Introduction

microRNAs (miRNAs) are small non-coding RNAs which regulate the expression of coding genes at a post-transcriptional level through inhibition and destabilization of messenger RNAs [1]. MicroRNAs participate in diverse biological processes like cell division, proliferation, differentiation, death, growth and development, stem cell regulation, metabolism and stress response [2,3].

Aberrant expression of microRNAs is related with the development of different pathologies, including cancer, where they can act either as tumor suppressors or as “oncomirs” (oncogenes). Approximately 50% of the microRNA genes are located in regions commonly affected by chromosomal alterations (amplification, deletions and fragile sites) in the cancer genome [4]. As in the case of messenger RNA expression patterns, microRNA expression signatures can also be used to classify human tumors and to identify molecular signatures associated with relevant clinical characteristics [5,6]. During the biogenesis of microRNAs, an intermediate RNA duplex represents an obligatory intermediate. After the maturation process, the mature, leading strand is preferentially incorporated into the silencing complex, while the role of the other strand (miRNA*) was regarded as a simple passenger. However bioinformatic and functional analyses have identified important regulatory roles of the miRNA*, both in normal and in pathological states, including cancer [7].

In breast cancer, analysis of microRNA expression patterns has led to the identification of signatures which can distinguish tumor from normal tissues [8,9]. Analysis of the messenger RNA targets of microRNAs with differential expression in normal and tumor breast tissues, indicates that their aberrant expression impact the regulation of important cellular networks known to drive breast cancer [10]. This is supported by the observation that several clinically relevant breast tumor features, such as tumor size, nodal involvement, vascular invasion, hormone receptor and HER2 status, are also related to the expression of particular microRNAs [8,9,11]. Additionally, microRNAs might be used as markers of the metastatic potential of primary breast tumors [12].

In order to further analyze the differences in microRNA expression patterns in breast tumors, we evaluated the expression profile of 667 microRNAs in 29 breast tumors compared to 21 adjacent normal tissues. We also compared the expression patterns between four fresh frozen and their corresponding paraffin
embedded tumor tissue pairs, in order to evaluate the robustness of the TaqMan low density array platform as a tool for retrospective studies.

Our analyses identified 130 differentially expressed microRNAs between the tumor and normal tissues, 43 of them whose involvement in breast cancer has not been previously described, to our best knowledge. Differential expression of 14 of these microRNAs was validated in an independent set of normal and tumor samples, suggesting that their aberrant expression might play important roles in breast cancer. Several evolutionary conserved miRNAs were included in this expression signature, showing expression rates similar to their mature strand, suggesting their potential regulatory role in breast tumors.

**Results**

**MicroRNA expression profiles in breast tumors**

Comparative Ct analysis (2-ΔΔCt) was used to identify a set of 130 microRNAs that were differentially expressed between the normal and tumor tissue (adjusted P value ≤ 0.05, fold change: 2). 17 were over-expressed and 113 were down-regulated in the tumor compared to the normal tissue (Table 1 shows the microRNAs with the highest differential expression values, a complete list including all 113 is shown in Table S1). This is in accordance with other studies performed using different types of platforms, such as bead-based flow cytometry [5] or miRNA microarrays [13].

Unsupervised hierarchical clustering analysis of the log-transformed delta Ct values of the differentially expressed microRNAs, showed that this set of markers is able to differentiate the tumors from the normal breast tissues (Figure 1, figure S1). We observed an overlap between microRNAs expressed in normal and tumor samples, indicating that miRNA expression levels, rather than a differential tissue-specific pattern, is driving the separation between normal and tumor tissue.

**Identification of microRNAs with previously unknown involvement in breast cancer**

Out of the 130 differentially expressed microRNAs we detected in this study, 43 (30%) have not been previously reported in the literature as involved in breast cancer, to our best knowledge (Table 2). Some of these represent the passenger strand (miRNA*) of pre-miRNAs. In some cases, like miR-10b* and miR-145*, their corresponding leading strand have important, proven roles in breast cancer, but the role of the star strand has not been explored. Interestingly, for most of the microRNA* included in our profile, their guiding opposite strand is differentially expressed in the tumor tissue, in most cases with a very similar expression rate (Figure 2).

The published and validated transcriptional targets (oncogenes, estrogen regulators, tumor suppressors, miRNA biogenesis machinery and epigenetic master regulators) of the miRNAs with no previous involvement in breast cancer, indicate that they might have important biological implications in breast tumor biology (Table 2).

**Evaluation of the reproducibility of the microRNA screening method**

microRNA expression profiles were analyzed in a set of 29 breast tumor tissues compared to 21 normal, non-paired, adjacent tissues (clinical characteristics of these samples is presented in Table 3). 23 tumors and two pools of normal samples were run in triplicate to assess the reproducibility of the TaqMan low-density array. The standard deviation between the technical triplicates was 0.1675507, while the Spearman Correlation was 97% (Max: 99%, Min: 96%). The Spearman correlation as well as the standard deviation values between our triplicates, showed a high correlation between each technical and biological replicates (Figure S2).

**Hormone receptor status is associated to the expression of different microRNAs**

Delta Ct analysis identified differential microRNA expression signatures related with ER and PR status in the tumor samples. ER+ tumors showed differential expression of miR-342-3p, miR-29c*, miR-29b-2*, miR-30e, miR-190b, miR-769-5p, miR-30d and miR-432, with a (P value ≤ 0.05) compared to ER- tumors (Table 4, figure S3). Differential expression of miR-145*, miR-
34a* and miR-193b* (adjusted P value ≤ 0.05) was able to discriminate between the PR+ from the PR- samples (Figure S4).

Analysis of the miRNA/miRNA* sequence conservation

Most of the miRNA/miRNA* pairs included in the expression profile were conserved in the seed sequence region across the five different vertebrate genomes we analyzed (Figure S5). Some of the miRNA*, like miR-10b* and miR-30a*, presented divergence in its sequence, reflected in the percentage of nucleotide substitutions. However, most of the miRNA/miRNA* showed a high degree of evolutionary conservation of the passenger miRNA* strand with a low percentage of nucleotide substitutions (miR-19b, miR-19b*, miR-125, miR-125*, miR-26b, miR-26b*, miR-145, miR-145*, miR-335, miR-335*, miR-214 and miR-214) (Figure S6). This analysis determined that the miRNA* detected in our analyses is both differentially expressed between the normal and tumor tissues, in most cases with a very similar expression pattern compared to the corresponding leading strand and is also evolutionary conserved, suggesting that they might have a biological role in breast cancer.
Table 2. Differentially expressed microRNAs with no previous involvement in breast cancer and their validated transcript targets.

| miRNA   | Validated targets         | miRNA   | Validated targets         |
|---------|--------------------------|---------|--------------------------|
| miR-379 | None reported            | miR-656 | None reported            |
| miR-433 | FGFFR1, FGF20, HDAC6, GRB2 | miR-543 | None reported            |
| miR-655 | None reported            | miR-10b*| DICER1, ERBB2, EGFR, RHOC|
| miR-99a | NFKB1, SLC12A2, DICER1, AKT1, MYC, IGF1 | miR-145*| DGC8R8, KRAS, ADAM17, BCL2, ERBB2, DICER1, |
| miR-758 | None reported            | miR-26b*| CDDN1, BRCA1, NOTCH1, EZH2, DICER1, MYC, EPHA2 |
| miR-874 | None reported            | miR-30e*| ERBB2, TGFBR2, CCND1     |
| miR-127-3p | BCL2, BCL6, MYC, NOTCH1,  | miR-30a*| DICER1, ARL2, PRKCI, NOTCH1, SOX2 |
| miR-135a | JAK2, STAT3, PI3         | miR-99a*| None reported            |
| miR-518b | None reported            | miR-214*| VEGFA, EZH2, MYC, PAK1, TWIST1, DICER1 |
| miR-382 | POMC, RUNX1, MECP2       | miR-92a-1*| TGFBI, IGFRI, BRCA2, MYC |
| miR-134 | CREB1, YY1, SOX2, MDM4   | miR-129-3p| SOX4, MLH1               |
| miR-889 | None reported            | miR-215 | ZEB2, ALCAM, MCL-1       |
| miR-338-3p | DICER1, ERCC4, HES5, MCM2, BRAF | miR-488 | None reported            |
| miR-487b | None reported            | miR-668 | None reported            |
| miR-455-5p | TLR4, UCP1, MAPK3       | miR-222*| ETS1, TNF, AKT1, BCL2, DICER1, EPHB2 |
| miR-361-5p | TGFBI, TLR4, AKT1, IRS1 | miR-454*| None reported            |
| miR-517a | None reported            | miR-148b*| IL6, TLR3, TLR4, APC     |
| miR-539 | HLC5                     | miR-877 | RNASEN                   |
| miR-512-3p | CFLAR, CDKN1A, DNMT1    | miR-183*| PIK3CA, DICER1, FOXO1, PTEN, SAG |
| miR-491-5p | FGFRI, NCOA6, FOSB, TNF | miR-592 | DICER1                   |
| miR-331-5p | MDM2, SOAT1, ERBB2, JAG1 | miR-425 | DICER1, SMAD3, PDGF/C    |
| miR-136* | CD36                     |         |                          |

Figure 2. Comparison of the expression rate between miRNA-miRNA*. The bars show the normalized expression values of the microRNA pairs: driver (microRNA) and passenger (miRNA*) strands present in the breast cancer profile.
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Validation of differentially expressed microRNAs in an independent set of breast tumor tissues

A set of 17 microRNAs was selected for further analysis in an independent set of samples through evaluation of their expression using independent TaqMan assays. This set included 13 micro-RNAs with differential expression between the tumor and normal tissues and 4 with non-differential expression. Expression profiles were concordant in 14/17 (82.3%) of the selected microRNAs, and only three failed to replicate. A similar expression value was obtained for the same microRNA in both of the TLDA and the single probe assay. (Pearson Correlation: 97.3153%) (Table 5).

Deregulated microRNAs and their putative transcriptional targets

To define potential mRNA targets of the differentially expressed microRNAs, and their impact on cellular pathways, we performed an mRNA target prediction analysis with at least 3 different algorithms (Tables 6–7) followed by enrichment analysis of the predicted mRNA targets using Diana, mir-Path and the Reactome databases. The list of the top pathways ranked by the enrichment P-value is presented in Table 8.

Comparison of microRNA profiles between Paraffin embedded and fresh-frozen tissues

Formalin fixed paraffin embedded (FFPE) tissue represents a major source of potentially useful biological material for retrospective analysis. To determine the performance and robustness of the microRNA TLDA system in the analysis of microRNAs obtained from FFPE tissues, we compared the expression patterns of pairs of fresh-frozen and FFPE tissues from the same patient. Correlation between these results was analyzed using Spearman correlation and unsupervised hierarchical clustering. We observed cluster aggregation, as well as a high correlation value between the fresh and the FFPE tissues obtained from the same patient (average 93.75%, minimum of 90%; maximum of 98%), indicating that results obtained from RNA isolated from FFPE tissues retain the same expression signature as the fresh frozen tissue (Figure 3).

Discussion

Differentially expressed microRNAs with no previous involvement and potential relevance in breast cancer

We have analyzed the microRNA expression patterns in a set of breast cancer tumors and compared them to normal tissue. Our
analyses identified a set of differentially expressed microRNAs whose role in breast cancer has not been previously described, to our best knowledge. Several evolutionary conserved miRNA* were included in this expression signature, showing expression rates similar to their mature strand. Differential expression of a set of these microRNAs was validated in an independent set of tumor and normal breast tissues, suggesting their potential regulatory role in breast tumors. Finally, we evaluated the performance of the TaqMan low-density array through the comparison of microRNA expression patterns obtained from fresh-frozen and FFPE tissue pairs, obtaining similar results in both cases.

### Table 4. Differentially expressed miRNAs associated with hormone receptor status.

| miRNA | Fold Change | p.value | Pathways of the mRNA targets |
|-------|-------------|---------|-----------------------------|
| miR-342-5p | 1.576048754 | 0.002539134 | Cell cycle |
| miR-29c* | −2.631736726 | 0.007378817 | DNA replication |
| miR-30e | −1.517276999 | 0.003742107 | Cell surface interactions |
| miR-190b | 3.822193954 | 0.001163848 | Apoptosis |
| miR-30d | 1.305024162 | 0.005495711 | |
| miR-432 | 1.110681182 | 0.003739888 | Cell cycle check points. |

| miRNA | Fold Change | p.value | Pathways of the mRNA targets |
|-------|-------------|---------|-----------------------------|
| miR-145* | 3.171062506 | 0.043296329 | Apoptosis |
| miR-34a* | 2.12616702 | 0.013542569 | DNA repair |
| miR-193b* | 3.125460441 | 0.003739888 | Immune system |

**Estrogen Receptor**

**Progesterone Receptor**

### Table 5. miRNAs selected for validation in an independent set of tumor and normal breast tissues.

| miRNA | Independent Assay | TLDA |
|-------|--------------------|------|
| RQ    | P-Value            | RQ   | Adj.P-Value |
| miR-129-3p | −22.241252466 | 0.0509 | −23.8634845 | 0.0506 |
| miR-136* | 5.641025641 | 0.0078 | 2.708003 | 3.85E-06 |
| miR-99a | 3.8334934654 | 0.0082 | 1.303934 | 0.001342569 |
| miR-10b | 5.980861244 | 0.0484 | 2.990468209 | 0.000109373 |
| miR-206 | 2.070966521 | 0.009 | 2.355497628 | 0.0077 |
| miR-27a | 3.58347292 | 0.0142 | 1.982850027 | 0.009750279 |
| miR-145 | 2.671988854 | 0.0414 | 2.90916182 | 0.001369377 |
| miR-21 | 2.054 | 0.0274 | 1.271732126 | 0.052188036 |
| miR-184 | 6.672 | 0.2183 | 3.3103 | 0.169 |
| miR-24 | 1.804077215 | 0.1159 | 1.857351256 | 0.079 |
| miR-492 | 3.2652 | 0.0724 | 2.3677 | 0.1465 |
| miR-326 | 2.419549964 | 0.2573 | 2.578 | 0.678 |
| miR-488 | 6.954102921 | 0.0063 | 5.61731454 | 6.33E-06 |
| miR-668 | 15.87301538 | 0.04 | 14.15451895 | 0.0019 |
| miR-25* | 3.4012 | 0.2661 | 20.581 | 0.0133 |
| miR-431 | 8.605851979 | 0.1159 | 2.2719 | 0.0576 |
| miR-149* | 8.271298594 | 0.0033 | 10.269 | 0.0298 |

DR: down-regulated, OR: over-expressed, DR*: down Regulated, but expressed in 78% of the samples, FP: False Positive.
Between the microRNAs without previous involvement in breast cancer, we identified the down-regulation of miR-129-3p. In mouse lung epithelial cells, lentiviral mediated expression of miR-129-3p results in G1 phase arrest and cell death through down-regulation of CDK6, ERK1 and ERK2, indicating its activity in cell proliferation. Epigenetic repression of miR-129 also

| miRNA   | Status       | Cellular pathways related with cancer                                                                 |
|---------|--------------|-------------------------------------------------------------------------------------------------------|
| miR-129-3p | Down-regulated | Regulation of BAD phosphorylation, IGF-1, ERBB2 in signal transduction and oncology, breast cancer resistance to antimicrotubule agents and influence of RAS and RHO proteins on G1 to S transition |
| miR-488 | Down-regulated | CAR1 and regulation of the Estrogen Receptor                                                        |
| miR-139-5p | Down-regulated | Apoptosis, chromosome maintenance and transmembrane transport of small molecules.                   |
| miR-655 | Down-regulated | Apoptosis, signaling by VEGF, cell cycle membrane, metabolism of protein and signaling by insulin receptor. |
| miR-134 | Down-regulated | Transmembrane transport of small molecules and transcription                                         |
| miR-136* | Down-regulated | Signaling by EGFR, signaling by Wnt, apoptosis, transcription, cell junction organization and signaling by VEGF. * |
| miR-874 | Down-regulated | Signalling by Notch, gene expression, cell cycle, mitotic and immune system**                       |
| miR-539 | Down-regulated | Apoptosis, signaling by insulin receptor, pyruvate metabolism and citric Acid (TCA) cycle, signaling of EGFR, steroid hormones, cell cycle, mitotic and signaling by Notch. |
| miR-491-5p | Down-regulated | DNA replication, Signaling by notch, regulatory RNA pathway and signaling by EGFR                    |
| miR-889 | Down-regulated | Cell cycle, cell junction organization, membrane trafficking, metabolism of hormones and DNA repair** |
| miR-222* | Down-regulated | Transmembrane transport of small molecules, apoptosis, cell cycle, signaling by EGFR and cell cycle checkpoint *** |
| miR-877 | Over-expressed | Signaling by EGFR and cell junctions Organization                                                    |
| miR-425 | Over-expressed | EXT2 (possible tumor suppressor), MET receptor, PAK4. Cell cycle signaling, cell signaling checkpoints and transport of small molecules and biological oxidation. |
| miR-454* | Over-expressed | Transmembrane transport of small molecules, membrane trafficking, apoptosis, cell cycle, and DNA replication* |
| miR-592 | Over-expressed | ERBB4, CD200, ST7. DNA replication, apoptosis, miRNA processing, transcription and metabolism of lipids |
| let-7g* | Over-expressed | Cell junction organization, apoptosis, Immune system, signaling by TGF beta and integration of energy metabolism** |
| miR-183* | Over-expressed | Transmembrane transport of small molecules, cell junction organization, signaling by VEGFR, and integration of energy metabolism** |

*mRNA targets predicted only with Miranda,
**mRNA targets predicted with Miranda and Targetscan,
***mRNA targets predicted with Miranda and Pictar.

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Table 7. Previously reported miRNAs with differential expression in breast cancer, some of their mRNA targets and the cellular pathways where they participate.

| miRNA   | Status       | Targets and cellular pathways related with cancer                                                                 | Bibliography |
|---------|--------------|---------------------------------------------------------------------------------------------------------------|--------------|
| miR-125a/b | Down-regulated | Oncogene: ERBB2 and ERBB3. Signal transduction: MAP3K10 and MAP3K11. c-raf-1 | [32]          |
| miR-10b  | Down-regulated | Growth factor: FLT and BDNF. Transducing factor :SHC1. Oncogene: Rho. Homeobox gene: HOXD10 | [13]          |
| let-7    | Down-regulated | Oncogene: RAS. Architectural factor: HMG12       | [13]          |
| miR-205  | Down-regulated | Growth factor: TGF-β. HER3 phenotype. Oncogene: ErbB3 and Zeb1 | [61]          |
| miR-145  | Down-regulated | Kinase: RAF1. Oncogene: YES | [13,62] |
| miR-31   | Down-regulated | Oncogene: Rho, Metastasis-promoting genes FZAD3, ITGAS, M-RIP. Regulate invasion and metastasis. Patients with higher miR-31 expression, had prolonged survival. | [63]          |
| miR-335  | Down-regulated | Oncogene: SOX4 and TNC. TNC (Tenascin C): responsible for the acquisition of metastatic properties | [64]          |
| miR-126  | Down-regulated | Growth factor: VEGF. Insulin receptor tyrosine kinase: IRS-1 | [64]          |
| miR-101  | Down-regulated | EZH2, oncogenic and metastatic activity | [65]          |
| miR-206  | Down-regulated | CAR1 and Regulation of the Estrogen Receptor, T Cell Receptor Signaling Pathway, Regulation of BAD phosphorylation. | [66]          |
| miR-222  | Down-regulated | ERs negatively modulates miR-222. Confers proliferative advantage and migratory activity to cells and promote the transition from ER-positive to ER-negative tumors | [32]          |
| miR-21   | Over-expressed | BCL-2, PTEN, MASP2, involved in apoptosis | [38]          |
| miR-210  | Over-expressed | Overexpression is induced by hypoxia in a HIF-1α- and VHL-dependent manner. miR-210 influences the hypoxia response by targeting a transcriptional repressor of the MYC-MAX pathway | [67]          |

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leads to over-expression of the SOX1 oncogene in gastric and endometrial cancer [14]. In breast cancer, SOX1 is activated due to the loss of miR-335, which was also found down-regulated in endometrial cancer [14]. In breast cancer, SOX1 is activated due to the loss of miR-335, which was also found down-regulated in endometrial cancer [14].

Increased expression of miR-215, down-regulated in our expression profile, has been associated with cell proliferation rate and has been proposed as a tumor suppressor candidate in colon cancer. miR-215 reduces cell proliferation and cell cycle G2-arrest through regulation of dihydrofolate reductase thymidylate synthase (DTL), and increased expression of TP53 and p21 [16]. DTL has been implicated in cell proliferation, cell cycle arrest and cell invasion in diverse tumor types, including hepatocellular carcinoma and breast cancer [17].

We found down-regulation of some of the miR-99 family members, including miR-99a, which has also been described in advanced prostate cancer cell lines and tissues. The direct targets of the miR-99 family are the chromatin remodeling factors SMARCA5, SMARCD1 and the growth regulatory kinase mTOR, which is also an important pathway activated in breast cancer [18]. miR-99a has also been found downregulated in serous ovarian carcinoma [19].

Senescence represents a potent tumor suppressive mechanism, and involvement of microRNAs in this process has been already described. miR-668, down-regulated in head and neck squamous cell carcinoma cell lines and in our expression profile, has been identified as a senescence-inducing microRNAs, playing an important role not only in cancer pathogenesis, but also as an interesting target for the development of new therapeutic targets [20].

Over-expression of miR-425 has been described in several human cancer cells [21] and its pri-miRNA sequence is evolutionarily conserved in different mammals (human, mouse, dog and opossum) supporting the idea that miR-425 plays a regulatory role in eukaryotic cells [22]. TargetScan, MirTarget2, Miranda, miRTar, PITA and RNA hybrid algorithms, predicted DICER1 and SMAD2 as potential targets of miR-425. SMAD is involved in the regulation of DROSHA, another key player in small RNA processing [23], indicating that aberrant expression of miR-425 might have important effects in the biogenesis of small RNAs.

miR-592 was over-expressed in our tumor dataset. This microRNA has been found differentially expressed between DNA mismatch repair deficient and proficient colon tumors. The interactions between miR-592 and genes associated with the mismatch repair system suggest an oncogenic role of this miRNA, possibly acting through inhibition of tumor suppressor genes [24]. Upregulation of miR-592 has also been reported as part of the microRNA signature of kidney cancer [25].

miR-877 is a DROSHA independent intronic microRNA, up-regulated in our breast tumor samples. It is part of the coding region of the ATP-binding cassette subfamily F member 1 (ABCF1), which is over expressed in breast cancer. ABCF1 is a transporter of molecules through membranes, and has been associated to drug resistance and the development of some types of cancer [26].

Together with this set of novel microRNAs, we also confirmed the differential expression of microRNAs whose role and biological targets in breast cancer have been well described (Table 5). This is the case of the down-regulation of miR-125b [13,27], let-7

### Table 8. Statistically significant biological pathways potentially affected by the differentially expressed microRNAs in breast cancer.

| Pathway                        | # of Genes (Union) | p-value        | Description (KEGG Pathway Database)                                                                 |
|--------------------------------|--------------------|----------------|-----------------------------------------------------------------------------------------------|
| MAPK signaling pathway        | 130                | 0.0002488      | Involved in cellular functions: cell proliferation, differentiation and migration.              |
| Wnt signaling pathway         | 85                 | 0.0000056      | Required for developmental processes, cell proliferation and cell division.                   |
| Focal adhesion                 | 100                | 0.00000193     | Cell motility, cell proliferation, cell differentiation, regulation of gene expression and cell survival. |
| Adherens junction              | 47                 | 0.0001819      | Important for maintaining tissue architecture and cell polarity                              |
| TGF-beta signaling pathway    | 54                 | 0.00001657     | Regulates proliferation, apoptosis, differentiation and migration.                            |
| Insulin signaling pathway     | 73                 | 0.00001462     | Allows Tyrosine phosphorylation of insulin receptor substrates                                |
| Regulation of actin cytoskeleton | 100                | 0.0001411     | Cell-matrix adhesions: cell motility, cell proliferation, cell differentiation, regulation of gene expression and cell survival |
| Ebr signaling pathway         | 49                 | 0.0001177      | Regulates proliferation, differentiation, cell motility, and survival.                       |
| Ubiquitin mediated proteolysis| 66                 | 0.0000034      | Signal for protein degradation                                                               |
| Gap junction                   | 48                 | 0.000826       | Intercellular channels to communicate the cytosolic compartments with neighbor cells         |
| Basal cell carcinoma           | 29                 | 0.0000621      | Common cancer related with epithelia                                                          |
| Calcium signaling pathway     | 71                 | 0.0000547      | Electrochemical gradient across the plasma membrane                                           |
| VEGF signaling pathway         | 34                 | 0.000047       | Mediator of VEGF-driven responses, is a crucial signaling pathologic angiogenesis             |
| Androgen and estrogen metabolism | 8               | 0.0000361      | Sexual hormones                                                                               |
| Glycerophospholipid metabolism| 31                 | 0.000344       | Lipid metabolism                                                                              |
| Hedgehog signaling pathway    | 26                 | 0.0000332      | Regulates stem cell proliferation                                                             |
| Cell Communication            | 33                 | 0.00000329     | Intercommunication between cells                                                              |
| Jak-STAT signaling pathway    | 59                 | 0.000213       | Signaling mechanism of cytokines and growth factors                                           |
| Tight junction                 | 52                 | 0.00186        | Mediate cell adhesion                                                                          |
| TP53 signaling pathway        | 28                 | 0.0000157      | Responses to stress signals, DNA damage, oxidative stress and activated oncogenes           |

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[5,13,28] miR-205 [29–31], miR-145 [13,31], miR-10b [13], miR-222 [32], miR-34a [33], miR-31[34], miR-206 [12,13,35]; and over-expression of miR-210 [13,36,37] and miR-21 [13,28,38] (check reference [39] for a review).

As part of our expression profile we found an important presence of miRNAs*. Recent bioinformatic and experimental data show a high degree of conservation over vertebrate evolution, particularly in the seed regions of expressed miRNAs* [7]. The miRNA/miRNA* ratios also change in different developmental stages [40] and have been involved in the regulation of different biological networks in normal physiological conditions [41]. In cancer, miRNA* expression has been detected in childhood acute lymphoblastic leukemia [42], myelodysplastic syndrome [43], cell lines of tumors of the female reproductive tract [44] and melanoma [45] indicating their potential role in cellular transformation. The set of miRNA* we found differentially expressed between the normal and tumor breast tissues show a high degree of evolutionary conservation, according to our analysis in five animal genomes, as well as a similar expression rate than their corresponding leading strand. These results suggest that these miRNA* might be playing regulatory roles in breast cancer.

Breast cancer etiology includes genomic alterations that drive cancer cell development, like loss of heterozygosity, amplifications, deletions and fragile sites, which can promote oncogene activity or repress the expression of tumor suppressors [46]. More than half of the human miRNAs (60%) are located in regions commonly affected in the cancer genome, a situation that might affect their expression [4]. Our analysis identified down-regulation of 22 microRNAs located in the 14q32 region, which is deleted in approx. 10% of breast tumors [20], and has been reported as a chromosomal region where several breast cancer-related microRNAs are located [47] (Figure S7), suggesting that loss of this chromosomal region and the down-regulation of the miRNAs codified in this locus, might be correlated in a fraction of breast tumors.

Hormone receptor status is an important tumor characteristic to classify breast cancer and to determine clinical treatment. However, there is limited information about the genetic mechanisms regulating the expression of hormone receptors. We identified a set of miRNAs which can differentiate hormone receptor positive and negative tumors. Analysis of the miRNA targets of these miRNAs identified biological pathways relevant to breast cancer, like apoptosis, DNA repair, cell cycle check-points, etc. (Table 3). The miRNAs in the ER signature can directly regulate transcripts like ESR1 (miR-342-5p, miR-190b, miR-432), ESRRG (miR-30d, miR-30e), ESRRB (miR-432); ERN2 (miR-342-5p), which induces translational repression in response to ER stress, coactivators of the estrogen receptor like PELP1 (miR-342-5p) and SRC (miR-342-5p); mediators of cell cycle through estrogen activation like E2F (miR-30d, miR-30e and miR-432) and transcription factors like DFI (miR-30c). While miRNAs of the PR profile can regulate the activity of the progesterone receptors PRDM4 (miR-34a*), PRDM16 (miR-145*) and co-activators of the progesterone pathway like SRC (miR-34a*, mir-145*) and MAPK (miR-145*, miR-193*, miR-34a*), miR-190 and miR-345 have already been reported as discriminators of ER status [47], suggesting its importance in the establishment of this phenotype.

Deregulation of microRNA expression might potentially affect the regulation of multiple cancer-related genes; for this reason it’s important to define the biological networks affected by differentially expressed microRNAs and their transcriptional targets. Pathway analysis of our expression profile determined different transcripts and protein-protein interactions, which can be activated or repressed by these miRNAs. Examples of these pathways are ERBB signaling, which plays a determinant role in breast cancer [48] through its contribution to tumor development, cellular transformation, involvement in the development of central nervous system metastases and targeted therapy [49]. Another important pathway affected by the differentially expressed miRNAs is the mitogen-activated protein kinase (MAPK), which is activated in breast cancer and is involved in the initiation and pathogenesis of breast tumors [50]. Analysis of the targets affected by the miRNAs with no previous relation with breast cancer included in our signature, also identified several cancer-related pathways, including KRAS, EGFR, MAPK, VEGF, ERBB, PTEN, FOS, AKT1, etc. (Table 6, table 8, figure S8).

Finally, in order to evaluate the potential application of the TLDA platform in the retrospective evaluation of miRNA expression patterns in breast cancer, we carried out a comparison between results obtained from fresh frozen and FFPE tissue. Results of the comparison showed a high correlation between the two tissues, indicating that the platform can be used in retrospective studies using FFPE tissue [51].

Materials and Methods

Breast tissue samples and RNA extraction

After obtaining the written patient’s informed consent, tumor and normal adjacent breast samples were collected during surgery at the Institute of Breast Diseases (FUCAM) in Mexico City. The protocol was reviewed and approved by the Ethics and Research committees of the National Institute of Genomic Medicine and the Institute of Breast Diseases in Mexico City (CE2009/11). Tissues were macroscopically analyzed by a trained pathologist and stored at −80°C until further processing. Frozen sample sections were evaluated histologically to assure that only samples with more than 80% of tumor cells were included in our analyses. Frozen tissues were disrupted with a Tissue Ruptor (Qiagen Inc., Valencia, CA) and total RNA was obtained using the Trizol protocol (Invitrogen, Carlsbad, CA). For the FFPE tissues, total RNA was isolated with the Recoverall kit (Ambion, Austin, USA) according to the manufacturer’s protocol. Briefly, 10-8 µm sections were incubated in xylene for 3 minutes at 50°C for de-paraffinization, followed by two brief washes in 100% ethanol. Once ethanol was evaporated, RNA extraction was done as described in the kit’s protocol. Total RNA concentration was evaluated by spectrophotometry (NanoDrop Technologies, Wilmington, Delaware). Total RNA integrity from the frozen samples was analyzed using the Agilent 2100 Bioanalyzer with the Nano-Eukariotic chip.

MicroRNA expression analysis

The Megaplex TLDA, v2.0 (TaqMan® Low Density Array, Applied Biosystems (ABI), Foster City, CA) platform was used to measure miRNA expression. There are two plates in this system: plate A, containing well-characterized and widely expressed microRNAs, while plate B presents less characterized microRNAs. The combined plates evaluate the expression of 667 unique human specific microRNAs (present in V14 of the Sanger miBase) in parallel. Briefly, the procedure begins with the retro-transcription of 70 ng of total RNA with stem-loop primers.
to obtain a cDNA template. A pre-amplification step was included in order to increase the concentration of the original material and to detect microRNAs that are expressed at low levels. The pre-amplified product was loaded into the TaqMan® Low Density Arrays and amplification signal detection was carried out using the 7900 FAST real time thermal cycler (AB). A total of 29 tumor and 21 normal samples (two pools; one containing five normal samples, other containing 12 normal samples, plus 4 independent normal samples) were analyzed. 23 tumors and the two normal pools were processed by triplicate, representing 92% of the total samples. Raw microRNA expression data is available at the Gene Expression Omnibus (GEO), with accession number GSE33412.

**Statistical analysis**

To determine the expression level of each microRNA, the comparative Ct (2^(-ΔΔCt)) method was used. RNU44 and RNU48 showed the most stable expression between samples and were selected for normalization across all experiments [52]. All analyses were done using R [HTqPCR, gplots-biocductor]. The Ct raw data (fractional cycles numbers at which the fluorescence cross the threshold) was determined using an automatic baseline and a threshold of 0.2. Samples with a Ct value of <36 cycles were excluded from the analyses and the normal tissue samples were used as calibrators. A geometric mean was used to obtain the expression differences in the Ct values between controls and tumors as well as between hormone receptor positive and negative tumors. Only microRNAs with an adjusted t-test value of 2 and consistent expression in at least 80% of the samples were considered as differentially expressed. Unsupervised clustering analysis, using Spearman correlation and average linkage, was used to identify different sub-groups defined by microRNA expression profiles. The rank-invariant normalized data was evaluated through Spearman correlation analysis between biological and technical replicates.

**Analysis of potential mRNAs targeted by differentially expressed microRNAs**

Possible mRNA targets of the differentially expressed microRNAs were identified using the mirDIP [2011] [53] and miRwalk databases [2011] [54], through an integrative evaluation with different algorithms: TargetScan v5.1 (http://www.targetscan. org), PicTar (http://picTar.mede-berlin.de), miRanda (www. mirorna.org/mirorna/getGeneForm.do), and Pita (Gene:weizmann.ac.il/pubs/mir07/mir07_data.html). We only considered as potential mRNA targets those who were predicted by three of these algorithms. Gene ontology and cellular pathway analysis altered by the aberrant expression of the microRNAs was done using the Reactome [55] and DIANA lab software [56], which obtained the information from TargetScan 5 (2009) [57], PicTar 4-way (2007) [58], and visualized in Wikipathways [59].

**Biological replication of differentially expressed microRNAs**

Independent RT-PCR analysis using specific TaqMan microRNA assays was performed to replicate the expression of 17 microRNAs (miR-10b, miR-668, miR-431, miR-136*, miR-129-3p, miR-488, miR-99a, miR-25*, miR-27a, miR-149*, miR-206, miR-492, miR-21) in an independent set of 20 normal and 55 fresh-frozen breast tumor samples. All assays were run in duplicate. These microRNAs were chosen based on differential expression between tumors and normal tissues and statistical confidence. Of this set of microRNAs, there is no previous information regarding the expression of miR-136*, miR-99a, miR-488 and miR-668, in breast tumors. MiR-184, miR-24, miR-492 and miR-326, whose expression did not change significantly between tumors and normal tissues in the TLDA assay, were selected as experimental controls, while RNU-44 and RNU-48 were used as endogenous controls for normalization.

**Conservation of mature microRNA/miRNA* sequence analysis**

We made a comprehensive computational survey of microRNA conservation sequence across 5 animal genomes: Gasterosteus aculeatus (fish), Xenopus tropicalis (frog), Anolis carolinensis (reptile), Gallus gallus (bird), Monodelphis domestica (marsupial), Mus musculus (rodent) and Homo sapiens. We used the miROrtho database [60], to make multiple ortholog alignments and evaluate the conservation of the RNA secondary structure. For this analysis, we blasted the hairpin structure sequence of each microRNA obtained from miRBase.

**Correlation between microRNA profiles in FFPE tissues and fresh frozen samples**

Four fresh-frozen samples and their corresponding FFPE tissues obtained from the same patient’s tumor were analyzed with the TLDA platform (grade A) to evaluate the effect of formalin fixation and paraffin embedding process on the microRNA expression patterns. Correlation between the fresh-frozen and the FFPE results was evaluated using Spearman Correlation. For all statistical analysis, the log transformed delta Ct values were used. Non-supervised clustering analysis was done using Euclidean distance and average linkage including all samples. The results were visualized in a heat map.

In conclusion, our analysis identified a set of microRNAs with no previously known involvement in breast cancer, whose altered expression target relevant cellular pathways. The identification of a set of evolutionary conserved microRNA* showing differential expression between the normal and tumor tissues interesting research opportunities to study the role of the passenger microRNA strand in cancer.

**Supporting Information**

**Figure S1 Principal Component analysis based in the microRNA differential expression profile.** The two most informative components were plotted. Clustering of the normal tissues and tumor tissues is observed. (TIF)

**Figure S2 Signal correlation Plot between the biological and technical samples analyzed.** A) Scarlet plots of the correlation between expression values between control samples evaluated by Spearman correlation (correlation: 100-93%) B) Scarlet plots of the correlation between expression values between breast tumor tissues (correlation: 100-84%). (TIF)

**Figure S3 Unsupervised hierarchical clustering using the differentially expressed microRNAs between Estrogen Receptor (ER) positive and ER negative samples.** The heatmap (Spearman correlation, Euclidean distance, complete linkage) represents Delta Ct values. Heat map colors correspond to microRNA expression as indicated in the color key: red over-
expressed and green down-regulated. Salmon line: PR negative and Dark blue line: PR positive. (TIF)

Figure S4 Unsupervised hierarchical clustering using the differentially expressed miRNAs in Progesterone Receptor (PR) positive and PR negative samples. The heatmap (Spearman correlation, Euclidean distance, complete linkage) represents Delta Ct values. Heat map colors correspond to miRNA expression as indicated in the color key: red over-expressed and green down-regulated. Salmon line: PR negative and Dark blue line: PR positive. (TIF)

Figure S5 Analysis of evolutionary conservation by multiple sequence alignments. The upper panel shows sequence alignments with the consensups hairpin sequence and the conservation profile displayed in the grey histogram. The mature miRNA sequence is underlined. The miRNA sequence is located at the left side of the aligned sequence while the miRNA* is at the right. The inferior panel shows the consensus secondary structure of the orthologous sequence. The color-coding of the nucleotide changes is shown in the box. (TIF)

Figure S6 Consensus secondary structure of the orthologous sequence. Percentage of the miRNAs nucleotide substitutions in each miRNA/miRNA* of the seed regions (2–8 nucleotide). The blue bars represents the miRNA strand, the red bars represents the miRNA* strand. (TIF)

References
1. Rana TM (2007) Illuminating the silence: understanding the structure and function of small RNAs. Nat Rev Mol Cell Biol 8: 23–36.
2. Kroj J, Lodiego I, Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11: 597–610.
3. Cheng AM, Byrom NW, Seshen J, Ford LP (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic acids research 33: 1290–1297.
4. Zhang L, Huang J, Yang N, Greshock J, Megraw MS, et al. (2006) microRNA exhibit high frequency genomic alterations in human cancer. Proceedings of the National Academy of Sciences of the United States of America 103: 9136–9141.
5. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, et al. (2005) MicroRNA expression profiles classify cancers. Nature 435: 834–838.
6. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, et al. (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 103: 2257–2261.
7. Yang JS, Phillips MD, Bettei D, Mu P, Ventura A, et al. (2010) Widespread regulatory activity of vertebrate microRNAs species. RNA 17: 312–326.
8. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065–7070.
9. Bleulikin C, Goldstein LD, Thorne NP, Sipeter I, Chiu SF, et al. (2007) MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol 8: R214.
10. O’Day E, Li (2010) MicroRNAs and their target gene networks in breast cancer. Breast Cancer Res 12: 201.
11. Lowery AJ, Miller N, Devaney A, McNell RE, Davoren PA, et al. (2009) MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/new receptor status in breast cancer. Breast Cancer Res 11: R27.
12. Tavaozie SF, Alarcon G, Oskarson T, Padua D, Wang Q, et al. (2008) Endogenous human microRNAs that suppress breast cancer metastasis. Nature 451: 147–157.
13. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065–7070.
14. Huang YW, Liu JC, Dearthridge DE, Luo J, Mutch DG, et al. (2009) Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 oncogene in endometrial cancer. Cancer Res 69: 9030–9046.
15. Negrimi M, Calin GA (2008) Breast cancer metastasis: a microRNA story. Breast Cancer Res 10: 203.
16. Karayayz M, Pal T, Song B, Zhang C, Georgakopoulos P, et al. (2011) Prognostic Significance of miR-215 in Colon Cancer. Clinical colorectal cancer.

Figure S7 Chromosome 14 and policistronic miRNAs. A) Number of miRNAs included in the expression profile and their chromosomal location. Asterisks indicate chromosomes with the higher numbers of differentially expressed miRNAs. B) microRNA with differential expression in chromosome 14. Green lines indicate the miRNAs that are included in our differential profile. (TIF)

Figure S8 Gene Ontology analysis of the pathways affected by the differentially expressed novel microRNAs. Enrichment analysis made with the miRNA targets of the not previously reported miRNAs. Yellow circles indicate the pathways associated with breast cancer. (TIF)

Table S1 Complete list of the 130 differentially expressed miRNAs between normal and breast tumors. (DOC)

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Author Contributions
Conceived and designed the experiments: AHM S. Romero-Cordoba S. Rodriguez-Cuevas. Performed the experiments: AHM S. Romero-Cordoba RRV VQJ VBP RAL. Analyzed the data: AHM S. Romero-Cordoba VQJ VBP RAL AMA GJS. Wrote the paper: AHM S. Romero-Cordoba.

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30. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsokui A, et al. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nature cell biology 10: 593–601.

31. Semper LF, Christensen M, Salazarzeh A, Bak M, Heath CV, et al. (2007) Altered MicroRNA expression confined to specific epithelial cell subpopulations in breast cancer. Cancer research 67: 11612–11620.

32. Di Leva G, Gasparini P, Piovan C, Ngeku A, Garofalo M, et al. (2010) MicroRNA cluster 211–222 and estrogen receptor alpha interactions in breast cancer. Journal of the National Cancer Institute 102: 796–797.

33. Gaur A, Jewell DA, Liang Y, Ridzon D, Moore JH, et al. (2009) A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. Cell 137: 1032–1046.

34. Valastyan S, Reinhardt F, Benaich N, Calogrias D, Szasz AM, et al. (2009) A microRNA cluster with tumor suppressive activity in breast cancer. Cancer research : BCR 11: 4566–4578.

35. Leivonen SK, Makela R, Ostling P, Kohonen P, Haapa-Paananen S, et al. (2008) miR-210 is downregulated by hypoxia and is an independent prognostic factor in breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 14: 1340–1348.

36. Foekens JA, Sieuwerts AM, Smid M, Look MP, de Weerd V, et al. (2008) Four microRNAs Not Previously Involved in Breast Cancer...