MicroRNA-128-3p-mediated depletion of Drosha promotes lung cancer cell migration.

RUNNING TITLE: miR-128-3p determines widespread miRNAs down-regulation

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ABSTRACT

Alteration in microRNAs (miRNAs) expression is a frequent finding in human cancers. In particular, global miRNAs down-regulation is a hallmark of malignant transformation through the mediation of Drosha and Dicer two key enzymes of global miRNAs processing. In the present report. ~~.~~ we~~Here we~~ showed that the miR-128-3p, which is up-regulated in lung cancer tissues, has ~~targeted two key enzymes of global miRNA processing, such as~~ Drosha and Dicer as the main modulation targets~~.~~ allowing the ~~This led to a~~ global down-regulation of miRNA expression. We observed that the global downregulation induced by ~~Such~~ miR-128-3p ~~mediated effect~~ contributed to the tumorigenic properties to the lung cancer cells. In particular miR-128-3p-mediated miRNAs dowregulation contributed to aberrant SNAIL and ZEB1 expression thereby promoting the epithelial-to-mesenchymal transition (EMT) program. Moreover, Drosha resulted to be implicated in the control of migratory phenotype as its expression counteracted miR-128-3p functional effects. Our study provides mechanistic insights into the function of miR-128-3p as a key regulator of the malignant phenotype of lung cancer cells. This also enforces the remarkable impact of Drosha and Dicer alteration in cancer, and in particular it highlights a role for Drosha in NSCLC cells migration.

KEY WORDS: miR-128-3p, Drosha, Dicer, lung cancer

Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs regulating gene expression at the post-transcriptional level. MiRNAs are transcribed by RNA polymerase II as primary miRNA (pri-miRNA) and then processed into mature double-stranded miRNA by two major enzymes, Drosha and Dicer, which belong to the class of RNase III endonucleases. Mature miRNAs, through the RISC complex, can interact with the 3' untranslated region (UTR) of mRNA targets, causing translational repression or mRNA deadenylation, depending on affinity of the miRNA to its mRNA target (1).

MiRNAs are fine tuners of many biological processes, due to the multiplicity of mRNA targets for each miRNA (2). Altered miRNA biosynthesis has been associated with the occurrence of several diseases, among which cancer (3). In detail, deregulation of miRNAs expression was shown to promote cell proliferation, metastasis and chemoresistance (3). A large body of evidences suggests

that a global reduction of miRNAs is a prevalent feature of human cancers (4-7). Deregulation of key components of the mRNA machinery can impinge on the global miRNAs down-regulation observed in cancer tissues (4,8). This is strongly supported by clinical correlative observations, which suggest prognostic value for altered levels of miRNA processors (9-12). In detail, Drosha and Dicer abrogation in cancer was ascribed to occurrence of somatic missense mutations, or deletions, or to transcriptional repression at the promoter level (13-15). More recently, evidences emerged pointing to a miRNA-mediated post-transcriptional modulation of Dicer by some miRNAs resulting into oncogenic features (16-18).

MiR-128-3p is an intronic miRNA, that can be encoded both by miR-128-1 gene, located on human chromosome 2q21.3 into R3HDM1 gene, and by miR-128-2 gene, located on chromosome 3p22.3 into ARPP-21 gene. Altered expression of miR-128 gene was reported in several types of human cancers, implying an important role in tumorigenesis (19). Its functions range from pro-tumorigenic to tumor suppressive, depending on the tissue analysed. In line with this, we have demonstrated that miR-128-3p is induced by mutant p53, contributing to mutant-p53-mediated chemoresistance in non-small-cell lung cancer (NSCLC), indicating an oncogenic role of miR-128-3p in lung cancer (20).

In this study we demonstrated a direct binding and inhibitory effect of miR-128-3p on Drosha and Dicer 3'UTRs, causing a global down-regulation of miRNAs expression in NSCLC cells. Ectopic expression of the miR-128-3p reduced the levels of miRNAs targeting key EMT factors. This, ultimately, stimulated the invasive properties of the transfected cells. Moreover, reintroduction of Drosha in such a cellular context, determined a reversion of the migratory phenotype, suggesting a significant role of Drosha in the control of lung cancer cells migration.

Collectively, these findings suggest that miR-128-3p-mediated ablation of Drosha and Dicer expression might contribute to the acquisition of a malignant phenotype of lung cancer cells by indirectly altering the levels of multiple effector miRNAs.

Materials and methods

Cell cultures and treatments

Human cell lines H1299 and A459 were grown in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS; all cell lines were grown at 37 °C in a balanced air humidified incubator with 5% CO2. All fresh cell lines were purchased from ATCC that has authenticated them by STR genotyping with Promega PowerPlex® 1.2 system and the Applied Biosystems Genotyper 2.0 software for analysis of the amplicons. The cells were maintained in culture no more than six passages. All the cell lines have been tested by PCR/IF for Mycoplasma presence.

Plasmids and transfections

For mature miR-128-3p expression, we used mirVana™ miRNA Mimic Negative Control #1 (Ambion) or hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion) at final concentration of 5nM. For miR-128-3p depletion we used mirVana™ miRNA Inhibitor Negative Control #1 or hsa-miR-128-3p mirVana™ miRNA Inhibitor (Ambion) at final concentration of 10nM. miR-128-3p expression was also abrogated using a lentiviral vector named TWEEN 3'-UTR (decoy vector), enclosing a multicloning site in the 3'-UTR of an GFP reporter gene, where we inserted two antisense sequences for miR-128-3p (decoy-miR-128-3p vector). H1299 and A459 cells were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. For Luciferase assay H1299 cells were co-transfected in 24-well dishes using Lipofectamine 2000 (Invitrogen) with 100ng of DROSHA-3'-UTR (wt and mutant)-Luciferase vectors (psiCHECK-2, Promega), 100ng of poli II vector and 20nM mirVana™ miRNA Mimic Negative Control #1 (Ambion) or hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion). Moreover, H1299 cells were co-transfected in 24-well dishes using Lipofectamine 2000 (Invitrogen) with 300ng of DICER-3'-UTR-(wt and mutant)-Luciferase vectors (a kind gift of Dr. Stefano Piccolo), and 20nM mirVana™ miRNA Mimic Negative Control #1 (Ambion) or hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion). 30ng of the transfection control Renilla vector (phRLTK, Promega) was used to normalize the firefly luciferase. Cells were harvested 48 hours post transfection and luciferase activities were analyzed by the dual-luciferase reporter assay system (Promega, Madison, WI) in the GloMax 96 Microplate Luminometer (Promega). Each sample was transfected in duplicate. Each experiment was repeated in triplicate. Drosha and Dicer mutants were made with the QuikChange site-directed mutagenesis kit (Stratagene) using the following primers:

- DROSHA mut a40c_ t42g

FW 5'-CATGCAAGTGTGGAGTATTTACTTGCTCAGTACAGGTGACTGTTGTCTATTG-3'

RV 5'-CAATAGACAACAGTCACCTGTACTGAGCAAGTAAATACTCCACACTTGCATG -3'

- DICER mut del 640-642

FW 5'-TGTCTTTTCTTTCCACGTTATATGTAAGGTGATGTTCCCG-3'

RV 5'-CGGGAACATCACCTTACATATAACGTGGAAAGAAAAGACA -3'

For siRNA experiments, H1299 and A459 were transfected with siSCR (5'-CUAUAACGGCGCUCGAUAU-3'), as a control or siDROSHA (5'-AACGAGUAGGCUUCGUGACUU-3') at 0.1uM for 48 or 72 hours.

For rescue experiments, H1299 cells were cotransfected with 500 ng of pcDNA3 (EV) or pcDNA3-DROSHA expression plasmid and 5nM of hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion) at the indicate time points.

RNA extraction, labelling and microarray hybridization.

RNA from FFPE samples was extracted using the miRneasy FFPE kit (QIAGEN) following the manufacturer's instructions. The concentration and purity of total RNA were assessed using a Nanodrop TM 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Total RNA (100ng) was labelled and hybridized to Human miRNA Microarray Rel 14 V2 (Agilent). Scanning and image analysis were performed using the Agilent DNA Microarray Scanner (P/N G2565BA) equipped with extended dynamic range (XDR) software according to the Agilent miRNA Microarray System with miRNA Complete Labeling and Hyb Kit Protocol manual. Feature Extraction Software (Version 10.5) was used for data extraction from raw microarray image files using the miRNA_105_Dec08 FE protocol.

Total RNA extraction from cells and reverse transcriptase.

Total RNA was extracted using the TRIZOL Reagents (GIBCO). One microgram of total RNA was reverse-transcribed at 37°C for 60 minutes in the presence of random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR analyses were carried out using oligonucleotides specific for the genes listed in Supplementary Table S3. Gene expressions were measured by real-time PCR using the Syber Green assay (Applied Biosystems, Carlsbad, CA, USA) on a StepOne instrument (Applied Biosystems).

Small amount of RNA (40ng) was reverse-transcribed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystem) and Real time-PCR of miRNA expression was carried out in a final volume of 10ul using ABI Prism 7000 Sequence Detection System (Applied Biosystems). The PCR Reactions were initiated with a 10 minutes incubation at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. qRT-PCR quantification of miRNA expression was performed using TaqMan MicroRNA® Assays (Applied Biosystems) according to the manufacturer's protocol. RNU19 and RNU48 were used as endogenous control to normalize miRNA expression. All reactions were performed in duplicate.

Lysate preparation and immunoblotting analysis.

Cells were lysed in buffer with 50mM Tris-HCl pH 8, with 1% NP-40 (Igepal AC-630) 150mM NaCl, 5mM EDTA and fresh protease inhibitors. Extracts were sonicated for 10 seconds and

centrifuged at 12000 ×rpm for 10 minutes to remove cell debris. Protein concentrations were determined by colorimetric assay (Bio-Rad). Western blotting was performed using the following primary antibodies: mouse monoclonal anti-Gapdh (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-Drosha (Cell Signaling), rabbit polyclonal anti-Dicer (Santa Cruz Biotechnology), rabbit monoclonal anti-Zeb1 (Cell Signaling), rabbit monoclonal anti-Snail (Cell Signaling) and mouse monoclonal anti B-actin (Santa Cruz Biotechnology sc-81178). Secondary antibodies used were goat anti-mouse and goat anti-rabbit, conjugated to horseradish peroxidase (Amersham Biosciences,Piscataway, NJ, USA). Immunostained bands were detected by chemiluminescent method (Pierce, Rockford, IL, USA).

Transwell migration assay.

Migration assay was performed using a 24-well plate with a non-coated 8-mm pore size filter in the insert chamber (Falcon). Cells were transfected with mirVana™ miRNA Mimic Negative Control #1 (Ambion) or hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion), for miR-128-3p depletion we used mirVana™ miRNA Inhibitor Negative Control #1 or hsa-miR-128-3p mirVana™ miRNA Inhibitor (Ambion) at final concentration of 10nM. For Drosha depletion, we used 0.1uM siSCR or siDrosha and for rescue of Drosha, we cotransfected 500 ng of pcDNA3 (EV) or pcDNA3-DROSHA expression plasmid and 5nM of hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion).

After 48 or 72 hours from transfection, cells were resuspended in RPMI media without FBS and seeded into the insert chamber. Cells were allowed to migrate for 12 h into the bottom chamber containing 0,7 ml RPMI media containing 5% or 1% FBS in a humidified incubator at 37°C in 5% CO2. Migrated cells that attached to the outside of the filter were visualized by staining with DAPI and counted.

Wound healing assay.

H1299 and A459 cell lines transfected with mirVana™ miRNA Mimic Negative Control #1 (Ambion) or hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion) or with mirVana™ miRNA Inhibitor Negative Control #1 or hsa-miR-128-3p mirVana™ miRNA Inhibitor (Ambion), or with siSCR or siDrosha, were grown to 80% confluence in 6-well tissue culture plates and wounded with a sterile 200ul pipet tip to remove cells by perpendicular linear scrapes. PBS 1x washing was used to remove loosely attached cells. The cells were incubated in full medium with 10% FBS for 24 h. The progression of migration was photographed immediately, at 24 h after wounding.

Scattering assays.

For scattering assay 1000 cells were seeded in six-well plates and allowed to settle. After 96h H1299 cells were transfected with mirVana™ miRNA Mimic Negative Control #1 (Ambion) or hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion). Following 48 h, it was captured phase-contrast images of scattered cells.

Immunofluorescence.

For immunofluorescence assay cells were transfected with mirVana™ miRNA Mimic Negative Control #1 (Ambion) or hsa-miR-128-3p mirVana™ miRNA Mimic (Ambion) or with siSCR or siDrosha. 72h after transfection cells were washed twice with PBS 1% and then were fixed with 4% formaldehyde in PBS. After that cells were washed twice with PBS 1% and incubated for 5 min with 0.25% Triton and 5% BSA in PBS 1%. Then cells were washed with PBS 1% and incubated o.n. with rabbit monoclonal N-cad (Santa Cruz Biotechnology) 1:400 in 0.25% Triton and 1% BSA in PBS 1%. The day after cells were washed three times with PBS 1% and followed by incubation with Alexa Flour 488 (rabbit) conjugated secondary antibodies (Molecular Probes Inc., Eugene, OR, USA) for 2 hours at RT. After washing three times with 0.02% Tween-20 and 1% BSA in PBS 1%, the coverslips were counterstained with DAPI 5 min and mounted with Vectashield (Vector Labs,Burlingame, CA, USA). Cells were examined under a Zeiss LSM 510 laser scanning fluorescence confocal microscope (Zeiss, Wetzlar, Germany).

EnSpire® cellular label-free platform.

H1299 cells were seeded in specially designed 384-well plate with highly precise optical sensors able to measure changes in light refraction resulting from dynamic mass redistribution (DMR) within the cell's monolayer. Change in the light refraction was indicated by a shift in wavelength.

Microarray data analysis.

Arrays were verified for quality control and extracted by Agilent Feature Extraction 10.7.3.1 software and entirely processed by MATLAB (The MathWorks Inc.) in house-built routines. All values lower than 1 were considered below detection and threshold to 1. The arrays were normalized by dividing by the mean intensity only using the 25th and 75th percentile range of the data, preventing large outliers from skewing the normalization. Data were log2-trasformed.

Deregulated miRNAs were established by permutation test and a false discovery procedure (Storey, 2002) used for multiple comparisons. Unsupervised hierarchical clustering was performed to individuate specific pattern of expression among samples and clusters of miRNAs.

Significance was defined at the $p<0.05$ level.

In silico miRNA targets identification.

Several prediction target tools were interrogated by using the web server tool MirWalk2 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/).

Bioinformatic analysis of TCGA dataset.

As validation set we used TCGA lung adenocarcinoma (TCGA Research Network: http://cancergenome.nih.gov/."). MiRNA deregulation was assessed by paired or unpaired Student's t-test. Significance was defined at the $p<0.05$ level.

Kaplan–Meier analysis.

For survival analysis of specific genes we used Kaplan-Meier plotter (http://kmplot.com/analysis/). Local recurrence-free survival (RFS) was evaluated by using Kaplan-Meier analysis and Cox proportional hazard regression model. Intensity levels of tumoral samples were z-score transformed and survival analysis was conducted by using those samples with absolute z-score higher than 0.5. The log-rank test was used to evaluate differences between curves. Significance was defined at the $p<0.05$ level.

Results

MiR-128-3p expression is up-regulated in NSCLC tissues.

We have previously shown that miR-128-3p was a transcriptional target of gain of function mutant-p53 proteins in lung cancer cells (20). To further investigate the oncogenic potential of miR-128-3p we evaluated the expression levels of miR-128-3p in a large lung adenocarcinoma patient cohort from The Cancer Genome Atlas (TCGA) database including data from XXX specimens. Interestingly, miR-128-3p resulted to be significantly up-regulated in 506 tumoral tissues when compared to 46 non-tumoral lung tissues (Figure 1A). This was independent of the tumor stage (Supplementary Figure S1). MiR-128-3p expression resulted to be significantly associated with TP53 status, exhibiting higher expression in tumor samples carrying TP53 mutations (Figure 1B). The analysis of recurrence-free survival of TCGA lung cancer patients showed a positive trend between high levels of miR-128-3p and probability of recurrence (Figure 1C). Altogether these data mirror an oncogenic role of miR-128-3p in lung cancer.

MiR-128-3p expression triggers a global down-regulation of miRNAs.

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The oncogenic properties of a miRNA may derive from altering miRNA homeostasis by targeting key processing enzymes. We explored whether such a mechanism may act in miR-128-3p-expressing NSCLC cells. We found that ectopic expression of miR-128-3p caused a pronounced global down-regulation of miRNAs in both H1299 and A459 cells (Figure 2A-B, Supplementary Figure S2A-B and Supplementary Table S1-S2). In detail, in H1299 cells, 106 out of the 247 expressed miRNAs were significantly down-regulated as compared to control cells (Figure 2A and Supplementary Figure S2A). Similarly, in A459 cells 151 out of the 254 expressed miRNAs were significantly down-regulated (Figure 2B and Supplementary Figure S2B). These findings accounted for a remarkable effect of miR-128-3p on global miRNAs expression. To support this observation, we analysed, by qRT-PCR, the expression levels of a set of representative miRNAs upon over-expression or depletion of miR-128-3p in H1299 and A549 cells (Figure 2C and Supplementary Figure S2C-D). This confirmed a widespread miRNAs down-regulation induced by miR-128-3p which was released by depleting the latter, thus enforcing a strong correlation between the miR-128-3p levels and those of multiple cognate miRNAs (Figure 2C and Supplementary Figure S2C-D). Additionally, we observed an accumulation of primary miRNAs (pri-miRs) in miR-128-3p over-expressing cells, suggesting a specific effect of miR-128-3p on miRNAs maturation steps rather than on miRNAs transcription (Figure 2D).

To rule out the possibility that the down-regulation of miRNAs observed could be ascribed to aspecific effects of the miR-128-3p agonist molecule, we transfected unrelated agonist molecules and we found this not to be the case (Figure 2E).

Collectively these findings suggested that the oncogenic activity of miR-128-3p in lung cancer might occur through a widespread miRNAs down-regulation.

The miRNA processing enzymes Drosha and Dicer are novel targets of miR-128-3p.

The unexpected global effect on the miRNA levels in miR-128-3p-expressing cells prompted us to investigate whether this may happen through targeting of miRNA processing factors. Intriguingly, *in silico* analysis revealed that, among the putative targets of miR-128-3p, Drosha (RNASEN) and Dicer were scoring high.

In agreement with the mentioned altered expression of Drosha and Dicer in many unrelated tumors and with their prognostic value (10-12), we found a significant positive correlation between high levels of Drosha and Dicer and overall survival in a cohort of 720 patients affected by lung adenocarcinoma (Figure 3A-B).

To assess if miR-128-3p was effectively able to bind Drosha and Dicer 3'UTRs, we used luciferase reporter constructs with the full-length 3'UTR of Drosha or Dicer, wild-type or mutated in miR-

128-3p-binding sites (Figure 3C). The activity of these reporters was evaluated in H1299 cells transiently transfected with miR-128-3p mimic or control mimic. As shown in Figure 3c, miR-128-3p significantly reduced the relative luciferase activity of the wild-type reporters but not that of mutant reporters.

Next, we evaluated the protein expression levels of Drosha and Dicer in NSCLC cell lines transiently transfected with the miR-128-3p agonist, and in H1299 cells depleted for miR-128-3p by means of an inhibitor molecule or a decoy vector (Figure 3D and Supplementary Figure S3). As shown in Figure 3D and in Supplementary Figure S2B, miR-128-3p ectopic expression reduced the protein levels of Drosha and Dicer, and, conversely, its depletion determined increased Drosha and Dicer protein levels.

These findings indicated a direct inhibitory effect of miR-128-3p on Drosha and Dicer expression. Moreover, Drosha or Dicer depletion in H1299 cells mimicked the effect of increasing the miR-128-3p on mature miRNAs expression (Figure 3E-F). This strongly supported that miR-128-3p-mediated down-regulation of miRNAs was a consequence of its inhibitory effect on Drosha and Dicer translation.

Ectopic expression of miR-128-3p promotes migratory phenotype in lung cancer cells.

To assess the functional relevance of the observed phenomena, we performed functional assays in NSCLC cells transfected with a miR-128-3p mimic, miR-128-3p inhibitor or decoy vector.

Firstly, we did not witness effects of miR-128-3p expression on clonogenicity or cell cycle progression (Supplementary Figure S4A-D).

On the other hand, by performing both trans-well and wound-healing migration assays in H1299 and A459 cells, we found that miR-128-3p-expressing cells were hypermigratory (Figure 4A-B and Supplementary Figure S5A-C). Conversely, miR-128-3p depletion strongly discouraged the migration of H1299 cells (Figure 4A-B and Supplementary Figure S5D).

Moreover, in NSCLC cells expressing ectopic miR-128-3p, we observed a striking change in cell morphology, consisting of a shift from a cobblestone shape, typical of epithelial phenotype, into a spindle-fibroblast-like morphology, with extensive cellular scattering (Figure 4C and Supplementary Figure S5E). Such a changes were quantitatively addressed by means of label-free assays and were not due to any alteration in cells viability (Figure 4D and Supplementary Figure S5F). Morphological changes were not detectable in H1299 cells depleted for miR-128-3p (Supplementary Figure S5G-H).

MiR-128-3p-mediated miRNAs down-regulation promotes EMT program.

EMT is an early and key step in the metastatic cascade in which epithelial cells acquire the motile and invasive characteristics of mesenchymal cells through a process involving cytoskeleton remodelling and cell morphologic changes (21). The observed morphological changes in NSCLC cells expressing ectopic miR-128-3p prompted us to study whether the molecular alterations typical of the EMT occurred in miR-128-expressing cells and could explain the effects of such a miRNA on cell migration.

Firstly, we found that the expression levels of two key transcription factors, SNAIL and ZEB1 (21) were significantly increased in H1299 cells upon miR-128-3p over-expression (Figure 5A). On the contrary, miR-128-3p depletion in H1299 cells determined a significant reduction of both SNAIL and ZEB1 mRNA and protein levels (Figure 5A). Moreover, we observed that miR-128-3p expression in H1299 cells determined a significant increase in the membrane localization of N-Cadherin protein, a typical feature of mesenchymal cells (Figure 5B and Supplementary Figure S6). As a further readout of the up-regulation of SNAIL and ZEB1 by miR-128-3p we observed increased mRNA levels of MMP9, a final effectors of the EMT-associated invasive phenotype (Figure 5C).

To explain the induction of SNAIL and ZEB1 expression by miR-128-3p, we analyzed the expression levels of a set of miRNAs previously reported to target SNAIL (miR-30b, miR-30e, miR-137) and ZEB1 (miR-96, miR-130b, miR-192) (22-24). As shown in Figure 5D, upon miR-128-3p ectopic expression, there was a significant decrease in the expression levels of all of the anti-EMT miRNAs that inversely correlated with SNAIL and ZEB1 induction.

In support of a key role for miR-128-3p in determining metastatic potential of lung cancer cells, we also had an *in vivo* evidence: in a casuistry of 13 primitive lung cancer tissues and their matched brain metastasis, that we previously profiled for miRNAs expression, we observed a significant up-regulation of miR-128-3p in brain metastases compared to primary lesions (Fig 5E) (25).

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All these findings indicated that miR-128-3p-mediated tumorigenic phenotype of lung cancer cells was in part sustained by Drosha and Dicer depletion and consequently by global miRNAs inhibition. In particular, miR-128-3p promoted epithelial plasticity through the regulation of mesenchymal genes expression, in part by inhibiting anti-EMT miRNAs.

Drosha depletion in lung cancer cells mimics miR-128-3p effects on cell migration and EMT.

Due to the established direct effect of miR-128-3p on Drosha and Dicer expression and consequently on global miRNAs expression, that in part promote a migratory phenotype, we further investigated the possible implication of Drosha and Dicer depletion in lung cancer cell migration and EMT promotion.

By performing migration assays in lung cancer cells depleted for Drosha or Dicer we observed a significant effect, at similar extent of miR-128-3p over-expression, only with Drosha silencing rather than Dicer depletion (Figure 6A-B and Supplementary Figure S7A-B). These data indicated an implication only for Drosha, rather than Dicer, in miR-128-3p-mediated migratory phenotype. In support of these results, ectopic expression of Drosha in miR-128-3p-expressing H1299 cells rescued the migratory phenotype (Figure 6C).

Looking at changes in cell morphology, in support to a mesenchymal phenotype, we observed a significant effect in H1299 cells depleted for Drosha (Figure 6D). Such a changes were quantitatively addressed by means of label-free assays and were not due to any alteration in cells viability (Figure 6D and Supplementary Figure S7C). Moreover, these morphological changes were rescued in miR-128-3p-expressing H1299 cells upon ectopic expression of Drosha (Figure 6E and Supplementary Figure S7D). All these data suggested an implication of Drosha in miR-128-3p-mediated EMT.

To deeper investigate Drosha involvement in EMT we looked at the same EMT effectors modulated by miR-128-3p. In particular, in H1299 cells upon Drosha depletion, we observed a localization of N-cadherin in membrane and an increase in its protein levels (Supplementary Figure S7E). Moreover, Drosha depletion determined an increase in SNAIL and ZEB1 protein levels concomitantly with a reduction in the expression of miRNAs targeting SNAIL or ZEB1 (Figure 6F-G). As showed for miR-128-3p, the final readout of EMT promotion by Drosha depletion was in part represented by the increase in MMP9 transcriptional levels (Supplementary Figure S7F).

Discussion

This study provides the first evidence for the ability of a single miRNA, miR-128-3p, to modulate, concurrently, the expression of two major players of miRNAs biosynthesis, Drosha and Dicer. This elicited a global miRNAs down-regulation that conferred a more aggressive tumoral phenotype to lung cancer cells.

Drosha and Dicer down-regulation is a widespread phenomenon in cancer and it is closely correlated to poor patients outcome (7,10,26-28). Notably, Karube and colleagues found that down-regulation of Dicer expression correlated with decreased post-operative survival in lung cancer patients (12). Moreover, Drosha and Dicer have been demonstrated to be positive prognostic factors in NSCLC patients with normal performance status (11). In agreement with these evidences, we observed ~~, by means of an on-line survival analysis software,~~ a significant positive correlation between high levels of Drosha and Dicer and overall survival in a cohort of 720 patients affected by lung adenocarcinoma (29).

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Understanding the molecular processes that determined Drosha or Dicer depletion in cancer is an emerging open field, due to their deep impact on global miRNAs expression. Despite some evidence for the occurrence of specific somatic mutations in Drosha and Dicer genes as determinants of their down-regulation in cancer (15,27,30,31), a post-transcriptional regulatory mechanism mediated by miRNAs that directly impinges on Dicer expression has been proposed (16-18,32). In detail, Martello et al., identified miR-103 and miR-107 as negative regulators of Dicer that consequently determined a global miRNAs down-regulation, promoting a higher metastatic potential of breast cancer cells (16). FurthermoreMoreover, Dicer has been reported to be a direct target of miR-630 and let-7 (17,18). More recently Sha et al., demonstrated that miR-18a upregulation decreases Dicer at mRNA and protein levels and conferred paclitaxel resistance in triple negative breast cancer (32). Till now, nothing was known about miRNAs-mediated regulation of Drosha.

Here we showed that miR-128-3p up-regulation is functionally relevant, in that it elicited a global miRNA down-regulation mediated by direct inhibitory effect on both Drosha and Dicer 3'UTRs.

It is well established that global down-regulation of miRNAs expression, caused by depletion of Drosha and Dicer, is a key determinant of the tumoral phenotype and it was observed in different cancers (6,7,16,26). For example, Chen and collaborators demonstrated that Dicer depletion in NSCLC cells, and the consequent global miRNA down-regulation, mainly impinged on tumor angiogenesis (6). Martello et al., reported that in breast cancer, miRNAs down-regulation, determined by Dicer depletion, mainly affected EMT driven metastatic pathways (16). It is evident that not all the pathways involved in tumorigenesis are affected by such a global miRNAs down-regulation at similar extent, probably for the occurrence of a sort of balance between antagonist miRNAs.

In our proposed model, up-regulation of the miR-128-3p could initiate a signaling cascade that leads to global miRNAs down-regulation and consequently to acquisition of pro-tumorigenic properties (Figure 7). In such a context, and in strict agreement with our previous observations, we found that Drosha depletion by siRNA mimicked the effects of miR-128-3p overexpression suggesting, again, a close correlation between the miR-128-3p mediated miRNAs down-regulation and the impact on cell motility. In particular, Drosha depletion seemed to be implicated in the promotion of a migratory phenotype in NSCLC.

In a previous study Hu J. et al, demonstrated a tumor suppressor activity of miR-128 gene in NSCLC cells (33). In particular they evaluated the functional effects of miR-128 precursor, by using a construct encoding for both miR-128-3p and its complementary miR (ref). On a different note, by using a specific mimic molecule, we exclusively evaluated the activity of mature miR-

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128-3p, whose effect on the motility of NSCLC were unprecedented.

We have previously demonstrated that miR-128-3p was a transcriptional target of the oncogenic mutant-p53 protein, and that its expression contributed to mutant-p53 gain of function through the promotion of chemoresistance in lung cancer (20). Additionally, in agreement with our previously reported data, we observed a significant correlation between miR-128-3p expression and TP53 mutations in lung cancer. By analysing miR-128-3p expression levels in lung cancer data sets from the TCGA consortium, we observed an up-regulation of miR-128-3p in tumor samples as compared to normal tissues was independent of the tumor stage. Here we speculate that keeping high levels of miR-128-3p might represent a cancer-specific adaptive mechanism to hijack the canonical miRNA biogenesis pathway, thereby leading to alternative mechanisms that generate functional oncogenic miRNAs (34-38). More recently, it has been demonstrated that intronic miRNAs can be processed by splicing enzymes avoiding Drosha and Dicer cleavage (34-38). It is possible that miR-128-3p, being located into ARPP-21 gene, is involved in this kind of mechanism.

This study further confirms how global miRNA down-regulation could be considered a hallmark of aggressive cancers. The identification of an oncogenic miRNA, such as miR-128-3p, that impinges simultaneously on the two major drivers of miRNAs biosynthesis, might pave the way for the development of novel targeted therapeutic strategies for lung cancer, especially for those patients exhibiting a global miRNAs down-regulation.

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FIGURE LEGENDS

Figure 1. MiR-128-3p expression in lung cancer casuistry.

A. MiR-128-3p expression levels distribution in normal lung tissues (N) and lung cancer samples (T) from TCGA casuistry. **B.** MiR-128-3p expression levels distribution accordingly to p53 protein status, wild type (wt) or mutant (mutP53), of lung cancer samples (T) and normal tissues (N) from TCGA casuistry. **C.** Kaplan-Meier curve of recurrence-free survival for lung adenocarcinoma patients from TCGA casuistry with miR-128-3p high (Z score >0.5; n=121) and miR-128-3p low (Z score <-0.5 ; n=128) expression.

Figure 2. Oncogenic miR-128-3p induces a global down-regulation of mature miRNAs in lung cancer cells.

A-B. Volcano plot of significance against the relative expression differences between the control- or miR-128-3p-mimics treated groups in H1299 (A) and A459 (B) lung cancer cells. Each dot represents one miRNAs that was filtered and had detectable expression upon miR-128-3p expression. The X-axis displays log2-transformed signal intensity differences between the control group and the miR-128-3p group; the Y-axis represents calculated p-values of statistical differences

between control and treated distributions. MiRNAs positioned in the left and right upper-lateral quadrants above red dot line represent significant down-regulation (↓) and up-regulation (↑), respectively. **C.** Heat Map relative to qRT-PCR analysis of 5 representative miRNAs of the identified signature (miR-16, miR-17, miR-28, miR-30a-3p miR-423) in three independent preparations of H1299 cells transiently transfected with miR-128-3p mimic or miR-128-3p inhibitor (p-value<0.05). **D.** qRT-PCR analysis of 5 representative primary miRNA (pri-miRs) used for array validation, in H1299 cells upon miR-128-3p expression. **E.** qRT-PCR analysis of 4 representative miRNAs of the identified signature (miR-16a, miR-26a, miR-17-5p, miR-106a) in H1299 cells transiently transfected with miR-128-3p, or miR-10b*,or miR-139-5p, or let-7c, or miR-98, or miR-34b, or miR-34c mimic molecules (miR-x). Histograms show the mean of three experiments.

Figure 3. MiR-128-3p targets the components of the miRNAs processing.

A. Kaplan-Meier curve of overall survival for lung adenocarcinoma patients with Drosha high (n=359) and Drosha low (n=361) expression. **B.** Kaplan-Meier curve of overall survival for lung adenocarcinoma patients with Dicer high (n=360) and Dicer low (n=360) expression. **C.** Schematic representation of plasmids used in luciferase experiments (upper panel) and firefly luciferase activity of Drosha-3'UTR-WT, Drosha-3'UTR-MUT, Dicer-3'UTR-WT and Dicer-3'UTR-MUT reporter genes in H1299 cells transiently transfected with miR-128-3p mimic or control mimic (lower panel). Results are presented as Firefly activity relative to total proteins and Renilla activity. Histograms show the mean of three experiments performed in duplicate. **D.** Western-blot analysis of Drosha and Dicer proteins expression levels in H1299 cells upon miR-128-3p over-expression and in H1299 cells depleted for miR-128-3p using miR-128-3p-inhibitor or miR-128-3p decoy vector. **E-F.** qRT-PCR analysis of the expression levels of a set of identified miRNAs in H1299 depleted for DROSHA (E) or DICER (F).

*p-value<0.05; **p value<0.01, ***p-value<0.001.

Figure 4. MiR-128-3p promotes lung cancer cells motility.

A. Transwell migration assay in H1299 cells transiently transfected with miR-128-3p mimic or miR-128-3p inhibitor. Fold increase in cell migration is represented by the histogram. **B.** Wound healing assays in H1299 cells transiently transfected with miR-128-3p mimic or miR-128-3p inhibitor. The histogram represents folds of wound width relative to control. **C.** Phase-contrast microscopy images (magnification x5) of cell scattering assay in H1299 cells transfected with miR-128-3p mimic or control mimic. **D.** Label-free assays in H1299 transiently transfected with miR-128-3p mimic or control mimic at the indicate time points. The change in impedance of H1299 cells

is represented by the graph (left panel). The right panel shows phase-contrast microscopy images (magnification x10) showing H1299 cells morphology after 72h from miR-128-3p mimic or control mimic transfection.
*p-value < 0.05; **p-value<0.01; ***p-value<0.001.

Figure 5. MiR-128-3p promotes EMT by miRNAs down-regulation.
A. qRT-PCR analysis (upper panel) and western-blot analysis (lower panel) of SNAIL and ZEB1 levels in H1299 cells transiently transfected with miR-128-3p mimic or miR-128-3p inhibitor. **B.** Graph representation of immunofluorescence assay for N-Cadherin protein in H1299 cells transiently transfected with miR-128-3p mimic or control mimic. **C.** qRT-PCR analysis of MMP9 expression levels H1299 cells transiently transfected with miR-128-3p mimic or control mimic. **D.** qRT-PCR analysis of the expression levels of miRNAs targeting SNAIL or ZEB1 3'UTR, in H1299 cells upon miR-128-3p over-expression. **E.** MiR-128-3p expression levels in 13 brain metastasis (BM) versus 13 matched primary lung cancer (PLC) using Agilent microarray platform.
*pvalue<0.05; **p-value<0.01; ***p-value<0.001.

Figure 6. Drosha counteracts miR-128-3p functional effects.
A. Transwell migration assay in H1299 cells depleted for Drosha. Protein levels of Drosha were analyzed by western-blot analysis (lower panel). Fold increase in cell migration is represented by the histogram (upper panel). **B.** Wound healing assays in H1299 cells depleted for Drosha. The histogram represents folds of wound width relative to control. **C.** Transwell migration assay in H1299 cells transiently transfected either with an empty vector (EV) or a DROSHA expression vector and miR-128-3p mimic or control mimic. Fold increase in cell migration is represented by the histogram.**: pvalue<0.01 vs control+EV ‡: pvalue<0.05 vs miR-128-3p+DROSHA. **D.** Label-free assays in H1299 depleted for Drosha at the indicate time points. The change in impedance of H1299 cells is represented by the graph (right panel). The left panel shows phase-contrast microscopy images (magnification x10) showing H1299 cells morphology upon silencing of Drosha or relative control (siSCR) after 72h from transfection. **E.** Label-free assays in H1299 cells transiently transfected either with an empty vector (EV) or a DROSHA expression vector and miR-128-3p mimic or control mimic at the indicate time points. The change in impedance of H1299 cells is represented by the graph (right panel). The left panel shows phase-contrast microscopy images (magnification x10) showing H1299 cells morphology after 48h from transfection. *: pvalue<0.05, **: pvalue<0.01 vs control+EV ‡: pvalue<0.05 vs miR-128-3p+DROSHA. ‡ ‡: pvalue<0.01 vs

miR-128-3p+DROSHA. **F.** Western-blot analysis of SNAIL and ZEB1 levels in H1299 cells depleted for Drosha. **G.** qRT-PCR analysis of the expression levels of miRNAs targeting SNAIL or ZEB1 3'UTR, in H1299 cells depleted for Drosha.
*pvalue<0.05; **p-value<0.01; ***p-value<0.001.

Figure 7. Schematic representation of the proposed molecular mechanism

In lung cancer cells, the oncogenic miR-128-3p directly binds to Drosha and Dicer 3'UTR determining the inhibition of their expression. The consequence is a significant alteration in miRNAs biogenesis, characterized by a global down-regulation in miRNAs expression, a feature known to promote tumorigenesis. Among the down-regulated miRNAs by miR-128-3p, there are a group of miRNAs that target SNAIL and ZEB1, two of the major players of EMT. This event leads to an up-regulation of SNAIL and ZEB1 and to a consequent induction of EMT that promotes metastatic potential of lung cancer cells. MiR-128-3p levels are kept high probably by alternative processing mechanisms that are Drosha/Dicer-independent.