is also associated with the maintenance of stem-cell properties (*Mani et al, 2008*). EMT is activated by key signalling pathways, including the TGF-β, Notch and FGF pathways, which converge in the stimulation of EMT activators, a group of transcription factors repressing epithelial gene expression. This group includes members of the Snail, the bHLH and the ZFH families (ZEB1 and ZEB2).

ZEB factors (ZEB1 and ZEB2, encoded by the *ZFHX1a* and *ZFHX1b* genes) are transcriptional repressors that induce EMT by suppressing the expression of many epithelial genes, including E-cadherin (*Vandewalle et al, 2009*). Their repressive function is exerted through binding to different co-repressors, such as CtBPs, HDACs and BRG1. A central activator of ZEB factors is the TGF-β signalling pathway, indicating that they are crucial intracellular mediators of TGF-β-induced EMT. Enforced expression of ZEB factors in epithelial cells results in a rapid EMT associated with a breakdown of cell polarity, loss of cell–cell adhesion and induction of cell motility. Vice versa, a knockdown of ZEB factors in undifferentiated cancer cells induces a mesenchymal- to-epithelial transition (MET). It is no

wonder that these potent factors, if aberrantly overexpressed, have central roles in tumour progression (*Brabletz et al, 2010*). Data from several research groups, all independently investigating EMT from different angles, pointed to the involvement of one family of miRNAs in the regulation of this central cellular processes, the mir-200 family. This family includes mir-200a, mir-200b, mir-200c, mir-141 and mir-429, the five miRNAs are located within two clusters on separate chromosomes (Fig 5). They can be further divided into two subgroups according to their seed sequences, subgroup I: mir- 141 and mir-200a; subgroup II: mir-200b,c and mir-429, which indicate slight differences in their target gene sets. All studies so far have described ZEB1 and ZEB2 as the crucial targets of mir-200 family members (*Burk et al, 2008; Korpal et al, 2008; Park et al, 2008*). the ZEB1 3′ UTR contains eight mir-200 binding sites (five for subgroup II members and three for subgroup I members), and the ZEB2 3′ UTR contains nine binding sites (six for subgroup II members and three for subgroup I members).

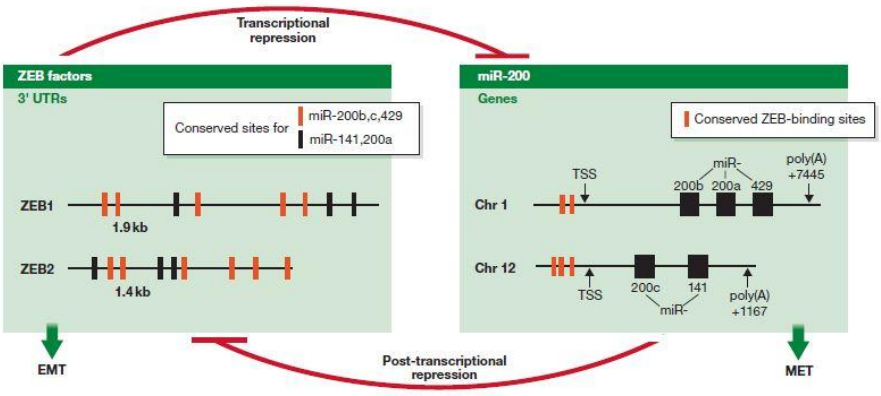


Figure 5 | The ZEB/miR-200 double-negative feedback loop. ZEB factors transcriptionally repress the genes of the miR-200 family members located in two clusters by binding to highly conserved recognition sequences in their promoters. miR-200 family members inhibit expression of ZEB at the post-transcriptional level by

binding to highly conserved target sites in their 3′ UTRs. (adapted from Brabletz S and Brabletz T, EMBO Rep, 2010).

Notably, in addition to the inhibitory effect of mir-200 on ZEB1, we also found a reverse interrelation. Knockdown of ZEB1 led to an increase in the expression of all mir-200 family members (*Burk et al, 2008*). We can demonstrate that ZEB1 directly inhibits transcription of mir-141 and mir-200c genes by binding to highly conserved sites in their common promoter (Fig 5). This finding was corroborated and extended by showing that all mir-200 members are transcriptional targets of ZEB1 and ZEB2 (*Bracken et al, 2008*).

Many investigators have established that in tumorigenesis, one of the fundamental roles of the miR-200 family is to maintain an epithelial phenotype (i.e., preventing epithelial-to- mesenchymal transition) via its gene targets ZEB1 and ZEB2, the transcriptional suppressors of E-cadherin, thus preventing a cancer cell from initiating the process of metastasis. When examined in the role of preventing cancer progression, investigators have shown that expression of this microRNA family can prevent a primary tumor from initiating metastasis by maintaining an epithelial phenotype (*Gregory et al, 2008*). However more recently there have also been studies suggesting that expression of the miR-200 family is associated with efficient metastatic colonization (*Dykxhoorn et al, 2009; Korpal et al, 2011*). In their isogenic mouse model Dykxhoorn and colleagues have shown that after cancer cells acquire the ability to metastasize, they cannot efficiently form metastatic lung colonies without the expression of the miR-200 family (*Elson-Schwab, 2010*). Furthermore Elson- Schwab et al have shown that expression of miR-200c confers a cellular morphology that favours invasion and metastasis (*Soond et al, 2011*). Finally Korpal and colleagues reported that miR-200s play a critical role in promoting the latter steps of metastatic colonization by targeting secretomes involved in metastasis

suppression. These studies highlight a biphasic role of miR-200 family members, they are downreguleted in primary cancer cells at the invasive front where they undergo EMT and they are upregulated in the resulting metastasis where MET facilitates colonization of a distant tissue (*Lussier et al, 2011, Paterson et al, 2013*). However, our knowledge about the role of the miR-200 family continues to evolve.

5. The proto-oncogene Jun and its role in cancer

JUN is the main component of AP1 complex and it was originally identified as the normal cellular counterpart of the avian sarcoma (ASV17) viral Jun oncoprotein (v-jun) (*Maki et al, 1987*). The Jun family consists of JUN, JUNB and JUND, and each protein has distinct characteristics. JUN is encoded by a 3.34 kb intronless gene, located on chromosome 1 (1p32-p31) and results in the expression of a 334 amino acid protein product composed of four main domains, which are involved in DNA binding, transcription and dimerization (*Ransone et al, 1990*) JUN is important for cell proliferation, survival and apoptosis, and accordingly mice lacking JUN die between day 12.5 and 13.5 of embryonal development owing to hepatic failure and heart defects (*Vogt et al, 2001*). JUN is an 'immediate early gene' and is responsive to mitogenic stimuli, as well as DNA damage and stress. JUN expression levels are tightly controlled by a combination of protein stability and a short mRNA half-life of 20–25 minutes, owing to an AU-rich RNA destabilizing element in the 3′-untranslated region. Post-translational modifications trigger a positive autoregulatory loop that involves the binding of AP1 dimers to a TPA response element (TRE; also known as a JUN1 site) and a cyclic AMP responsive element CRE (also known as a JUN2 site) in the JUN promoter resulting in increased

transcription (*Angel et al, 1988*).

The mammalian Activator Protein 1 complex (AP1) proteins are homodimers and heterodimers composed of proteins from the Jun (JUN, JUNB and JUND) and Fos (FOS, FOSB, FRA1 and FRA2) families, and the closely related activating transcription factor (Atf and Creb) subfamily and the Maf subfamily (*Angel et al, 1991*).

AP1 functions in almost all areas of eukaryotic cellular behaviour, from cell cycle proliferation and development to stress response and apoptosis (Fig 6). Indeed, AP1 is activated in response to a plethora of extracellular signals from cytokines and growth factors to stress and inflammation (*Eferl et al, 2003*).

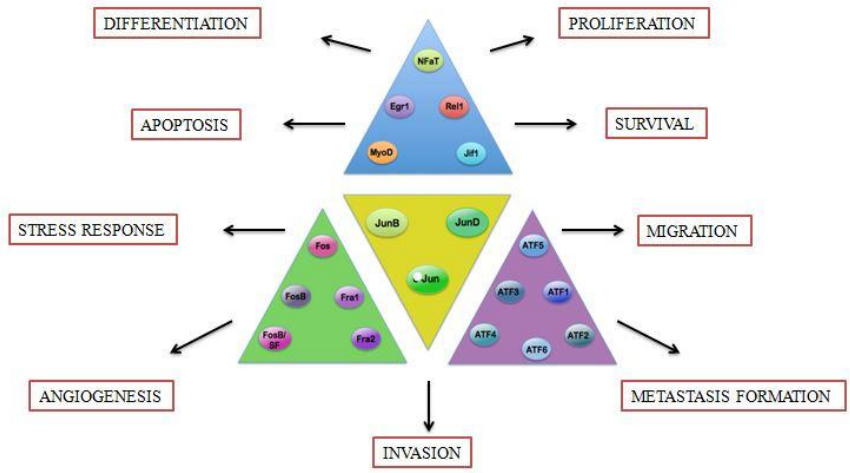


Figure 6 | Scheme showing AP1 complex composition and action. The diverse functions attributed to AP1 complexes have proved difficult to discern as each is dictated by the distinct heterodimeric combination that can be assembled from an array of potential complexes that these proteins can form. Differential dimerization between JUN, ATF2 and FOS with different family members is sufficient to alter their promoter-binding specificity, drastically changing the transcriptional capacity, protein stability and localization, and ultimately the transcriptional repertoire of these proteins[179]. Such

dimerization and consequent functional differences are largely attributed to tissue- and cell type-specific expression levels of the individual AP1 proteins and the degree of activation of upstream pathways such as MAPK or SAPK pathways. Accordingly, as the composition of the AP1 complexes is paramount to their function, deregulation of this composition in favour of more oncogenic partnerships may account for the transcriptional alterations observed during tumorigenesis. (adapted from Raivich G et al, Prog. Neurobiol. 2006).

The inherently diverse composition of AP1 complexes and their central role in transcriptional regulation places AP1 complexes at a functional epicenter for pathological signal relay in disease, particularly in the context of malignant cellular transformation in which AP1 proteins are often deregulated by oncoprotein signalling (*Lopez-bergami et al, 2010*).

AIMS

Is already known that alterations of microRNA expression are not exceptional but rather the rule in human cancer.

When Calin and colleagues in 2004 mapped all the known microRNA genes, they found many of them located in chromosomal loci prone to delections or amplifications, as was found in many different human tumors. Indeed, chromosomal regions encompassing microRNAs involved in the negative regulation of a transcript encoding a known tumor suppressor gene can be amplified in cancer development. This amplification would result in the increased expression of the microRNA and consequent silencing of the tumour suppressor gene (oncomiRNA). Conversely, microRNAs repressing oncogenes are often located in fragile loci, where deletions or mutations can occur and result in reduced microRNA levels and overexpression of the target oncogene (tumor suppressor miRNA, tsmiRNA).

For many microRNAs the role as oncomiRNA or tsmiRNA is already known, however, we have a number of microRNA with a still unclear expression profiling in tumor progression such as miR-200 family.

In spite of such extensive investigation, the expression and role of miR-200 in tumor formation and progression remains a point of contention. Available data indicate that these miRNAs are subject to dynamic changes depending on the stage of tumor progression, EMT, nuclear or cytoplasmic localization of interacting proteins and the cellular ROS content. Thus, with the aim of adding new knowledge about this microRNA family and clarify their role in cancer we chose this family for further investigations.

Effectively, miR-200 family members seem to be versatile players

in cancer so, establishing the role of miR-200 will certainly open new avenues for therapeutic intervention.

RESULTS

1. miR-200 family affect the expression of Jun proto-oncogene by opposite way

To better understand the possible role of miR-200 family in tumor progression, we searched for predicted mRNA targets. According to three independent target prediction algorithms, (TargetSCAN, www.targetscan.org; miRanda, www.microrna.org and PicTar pictar.mdc-berlin.de) we generated a lists of putative mRNA targets shared by all members of miR-200 family and searching for common putative targets we picked out 101 genes and along them we found the important proto-oncogene c-Jun (Fig 7A).

c-Jun mRNA has two predicted seed matches in its 3' untranslated region (UTR), one for miR-200a/141 and one for miR-200b/200c/429 (Fig 7B). 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 mRNA 3'UTR of c-Jun, we co-transfected a c-Jun reporter construct, in which the human c-Jun 3'UTR WT was fused to Renilla Luciferase (RLuc) and a microRNA overexpression plasmid, into the miR-200 low-expressing cell line HEK 293T (Fig 7C). Luciferase activity was strongly repressed in cells transfected with the miR-200b overexpressing plasmid (*Bracken et al, 2014; Jadhav et al, 2014*) (Fig 7D), as expected from a targeting microRNA but surprisingly the overexpression of miR-200a increased the RLuc activity. When both miRNAs were over-expressed together the luciferase activity didn't change compared to control.

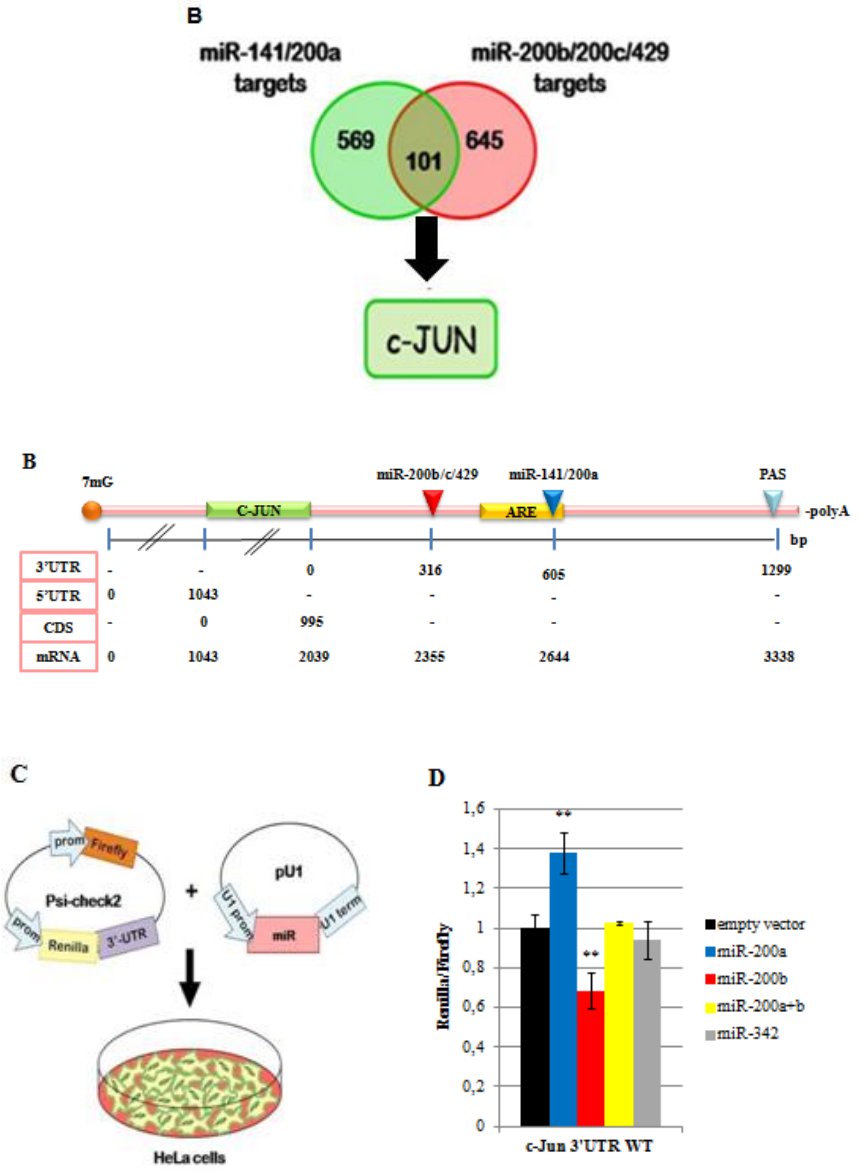
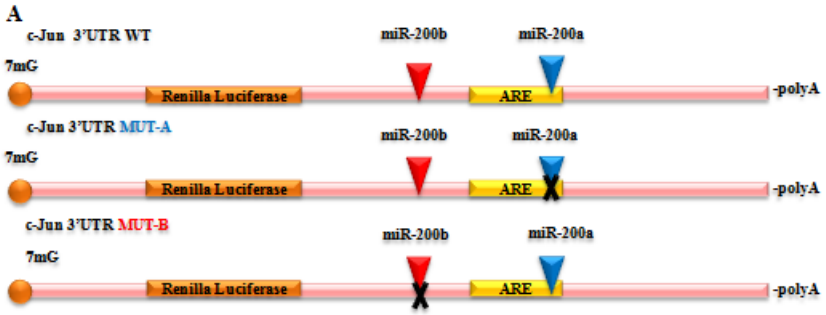


FIGURE 7 | **(A)** MiRNA target predictions were performed using Targetscan v5.2, PicTar and miRanda algorithms. Venn diagrams show the overlap among mRNAs

targeted putatively by different miRNAs. The numbers in the circles represent the number of mRNAs predicted at least by two algorithms as target of miR-200 family. DAVID 6. was interrogated for mRNA target functional annotation. **(B)** Schematic of the c-Jun 3'UTR with the location of the predicted miR-200a/141 and miR-200b/c/429 target sites. **(C)** Schematic representation of co-transfection strategy with Psi-check2 vector and miRNA overexpression plasmid pU1. **(D)** Luciferase assay with HEK 293T co-transfected with reporter construct, c-Jun 3'UTR WT, and a microRNA overexpression 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 1. Data represented the mean $\pm$ SD and asterisks (*) indicate statistically significant modulations respect to empty vector according to paired Student's test. * $p< 0.05$; ** $p < 0.01$.

In order to verify that the effect we observed 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); (Fig 8A). The two mutants were obtained by the replacement of three nucleotides of c-Jun 3'UTR pairing, from the 2^{nd} to the 4^{th} nucleotide of miR-200 seeds, with 5'-GUC-3' (Fig 8B). Using the MUT-B 3'UTR we demonstrated that it was completely resistant to the suppressing activity of miR-200b while the inductive effect of miR-200a was preserved (Fig 8C). The opposite happened with the MUT-A 3'UTR, using this construct the inductive effect of miR-200a was lost but we could still observe the suppressive action of miR-200b (Fig 8C). We generated also an overexpression plasmid carrying the sequence for miR-200a pre-microRNA with a mutation in the seed sequence. We changed three nucleotide in the seed sequence (Fig 8D) (ACA -> GAC) to allow the binding of this mutated miR-200a, named 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 8E). This was a confirmation that the non-canonical event we observed was mediates by direct pairing between the microRNA and the c-Jun 3'UTR.



B

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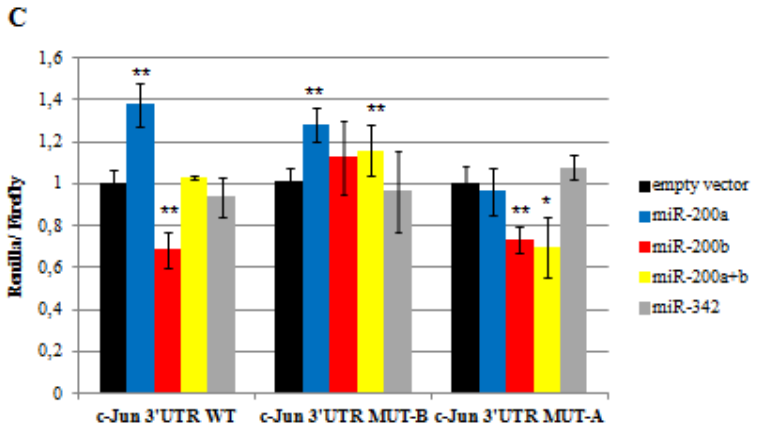
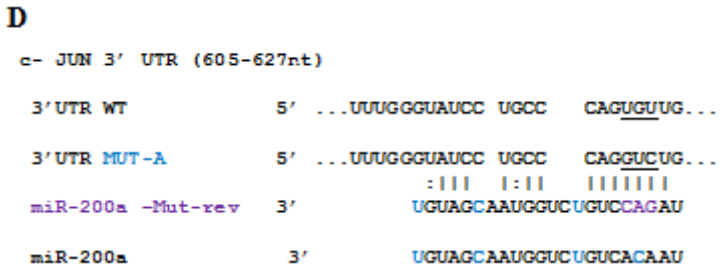
c-JUN 3'UTR (316-338nt)
3'UTR MUT-B 5'...ACAUUCGAUCUCAUUCAGGUCUA...
3'UTR WT    5'...ACAUUCGAUCUCAUUCAGUAUUA...
                 ||: | :||  ||||||||
miR-200b    3'    AGUAGUAAUGGUCCGUCAUAAU

c-JUN 3'UTR (605-627nt)
3'UTR MUT-A 5'...UUUGGGUAUCC UGCC   CAGGUCUG...
3'UTR WT    5'...UUUGGGUAUCC UGCC   CAGUGUUG...
                  :|||  |:||   |||||||
miR-200a    3'        UGUAGCAAUGGUCUGUCACAAU
```





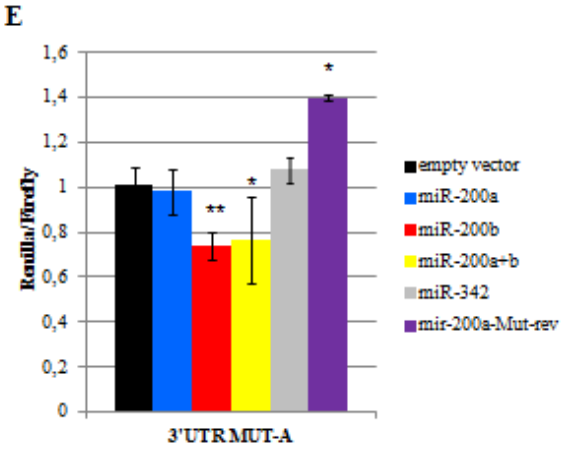


FIGURE 8 | (A) Schematic of the Luciferase construct with c-Jun 3'UTR WT, mutants

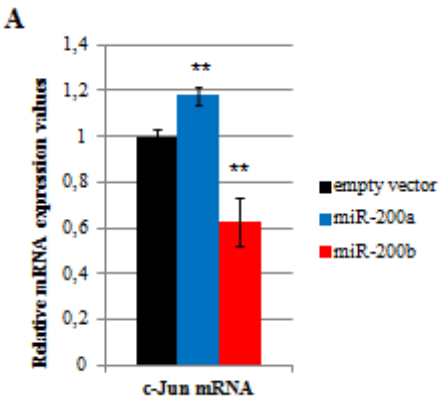
A or B. **(B)** Schematic representation of alignment between c-Jun 3'UTR WT or mutated (MUT-B or MUT-A) and seed region of miR-200b or miR-200a, the mutated nucleotides are underlined. **(C)** Luciferase assay with HEK 293T co-transfected with reporter construct (c-Jun 3'UTR WT, c-Jun 3'UTR MUT-A, c-Jun 3'UTR MUT-B) and a microRNA overexpression 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 1. **(D)** Schematic of the alignment between c-Jun 3'UTR MUT-A and the seed region of miR-200a-comp-mut, the mutated nucleotides are colored in violet. **(E)** Luciferase assay with HEK 293T, co-transfected with reporter construct c-Jun 3'UTR MUT-A and microRNA overexpression plasmids (miR-200amiR-200b, miR-342, miR-200a-comp-mut), showed as, with the compensatory mutation in the seed sequence of miR-200a, we were able to recover the inductive effect of the miRNA on the c-Jun 3'UTR. The mean value of the corresponding empty vector was set to 1. Data represented the mean $\pm$ SD and asterisks (*) indicate statistically significant modulations respect to empty vector according to paired Student's test. * $p < 0.05$; ** $p < 0.01$.

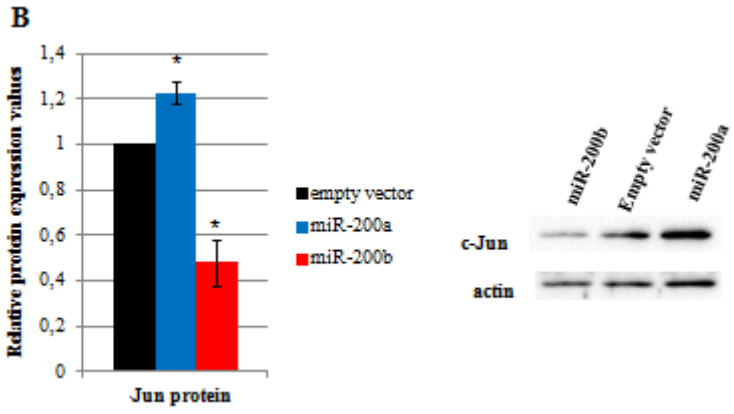
1.1 miR-200 family regulates the endogenous level c-Jun as well as the expression of AP1 regulated genes.

We know that Jun is the main component of the dimeric AP-1 transcription factor, this complex is involved in almost all areas of eukaryotic cell behaviour 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 oncoprotein signals (*Lopez-Bergami et al, 2010*).

To investigate the effect of miR-200a and miR-200b overexpression on endogenous Jun, we performed a quantitative Real Time PCR (qRT-PCR) to measure the expression level of c-Jun mRNA (Fig 9A) and a western blot assay for the protein (Fig 9B). We noticed that in the presence of miR-200b we obtained a reduction in both mRNA and protein of c-Jun, compare to the

cells transfected with the empty vector. The opposite happened when we transfected the HEK 293T cells with miR-200a overexpression plasmid, the level of both c-Jun mRNA and Jun protein were increased.





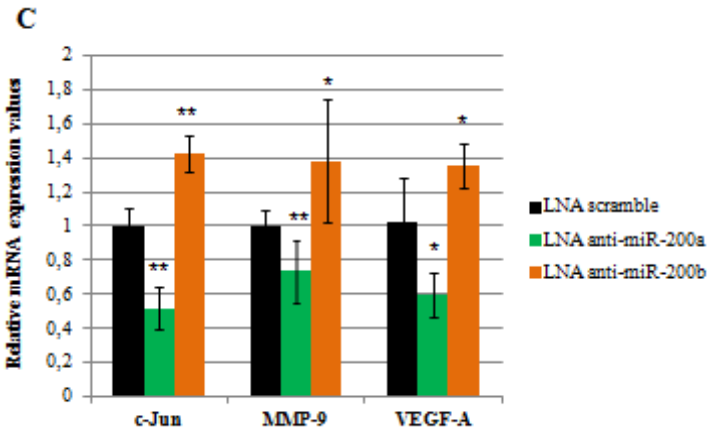


FIGURE 9 | (A) qRT-PCR of c-Jun mRNA showed an opposite effect of the two microRNA , miR-200a and miR-200b, on the expression level of endogenous c-Jun mRNA. The mean values of the corresponding empty vector were set to 1, data represented the mean ± SD of two independent experiments **(B)** Jun protein level measured by western blotting, the graphic on the left represented the densitometry analysis of three independent experiments on the right there was only one representative WB experiment, the empty vector was set to 1. **(C)** 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.
Data represented the mean ± SD and asterisks (*) in all the figures indicate statistically significant modulations respect to empty vector according to paired Student's test, * $p < 0.05$; ** $p < 0.01$.

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 non metastatic breast cancer cell line, MCF7, that still express 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 hours 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, compare 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. These genes are transcriptionally regulated by the AP-1 complex (Fig 9C). 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 main component.

1.2 miR-200b inhibits the migration capability of MDA-MB-231 cells by targeting c-Jun mRNA.

Previous works have demonstrated that ZEB1 and ZEB2, that 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; Burk et al, 2008*) this is the reason why this microRNA family is often associate 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 generally associated with metastatic competence. To do that, we transfected a metastatic breast cancer cells line, MDA-MB-231, with the overexression plasmid for miR-200b, miR-200a or with the empty vector. 24 hours after transfection we measured cells migration rate through a scratch test in the cells monolayer (Fig 10A). The cells transfected with the empty vector closed the scratch in 24 hours whereas the cells transfected with the miR-200b overexpression plasmid still maintained the scratch after 24h. No differences were observed for the cells transfected with miR-200a with respect to the empty vector control.

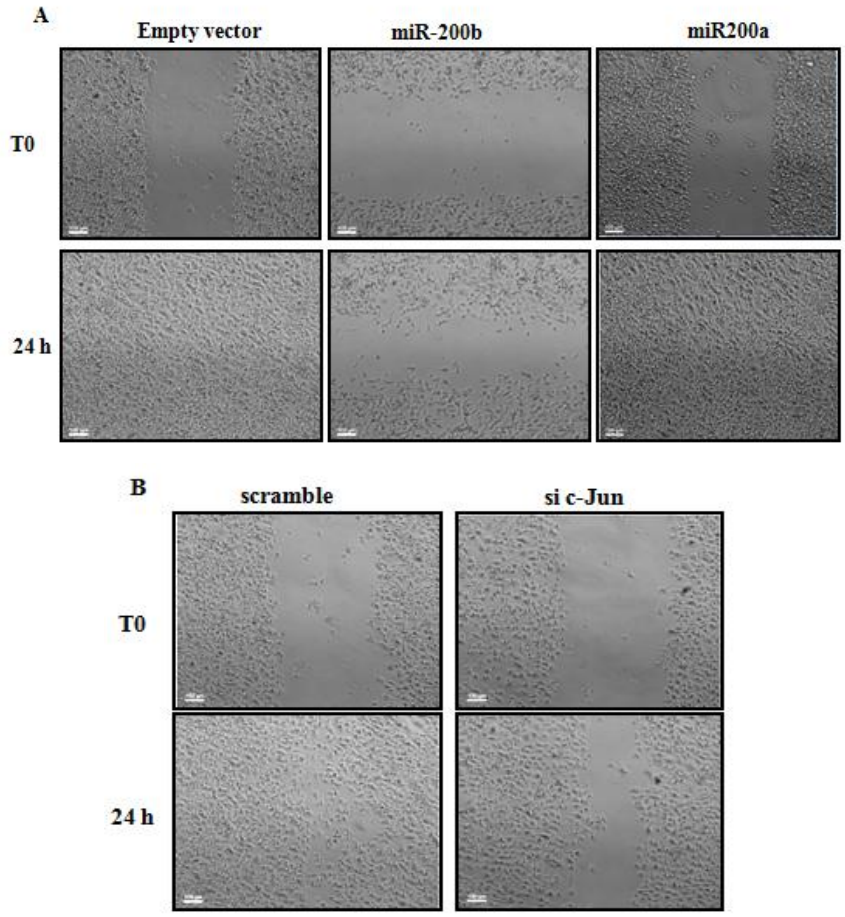


FIGURE 10 | Wound healing assay with metastatic breast cancer cells line, MDA-MB-231. **(A)** Cells were transfected with empty vector or miR-200b. 24h after transfection we generated a scratch in the cells monolayer and we observe the cells migration rate hourly, for 24h. **(B)** Cells were transfected with scramble RNA or c-Jun siRNA. 24h after transfection we generated a scratch in the cells monolayer and we observe the cells migration rate hourly, for 24h.The white bar in the panels represent 100μm.

We observed the same effect when we transfected the MDA-MB-231 cells with a siRNA against c-Jun. After 24 hours of monitoring, the cells transfected with a control siRNA closed the scratch but the cells transfected with siJun are still separated by the scratch (Fig 10B). These experiments show for the first time that the miR-200b effect on c-Jun expression is sufficient to inhibit migration of metastatic cells.

2. The miR-200a triggers a microRNA-mediated stabilization of c-Jun mRNA promoting the binding of HuR

We tried to understand how miR-200a could enhance c-Jun expression. To explain this unconventional effect, we formulated two hypothesis, a) miR-200a could compete with a destabilizing factor to bind the c-Jun 3'UTR, or b) miR-200a could cooperate with a stabilizing factor on c-Jun mRNA. We noticed that the c-Jun mRNA 3'UTR contained a well characterized ARE sequence (*Peng et al, 1998*) and that miR-200a binding site is located into 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.
The AREs elements are sequences rich in adenines and uracils, they are often bounded by specific RNA-binding proteins (RBPs). Systematic study of several RBPs implicated in post-transcriptional gene regulation revealed that ELAVL1, hereafter named HuR, could be a good candidate for our model. HuR is known as a stabilizer of ARE-bearing mRNAs, in rare cases it inhibits mRNAs; it is ubiquitously expressed in the cells and regulates the stability and translation of many ARE-containing mRNAs , indeed, 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 a small interfering RNA (siRNA) against the HuR mRNA or with a control siRNA. After 24h the cells were co-transfected with a luciferase construct and microRNA overexpression plasmid, as previously described. The luciferase assay reveals that after the depletion of HuR our reporter construct was completely unaffected by the inductive effect of miR-200a but it still influenced by the miR-200b overexpression (Fig. 11A). This result suggests an involvement of HuR in the non-canonical action of miR-200a we observed.

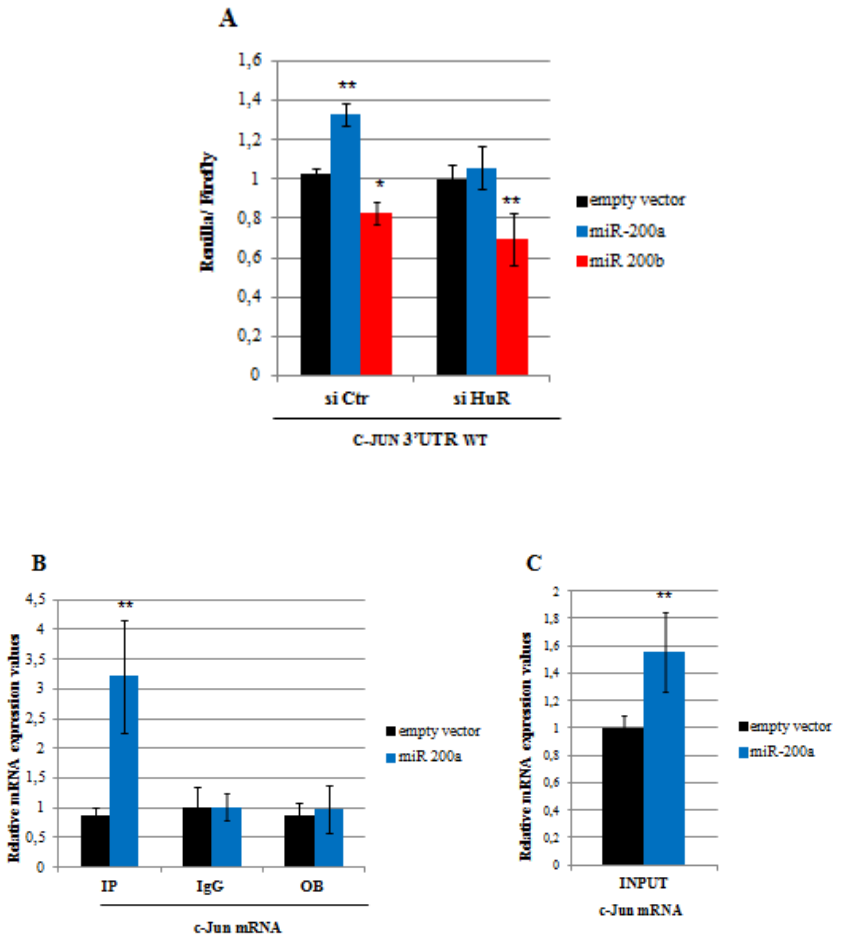
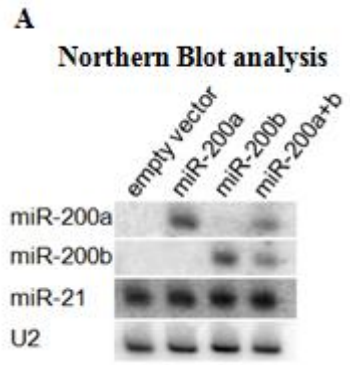
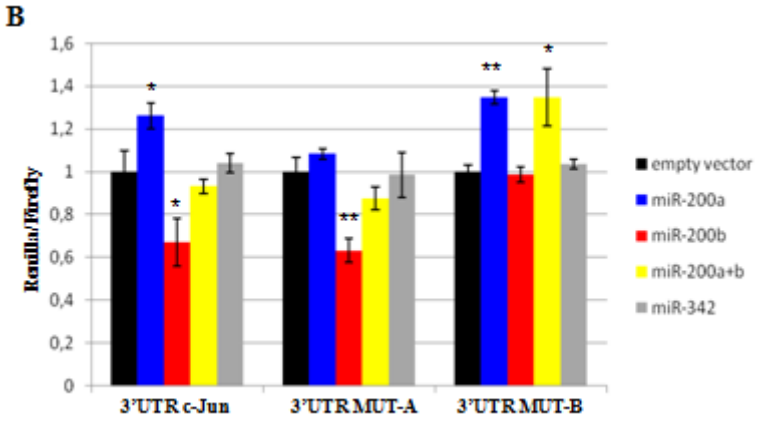


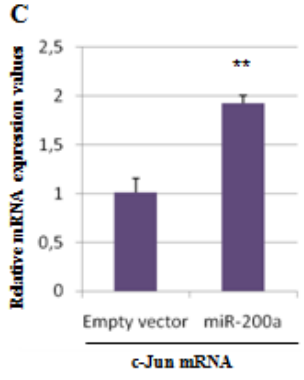
FIGURE 11 | (A) Luciferase assay with HEK 293T cells transfected with a HuR or a scramble siRNA shows that HuR is involved in the non canonical regulation mediated by miR-200a on the c-Jun 3'UTR WT. The mean values of the corresponding empty vector were set to 1. **(B)** qRT-PCR on RNA immunoprecipitation samples with an antibody against HuR (IP), reveals an increase affinity of HuR for c-Jun mRNA in presence of miR-200a, in HEK 293T cells. The mean values of the corresponding IgG were set to 1, OB (only beads) was a negative control. **(C)** qRT-PCR of input samples from the RIP assay in HEK 293T cells revealed that c-Jun mRNA was more abundant in the cells transfected with the miR-200a overexpression plasmid compare to the cells transfected with the empty vector. The mean values of the corresponding empty vector

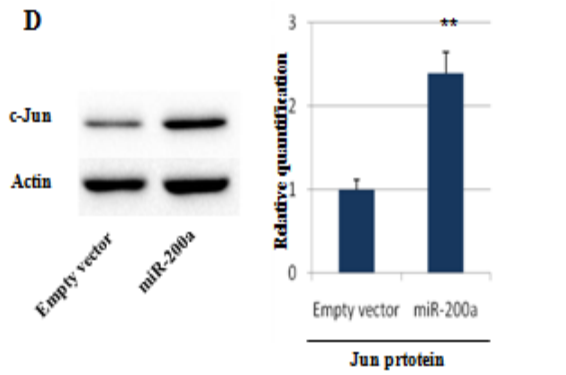
were set to 1. Data represented the mean ± SD and asterisks (*) indicate statistically significant modulations respect to empty vector according to paired Student's test * $p < 0.05$; ** $p < 0.01$.

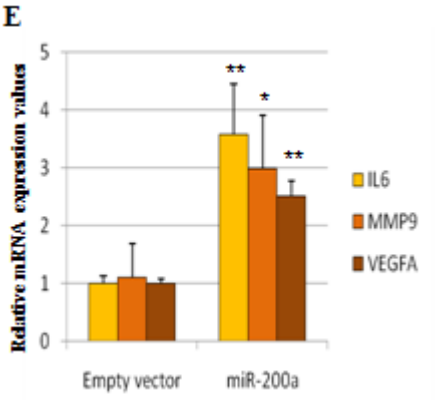
To determinate if miR-200a could actually enhance the binding affinity of HuR to c-Jun 3'UTR, we performed a RNA immunoprecipitation (RIP) to study the association of endogenous HuR with endogenous c-Jun mRNAs, using cytoplasmic fractions of cells transfected with empty vector or miR-200a overexpression plasmid. When the lysate from cells overexpressing miR-200a was processed, the RT-PCR assay revealed an enrichment in c-Jun mRNA level in the IP sample (with anti-HuR antibody), in the presence of miR-200a, relative to the IgG control (Fig 11B). In the presence of empty vector, we observed a similar c-Jun mRNA level in the IP sample compared to the IgG control, this result confirms that normally, HuR can't bind the c-Jun ARE sequence with high affinity. As a negative control we observe the abundance of c-Jun mRNA in only beads (OB) samples and it remained unchanged in presence or absence of miR-200a. In the input samples, we observe an increase in the endogenous level of c-Jun mRNA expression in the cells transfected with a miR-200a overexpression plasmid compare to the cells transfected with the empty vector (Fig 11C) as we previously observe (Fig 9A). Finally, to exclude the fact that such an unusual feature of regulation could be ascribed to a cellular background, we repeated most of the experiments in HeLa cells with identical results (Fig 12).


A
Northern Blot analysis
empty vector
miR-200a
miR-200b
miR-200a+b
miR-200a
miR-200b
miR-21
U2


B
Renilla/Firefly
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
3'UTR c-Jun
3'UTR MUT-A
3'UTR MUT-B
empty vector
miR-200a
miR-200b
miR-200a+b
miR-342


C
Relative mRNA expression values
2,5
2
1,5
1
0,5
0
Empty vector
miR-200a
c-Jun mRNA


D
c-Jun
Actin
Empty vector
miR-200a
Relative quantification
Empty vector
miR-200a
Jun prtotein


E
Relative mRNA expression values
IL6
MMP9
VEGFA
Empty vector
miR-200a

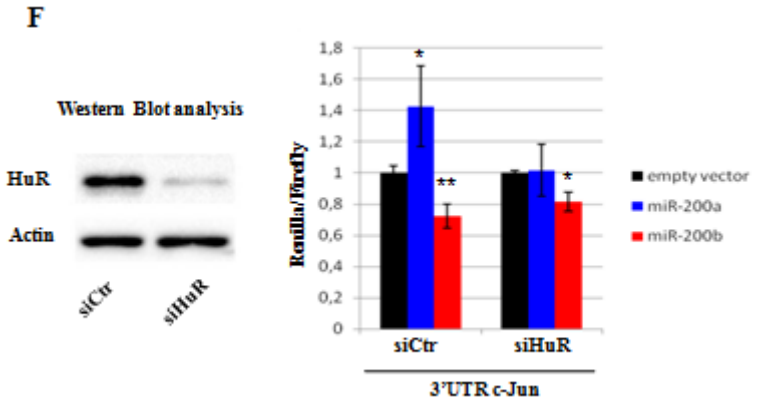

F
Western Blot analysis
HuR
Actin
siCtr
siHuR
Renilla/Firefly
empty vector
miR-200a
miR-200b
siCtr
siHuR
3'UTR c-Jun

FIGURE 12 | (A) Northern Blot analysis showing the expression levels of miR-200a and miR-200b upon transfection of empty vector or miR-200a or miR-200b coding plasmids in HeLa cells. miR-21 expression was verified as positive control. U2 was used to normalize. A representative picture is reported. **(B)** Histograms of luciferase assay results show the non-canonical and canonical regulation of c-Jun in HeLa cells, mediated respectively by miR-200a and miR-200b. Data are presented as means±SD from three replicates in one of at least two independent experiments. (C-D-E) miR-200a affects mRNA and protein levels of c-Jun as well as c-Jun activity. mRNA expression levels of both c-Jun **(C)** and c-Jun-regulated genes **(E)** were observed 24h after miR-200a coding plasmid transfection by qRT-PCR experiments. The empty vector was used as negative control. GAPDH was used as endogenous control. Relative quantification was performed using the comparative cycle threshold (ΔΔCt) method. Error bars are SD of three biological replicates. **(D)** Western blot with anti-c-Jun and anti-actin antibodies was performed on protein extracts from HeLa transfected with miR-200a over-expression plasmid or empty vector. Relative quantification to actin is shown in the histogram on the right. Error bars are SD of four biological replicates. **(F)** Luciferase assay measurements upon HuR depletion indicating that miR-200a-mediated regulation of c-Jun is HuR-dependent. (F left) Western blot analysis of protein extracts from HeLa cells transfected with 50nM siRNA against HuR (siHuR) or negative control (siCtr) two times at 24h interval, confirming HuR depletion.
For all experiments, asterisks (*) indicate statistically significant modulations with respect to the empty vector according to unpaired Student's t test, *$p<0.05$, **$p<0.01$.

Taken together, these observations indicate that HuR is involved in the inductive action of miR-200a and that HuR associates more prominently with the c-Jun mRNA after miR-200a overexpression.

2.1 miR-200a binding could induce a conformational change in the c-Jun 3'UTR

A lot of studies underline the importance of the 3'UTR secondary structure in the RNA-RNA and protein-RNA interactions. In fact target site accessibility plays a fundamental role in both miRNA and RBP mRNA binding (*Uren et al, 2011; Keddle et al, 2010*).

We wondered whether mRNA structural modifications might be involved in the non-canonical action mediated by miR-200a on the c-JUN 3'UTR. First of all, we analyzed the predicted

secondary structure of the complete c-JUN 3'UTR, downloaded by UCSC genome browser website (http://genome.ucsc.edu). We observed that miR- 200b target site is predicted within a loop (Fig 13A, red box) while miR-200a binding site is into a stem and loop portion (Fig 13A, green box) so, it seems to be less available for the miRNA binding than miR-200b target site. Indeed, if we look at the c-Jun ARE sequence more in detail we can make further considerations.

We know that the 152nt-long c-JUN's ARE consists of three functional domains, as described by *Peng et al, 1996* (Fig 13B):

- domain I, which is composed by two subdomains, I.1 and I.2;

- domain II, which is the least AU-rich (A+U= 58%) among the three domains and is composed by two subdomains, II.3 and II.4;

- domain III, also named III.5, which is rich in GU and contains miR-200a binding site.

In Peng and colleagues works we found that HuR had a low afinity for c-Jun ARE sequence and it could be, partially explain by the domain III rearrangement. This domain was the richest in GU and was also reach in stem (Fig 6A) structure. Because of HuR low affinity for double strand RNA (dsRNA) sequences the c-Jun ARE secondary structure may, in part, explain the inefficient bond by HuR.

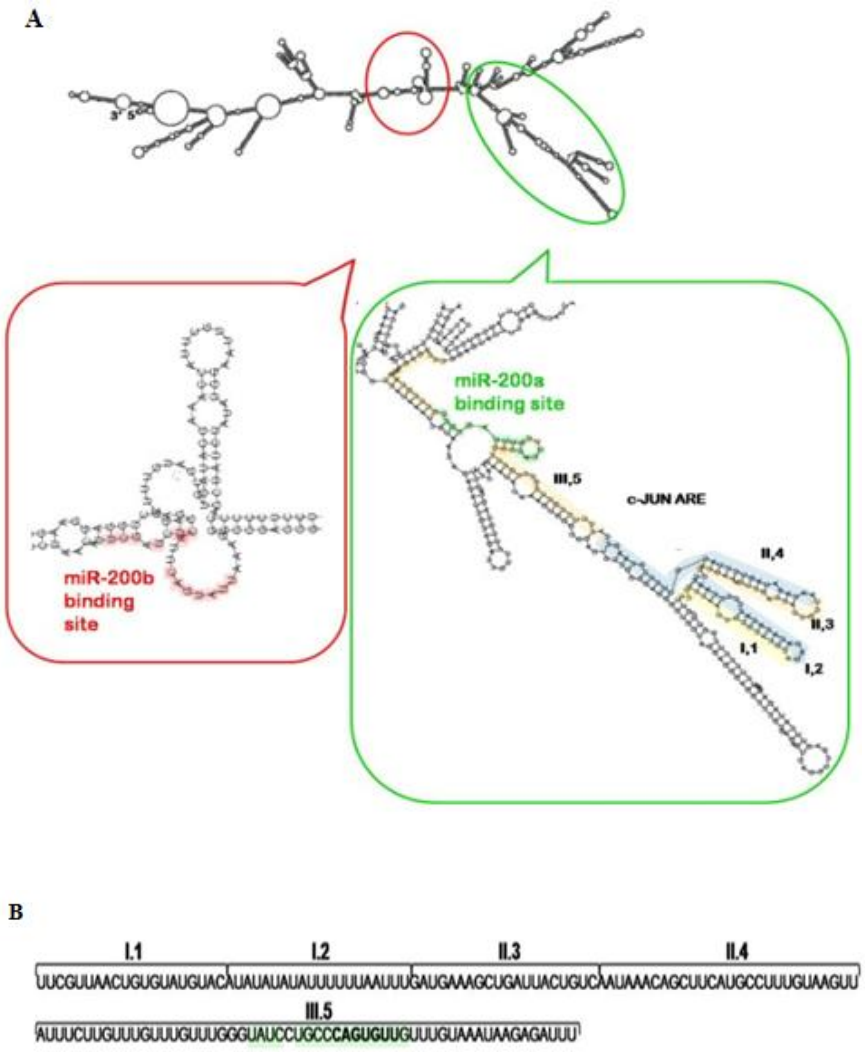
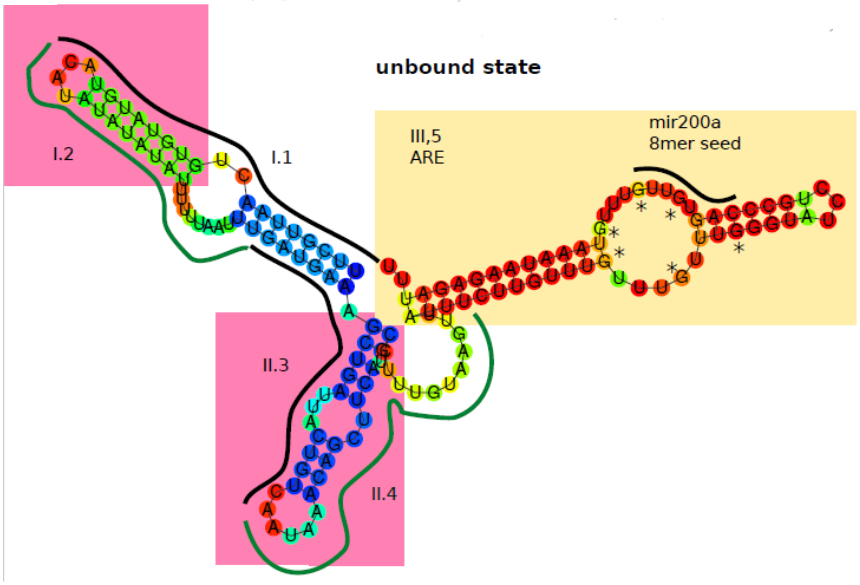


FIGURE 13 | (A) 2D prediction of the entire c-Jun 3'UTR available on UCSC (Energy= -386.00 kcal/mol). miR-200b binding region is zoomed into the red box. c-Jun ARE containing miR-200a binding site is into the green box. ARE domains and subdomains are reported and indicate alternatively in blue and yellow. **(B)** RNA sequence of the c-Jun ARE. miR-200a binding site is highlight in green.

In order to clarify the mechanism by which miR-200a could enhance the HuR affinity for c-Jun 3'UTR, we used RNA-fold algorithm to predict c-JUN ARE local conformation. In fact, local folding is more accurate than the global approach because a mRNA is usually regulated by a plethora of molecules and these can influence its global conformation (*Lange et al, 2012*). We noticed that the ARE domain III.5 could form a stem-loop-stem-loop structure with considerable base-pair probability (Fig 14A). miR-200a binding to the target sequence could promote the opening of the second stem, generating only one huger loop (Fig 14B). In this new rearrangement HuR could gain the access to c-Jun ARE. In contrast, the structure of I.1, I.2, II.3, II.4 domains didn't change upon miR-200a binding (Fig 14A, B). Taken together these predictions and previous experiments suggest that the binding of miR-200a might induce local changes in c-Jun 3'-UTR structure and favours association with HuR.

A


unbound state
I.2
I.1
III,5
ARE
mir200a
8mer seed
II.3
II.4

B

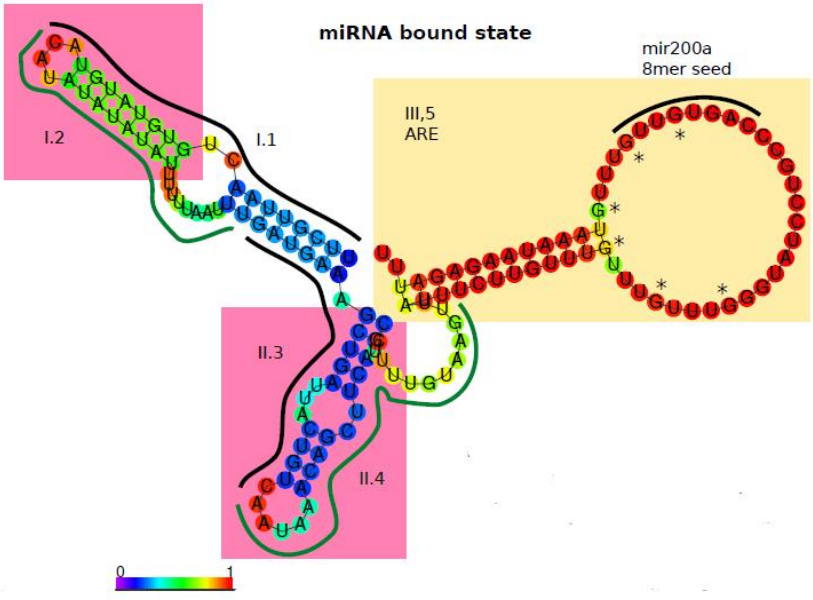

miRNA bound state
I.2
I.1
III,5
ARE
mir200a
8mer seed
II.3
II.4
0
1

FIGURE 14. (A) Schematic representation of the conformation of c-Jun ARE unbound **(B)** or bound by miR-200a, predicted using RNA-fold algorithm. Base-pair probability is reported in a color scale. Modifier letter up arrowheads (ˆ) 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 *Peng et al*; asterisks (*) indicate G->C mutations that increase mRNA stability as Vlasova et al, 2008 described.

2.2 The non-canonical effect mediated by miR-200a is not miRNA specific but is miRNA-binding site position specific

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 15A). 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 15B). With the 3'UTR MUT-INV we performed also a luciferase assay with a HuR siRNA and we observe 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 15C), as we previously observed for the c-Jun 3'UTR WT in presence of miR-200a (Fig 11A). These results suggest that the non-canonical effect is HuR dependent and it is due to the localization of microRNA binding site.

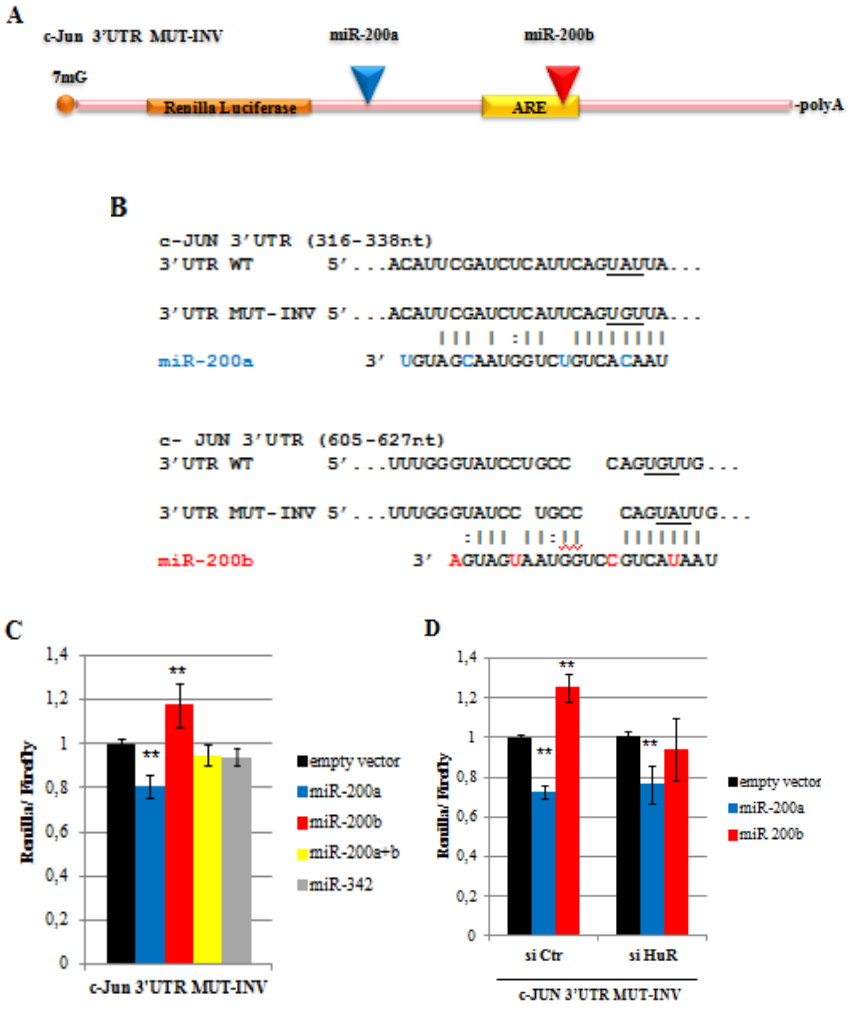


FIGURE 15. (A) Schematic of the Luciferase construct with c-Jun 3'UTR MUT-INV that contains a reciprocal exchange in the sequence bound by the seed region of miR-200a and miR-200b. The mutated nucleotides are underlined. **(B)** Schematic representation of alignment between c-Jun 3'UTR INV and the microRNA 200a or 200b. **(C)** 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 values of the corresponding empty vector were set to 1. **(D)** Luciferase assay performed with HEK 293T cells transfected with a HuR or a scramble siRNA reveals that also with the 3'UTR MUT-INV there was an involvement

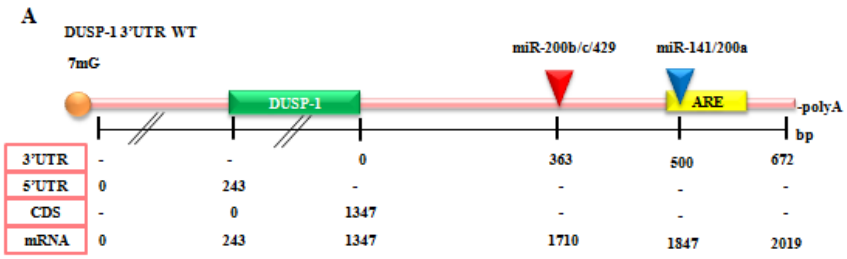
of HuR in the non canonical effect. The mean values of the corresponding empty vector were set to 1.
Data represented the mean ± SD and asterisks (*) indicate statistically significant modulations respect to empty vector according to paired Student's test * $p < 0.05$; ** $p < 0.01$.

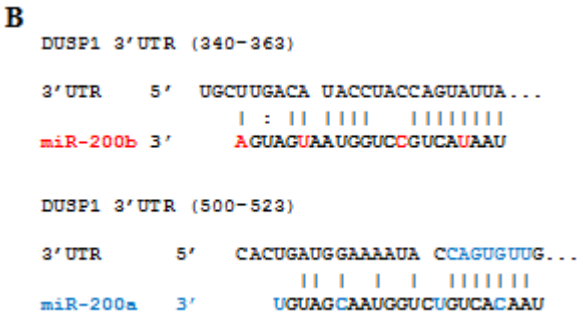
3. The non canonical regulation mediated by miR-200a involves also the DUSP1 mRNA.

Finally, we wondered whether this non canonical action of a microRNA could encompass other mRNAs. First of all, we looked at the mRNAs targeted by miR-200 family and we tried to found a mRNAs with a similar 3'UTR organization of c-Jun. We found that Dual Specificity Phosphatase 1 (DUSP1) mRNA 3'UTR contain an extended ARE *sequence* (*Kuwano et al, 2008*) and it had two binding site for miR-200 family members, one for miR-200a/141 and one for miR-200b/200c/429 and the miR-200a binding site was into the AU-rich element (Fig 16A, B) as we previously observed for c-Jun 3'UTR (fig 7B). DUSP1 is an inducible immediate early gene expressed in response to stressors like heat shock. Hypoxia, UV light and oxidants. The function of this phosphatases is to dephosphorylate and therefore inactivate the MAP kinases such as p38 and JNKs (*Franklin et al, 1997*). However, little is known about the posttranscriptional regulation of DUSP-1, despite the fact that it is an early response gene and, thus, is likely to be encoded by a short-lived mRNA (*Charles et al, 1992, Sun et al, 1993*).

To determine whether miR-200s could target the mRNA 3'UTR of DUSP1, we performed a luciferase assay, in HEK 293T cells, with the reporter construct carrying the DUSP1 3'UTR, cotransfected with the microRNA overexpression plasmids and we obtained the same non-canonical event. The overexpression of miR-200a increased the RLuc activity compare with the empty vector whereas miR-200b targeted the DUSP-1 mRNA and the

luciferase activity was repressed (Fig 16C). With a quantitative real time PCR we analyzed also the endogenous mRNA level of DUSP-1 and we found that with the overexpression of miR-200a the mRNA level of DUSP1 was enhanced compare to the empty vector but with miR-200b overexpression the DUSP1 mRNA level was decreased (Fig 16D).





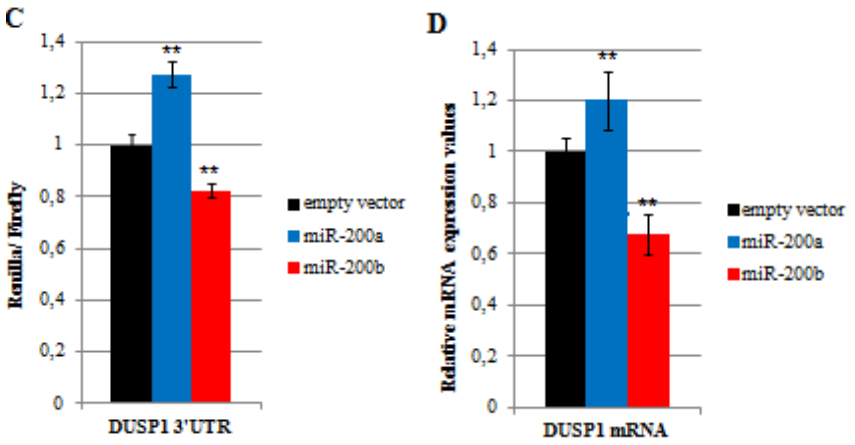


FIGURE 16. (A) Schematic of the DUSP1 3'UTR with the location of the predicted miR-200a/14 and miR-200b/c/429 target sites. **(B)** Schematic representation of alignment between DUSP1 3'UTR and the microRNA 200a or 200b. **(C)** Luciferase assay with HEK 293T co-transfected with reporter construct DUSP1 3'UTR and microRNA overexpression plasmids (miR-200a, miR-200b) showed an antithetical effect of the two microRNA, miR-200a enhances whereas miR-200b decreases the RLuc activity. **(D)** qRT-PCR of DUSP1 mRNA showed an opposite effect of the two microRNA , miR-200a and miR-200b, also on the expression level of endogenous DUSP1 mRNA, in HEK 293T cells. Data represented the mean $\pm$ SD (of two independent experiments in the right panel). In both experiments the mean values of the corresponding empty vector were set to 1. The asterisks (*) indicate statistically significant modulations respect to empty vector according to paired Student's test **$p < 0.01$.

These observations open up the possibility of identifying and characterizing a new regulatory mechanism mediated by HuR and microRNA.

DISCUSSION

There are five major steps for cancer development: initiation, promotion, malignant conversion, progression, and metastasis.
Many factors influence the development of cancers: some inhibit tumor development (tumor suppressors), and some promote cancer development (cancer inducers). The formation of cancer is the combined interaction of both tumor suppressors and cancer inducers (*Zhang et al, 2006*). In this already complicated scenario we have to consider also the role of microRNAs. miRNAs are major regulators of gene expression, with roles in nearly every area of cell behaviour, development and survival; therefore, it is not surprising that miRNAs are actively altered in all types of cancers.

miRNA dysregulation is mediated by genetic or epigenetic event and can potentially lead to cancer development. Genetic mechanisms are usually correlated with chromosomal abnormalities such as deletion, amplification, or translocation of miRNA sequences. In fact, greater than 50% of miRNA loci are at or near fragile genomic sites which are prone to breakage and rearrangement in cancer cells (*Calin et al, 2004*). For example, Dr. Croce' s group showed that miR-15a/16-1 cluster is frequently deleted in chronic lymphocytic leukemia (CLL), implicating these miRNAs as tumor suppressors. Alternatively, genomic amplification of oncogenic miR-21 was found in breast cancer (*Haverty et al, 2008*). Finally, also the epigenetic alterations can potentiate microRNAome changes. A number of miRNAs have been detected in the vicinity of CpG islands, and it has been found that the methylation status of these CpG islands may have a drastic influence on the expression of miRNAs (*Kourbash et al, 2010*).

Moreover for some microRNAs the role as oncomiRNA or tumor -suppressor miRNA is well defined, there are a number of

miRNAs that are overexpressed in one type of cancer and down-regulated in another, along them we have the miR-200 family.

On one hand, the overexpression of miR-200 family induces the suppression of Zeb1/2 transcription factors and inhibits the epithelial-to-mesenchymal transition (EMT), implicating it as a tumor suppressor (*Bracken et al, 2008; Burk et al, 2008; Park et al, 2008*). On the other hand, miR-200 family has been found to promote oncogenesis by promoting the reversal of EMT, the mesenchymal-to-epithelial transition, allowing invasive cells to revert back to a phenotype more conducive to metastatic colonization (*Dykxhoorn et al, 2009; Korpal et al, 2011*). In order to investigate deeply on the role of this family in tumor progression and metastasis formation we choose this family for further analysis. We found an interesting target for this miRNA family, the important proto-oncogene c-Jun.

In the experiments we present here, miR-200a and miR-200b regulate the production of c-Jun by opposite way. miR-200b is able to destabilize c-Jun, as expected from a targeting microRNA, but, surprisingly, mir-200a has a stabilizing effect (Fig 7). The miR-200b downregulating effect on c-Jun mRNA and protein level is sufficient, alone, to prevent the migration of MDA-MB-231 cells in a scratch test (Fig 9, 10). Otherwise, the increase of endogenous level of c-Jun, mediated by miR-200a, induce the AP1 transcription factor activity, resulting in increased level of mRNA transcriptionally regulated by AP1 (Fig 9).
About the non-canonical mechanism we observed, we noticed that the c-Jun's miR-200a binding site is into a well known ARE sequence (Peng et al, 1998) and we know that 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 we wondered whether the factors involved in the two processes may interact with each other.

Among the ARE binding proteins, HuR/Elav1 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 mRNA 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 (reviewed by *Simone and Keene, 2013*). Still, there does not appear to be a single general mechanism by which HuR antagonizes miRNPs: for example, HuR has been demonstrated 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 (reviewed by *van Kouwenhove and Agami*) 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 a specific proteins. Most of the evidences come 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 non-canonical, 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 order to verify the involvement of HuR in the non-canonical mechanism we observed, we perform a luciferase assay in HEK 293 cells upon HuR depletion. The results show that the non-canonical event mediated by miR-200a depends on HuR presence, indeed, with a RNA Immunoprecipitation experiment, we can

appreciate an increase in the amount of c-Jun mRNA bounded by HuR in presence of miR-200a (Fig 11).
In our experiment we find out also that the non-canonical effect we observed 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. Infact, when mir-200a site on the 3'UTR is mutated to recognize mir-200b, this microRNA is now able to stabilize the mRNA (Fig 15). Remarkably, the binding site of mir-200a is located inside the ARE recognized by HuR, so the action of the micro-RNA 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 14A, B). At this regard, 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 (*Pen 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 (*Sharma et al, 2013*) indicate that the presence of a human microRNA, miR3134, can affect the binding of HuR on a subset of human ARE bearing transcripts including vegf, sox9 and EGFR. 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 co-factors). In the case of 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 (*Bracken et al, 2014*) performed a genome wide analysis of mRNAs targeted by mir-200 family

members in different cell lines. 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 into 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. The mechanism we described adds new knowledge about the mechanisms of post-transcriptional regulation mediated by competition or cooperation between microRNA and RBP (Fig 17). Finally, this coordinated process open new insights on the contribution of these factors to development, differentiation and carcinogenesis.

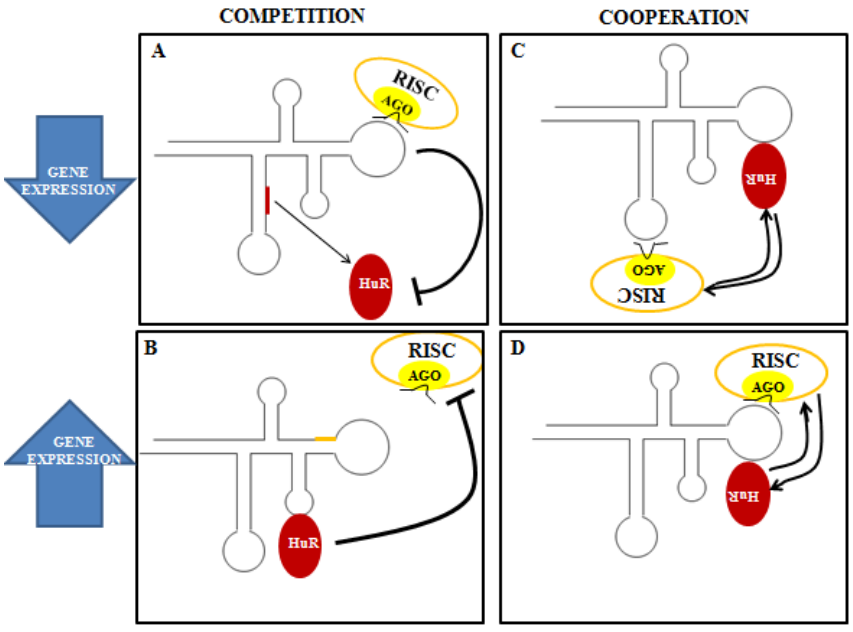


FIGURE 17 | Schematic of all competitive/cooperative interactions between HuR and microRNAs on shared target mRNAs. (A) In the 'competition' model, binding of miRNA-RISC may trigger a conformational change that hides the site of HuR binding to the mRNA leading to a lower expression of the target mRNA; **(B)** conversely, HuR binding may trigger changes in RNA structure that make inaccesible the site of interaction with miRNA-RISC, causing increased expression of the mRNA. **(C)** in the 'cooperation' model, binding of microRNA-RISC may trigger conformational changes that allow binding of HuR (or vice-versa) resulting in repression of the mRNA **(D)** or resulting in induction of mRNA expression.

MATERIAL AND METHODS

Cell Culture – All cell lines (HEK 293T, HeLa, MCF7, MDA-MB-231) were coultered 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 overexpressing 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 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, subsequently, were used as templates for PCR to generate complete mutated 3'UTRs. They were, finally, cloned as described for wild type. Specifically, MUT-A and MUT-B 3'UTRs were generated by replacement of three nucleotides of JUN 3'UTR pairing from the 2^{nd} to the 4^{th} 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 JUN 3'UTR (Fig. 3A). The compensatory seed mutation (ACA->GAC) in miR-200a overexpression 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 the table.

miR-200a fw	GGAAGATCTTATTGCGATGCATATACGGTCTC
miR-200a rev	ATTCTCGAGCTGTAGAGCTGAGACAGGCCCT
miR-200b fw	GGAAGATCTAATGCTCTGGGGATTAGGACAC
miR-200b rev	ATTCTCGAGTGCCCCATAGCCCTACCTT
c-Jun 3'UTR fw	ATTCTCGAGTGAGAACTTGACAAGTTGCGAC
c-Jun 3'UTR rev	ATTCTCGAG GATTCCAAATTGGAAGGATACATC
DUSP1 3'UTR fw	TATCTCGAGGAGTTCCTCTGGGTTTCTAAGCA
DUSP1 3'UTR rev	TATGCGGCCGCCAGCAAACATACAACTGTTGG
c-Jun 3'UTR MUT-B fw	ACCTAACATTCGAATCTCATTCAGGTCTAAAGGG
c-Jun 3'UTR MUT-B rev	CCCTTTAGACCTGAATGAGATCGAATGTTAGGT
c-Jun 3'UTR MUT-A fw	GGGTATCCTGCCCAGGTCTGTTTGTAAATAAGA
c-Jun 3'UTR MUT-A rev	TCTTATTTACAAACAGACCTGGGCAGGATACCC
c-Jun 3'UTR MUT-INV miR-200a fw	GATTTGGAGCACTCTGAGTTTACCATTTGTAAT
c-Jun 3'UTR MUT-INV miR-200a rev	TCTTATTTACAAACAATACTGGGCAGGATACCC
c-Jun 3'UTR MUT-INV miR-200b fw	ACCTAACATTCGATCTCATTCAGTGTTAAAGGG

c-Jun 3'UTR MUT-INV miR-200b rev	CCATGCAGTTCTTGGTCAATGTTAACGAA
miR-200a–comp-mut fw	CTTGACTCTAGACCTGTCTGGTAACGATGTT
miR-200a–comp-mut rev	CCAAGAAATCCAGCACTGTCCGGTA

Transfection and Reporter Assays – For reporter assays 1 X 10^5 HEK 293T cells or 8 X 10^4 HeLa cells were plated in 24- well plates and co-transfected with 100 ng of c-Jun 3'UTR reporter plasmid (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 overexpression plasmid using Lipofectamine 2000 (Invitrogen). 24 h after transfection, cells were lysed in Passive Lysis Buffer (Promega), and 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 activites (avereged ratios of RLuc/FLuc) and combained from at least three separate experiments. For siRNA experiments, cells were transfected with 30nM control or HuR (Dharmacon) siRNAs and in some cases after 24 h the cells were also cotransfected with reporter construct and microRNA overexpression plasmid, as previously described. Cells were harvested 48h later 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, cells were lysed and processed as previously described.

Quantitative Real-Time PCR **-** Total RNA was isolated using 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 un

paired Student's t-test. 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. Protein were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membrane (Whatman, Scheleicher & Schuell, Springfield Mill, Maidstone, Kent, UK). The immunoblots were incubated with the following antibodies: anti-actin (A2066, Sigma Aldrich), anti-c-Jun (ab31415, Abcam). The densitometric analysis was performed using Image Lab software (Bio-Rad).

Wound healing assay – 5 X 10^5 MDA MB 231 cells were plated in the uncoated μ-slide 2 well (ibidi) and transfected with 2μg of miR-200a or miR-200b overexpression plasmid or with 30nM of c-Jun (Dharmacon) siRNAs, using Lipofectamine 2000 (Invitrogen). 24h after transfection the cell monolayer was scraped in a straight line with a p200 pipet tip. The time-lapse immaging of living cells were acquired with a Nikon Eclipse Ti inverted microscope hourly for 24h.

RNA IP assays **-** RIP was performed by incubating 20 mg 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 hr at 4° C. HEK 293T cytoplasmatic lysates were prepared from cells cultured in complete medium for 24 h, after microRNA overexpression plasmid transfection, with 100 ml of

passive lysis buffer (PLB). 200 mg of each lysate was used for each RIP assay. Samples were precleared for 1 hr 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 hr at 4° C. Beads were washed with NT2 buffer five times and split for protein (1/3) and RNA analysis (2/3). 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 weighting 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 152nt-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 really accurate for short sequences (max 150nt) and RNAplfold is the preferred method for long sequence 2D folding prediction. Thus, RNAplfold was used take the influence of the context sequence into account and to check the structure calculated with RNAfold. Both algorithms gave similar results.

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LIST OF PUBBLICATIONS

"The RNA-Binding protein HuR and the members of miR-200 family play an unconventional role in the regulation of c-Jun mRNA stability". Del Vecchio G, De Vito F, Saunders SJ, Risi A, Mannironi C, Bozzoni i, Presutti C. RNA. **Submitted**

MicroRNA-335-5p modulates spatial memory and hippocampal synaptic plasticity". Capitano F, Camon J, Licursi V, Ferretti V, Maggi L, Scianni M, Del Vecchio G, Mannironi C, Presutti C, Mele A. Learning and Memory. **Under revision**

Congress:

- "Individual variability of miRNAs in prefrontal cortex in an animal model of PTSD"

  Rajendra S, Dasani RR, Del Vecchio G, Presutti C, Mele A, Mannironi C, Rinaldi A

  EBBS & EBPS Meeting, September 12-15, 2015, Verona, Italy

- "miR-200 family and HuR play a new game in the regulation of the proto-oncogene c-Jun".

  Del Vecchio G, De Vito F, Risi A, Bozzoni I, Presutti C

  SIBBM From Genomes to Function, Turin, Italy. 1- 3 July 2015 .

- “miR-200 family and HuR play a new game in the regulation of the proto-oncogene c-Jun”.

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  20th Annual Meeting of RNA Society, May 26-31 2015, Madison, Wisconsin, USA.