New insights into radioresistance in breast cancer identify a dual function of miR-122 as a tumor suppressor and oncomiR

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List of abbreviations

Gy: Gray
KRAB: Krüppel associated box
miRNAs: MicroRNAs
oncomiRs: Oncogenic miRNAs
RT-qPCR: Retrotranscription-quantitative Polymerase Chain Reaction
RFS: Relapse-free survival
RNA-Seq: RNA sequencing
Rpm: Reads per million
SF: Surviving fraction
TLDAs: TaqMan Low-Density Arrays
TCGA: The Cancer Genome Atlas

TNBC: Triple negative breast cancer

**Abstract**

Radioresistance of tumor cells gives rise to local recurrence and disease progression in many patients. MicroRNAs (miRNAs) are master regulators of gene expression that control oncogenic pathways to modulate the radiotherapy-response of cells. In this study, differential expression profiling assays identifies 16 deregulated miRNAs in acquired-radioresistant breast cancer cells, of which miR-122 was observed to be up-regulated. Functional analysis revealed that miR-122 has a role as a tumor suppressor in parental cells by decreasing survival and promoting radiosensitivity. However, in radioresistant cells miR-122 functions as an oncomiR by promoting survival. The transcriptomic landscape resulting from knockdown of miR-122 in radioresistant cells showed modulation of the ZNF611, ZNF304, RIPK1, HRAS, DUSP8, and TNFRSF21 genes. Moreover, miR-122 and the set of affected genes are prognostic factors in breast cancer patients treated with radiotherapy. Our data indicate that up-regulation of miR-122 promotes cell survival in acquired radioresistance of breast cancer cells, and suggest that miR-122 differentially controls the response to radiotherapy by a dual function as tumor suppressor and oncomiR dependent on cell phenotype.

**1. Introduction**

The radiotherapy, in addition to surgery and chemotherapy, remains the core of current clinical management of breast cancer. Although radiotherapy is effective in most patients, some of them will develop recurrent disease because of radioresistant tumor cells (Jameel *et al.*, 2004). Radiotherapy is an extrinsic factor that affects the behavior of breast cancer cells themselves, when cells avoid the effect cytotoxic of
radiation it is induce cell growth and spread resulting in progression or recurrence of tumors at the patients (Moran and Haffty, 2002; Torres-Roca et al., 2015). To overcome this problem, it is necessary to elucidate the mechanisms of resistance to radiotherapy. Several studies have demonstrated that tumor recurrence and progression as a consequence of radioresistance can be regulated by miRNAs (Metheetraitur and Slack 2013; Arechaga-Ocampo et al., 2017). miRNAs are master regulators of gene expression; moreover, they have a role in the regulation of carcinogenesis and the control of response to chemo and radiotherapy in breast cancer (Zhang et al., 2014). miRNAs are short, 18-25 nucleotide-long, noncoding RNA molecules that regulate gene expression by suppressing mRNA translation and reducing mRNA stability, usually through imperfect complementary base pairing to the 3´-untranslated region (Bartel, 2004). miRNAs in cancer are classically categorized either tumor suppressive or oncogenic. Generally, oncogenic miRNAs (oncomiRs) are overexpressed in tumors while tumor-suppressive miRNAs are repressed. When these tumor-suppressor miRNAs or oncomiRs are stimulated or inhibited, respectively, cancer cell growth, proliferation, metastasis, and survival may be significantly reduced through the control of pro-oncogenic factors (Svoronos et al., 2016). Mainly, miR-122 is frequently down-regulated in breast cancer and it has been related with a tumor suppressor activity in breast cancer. Up regulation of miR-122 suppressed cell growth and cell-cycle progression in breast cancer cell lines and suppressed tumorigenesis in vivo through targeting IGF1R and regulating PI3K/Akt/mTOR/p70S6K pathway (Wang et al., 2012). However, the crucial roles and underlying mechanisms of miR-122 in radioresistance of the breast cancer remain unclear. In this study, we report the generation of an isogenic model of acquired-radioresistant human breast cancer cells and functional approaches to
identify molecular changes in miRNAs that may explain this phenotype. We demonstrate that miR-122 has a dual function in breast cancer, since it has a tumor suppressor activity by sensitizes parental cells to radiation, but it functions as an oncomiR in radioresistant breast cancer cells by promote cell survival.

2. Materials and methods

2.1. Cell lines

Human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from ATCC (# HTB-22 and HTB-26). MCF-7, MCF-7RR, MDA-MB-231 and MDA-MB-231RR cell lines were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO₂ atmosphere.

2.2. Establishment of radioresistant breast cancer cells

MCF-7RR and MDA-MB-231RR cell lines were established from their parental MCF-7 and MDA-MB-231 cells. 1x10⁶ parental cells were irradiated with a linear accelerator (Clinac 600, Varian) available at the National Institute of Cancer in Mexico City. Cells received 15 sequential fractions of 2 Gy/wk allowing irradiated cell populations a period to recover between exposures. Non-irradiated controls were handled identically to the irradiate cells without radiation exposure. All experiments were performed within 4 to 10 passages after the final irradiation.

2.3. Clonogenic survival assay

3 x10⁵ cells were irradiated and after 24 h of radiation, 1000 cells per well were seeded in 6-well tissue culture plates. The cells were cultured for 10 to 12 days. Colonies were fixed with 7:1 methanol/acetic acid, stained with 0.05% crystal violet
and counted. The surviving fraction (SF) were calculated according to Franken et al., (2006). The SF of cells was further plotted in log scale.

2.4. miRNAs expression profile analysis
Expression of 667 miRNAs was analyzed by RT-qPCR using the Megaplex TaqMan Low-Density Arrays (TLDAs) v2.0 system (Applied Biosystems. Foster City, CA). Briefly, 100 ng of total RNA was retro-transcribed using stem-loop primers and a pre-amplification step was added so that the minimum amounts of miRNAs were detected. qPCR assays were performed in the GeneAmp System 9700 (Applied Biosystems).

2.5. RT-qPCR
The expression of individual miRNAs was evaluated by RT-qPCR using the Stem-loop RT miRNA assay (Applied Biosystems). 100 ng of total RNA were retro-transcribed using the looped RT primer (Applied Biosystems) according to the manufacturer’s protocol. Detection for miR-122, miR-10a, miR-222, miR-222*, miR135b, miR-135b*, miR-196b and miR-934 was performed using TaqMan Universal PCR Master Mix (Applied Biosystems). qPCR was carried out in 7500 Real-Time PCR System (Applied Biosystems). The expression of miRNA was determined using the comparative Ct (2−ΔΔCt) method. RNU44 was used as a control for normalization of data.
2.6. Transfections

MCF-7 and MDA-MB-231 cells were transfected with mimic-miR122 (Ambion) 10nM, while MCF-7RR and MDA-MB-231RR cells were transfected with antagomiR-122 (Ambion) 30nM. Mimic-miR122 and antagomiR-122 were diluted in Opti-Mem (Invitrogen), scramble sequence was used as a control and Lipofectamine 2000 (Invitrogen) was used as transfection agent. The expression of miR-122 was evaluated 48 h post-transfection by RT-qPCR. After transfection cells were irradiated with 4 Gy of IR. The subsequent clonogenic assay was performed as previously described.

2.7. Microarray processing and data analysis

Total RNA was obtained from MCF-7RR and MCF-7RR cells transfected with antagomiR-122. Equimolar concentrations of total RNA from 3 independent experiments were mixed and the transcriptional profiles were analyzed using the Affymetrix GeneChip Human Gene 1.0 ST array, following the manufacturer’s instructions. Arrays were scanned using a Genechip Scanner 3000 7G (Affmetrix, Santa Clara CA, USA). The data were analyzed with Robust Multichip Analysis algorithm using Affymetrix’s default analysis settings and global scaling as normalization method. To define the differential expression profile, Affymetrix Transcriptome Analysis Console software was used. Genes with fold change >1.3 or < –1.3 and with an ANOVA p-value < 0.05 were considered significantly altered between the conditions (MCF-7RR and MCF-7RR cells transfected with antagomiR-122). Microarray raw data tables have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120171).
2.8. Bioinformatic analysis

Validated miRNAs targets were obtained by miRTarBase and predicted miRNAs targets from miRWalk database. Only miRNA-target interactions identified by at least three algorithms were considered. DAVID 6.7 and PANTHER Pathway programs were used to identify components of signaling pathways and Gene Ontology (GO) for biological processes or molecular functions. The analysis of biological network enrichment of the modulated genes obtained by microarrays assays was perform by Cytoscape using the Key Pathway Miner App (Alcaraz et al., 2014). In this analysis a K= 6 value was used.

2.9. The Cancer Genome Atlas data analysis (TCGA)

The RNA sequencing (RNA-Seq) data from samples of 491 breast cancer patients were downloaded from the TCGA database. First line treatment and/or additional radiotherapy, tumor status and follow-up days were considered. Total population was stratified according to low or high expression of mir-122, ZNF611, ZNF304, RIPK1, TNFRSF21, DUSP8 and HRAS. Kaplan-Meier was used for RFS curves and log-rank tests to analyze the differences between curves. Results were confirmed by Cox proportional-Hazard regression analyses.

2.10. Western blot

Following cell transfection, total protein was extracted, separated on SDS-PAGE and blotted onto nitrocellulose membranes (Bio-Rad). Membranes were probed with specific primary antibodies [ZNF611 (Abcam); ZNF304 (Abcam); RIPK1 (BD Transduction Laboratories); DUSP8 (Santa Cruz), HRAS (Abcam), TNFR21 (Santa Cruz) and β-actin (Cell Signaling)], followed by HRP-conjugated secondary
antibodies anti-mouse (Zymed) or anti-rabbit (Zymed). Immunodetection was by chemiluminescence (Super Signal® West Femto, Thermo Scientific). Densitometry analysis was performed using the software ImageJ software version 1.45 (National Institute of Health, USA).

2.11. Statistical analysis.
All results were derived from three independent experiments, which were plotted as mean ± standard deviation (SD). The comparison between the groups was performed using ANOVA test for all analyzes, p ≤ 0.05 was considered as statistically significant. All statistical analyzes were performed using the statistical software package SPSS 17.0.

3. Results
3.1. Establishment of radioresistant MCF-7 and MDA-MB-231 breast cancer cells
In order to determine a mean lethal dose of irradiation, MCF-7 and MDA-MB-231 cell was evaluated by MTT and clonogenic assays following increased radiation dose (2-8 Gy). Results showed that cell proliferation was significantly reduced at different time points (0, 24, 48 and 72 h) and at increased radiation dose (0, 2, 4, 8 Gy) compared to control cells. Proliferation diminished to 53% (+/-0.07) in MCF-7 (Fig. 1A) and 58% (+/-0.05) in MDA-MB-231 (Fig. 1B) cells at 4 Gy at 48 h. Results from clonogenic assays showed a significantly survival reduction (Fig. 1C) of MCF-7 (0.48+/−0.005) and MDA-MB-231 (0.44+/−0.005) cells at 4 Gy compared to non-irradiated cells. These results indicated that 4 Gy of radiation is a mean lethal dose for MCF-7 cells and MDA-MB-231 cells. Therefore, 4 Gy of radiation dose was used
for all following experiments. To establish the isogenic model of radioresistance, MCF-7 and MDA-MB-231 cells were exposed to 2 Gy-fractionated irradiations to a cumulative dose of 30 Gy (Fig. 1D). At the end of this process, radioresistance of the cell population resultant designated as MCF-7RR and MDA-MB-231RR was confirmed by clonogenic assays after single doses of 4 Gy of radiation. Acquired radioresistance of MCF-7RR and MDA-MB-231RR cells was further evidenced by an increase of survival after 4 Gy of radiation compared to parental cells. The SF of the MCF-7RR (Fig. 1E) and MDA-MB-231RR (Fig. 1F) cells was 0.70 and 0.75 respectively; whereas for parental MCF-7 and MDA-MB-231 cells was 0.47 and 0.45 respectively. These data confirmed that the population resultant of the cells exposed to long-term therapeutic fractionated irradiation developed a radioresistance phenotype.

3.2. miR-122 is overexpressed in therapy-induced radioresistant breast cancer cells

To identify miRNAs associated to radioresistance in breast cancer, we analyzed the expression of 667 miRNAs by PCR array analysis in MCF-7RR cells. Expression of 16 miRNAs was modulated (Fig. 2A). miR-135b*, miR-934, miR-223*, miR-222*, miR-122, miR-135b, miR-184, miR-411, miR-449b, miR-424*, miR-10a, miR-218, miR-222 miRNAs were significantly overexpressed (Fold change ≥ 1.5). While miR-181a-2*, miR-146a and miR-196b were down-regulated (Fold Change ≤ -1.5) (Table 1). Individual RT-qPCR assays using RNA samples from different clones of MCF-7RR cells further confirmed the array result (Supplementary Fig. S1). Validated target genes of miRNAs were obtained from databases and published papers (Table 2). Gene ontology and enrichment analysis indicated that set of miRNAs could
modulate biological pathways as cell migration, signal transduction, apoptosis and survival (Fig. 2B). The expression of the most significantly modulated miRNAs identified in MCF-7RR cells was also evaluated in MDA-MB-231RR cells. Remarkably, overexpression of miR-122, miR-222 and miR-135b; and downregulation of miR-196b were likewise observed in MDA-MB-231RR cells; conversely, miR-222* and miR-934 were deeply suppressed in MDA-MB-231RR cells (Fig. 2C). These results suggested that variation of mir-122, miR-222, miR-135b and miR-196b expression might be a relevant phenomenon in acquired radiotherapy resistance of the breast cancer cells.

### 3.3. **miR-122 increase radiosensitivity in parental breast cancer cells and in breast cancer patients treated with radiotherapy**

miR-122 has been described as a tumor suppressor and its down-regulation is a common event in breast cancer (Wang et al., 2012). Conversely, in our study, overexpression of miR-122 was observed in both radioresistant breast cancer cells. To investigate whether miR-122 might increase the radioresistance of breast cancer cells, we performed assays of gain-of-function in parental MCF-7 and MDA-MB-231 cells. We were able to overexpressed miR-122 in parental MCF-7 (Fig. 3A) and MDA-MB-231 cells (Fig. 3B) by using a mimic-miR122. Then we evaluated the surviving potential of these cells in response to radiotherapy by clonogenic assays. Results showed that in non-irradiated cells the overexpression of miR-122 alone, but not scrambled transfected or control untransfected, significantly decrease the survival in MCF-7 (SF=0.63) (Fig. 3C); and MDA-MB-231 (SF=0.4) (Fig. 3D) cells. Remarkably, results showed that the cells transfected with mimic-miR122 treated with radiotherapy significantly diminished its surviving potential [MCF-7 (SF=0.12);
MDA-MB-231 (SF=0.07]) (Fig. 3 C and D). These results revealed that miR-122 is capable to sensitize the breast cancer cells to radiotherapy. To investigate the clinical relevance of the miR-122 expression levels, we performed Kaplan-Meier analysis for RFS of breast cancer patient treated with radiotherapy. Data of 102 patients that received radiotherapy as a first line treatment obtained from TCGA database were randomly categorized in two groups according it positive or null expression of miR-122. Results revealed that patients with positive expression of miR-122 and that received radiotherapy had significantly much better RFS than those with negative expression of miR-122 (Fig. 3E), suggesting that patients with null expression of miR-122 were significantly associated with poor prognoses after radiotherapy. These results were according to our findings in vitro, in which miR-122 sensitized breast cancer cells to irradiation. Furthermore, results in vivo suggested that expression of miR-122 might be predictor biomarker for RFS in breast cancer patients treated with radiotherapy.

3.4. miR-122 knockdown overcomes acquired radioresistance in breast cancer cells

We have demonstrated that miR-122 is up-regulated in both radioresistant breast tumor cells. To get more insights into the biological function of miR-122 in acquired radioresistance we performed loss-of-function assays in MCF-7RR and MDA-MB-231RR cells. We efficiently inhibit the expression of miR-122 in MCF-7RR (Fig. 4A) and MDA-MB-231RR (Fig. 4B) cells through use antagomiR-122. These transfected cells were evaluated for radioresistance by clonogenic assays. As expected, the radioresistant cell lines with deficiency in miR-122 had significantly reduced survival efficiency. Our evidence showed that knockdown of miR-122 alone (non-irradiated
cells) but not in transfected cells with scrambled control had significantly negative effect on survival rates in both MCF-7RR (SF=0.87) (Fig. 4C) and MDA-MB-231RR (SF=0.70) (Fig. 4D). This negative effect on survival was higher when cells were irradiated [MCF-7RR (SF=0.43) (Fig. 4C); MDA-MB-231RR (SF=0.26) (Fig. 4D)]. Hence, the knockdown of miR-122 in radiotherapy-induced resistant cells is able to revert radioresistance of the cells through delayed cell survival. Furthermore, these results suggested that miR-122 promotes survival pathways to maintaining a radioresistant phenotype in breast cancer cells. In this sense, we evaluate the expression of miR-122 in parental cells treated with radiotherapy. Results showed that radiation promote overexpression of miR-122 in parental breast cancer cell (Fig. 4E). We hypothesized that overexpression of miR-122 was maintained during the adaptive biological reprogramming in response to continuous application of radiation, that is, during the transition from a cancer cell to a radioresistant cancer cell (Fig. 4F). Moreover it likely that miR-122 could gain an oncogenic role in radioresistant cells, therefore having a dual function in breast cancer cells, acting either as a tumor suppressor or an oncogene depending on the cellular context (Fig. 4F). The adaptive transcriptional reprogramming includes the possibility that target genes of the miRNAs could be also change, producing oncogenic or tumor suppressive effect. In this way, miR-122 should regulate different target genes to act as a positive regulator of the survival pathways to favoring an oncogenic function in radioresistant cells. In order to explore this hypothesis, we evaluated the expression of IGF1R, which has been previously reported as a target gene of miR-122 in breast cancer (Wang et al., 2012). Remarkably, results obtained by RT-qPCR assays showed that IGF1R is down-regulated in parental MCF-7 cells with gain-of-function of miR-122 and up-regulated in MCF-7RR with loss-of-function of miR-122 (Fig. 4G), consistent with
targeted activity of miR-122. These results suggested that radiosensitivity observed in MCF-7RR cells with loss-of-function of miR-122 could be independent of the IGF1R function. Taken together, these results indicate that miR-122 have an oncogenic role in acquired radioresistance of breast cancer cells.

3.5. Transcriptomic landscape of the radioresistant breast cancer cells with loss-of-function of miR-122

To obtain a comprehensive molecular understanding of the oncogenic role of miR-122 in acquired radioresistance, we utilized a gene expression profiling approach by microarrays to systematically identify genes associated to loss-of-function of miR-122 in radioresistant cells. A total of 158 genes differentially expressed were identified in hierarchical clustering analysis (Fig. 5A). Twenty-seven genes were up-regulated (Fold Change ≥ 1.3) and 131 genes were down-regulated (Fold Change ≤ -1.3); (Supplementary Table 1). Using the Key Pathway Miner application of Cytoscape to determine interconnected pathways by protein-protein interaction in silico, we reveal products of genes that were not observed in the microarrays assays (outliers) such as EGFR, APP, MOV10, EWSR1, SIRT7 and ELAVL1 (Fig. 5B). Our analysis by functional protein association networks, gene ontology and biological pathways (Fig. 5C) showed that gene signature in knockdown miR-122-MCF-7RR could be associated to regulation of transcription (SSX8, ZNF611, ZNF18, EGR4, TFCP2L1, ZNF684, ZNF793, CITED4, ZNF616, ZNF304, BHLHA9, LTF, SP7, RBPJL, FOXD4L6), G-protein coupled receptor signaling pathway (CCL25, OR211P, OR1L8, OR5AP2, OR8B4, OR4D10, AREG, VIPR2, HTR1E), TNF pathway (TNFRSF21, CCL25, RIPK1), Ras-MAPK pathway (IL1R2, HRAS, MAP4K1, DUSP8) and inflammatory response (TNFRSF21, CCL25, IL1R2, IL13) (Fig. 5D). To
identify potential genes that could be directly regulated by miR-122, we performed \textit{in silico} analysis of 3'-UTR-binding sites for miR-122 in modulated genes. We found 9 up-regulated and 29 down-regulated genes containing canonical 3'-UTR-binding sites for miR-122 (Table 3), among which highlight up-regulated \textit{IGLON5, NUP62CL, ACAA1, KLHL5, FBXO48, ZNF304, VIPR2, CCDC127} and \textit{ZNF611}, and down-regulated \textit{DUSP8, DDR2, IL1R2, DEAF1} and \textit{RIPK1}. Therefore, microarrays analysis revealed that radioresistant cells with loss-of-function of miR-122 are highly enriched for genes encoding signaling pathways and transcriptional processes, suggesting that a major influence of miR-122 function on acquired radioresistance is related to maintaining survival networks.

3.6. Genes associated to loss-of-function of miR-122 are involved in the outcome of breast cancer patients treated with radiotherapy.

To determine the prognostic value of genes associated to loss-of-function of miR-122, we evaluated RFS in 491 breast cancer patients treated with radiotherapy obtained from the TCGA database. Patients were categorized according to median expression of each gene; therefore, we obtain different group of patients according to low and high expression of individual gene. Characteristic populations are shown in supplementary tables 2 to 7. In the analysis for RFS, differential expression of \textit{ZNF611, ZNF304, RIPK1, DUSP8, TNFRSF21} and \textit{HRAS} genes was associated to outcome of the breast cancer patients who received radiotherapy. Kaplan-Meier curves showed that increase expression of \textit{ZNF611} (\(P = 0.0269; \text{Fig. 5E}\)) and \textit{ZNF304} (\(P = 0.0081; \text{Fig. 5F}\)) and lower expression of \textit{RIPK1} (\(P = 0.0047; \text{Fig. 5G}\)), \textit{TNFRSF21} (\(P = 0.0058; \text{Fig. 5H}\)), \textit{DUSP8} (\(P = 0.048; \text{Fig. 5I}\)) and \textit{HRAS} (\(P = 0.0138; \text{Fig. 5J}\)) were associated with longer RFS. These results were according to the
experimental evidence in MCF-7RR cells in which increase of ZNF611 and ZNF304 in addition to decrease of RIPK1, TNFSF21, DUSP8 and HRAS expression inducing by knockdown miR-122 correlated with a radiosensitivity in vitro.

3.7. miR-122 differentially controls levels of ZNF611, ZNF304, RIPK1, DUSP8, HRAS and TNFRS21 protein in radioresistant breast cancer cells

Our findings obtained by Kaplan-Meier analysis revealed that ZNF611, ZNF304, RIPK1, DUSP8, TNFRSF21 and HRAS genes have a prognostic value in patients treated with radiotherapy. Among these genes, ZNF611 (positions 2916-2922), ZNF304 (positions 2676-2682), RIPK1 (positions 1680-1684) and DUSP8 (positions 1631-1634) genes contain canonical miR-122 3’-UTR binding sites (Fig. 6A). For experimental validation of transcriptome results, and to test whether modulation of ZNF611, ZNF304, RIPK1, DUSP8, HRAS and TNFRS21 following knockdown of miR-122 could be a common event in radioresistant breast cancer cell we performed Western blot assays in transfected MCF-7RR and MDA-MB-231RR cells with antagomiR-122. As expected, we validate the results obtained by microarrays assays in MCF-7RR cells. Levels of proteins ZNF304 and ZNF611 were up-regulated, while RIPK1 and DUSP8 were repressed when we inhibit miR-122 (Fig. 6B). Levels of HRAS and TNFRS21 levels were observed with non-significantly changes. These results showed a direct correlation among the expression of miR-122 and ZNF304, ZNF611, RIPK1 and DUSP8 levels. As a control we compared the abundance of these proteins in parental MCF-7 cells transfected with mimic-miR122 (Fig. 6C). Results showed that expression of ZNF304, RIPK1, DUSP8 and TNFRS21 have not changes when we forced overexpression of miR-122, contrary the expression of HRAS was higher compared to non-transfected and scrambled.
transfected cells (Fig. 6C). Notably, \textit{ZNF611} was down-regulated when \textit{miR-122} was overexpressed (Fig. 6C). These findings suggested that \textit{miR-122} could target \textit{ZNF611} but was unable to modulate \textit{ZNF304, RIPK1, DUSP8} and \textit{TNFRS21} protein levels in parental MCF-7 cells. Although not all Western blot results were similar in MDA-MB-231RR compared to MCF-7RR, we observed that in MDA-MB-231RR cells knockdown of \textit{miR-122} correlated to up-regulation of \textit{ZNF611, DUSP8} and \textit{HRAS} (Fig. 6D). Moreover, in parental MDA-MB-231 cells, forced overexpression of \textit{miR-122} also correlated with inhibition of \textit{ZNF611, DUSP8} and \textit{HRAS} levels (Fig. 6E). These results suggested that \textit{ZNF611} and \textit{DUSP8} might be targeted by \textit{miR-122} in MDA-MB-231RR model. It should be noted that \textit{miR-122} could modulate dissimilar pathways in radioresistant breast cancer cells compared to parental breast cancer cells, which might partially explain the dual function as a tumor suppressor or oncomiR according to cell context. On the other hand, the variability of the results among the radioresistant cells could be due to the difference in cell context origin of the cell lines because of MDA-MB-231 is a model of triple negative breast cancer (TNBC) subtype, while MCF-7 is a model of luminal tumor. In this sense, we evaluate the prognostic value of the \textit{ZNF611, ZNF304, RIPK1, TNFRSF21, DUSP8} and \textit{HRAS} genes according to tumor subtypes. The RFS of patients with luminal breast cancer and TNBC treated with radiotherapy was evaluated by Kaplan-Meier. Results showed that higher levels of \textit{ZNF611} \((P=0.0338)\) and lower levels of \textit{RIPK1} \((P=0.0024)\) and \textit{DUSP8} \((P=0.0165)\) were associated with longer RFS in luminal subtypes (Fig. 6F). However, although these genes were not significantly associated to RFS in TNBC subtypes, higher levels of \textit{ZNF611} and \textit{ZNF304} and lower levels of \textit{HRAS} showed a trend for associate with longer RFS (Fig. 6G).
4. Discussion

In order to gain insight into the molecular adaptation underpinning radiation resistance of breast cancer cells, in this study we report the development of isogenic radioresistant breast cancer cell lines, miRNA landscape analysis and functional analysis of miR-122. Isogenic model of radioresistance was developed through cumulative exposure of MCF-7 and MDA-MB-231 cells to 30 Gy-fractionated radiation resulted in the generation of a sub-lines with a significantly increased survival potential than sham control cells, as we previously reported in a model of radioresistant lung cancer cells (Arechaga-Ocampo et al., 2017). The development of isogenic chemo and radioresistant cell lines have been successfully used to understanding the molecular changes associated with acquired resistance to therapies and tumor aggressiveness in cancer (McDermott et al., 2014). Exposure of tumors to fractionated radiation schedules can select a cancer cell subpopulation with an increased capacity to overcome the anti-proliferative effects of radiotherapy (Zaider and Hanin, 2011) by modulating the abundance and function of molecules, including miRNAs (Metheetraitrut and Slack, 2013; Arechaga-Ocampo et al., 2017; Zhang et al., 2014). Here we identify a set of miRNAs related to acquired radioresistance. Wang et al., (2014) have been shown that expression of a group of miRNAs establishes a useful molecular signature to differentiate radioresistant from non-radioresistant tumors, even though they come from the same histological type. In this sense, we identify the miRNAome of the isogenic MCF-7RR cell line compared to parental MCF-7 cell line and we found 16 miRNAs differentially expressed (13 up- and 3 down-regulated). Among them, miR-184 (Fang et al., 2017), miR-424* (Zhang et al., 2017), miR-218 (Wang et al., 2017), miR-222 (Wei et al., 2017) and miR-10a (Rong et al., 2016) have been reported in resistance to chemotherapy in several
types of tumors. Others as miR-135b* (Wang et al., 2014); miR-223* (de Melo et al., 2016); miR-135b (Han et al., 2017) and miR-196b (Ren et al., 2017) have been reported as oncomiRs. The aberrant expression of miRNAs is a known event in radioresistance of human tumors because of their function as negative regulators of the gene expression. miRNAs can control the expression of genes that are components of cell survival pathways, apoptosis, immune response, cell differentiation, between other (Bartel, 2004). Our results of bioinformatic analysis of the gene ontology and biological pathways revealed that miRNAs set are implicated in proliferation and survival pathways, immune response and transcriptional control, suggesting that, signaling pathways involved in acquired radioresistance can be directed by the coordinated action of these molecules. It is remarkable that deregulation of miRNAs identified in MCF-7RR cells is conserved in MDA-MB-231RR cells. Specially, miR-196b, miR-222 and miR-122 have a similar expression in both radioresistant cell lines, which suggested that they might have a significant role in the phenotypic evolution of the cancer cells to acquired radioresistance. Notably, it is known that miR-122 act as tumor suppressor in breast cancer by targeting IGF1R (Wang et al., 2012) and ADAM10 genes (Ergün et al., 2015). In addition, miR-122 have the same role in liver and glioma tumors in which suppresses mechanisms to promote tumor progression and survival (Wang et al., 2014). In this work we demonstrate that miR-122 significantly reduced survival of the parental cells, but this effect was enhanced when the cells were irradiated. This result is consistent with previously reports of the tumor suppressor function of miR-122 in breast cancer (Wang et al., 2012; Ergün et al., 2015) and it also shows its potential as radiosensitizer. Similarly, a previous report showed that miR-122 induces radiosensitivity in lung cancer cells exposed to different doses of radiation. In this
report, the ectopic overexpression of miR-122 in lung cancer cells decreases anchorage-dependent invasion and inhibits cell growth by knockdown target genes related to tumor survival and cellular stress response (Ma et al, 2015). The role of miR-122 as radiosensitizer reported in lung cancer is consistent with our findings in breast cancer. Recently Zhang et al (2017), reported the function of miR-122-3p in response to radiation in triple negative breast cancer cells. Zhang´s group demonstrate that miR-122-3p promotes sensitivity to radiation by modify cellular apoptosis, migration and invasion through modulation of PTEN/PI3K/AKT pathway. It should be noted that miR-122-3p (miR-122*) is the passenger strand of leading strand miR-122-5p (miR-122); therefore, miR-122-3p could target different genes, which give it an independent function of miR-122-5p. The Zhang´s group results and our results suggest that miR-122* and miR-122 might function cooperatively to promote the sensitivity of the breast cancer cells exposed to radiation. However, in our isogenic model of acquired-radioresistant breast cancer cells we did not observed the aberrant expression of miR-122-3p, so the role of miR-122-3p in the acquired-radioresistance in the breast cancer is still unknown.

Besides its function in vitro we show that expression of miR-122 in breast tumors was significantly associated with a favorable response to radiotherapy of patients compared to those who did not express miR-122. In those group of patients, miR-122 has a tumor suppressive role due to patients has a treatment-naïve tumors; that is, patients have not yet received radiation therapy, therefore radioresistance has not yet developed. With these results, we demonstrate that miR-122 is related to sensitivity to radiotherapy in parental breast cancer cells in vitro and in vivo in a set of breast tumors, of which has been no reported previously to our findings.
Surprising, in MCF-7RR and MDA-MB-231RR cells we observed over-expression of miR-122. Its down-regulation by antagomiR-122 was able to revert the resistance to radiotherapy by counteract cell survival. Contrary to the function as tumor suppressor, it seems that miR-122 have an oncogenic role in breast cancer cells that have acquired radioresistance. It is known that miRNAs could act as tumor suppressor or oncogenes depending to scenario. Svoronos et al. (2016), publish an excellent review in which they discuss the dual role of miRNAs in cancer cells themselves and by extrinsic factors. They reviewed that dual function could be dependent of different cell phenotypes, tumor microenvironmental, immune evasion and by the selective pressure inducing by therapy treatments including radiotherapy. We showed that miR-122 is up-regulated in response to radiation treatment suggesting that continuous fractionated irradiation maintain the overexpression of miR-122 until attaining a radioresistant phenotype. Based on our results we propose that miR-122 could acquire an oncogenic function under the pressure exerted by radiation in breast cancer, which allows the evolution of the cell for to adapted to radiotherapy. To explore genes and biological pathways modulated by miR-122 in radioresistant cells we analyzed the transcriptome of MCF-7RR cells with loss-of-function of miR-122. Results shown 158 genes differentially modulated, of which 27 were increased and 131 decreased. Analyses of biological network by cytoscape revealed protein outliers which have been reported in radiotherapy-resistance in cancer and survival, proliferation and epigenetics regulation such as EGFR (Lee et al., 2011); MOV10 (El Messaoudi-Aubert et al., 2010); ELAVL1 (Mehta et al., 2016); SIRT7 (Chen et al., 2017; Tang et al., 2017); APP (Lim et al., 2014) and EWSR1 (Suzuki et al., 2012). Many reports have shown that radiotherapy can induce a transcriptional reprogramming to acquire resistance and avoid the toxicity trigger by
radiotherapy (Doan et al., 2018; Ma et al., 2013). We determined that miR-122 modulates genes related to molecular processes as transcriptional regulation, signaling of receptors coupled to G proteins, MAPK and TNF pathways. This diversity of cellular processes is consistent with numerous regulatory mechanisms associated to response to radiation and radioresistance in breast cancer (Kaidar-Person et al., 2013). In the transcriptomic landscape induced by knockdown miR-122 we identify genes involved in these cellular processes such as ZNF611, ZNF304, RIPK1, DUSP8, HRAS and TNFRSF21. Moreover, these genes were prognostic factors in breast cancer patients treated with radiotherapy. These clinical findings were according to results of survival assays in vitro. Among set of genes up-regulated we identify that ZNF611 and ZNF304 genes contain canonical miR-122-binding sites in their 3'-UTR region, besides, we validated that protein levels of these transcription factors also increase when miR-122 is inhibited in MCF-7RR cells. ZNF304 and ZNF611 are transcription factors that belong to the C2H2 zinc finger family possess Krüppel associated box (KRAB), which are related to transcriptional silencing by to recruit epigenetic complexes (Aslan et al., 2015; Pengue and Lania, 1996). ZNF304 have been reported as regulator of RAS pathway by recruits an epigenetic silencing complex in tumor suppressor genes in colorectal cancer (Serra et al., 2014), and a promoter of cell survival in ovarian cancer (Aslan et al., 2015). ZNF611 has not yet been reported in cancer. On the other hand, RIPK1, DUSP8, HRAS and TNFRS21 were down-regulate when we knocked-down miR-122. RIPK1 (Miyamoto, 2011) and TNFRS21 (Benschop et al., 2009) are component of the TNF pathway, while DUSP8 (Keyse, 2008) and HRAS (Knobbe et al., 2004) acts on the RAS-MAPK pathway. Alteration in the activity of transcription factors could be critical in the development and maintenance of radioresistance in breast cancer by control
genes of survival pathways as RAS-MAPK and TNF pathways (Ishihara et al., 2015; Zhao et al., 2018). Many genes that we observed down-regulated in the transcriptome analysis might be part of an epigenetic network of transcriptional silencing driven by axis miR-122-ZNF611 or miR-122-ZNF304. This is a research topic that is currently under investigation; however, we obtained preliminary data that strongly suggest that RIPK1 and DUSP8 possess elements of response to ZNF611 and ZNF304 in their promoter regions (data no shown). Therefore, we propose that dual function of miR-122 in the isogenic model of radioresistant breast cancer cells could be result of the transcriptional reprogramming controlled by the modulation of ZNF611 and ZNF304 by miR-122.

5. Conclusion
In conclusion, our data contribute to understand the mechanisms of molecular adaptation to radiotherapy in breast cancer cells. Our evidence describes aberrant expression of a set of miRNAs linked to carcinogenesis and molecular control of the pathways related to therapies response in cancer. Particularly, the overexpression of miR-122 is determinant to maintain the radioresistant phenotype in breast cancer cells by promote cell survival from the regulation of several genes to the downstream effects of these genes; which confer it an oncogenic function (Fig. 7).

Data accessibility
Microarray raw data tables have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120171.
Author contributions

Conception and design: IXPA, EAO, CHGR; Development of methodology: IXPA, EAO, CHGR, FOBA Acquisition of data: IXPA, EAO, FOBA, MSA, OAZ. Analysis and interpretation of data: IXPA, EAO, CHGR, ESRS, FOBA Writing, review, and/or revision of the manuscript: IXPA, EAO, CHGR, ESRS, FOBA, DALH, MCS. Administrative, technical, or material support: EAO, NVS, JAGB, CHGR, ODMH, ESRS AGC; Study supervision: IXPA, EAO, CHGR, ESRS

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Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. All coauthors have seen a draft copy of the manuscript and agree with its publication. The work has not been published elsewhere, either completely, in part, or in any other form.

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Supporting information

Figure S1. Validation of the expression of a set of miRNAs deregulated in MCF-7RR cells.

Table S1. Differentially expressed genes in the MCF-7RR cells transfected with antagomiR-122.

Table S2. Characteristics of the population of breast cancer patients based on expression of ZNF611 gene.

Table S3. Characteristics of the population of breast cancer patients based on expression of ZNF304 gene.

Table S4. Characteristics of the population of breast cancer patients based on expression of RIPK1 gene.

Table S5. Characteristics of the population of breast cancer patients based on expression of TNFRSF21 gene.
Table S6. Characteristics of the population of breast cancer patients based on expression of DUSP8 gene.

Table S7. Characteristics of the population of breast cancer patients based on expression of HRAS gene.

Figure legends

Figure 1. Establishment of isogenic model of acquired radioresistant breast cancer cells. Proliferation of parental (A) MCF-7 and (B) MDA-MB-231 cells was assessed by MTT assay for 24, 48 and 72 h after irradiation with increasing doses (0, 2, 4 and 8 Gy) of x-ray. (C) Clonogenic survival of parental MCF-7 and MDA-MB-231 cells was assessed by colony formation in response to treatment with increasing doses (0, 2, 4 and 8 Gy) of x-ray. (D) Schematic overview of fractionated treatment schedule for establishment of radioresistant breast cancer cells. Parental cells were exposed to treatment of 2 Gy of IR to reach 30 Gy of total doses. Radioresistance of MCF-7RR and MDA-MB-231RR cells was confirmed by clonogenic assays after irradiation with 4 Gy of x-ray. SF of irradiated (IR+) MCF-7RR (E) and MDA-MB-231RR (F) cells was normalized by survival fraction of non-irradiated (IR−) cells. Representative image of clonogenic assays results of MCF-7RR and MDA-MB-231RR cells are show in E and F. Error bar, SD from three independent experiments. **p < 0.01; *p < 0.05 by Student’s t-test.

Figure 2. Radioresistant breast cancer cells show differential expression profile of miRNAs. (A) miRNAs expression profile in MCF-7RR cells. (B) Gene ontology, signaling pathways and biological processes controlled by deregulated miRNAs in MCF-7RR cells. (C) Validation of the expression of a set of miRNAs in
MCF-7RR and MDA-MB-231RR cells was evaluated by RT-qPCR. All values were normalized using RNU44 as internal control. The expression data were normalized using the parental MCF-7 and MDA-MB-231 cell. Dotted line indicates the threshold of the normalized data. Data are presented graphically as the mean ± SD of three independent experiments. *p < 0.01 by ANOVA test.

Figure 3. MiR-122 promotes radiosensitivity in parental breast cancer cells. Increased expression of miR-122 in parental (A) MCF-7 and (B) MDA-MB-231 cells transfected with mimic-miR122 was verified by RT-qPCR assays. All values were normalized using RNU44 as an internal control. Mimic-miR122-transfected cells were evaluated for radioresponse by clonogenic survival. Graphical data of survival fraction of transfected (C) MCF-7 and (D) MDA-MB-231 cells irradiated (+IR) with 4 Gy of x-ray are shown. Data normalized was by using non-irradiated cells (-IR). Representative image of clonogenic assays results of MCF-7 and MDA-MB-231 cells are shown in C and D. Data are presented graphically as the mean ± SD of three independent experiments. *p < 0.05; **p < 0.01 by ANOVA test. (E) Kaplan-Meier analysis of the breast cancer patients with tumors positive or negative for miR-122 expression who received radiotherapy treatment. Curves were compared using Log-rank test *p < 0.01. Rpm (Reads per million).

Figure 4. miR-122 is overexpressed in radioresistant breast cancer cells and its inhibition revert radioresistant phenotype. Knockdown of miR-122 in radioresistant (A) MCF-7RR and (B) MDA-MB-231RR cells transfected with antagomiR-122 was verified by RT-qPCR assays. All values were normalized using RNU44 as an internal control. AntagomiR-122-transfected cells were evaluated for
radioresponse by clonogenic survival. Graphical data of survival fraction of transfected (C) MCF-7RR and (D) MDA-MB-231RR cells irradiated (+IR) with 4 Gy of x-ray are shown. Data were normalized by using non-irradiated cells (-IR). Representative image of clonogenic assays results of MCF-7RR and MDA-MB-231RR cells are shown in C and D. (E) Overexpression of miR-122 in parental MCF-7 and MDA-MB-231 induced by treatment with 4 Gy of x-ray was evaluated by RT-qPCR assays. The expression data were normalized using the parental MCF-7 and MDA-MB-231 cell. All values were normalized using RNU44 as an internal control. (F) Schematic representation of the role of miR-122 as tumor suppressor miRNA in parental breast cancer cells and its oncogenic role during the transition from a cancer cell to a radioresistant cancer cell. (G) Expression of IGF1R in MCF-7 and MCF-7RR cells transfected with mimic-miR122 and antagomiR-122 respectively was evaluated by RT-qPCR. All values were normalized using GAPDH as an internal control. All Data are presented graphically as the mean +/- SD of three independent experiments. *p= <0.05; **p<0.01 by ANOVA test.

Figure 5. Transcriptome analysis of MCF-7RR cells with knockdown of miR-122 and analysis of prognostic factor genes to breast cancer patients. (A) Heat map showing different expression pattern of 158 genes in MCF-7RR cells transfected with antangomiR-122. The heat map indicates 131 genes up-regulated (red) and 27 genes down-regulated (green). The columns represent duplicate of individual samples of MCF-7RR cells with knockdown of miR-122 as well as MCF-7RR untransfected cells. The rows represent individual genes. Arrows show genes with prognostic value in breast cancer patients treated with radiotherapy. (B) The interactome generated from protein-protein interaction data by Key Pathway

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Cyto[11]. 36 genes in green are the modulated genes by knockdown of miR-122, the 6 genes in pink are linker genes that connect the 36 genes from transcriptome. Number of connections in the network are show as nodes. (C) Bubble chart shows the enriched GO terms of the genes modulated by the knockdown of miR-122. In the Y axis are shown the biological processes. Color and size of the bubble represents the number of genes involved in each GO/network process and significance, respectively. (D) Chart of GO and biological processes of the genes modulated by the knockdown of miR-122. Genes symbol are shown; bold-labeled indicate genes containing miR-122 binding sites in their 3’-UTR. Kaplan-Meier curves of RFS. Survival curves of high vs low expression of (E) ZNF611; (F) ZNF304; (G) RIPK1; (H) TNFRSF21; (I) DUSP8 and (J) HRAS of patients with breast cancer treated with radiotherapy. High or low gene expression levels according to > median or ≤ median expression levels each gene. Curves were compared using Log-rank test *p= < 0.05; ** p= < 0.01. Rpm (Reads per million).

Figure 6. Levels of ZNF611, ZNF304, RIPK1, TNFRSF21, DUSP8 and HRAS are differentially regulated by miR-122 in parental and radioresistant breast cancer cells. (A) Schematic illustration of the potential miR-122 binding site in 3’-UTR of ZNF611; ZNF304; RIPK1 and DUSP8 genes. letters blue-labeled indicate seed region. Levels of ZNF611, ZNF304, RIPK1, TNFRSF21, DUSP8 and HRAS proteins were evaluated by Western blot assays in (B) MCF-7RR transfected with antagomiR-122; (C) Parental MCF-7 transfected with mimic-miR122; (D) MDA-MB-231RR transfected with antagomiR-122 and (E) Parental MDA-MB-231 transfected with mimic-miR122. β-Actin was used as an internal control. Images are representative of three independent experiments. Kaplan-Meier curves of RFS.
Survival curves of high vs low expression of ZNF611; ZNF304; RIPK1; TNFRSF21; DUSP8 and HRAS of patients with (F) Luminal breast cancer and (G) Triple-negative breast cancer (TNBC) treated with radiotherapy. High or low gene expression levels according to > median or ≤ median expression levels each gene. Curves were compared using Log-rank test *p= < 0.05.

Figure 7. Schematic representation of the miR-122-mediated radioresistance in breast cancer cells. miR-122 have an oncogenic role in acquired radioresistance of luminal and triple-negative breast cancer (TNBC) cells by differentially control the expression of ZNF611 and ZNF304 transcription factors, and modulate the expression of genes involved in RAS-MAPK and TNF pathways to promote survival.

Supplementary Figure 1. Validation of the expression of a set of miRNAs deregulated in MCF-7RR cells. Expression of a set of miRNAs was evaluated by RT-qPCR in MCF-7RR cells. The expression data were normalized using the parental MCF-7 cells. All values were normalized using RNU44 as an internal control. Data of RT-qPCR assays were compared to results from global expression profiles by TLDAs system. Data are presented graphically as the mean ± SD of three independent experiments. *p < 0.01 by Student’s t-test.
Table 1. miRNAs with modulated expression in breast cancer cells MCF-7RR.

| miRNA       | Fold Change (log2) | R (P Value)* | Chromosome |
|-------------|--------------------|--------------|------------|
| Down-regulated |                    |              |            |
| hsa-miR-196b| -2.43              | 0.0113       | 7p15.2     |
| hsa-miR-146a| -2.13              | 0.05         | 5q34       |
| hsa-miR-181a-2*| -1.7             | 0.0091       | 9q33.3     |
| Up-regulated |                    |              |            |
| hsa-miR-222 | 1.53               | 0.0083       | Xp11.3     |
| hsa-miR-218 | 1.63               | 0.0097       | 4p15.31    |
| hsa-miR-10a | 1.88               | 0.032        | 17q21.32   |
| hsa-miR-424*| 1.9                | 0.0375       | Xq26.3     |
| hsa-miR-449b| 2.14               | 0.017        | 5q11.2     |
| hsa-miR-411 | 2.34               | 0.0452       | 14q32.31   |
| hsa-miR-184 | 2.64               | 0.017        | 15q25.1    |
| hsa-miR-135b| 3.23               | 0.0068       | 1q32.1     |
| hsa-miR-122 | 3.41               | 0.0282       | 18q21.31   |
| hsa-miR-222*| 3.7                | 0.0169       | Xp11.3     |
| hsa-miR-223*| 3.9                | 0.0014       | Xq12       |
| hsa-miR-934 | 7                  | 0.0529       | Xq26.3     |
| hsa-miR-135b*| 7.9              | 0.015        | 1q32.1     |

*P < 0.01 ANOVA test.
Table 2. Validated targets genes of miRNAs modulated in MCF-7RR cells.

| Down-regulated | Genes |
|----------------|-------|
| hsa-miR-196b   | HOXB8, HOXC8, CD8A, HOXA9, HOXA9, MEIS1, FAS, ETS2, RDX, HOXB7. |
| hsa-miR-146a   | CXCR4, TLR2, FADD, TRAF6, IRAK1, ROCK1, BRCA2, BRCA1, NFkB1, CDKN1A, EGFR, CD40LG, FAS, ERBB4, SMAD4, TLR4, WASF2, STAT1, UHRF1, L1CAM, SMN1, CARD10, COPS8, ELAVL1, NUMB, PTGS2, CCL5, PTGES2, CNOT6L, SIKE1, CXCL12, PRKCE, RAC1, LAMC2, COX2, RNF11. |
| hsa-miR-181a-2*| Non reported |
| Up-regulated   |       |
| hsa-miR-222    | STAT5A, CDKN1B, SOD2, MMP1, FOXO3, CDKN1C, KIT, PPP2R2A, TIMP3, FOS, ICAM1, ESR1, BBC3, PTEN, SELE, DIRAS3, ETS1, DICER1, RECK, TRPS1, CERS2, GJA1, SSX2IP, DKK2, VGLL4. |
| hsa-miR-218    | LAMB3, LASP1, IKBKB, SP1, VOPP1, BIRC6, ACTN1, STAM2, CDKN1B, BIRC5, GJA1, ROBO1, RICTOR, SOST, SFRP2, HOXB3, DKK2, TOB1, CD6, BM1, LEF1, MITF, PDKFRA, GLI2, OTUD7B, RUNX2, CDH2, EGFR, RET, SH3GL1. |
| hsa-miR-10a    | HOXA1, USF2, MAPK7, BTRC, SRSF1, TRA2B, CHL1, PTEN, PIK3CG. |
| hsa-miR-424*   | Non reported |
| hsa-miR-449b   | SIRT1, CCNE2, MET, GMNN, HDAC1. |
| hsa-miR-411    | Non reported |
| hsa-miR-135b   | APC, KLF4, MAFB, CASR, PPP2R5C, SMAD5, LZTS1, MID1, MTCH2, ACVR1B, BMP2, TGFB1. |
| hsa-miR-184    | AKT2, INPPL1, NFATC2, SOX7, EIF2C2, MYC, BCL2, EZR. |
| hsa-miR-122    | CYP7A1, IGF1R, SRF, RAC1, RHOA, ANK2, NFATC2IP, ENTPD4, ANXA11, ALDOA, RAB6B, RAB11FIP1, FOXP1, MECP2, NCAM1, UBA1, TXB19, AACS, DUSP2, ATP1A2, MAPK11, FUNDC2, AKT3, TP53L2, GALNT10, G6PC3, AP3M2, SLCL7A1, XPO6, FOXJ3, SLCL7A1, TRIB1, EGLN3, NUMBL, ADAM17, DTYK, FAM117B, BCL2L2, PRKAB1, ADAM10, ACVR1C, PRKRA, WNT1, PTTP1, NT5C3A, P4HA1, PKM, CLIC4, MEF2D, AXL, NOD2, FUT8. |
| hsa-miR-222*   | STAT5A, CDKN1B, SOD2, MMP1, FOXO3, CDKN1C, KIT, PPP2R2A, TIMP3, TNFSF10, FOS, ICA1, ESR1, BBC3, PTEN, SELE, DIRAS3, ETS1, DICER1, RECK, TRPS1, CERS2, GJA1, SSX2IP, DKK2, ADAM1A, MGMT, VGLL4. |
| hsa-miR-223*   | Non reported |
| hsa-miR-934    | Non reported |
| hsa-miR-135b*  | APC, KLF4, MAFB, CASR, PPP2R5C, SMAD5, LZTS1, MID1, MTCH2, ACVR1B, BMP2, TGFB1. |
Table 3. Genes differentially regulated in knockdown miR-122-MCF-7RR cells with 3’UTR-canonical binding site to miR-122

| Gene   | Function                                                                 |
|--------|--------------------------------------------------------------------------|
| **Up-regulated**                                                                 |                                                                                     |
| IGLON5 | Neuronal cell-adhesion protein                                            |
| NUP62CL| Nucleocytoplasmic transporter activity                                    |
| ACA1   | Acetyl-CoA C-acyltransferase activity                                     |
| KLHL5  | Ubiquitin-protein transferase activity                                    |
| FBXO48 | Decreased IL8 secretion                                                   |
| ZNF304 | Activation of KRAS and silencing of several tumor suppressor genes       |
| VIPR2  | Pituitary adenylate cyclase activation                                    |
| CCDC127| Cell surface transport                                                   |
| ZNF611 | Transcriptional regulation                                               |
| **Down-regulated**                                                               |                                                                                     |
| LCE2C  | Precursors of the cornified envelope of the stratum                      |
| PRH2   | Protective and reparative environment for dental enamel                   |
| RAB40AL| Mediates the ubiquitination and subsequent proteasomal degradation of target proteins |
| MICAL2 | Actin binding and flavin adenine dinucleotide binding                     |
| SEMA5A | Semaphorin gene family that encodes membrane proteins containing a semaphorin domain and several thrombospondin type-1 repeats. May promote angiogenesis by increasing endothelial cell proliferation and migration and inhibiting apoptosis |
| SLC25A18| Involved in the transport of glutamate across the inner mitochondrial membrane. |
| PODNL1 | Small leucine-rich repeat protein family                                  |
| DUSP8  | Phosphatase activity with synthetic phosphatase substrates and negatively regulates mitogen-activated protein kinase activity, |
| DDR2   | Tyrosine kinase that functions as cell surface receptor for fibrillar collagen and regulates cell differentiation, remodeling of the extracellular matrix, cell migration and cell proliferation. |
| C2CD4A | Involved in inflammatory process. May regulate cell architecture and adhesion. |
| KIAA0825| Cell surface transport                                                   |
| ZNF793 | Transcriptional regulation                                               |
| DLX4   | Play a role in determining the production of hemoglobin S. May act as a repressor. |
| LIN28A | Protein that acts as a posttranscriptional regulator of genes involved in developmental timing and self-renewal in embryonic stem cells. Disrupting the maturation of certain miRNAs |
| SP7    | Transcriptional activator essential for osteoblast differentiation.       |
| TFCP2L1| Transcriptional suppressor, cellular self-renewal                         |
| CITED4 | Acts as transcriptional coactivator for TFAP2/AP-2. Enhances estrogen-dependent transactivation mediated by estrogen receptors. |
| TRPM1  | Cation channel essential for the depolarizing photoresponse of retinal ON bipolar cells. It is part of the GRM6 signaling cascade. Metastasis in melanoma |
| IL1R2  | Cytokine receptor that belongs to the interleukin 1 receptor family       |
| VEPH1  | Downregulation of Wnt pathway after Wnt3A stimulation                   |
| ARHGAP23| GTPase activator for the Rho-type GTPases by converting them to an inactive GDP-bound state |

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**C4B**  Complement component 4B, including the ZB transcript in the same orientation, complexing with C2 to form the C3/C5 convertase, classical pathway

**CNIH3**  Regulates the trafficking and gating properties of AMPA-selective glutamate receptors

**DEAF1**  Cell proliferation, arresting cells in the G0 or G1 phase. Required for neural tube closure and skeletal patterning. Regulates epithelial cell proliferation and side-branching in the mammary gland.

**EPPIN**  Serine protease inhibitor that plays an essential role in male reproduction and fertility.

**HEATR3**  Role in ribosomal protein transport and in the assembly of the 5S ribonucleoprotein particle (5S RNP). The encoded protein also may be involved in NOD2-mediated NF-kappa B signaling.

**NUB1**  Protein that functions as a negative regulator of NEDD8, a ubiquitin-like protein that conjugates with cullin family members in order to regulate vital biological events.

**RIPK1**  Serine-threonine kinase which transduces inflammatory and cell-death signals (programmed necrosis) following death receptors ligation, activation of pathogen recognition receptors (PRRs), and DNA damage. Activates the MAP3K5-JNK apoptotic cascade

**KRT77**  Responsible for the structural integrity of epithelial cells
