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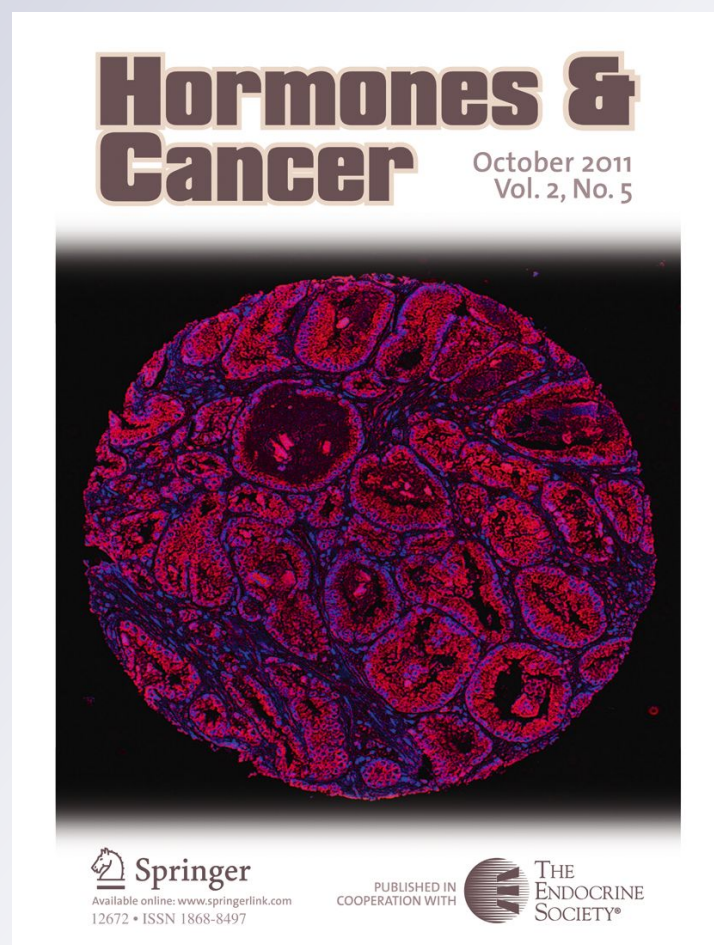
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Effects of Oestrogen on MicroRNA Expression in Hormone-Responsive Breast Cancer Cells

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Abstract Oestrogen receptor alpha (ER α) is a ligand-dependent transcription factor that mediates oestrogen effects in hormone-responsive cells. Following oestrogenic activation, ER α directly regulates the transcription of target genes via DNA binding. MicroRNAs (miRNAs) represent a class of small noncoding RNAs that function as negative regulators of protein-coding gene expression. They are found aberrantly expressed or mutated in cancer, suggesting their crucial role as either oncogenes or tumour suppressor

genes. Here, we analysed changes in miRNA expression in response to oestrogen in hormone-responsive breast cancer MCF-7 and ZR-75.1 cells by microarray-mediated expression profiling. This led to the identification of 172 miRNAs up- or down-regulated by ER α in response to 17 β -oestradiol, of which 52 are similarly regulated by the hormone in the two cell models investigated. To identify mechanisms by which ER α exerts its effects on oestrogen-responsive miRNA genes, the oestrogen-dependent miRNA

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expression profiles were integrated with global *in vivo* ER α binding site mapping in the genome by ChIP-Seq. In addition, data from miRNA and messenger RNA (mRNA) expression profiles obtained under identical experimental conditions were compared to identify relevant miRNA target transcripts. Results show that miRNAs modulated by ER α represent a novel genomic pathway to impact oestrogen-dependent processes that affect hormone-responsive breast cancer cell behaviour. MiRNome analysis in tumour tissues from breast cancer patients confirmed a strong association between expression of these small RNAs and clinical outcome of the disease, although this appears to involve only marginally the oestrogen-regulated miRNAs identified in this study.

Keywords Oestrogen receptor · Breast cancer · MicroRNA · Cell cycle · Gene expression

Introduction

The steroid hormone 17 β -oestradiol (E2) is a key regulator of growth and differentiation in the mammary gland [1, 2] where it is involved in the pathogenesis and clinical outcome of breast cancer (BC) [3]. In normal and transformed mammary epithelial cells, the biological effects of E2 are mediated primarily by oestrogen receptor alpha (ER α), a ligand-inducible transcription factor of the nuclear receptor gene superfamily. Following oestrogenic activation, ER α mediates transcription by interacting directly with specific oestrogen response elements (EREs) located in the promoter/enhancer region of its target genes; it can also interact with other transcription factor complexes like Fos/Jun [4] or SP-1 [5] influencing transcription of genes whose promoters do not harbour ERE (tethering). This leads to transcriptional activation or repression of target genes involved in important cellular function such as cell cycle control, differentiation and apoptosis [6–8]. Alternatively, oestrogens are able to trigger rapid and transient cellular responses via ER α crosstalk with different signal transduction pathways in the cytoplasm [9, 10]. The cellular response to oestrogens involves multiple biological events, including transcription, RNA stability and post-translational modifications [11]. MicroRNAs (miRNAs) are a class of small RNAs of 23 nucleotides (nt) in length, which coordinate a broad range of gene expression programs mainly through modulation of gene regulation [12]. There are over 1,700 identified miRNAs in the human genome that are, themselves, subject to regulation at both transcriptional and post-transcriptional level. MiRNAs are encoded in several regions of the genome, both in protein coding and non-coding transcription units. Approximately 50% of miRNAs are derived from non-coding RNA transcripts and have their own promoters,

while an additional 40% are located within the introns of protein coding genes and share the same transcriptional control of the host genes [13, 14]. Moreover, many miRNAs are encoded in the genome as clusters that can range from 2 to 19 miRNA hairpins, encoded in tandem and in close proximity to each other [15]. After being transcribed, miRNA carrier transcripts (pri-miRNA [16]) undergo a step-wise processing: The long miRNA transcript is cleaved into the nucleus by Drosha into pre-miRNA [17], exported into the cytoplasm and there cleaved by Dicer into a miRNA-miRNA* duplex [18]; mature miRNAs are loaded into microRNA-induced silencing complex (miRISC), which interferes with the transfer of transcriptome information into proteome output via RNA-induced gene silencing [19–21]. Regardless of the mechanisms, each miRNA can potentially regulate gene expression of hundreds of genes, and on the other hand, a single transcript can be targeted by multiple miRNAs [22, 23]. In fact, almost one third of the protein-coding genes are under the regulation of miRNAs, and, as a consequence, many miRNAs seem to play a crucial role in different biological processes such as differentiation, proliferation and cell death in a context-dependent way [24]. Not surprisingly, aberrant miRNA expression is a hallmark of several diseases, including cancer [25]. Several studies have established a role of miRNAs in the pathogenesis of BC, showing a link between E2/ERs and microRNAs expression either in BC cell lines or in cancerous breast tissues [26–30]. These studies indicate that miRNAs can act as either oncogenes or oncosuppressors [31]. In addition, identification of differential expression profiles of miRNAs between normal and neoplastic breast tissue or among human BC subgroups confirms the hypothesis of a possible involvement of miRNAs in tumour development and progression [32–35]. The expression of miRNAs has been examined in different BC cell lines and biopsies. Among these, the most consistently deregulated miRNAs following E2 treatment were miR-206, miR-125a/b, miR-17-5p, miR-34a and some member of let-7 family that may act as tumour suppressor genes [36]. Some recent reports, in fact, have shown that E2 treatment leads to alteration of miR-206 whose expression levels decrease in ER α -positive human BC tissues. This miRNA is able to suppress ESR1 expression and to inhibit growth of MCF-7 BC cells [37]. Moreover, miR-17-5p represses the translation of AIB1 mRNA, thereby inhibiting the function of E2F1 and ER α . Down-regulation of AIB1 by miR-17-5p results in the suppression of oestrogen-stimulated proliferation and oestrogen/ER-independent BC cell proliferation [38, 39]. MiRNA let-7, one of the first discovered members of let-7 family, is poorly expressed or deleted in many human cancers as well as miR-34a, which has been shown to be transcriptionally regulated also by p53 [40]. On the other hand, miR-21, miR-155 and miR-10b may act as oncogenes, being consistently found

over-expressed in cancer. Consistent with these findings, miR-21 was also shown to be highly up-regulated in breast tumours compared to the normal breast tissues, suggesting its oncogenic role [41]. Moreover, miR-21 together with let-7 and miR-98 are involved in a negative-regulatory loop that controls c-Myc, E2F1 and E2F2 protein levels [28].

A number of genes involved in BC progression have been identified by *in silico* analyses and then experimentally proven to be targets of miRNAs that are deregulated in breast tumours [42]. Furthermore, it has been shown that miRNA deregulation in BC can occur not only at the transcriptional level but also at the processing level. In fact, it was recently reported that E2 is able to up-regulate Dicer1 gene expression in ER α -positive BC cells [28]. Furthermore, some miRNAs, including miR-221/222 and miR-29a (highly expressed in ER α negative BC cells), directly repress ER α and Dicer1 expression; in contrast, miR-200c (highly expressed in ER α -positive BC cells) increases Dicer1 levels [43].

In this study, we performed a time-course analysis of oestrogen-regulated miRNAs in MCF-7 and ZR-75.1 cell lines, with the aim to identify all miRNAs showing identical kinetics and type of response to the hormone in this BC cell model. In order to identify relevant miRNA target transcripts, we then performed a functional analysis of the E2-regulated miRNAs by integrating data from both miRNA and mRNA expression profiles obtained under identical experimental conditions. To determine the mechanisms by which ER α exerts its effects on target miRNAs, data derived from global analyses of ER α *in vivo* binding sites to the genome upon E2 stimulation in MCF-7 cells were integrated with hormone-responsive miRNome data. Finally, to investigate the role of miRNA expression in primary breast tumours, we analysed *in silico* miRNomes from tumour specimens from patients with divergent clinical outcomes following surgical treatment to evaluate existing correlations between the expression patterns of oestrogen-responsive miRNAs identified in cellular models of the disease and clinical–pathological parameters of BC.

Materials and Methods

Cell Culture and Immunoblotting

Human hormone-responsive BC cells MCF-7 Tet-Off (Clontech-Takara, Saint-Germain-en-Laye, France) and ZR-75.1 (ATCC CRL-1500) were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% foetal bovine serum (HyClone, Cramlington, UK) and antibiotics: 100 U/ml penicillin, 100 mg/ml streptomycin, 250 ng/ml Amphotericin-B and 50 μ g/ml G418. Cells were routinely tested for mycoplasma

infection using MycoAlert mycoplasma detection kit (Cambrex BioScience, Rockland, ME, USA). For G0–G1 synchronisation, cells were plated at 20–40% confluence in steroid-free medium (phenol red-free Dulbecco's modified Eagle's medium with 5% foetal bovine serum, pre-treated with dextran-coated charcoal and antibiotics) and maintained for 4 days with replacement of the same fresh medium before stimulation with 10^{-8} M 17 β -estradiol (+E2) or EtOH (vehicle) as negative control. ER expression in cell lines was assayed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and immunoblotting of total protein extracts, using rabbit polyclonal anti-ER α (sc-543, Santa Cruz Biotechnology, Heidelberg, Germany) as previously described [44].

Patients

Thirty-two frozen tumour specimens were selected from a former cohort [45]. They were obtained from patients who underwent primary surgical treatment between 1988 and 2001 at a median age of 57 (range, 32–79). Twenty-two cases were oestrogen receptor (ER) positive and treated in the adjuvant setting with 20 mg tamoxifen daily for 5 years alone or in combination, while 10 cases were ER negative. The average follow-up was of 89 months. ER and PgR status was determined by immuno-histochemical stainings; samples were defined positives when tumours contained more than 10% positive cells. Details are provided as Supplementary Table S3.

RNA Purification

Total RNA was extracted from control (+EtOH, –E2) and from hormone-stimulated (+E2) cell cultures with Trizol (Invitrogen, Carlsbad, CA, USA), as described previously [46]. In each case, cells were collected from multiple parallel cultures and pooled before RNA extraction as described before [47]. For tumour samples, after surgical removal, total RNA was isolated with Concert Cytoplasmic RNA Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) from 20 to 50 mg tumour tissues, according to the manufacturer's guidelines. Frozen tumours were placed in this reagent and homogenised using a ball mill (MM200, Retsch, Düsseldorf, Germany). The suspension was centrifuged at 14,000 \times g for 5 min at 4°C, then lysed with 0.1 ml of 10% SDS followed by 0.3 ml of 5M sodium chloride and 0.2 ml of chloroform for milliliters of reagent. The lysate was centrifuged at 14,000 \times g for 15 min at 4°C, and the upper aqueous phase was removed and combined with 0.8 volume of isopropyl alcohol for 10 min at room temperature. The RNA was recovered by centrifugation, washed with 75% ethanol and finally dissolved in RNase-free water. Before use, RNA concentration in each sample was

determined with a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and quality assessed with Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA).

Microarray Analyses

For miRNA expression profiling, technical replicates were produced. For MCF-7 cells, 800 ng of total RNA were fluorescently labelled and amplified in triplicate to be then pooled for the hybridisation; for ZR-75.1 cells and breast tumour samples, the same concentration of RNA were fluorescently labelled, amplified and hybridised at least in duplicate. Hybridisation reactions were performed with Illumina v2 MicroRNA Expression BeadChips, according to the protocols provided by the array manufacturer (Illumina Inc., San Diego, CA, USA). The human microRNA panel used comprises 1,145 probes designed on miRNA sequence present in miRBase database (Release 12.0) and on additional novel content derived using Illumina sequencing technology. For mRNA expression profiling, 500 ng total RNA from MCF-7 and ZR-75.1 were reverse transcribed, as described previously [48, 49], used for synthesis of complementary DNA and biotinylated complementary RNA (cRNA), according to the Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX, USA; category number IL1791) protocol. For each sample, 700 ng of cRNA were hybridised for 18 h at 55°C on Illumina HumanWG-6 version 2.0 BeadChips containing 48701 probes (Illumina Inc., San Diego, CA, USA), according to the manufacturer's protocol. The BeadChips were scanned using Illumina BeadArray Reader 500 according to the manufacturer's standard methods.

MicroRNA Expression Profiling Data Analysis

For data analysis, the fluorescence intensity files were loaded into the Illumina GenomeStudio v2009.1 software for quality control and expression analysis. First, the quantile normalisation algorithm was applied on the raw datasets to correct systematic errors. This normalisation equalises distribution, median and mean of probe intensities among all samples, as the normalised distribution is chosen by averaging each quantile across samples. For differential expression analysis, technical replicates were grouped together, and miRNAs with a detection $p < 0.01$, corresponding to a false positive rate of 1%, were considered as expressed. Statistical significance was calculated with the Illumina DiffScore, a proprietary algorithm that uses the bead standard deviation to build an error model. Only miRNAs with a DiffScore ≤ -20 and ≥ 20 , corresponding to a $p = 0.01$, were considered as statistical significant. The microarray data

were deposited in the Array Express repository database with the following accession numbers: E-TABM-1194.

Breast Tumour Data Analysis

Raw data were normalised as described above using GenomeStudio v. 2009.1, and only probes with $p < 0.05$ were selected for further analysis. A total of 1,021 probes had at least one valid call in a tumour sample. Two tumour samples had < 10 valid data and were not included. Furthermore, to avoid unbalancing, only probes showing valid calls in at least 20 tumour samples were used in clustering and differential expression analysis (739 probes). Data were normalised to the median value in all tumour samples ($N = 30$) and converted to log (log ratio). Unsupervised hierarchical clustering was performed using TMEV package (MultiExperiment Viewer, at www.t4m.org/mev) using the HCL with Pearson's correlation with complete or average linkage, depending on sample/gene number. Differential expression analysis was carried out using the SAM routine in the same package, using 5,000 permutations and Tusher's S0 [50]. Clusters defined by the HLC were then compared in terms of overall survival (OS) or disease-free survival (DFS) using Kaplan–Meier log rank correlation. Survival curves were generated with PASW 18.0 statistical software.

Quantitative Real-time RT-PCR

Total RNA was extracted from MCF-7 cells before and after stimulation for 72 h with 10^{-8} M E2 as described above. Mature miRNAs were reverse transcribed using a miRNA-specific stem loops and reverse transcriptase before real-time PCR performed using Taqman microRNA assays (assay ID: 2439, 2445, 2441, 2126, 2174, 2440, 2333; Applied Biosystems), as described by Grober et al. [51]. RNU49, unaffected by hormone treatment (data not shown), was used as an internal control to normalise all data using the Taqman RNU49 assay (Applied Biosystems). All real-time PCR runs were performed on a MJ Research PTC-200 Opticon Instrument.

ChIP-Seq Data Analysis

For determining the ER α genome-wide binding sites, we have re-analysed the ChIP-Seq data (accession number E-MTAB-131) previously published by Cicatiello et al. [48]. Enriched regions from MCF-7 cells stimulated with 17 β -estradiol for 45min (+E2) were compared with the same from MCF-7 cultured maintained in steroid-free medium (–E2), with FindPeaks [52], setting the value of subpeaks parameter to 0.5. For selecting only the most relevant sites, we have applied the first quartile as statistical cut-off. For evaluating the nearest ER α binding sites to the miRNA genes within 50 kb, we have used windowBed of the suite programs BEDTools [53].

MicroRNA Target Prediction and Functional Analysis of Their Predicted mRNA Targets

For comprehensive prediction of miRNA target genes we used TargetScan, release 5.1 (www.targetscan.org). To identify statistically over-represented ‘biological process’ Gene Ontology terms among sets of selected mRNA targets, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov>) functional annotation tool [54, 55]. To this aim we used as background data coming from gene expression profiling experiments previously performed [48] on the same cell lines and under the same experimental conditions investigated in this study.

MiRNA Localisation in Host Genes

We annotated the genomic position and context of microRNAs in human genome. This analysis was performed with RegionMiner [56] application of the Genomatix software suite, which generates statistic annotation and data for chromosomal regions.

Results

Characterisation of ER α Positive Breast Cancer Cell Lines

The two ER α -positive breast carcinoma cell lines MCF-7 and ZR-75.1 were used as in vitro model of hormone-responsive BC; ER α expression was monitored by Western blot analysis, using as control extracts from ER α negative SKBR3 cells (Fig. 1a). In these cell lines, E2 deprivation induces G1 arrest [57], a quiescence status readily overrun by administration of physiological concentrations of E2. This determines resumption of cell cycle progression, mediated by regulation of cell cycle control pathways [58]. Indeed, analysis of hormone-deprived MCF-7 and ZR-75.1 cell cultures before and after oestrogen (E2 10⁻⁸ M) stimulation show the timed accumulation of cyclins that characterise cell cycle progression (Fig. 1b). In MCF-7 cells (upper panel), oestrogen-induced accumulation of the G1 cyclin D1 is detectable already after 4 h of stimulation, remaining high throughout the pre-replicative phase, and to a lower extent also during the S phase. Cyclin E2 concentration is also affected by hormone stimulation, showing a 1.5- to 2-fold induction after 10 h and lasting for up to 24 h. The cellular levels of the S-G2 phase cyclins A2 and B1 progressively accumulate in a latter time. The results were also confirmed in ZR-75.1 cells (lower panel of Fig. 1b), which show a good degree of oestrogen dependence and a similar pattern of cyclin gene activation occurring, however, faster than in MCF-7 cells. These molecular responses to E2, occurring in

both cell lines, confirm the direct stimulatory action of oestrogen under the conditions used for this study.

Identification of Oestrogen-Regulated MiRNAs

In order to study the effects of the oestrogen on miRNAs expression, we performed a time-course analysis following oestrogen in both BC cell lines selected for this study. To this aim, total RNA was extracted from MCF-7 and ZR-75.1 cells before and after different time points of stimulation

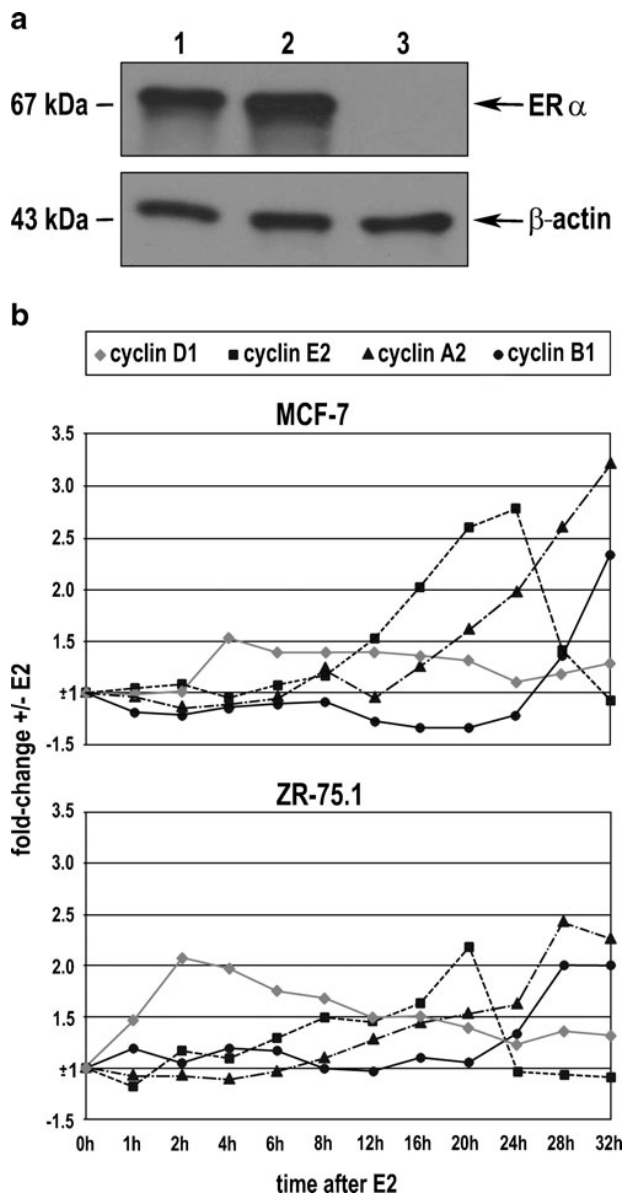


Fig. 1 Characterization of ER α -positive breast cancer cell lines. **a** Expression level of ER α by Western blot analysis of protein extracts from MCF-7 (lane 1), ZR-75.1 (lane 2) and SKBR3 (lane 3) cells. **b** Monitoring of cyclin gene expression in G1-synchronised MCF-7 and ZR-75.1 cell lines following stimulation with a mitogenic dose of E2

with a mitogenic dose of E2 (6, 12, 24 and 72 h), and a global analysis of miRNA expression profiles was carried out by microarray hybridisation as described in ‘[Materials and Methods](#)’, using a platform (Illumina MicroRNA Expression Beadchip) detecting most known and characterised miRNAs. Results indicate that, in agreement with what previously reported in this cell type [28, 30, 43, 48, 59, 60], this hormone can indeed affect significantly the intracellular concentration of specific miRNAs. Among all known miRNAs, we identified 230 showing significant changes in expression in response to E2 ($p \leq 0.01$), including 110 that changed in both cell lines (52 responding similarly and 58 showing opposite changes), 51 restricted to MCF-7 cells and 69 restricted to ZR-75.1 cells. The 52 miRNAs showing concordant regulation in the two cell lines, 25 down- and 27 up-regulated (Fig. 2 central panel), were considered good candidates to investigate miRNA involvement in oestrogen signalling and for this reason selected for further analysis. In Fig. 3 are displayed in graphic format the actual values of the fluctuations detected for 14 miRNAs showing representative expression profiles. Reliability of the microarray platform used for this study was thoroughly controlled in our laboratory in a previous study [30]; however, a test was performed by real-time RT-PCR on seven miRNAs in MCF-7 cells 72 h after hormone (Supplementary Fig. S1). The results confirm a good correlation between Q-PCR and microarray measurements.

Putative Targets of E2-Regulated miRNAs and Functional Analysis of their Predicted Targets

In order to evaluate the functional roles of the 52 miRNAs responsive to E2 treatment in both MCF-7 and ZR-75.1 cells, we performed an *in silico* functional analysis exploiting previously described mRNA expression profiling data obtained in the same cell lines under comparable experimental conditions [48]. To this aim, we first searched for mRNAs putative targets of these oestrogen-regulated miRNAs, results show that ~30% of all expressed mRNAs indeed represent potential targets of these small RNAs [48], and the same is true for 33% mRNAs found regulated by oestrogen in the same study. In order to identify biological processes likely to be influenced by ER α via miRNAs in our cell lines, a Gene Ontology analysis was performed by DAVID tool, using as background the list of expressed mRNAs identified previously [48]. The results reported in Supplementary Fig. S2 show how several cellular processes were found statistically enriched by ER α -responsive miRNAs, including those known to be affected by ER α , such as response to hormonal stimuli, regulation of transcription and cell proliferation, and other that represent key cellular processes in tumour cells, such as cell migration, adhesion and differentiation. Furthermore, starting from the assumption

that miRNA up-regulation might result in down-regulation of its mRNA targets, and vice versa, we investigated the existence of dynamic inverse relationships between miRNA and mRNA levels in oestrogen-stimulated cells. To evaluate this possibility, we first searched for the presence of perfect or imperfect matches between the seed sequence of each regulated miRNA and the untranslated region (UTR) of their putative mRNA targets. Interestingly, we could find only perfect complementarity between these two sequences, a finding that strongly supports the possibility of the existence of a pathway controlling mature mRNA half-life in hormone-responsive BC cells, whereby E2-activated ER α exerts a post-transcriptional control on its target gene activity via specific miRNAs. Indeed, when considering all mRNAs targeted by a single miRNA, we observed a clear inverse relationship between changes in miRNA concentration and that of the corresponding target mRNAs in about 50% of the cases 125 mRNAs of the 252 identified putative targets (listed in Supplementary Table S1).

E2-Regulated miRNAs Associated with ER α -Binding Sites or Located in the Intragenic Region of Oestrogen-Responsive Genes

Current understanding of microRNA biogenesis indicates that expression of these small RNAs can be modulated either during their transcription or through the multiple steps leading to their maturation. In order to identify the transcriptional mechanisms by which activated ER α exerts its effects on oestrogen-responsive miRNAs, we integrated two global genomic analyses of ER α *in vivo* binding sites [48, 61] with the miRNome expression profile obtained in this study. In this way, we could observe that some E2-responsive miRNAs indeed display ER α binding sites within 10 kb of the transcription unit (Table 1). In particular, six of these miRNA genes are associated with one or more ER α binding sites, suggesting a mechanism for direct regulation of RNA biogenesis exerted also in this case by chromatin-bound ER. However, recent findings suggest that the receptor is able to perform its action even when its binding site is not in close proximity of the regulated genes. In order to evaluate this possibility, we expanded our search to 50 kb around miRNA genes, leading to the identification of 18 more miRNA genes linked to one or more ER α -binding sites (Supplementary Table S2). As approximately 50% of miRNAs are transcribed from introns of protein-coding genes, while the others are intergenic [62], we searched for E-regulated protein-coding genes harbouring miRNA genes and aligned the results with those relative to E2-regulated miRNAs. The results are reported in Table 2 to show that six E2-regulated miRNAs are located within oestrogen-dependent genes. Interestingly, in most cases, the miRNA follows the same regulation trend of the harbouring gene.

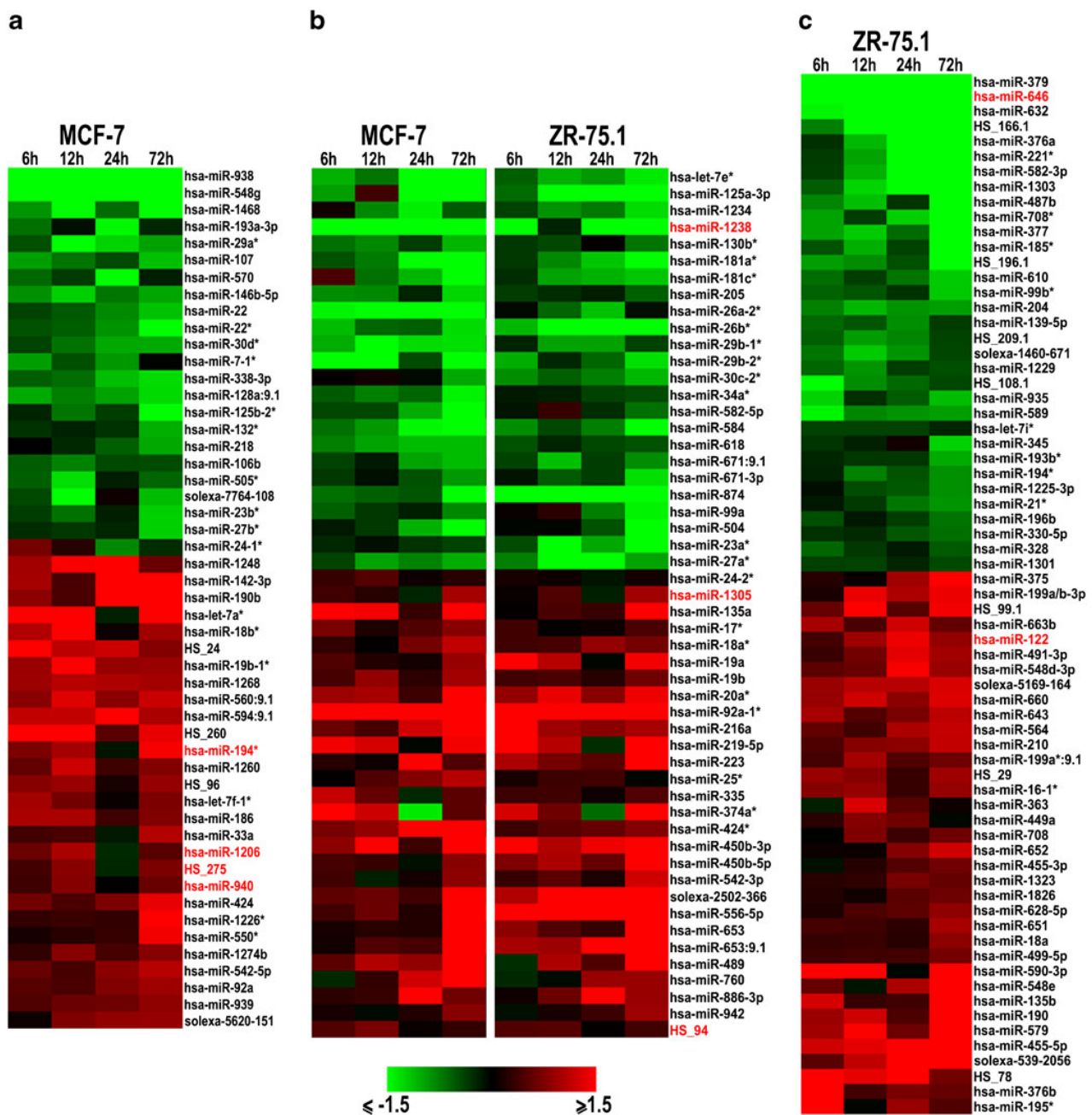


Fig. 2 Effects of ER α on miRNome of human breast cancer cell lines. Time-course analysis of miRNA expression profiles in MCF-7 and ZR-75.1 cell lines after cells exposure to E2 for the indicated times. Oestrogen-regulated miRNAs are grouped as follows: regulated in MCF-7 cells only (a), in both cell lines (b) or in ZR-75.1 cells only (c). Data displayed

represent the ratio between the fluorescence intensity values of each miRNA at the indicated time after exposure to 10^{-8} M E2 vs the corresponding 0 h time point. MicroRNAs marked in red represent those regulated in BC cell lines in vitro and those displaying differential expression between primary breast tumour subgroups (see Fig. 4)

Regulation of miRNA Expression in Breast Tumour Samples

We next evaluated expression of the hormone-regulated miRNAs identified here in primary BC tissue samples. For this, we performed miRNA expression profiling in

breast tumour samples as described in ‘Materials and Methods’. All miRNA probes that showed valid calls in at least 20 tumour samples were used in the statistical analysis for correlation with clinic–pathological parameters. Of these 739 probes, 161 were regulated by oestrogen in at least one cell line, whereas 49 were

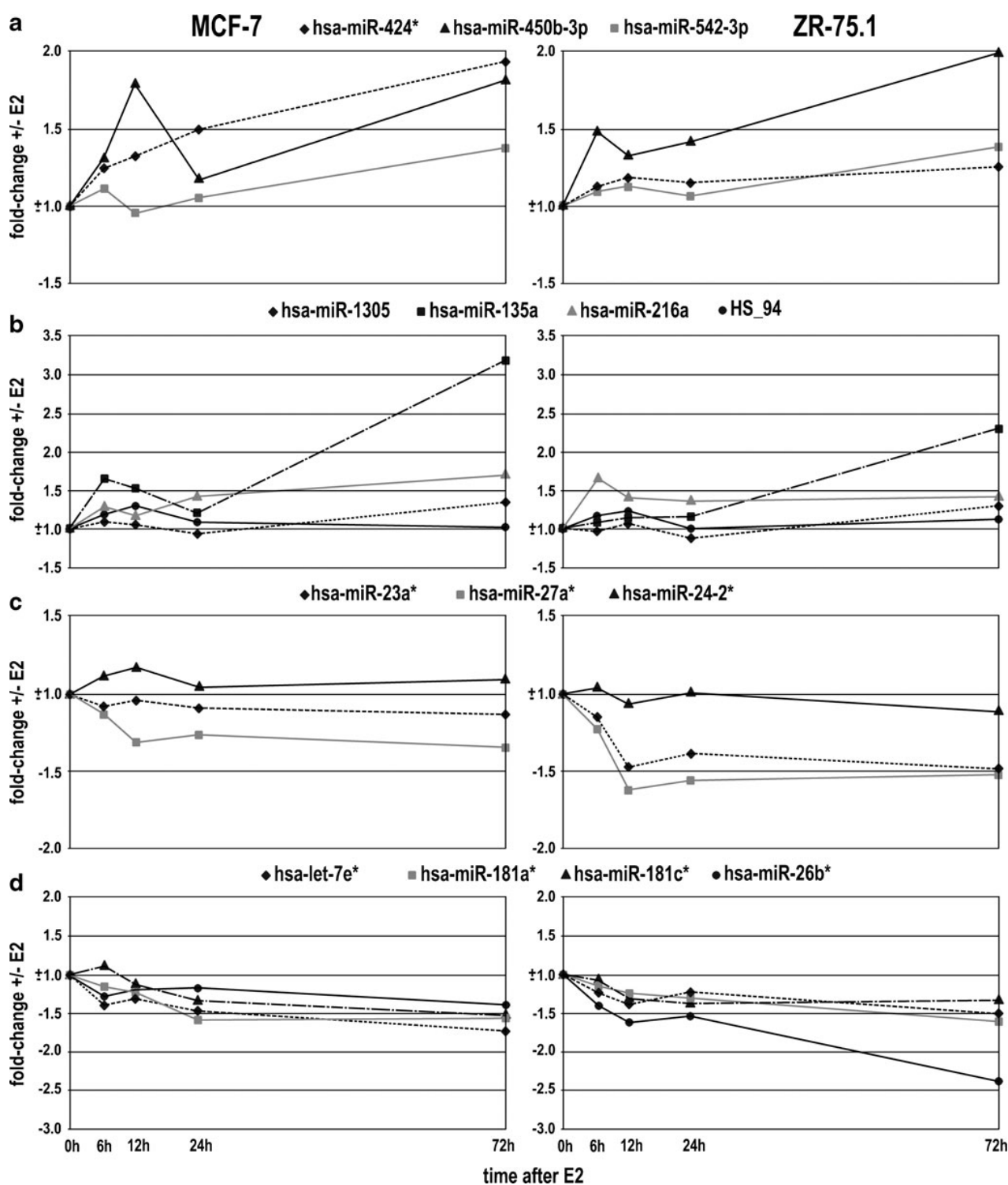


Fig. 3 Graphic representation of timed fold-change variations of selected E2-regulated miRNAs in MCF-7 and ZR-75.1 cell lines. MicroRNAs shown are members of clusters (a, c) or are encoded as independent transcript (b, d)

regulated in both cell lines examined. First, we considered whether the expression profile of *in vitro* regulated miRNAs (161 probes) could discriminate between ER-

positive and ER-negative breast tumours. However, both *t* test and SAM analysis failed to evidence any correlation. On the contrary, unsupervised hierarchical

Table 1 ER α -binding sites within 10 kb from loci encoding E2-regulated miRNAs

miRNA gene	Closest ER α binding site ^{a,b}	Closest ER α binding site ^{a,b}	Closest ER α binding site ^{a,c}	Closest ER α binding site ^{a,c}
hsa-miR-135a-2	–	4773 (chr12:96486593-7225)	–7265 (chr12:96474451-56)	8546 (chr12:96490366-0460)
hsa-miR-181c	–	–	–3445 (chr19:13842950-3068)	5623 (chr19:13852245-2362)
hsa-miR-23a	–6791 (chr19:13815263-6060)	191 (chr19:13807531-8210)	–7212 (chr19:13815685-99)	786 (chr19:13807588-7661)
hsa-miR-27a	–6933 (chr19:13815263-6060)	44 (chr19:13807531-8210)	–7354 (chr19:13815685-99)	639 (chr19:13807588-7661)
hsa-miR-24-2	0 (overlapping) (chr19:13807531-8210)	0 (overlapping) (chr19:13807531-8210)	–7649 (chr19:13815685-99)	486 (chr19:13807588-7661)
hsa-miR-26b	–	2814 (chr2:218978503-9023)	–	–

^a Distance in bps^b ChIP-Seq data from Cicatiello et al. [48]^c ChIP-Seq data from Fullwood et al. [61]

clustering of these probes gave two well-separated branches (Fig. 4a) that correlated with disease-free survival (disease-free or relapsing; DFS) when all the patients were considered ($P < 0.05$ in the case of DFS; Fig. 4b), while marginal significance was observed for overall survival (surviving and not surviving; OS). Limiting the analysis to the group of patients receiving Tamoxifen treatment did not increase separation of survival curves, whereas DFS retained marginal significance, this was lost for OS (Fig. 4c). A second kind of analysis was performed to see whether in vitro regulated probes were present among those differentially expressed in defined groups of samples. Again, SAM analysis failed to evidence differentially expressed probes in ER-positive versus ER-negative samples. On the contrary, groups defined by the DFS status and by the OS status were well differentiated. For DFS, we chose 47 probes differentially expressed (median false significant genes=7.84): of these, five were regulated in vitro in at least one cell line (hsa-miR-122, -194*, -1238, -1305 and HS_94). For OS, 63 probes were selected (median FSG06.3): Of these, nine were regulated in at least one cell line (hsa-miR-122, -194*, -646, -940, 1206, 1238, -1305, HS_94 and HS_275). MiRNAs belonging to these groups are shown in Supplementary Table S4.

Discussion

MicroRNAs represent a class of small non-coding RNAs that control gene expression by targeting mRNAs and thereby triggering either translation repression or RNA degradation. Among human diseases, it has been shown that miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may play a crucial role as a class of oncogenes or tumour suppressor genes. Multiple lines of evidence show the involvement of specific miRNAs in the pathogenesis of BC, where they may represent an alternative molecular mechanism that could impact the onset, development and progression of this hormone-responsive disease. In this study, we investigated the role of oestrogen and its nuclear receptor ER α in modulating miRNA expression in human BC cells. Once chosen robust and validated cellular models for our study, we performed a time-course profiling analysis to identify miRNAs whose levels are affected by E2 in both MCF-7 and ZR-75.1 cells. This led to the identification, among the 1,145 probes on the miRNA microarray used, of 172 E2-regulated miRNAs (15% of total). Of these small RNAs, 52 resulted commonly regulated in both cell lines, 51 were regulated only in MCF-7 and 69 regulated only in ZR-75.1 cells. These three sets, characterised by a defined kinetic of response to E2, cluster in two concordant groups of significantly down- or up-regulated miRNAs.

Table 2 E2-regulated miRNAs in MCF-7 and ZR-75.1 cell lines located in the intragenic region of hormone-regulated genes

miRNA gene	E2 effect on miRNA	Host gene ^a	E2 effect on host gene
hsa-miR-25 (chr7:99.529.199-202)	Up	MCM7 (chr7:99.528.340-99.537.363)	Up
hsa-miR-26a (chr12:56.504.649-742)	Down	CTDSP2 (chr12:56.499.977-56.527.014)	Down
hsa-miR-424 (chrX:133.508.310-407)	Up	MGC16121 (chrX:133.505.073-133.508.326)	Up
hsa-miR-618 (chr12:79.853.646-743)	Down	LIN7A (chr12:79.715.302-79.855.825)	Down
hsa-miR-760 (chr1:94.084.976-5055)	Up	BCAR3 (chr1:937.999.37-940.852.94)	Down
hsa-miR-942 (chr1:117.438.788-873)	Up	TTF2 (chr1:117.404.472-117.447.014)	Up

^a Oestrogen-dependent gene regulation data from Cicatiello et al. [48]

Interestingly, our microarray data reveal that the miRNA ‘star’ strand, until recently considered the carrier strand devoid of biological significance but now known to be fully functioning and independently controlled by the Ago 2, is most often regulated respect to the corresponding ‘non-star’ counterpart, which often do not show significant changes when compared to the control (0h-EtOH). The abundance of star sequences in our datasets could be explained by RISC incorporation of star arms, due to the thermodynamic stability of the miRNA–miRNA* duplex. These alternate mature forms share similar evolutionary and structural signatures, and show similar relationships with target 3’ UTRs. The results obtained here are also in agreement with the involvement of oestrogen and its receptors in miRNA maturation kinetics, as recently demonstrated for ER β in BC cells by Paris et al. [30]. As these alternate miRNA species target different transcripts respect to non-star strands, they increase the number of targets for each miRNA gene and may constitute also a powerful evolutionary mechanism for the emergence of new miRNAs.

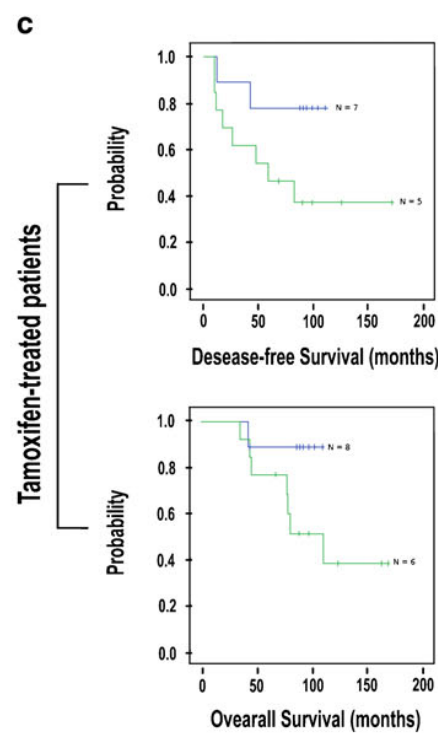
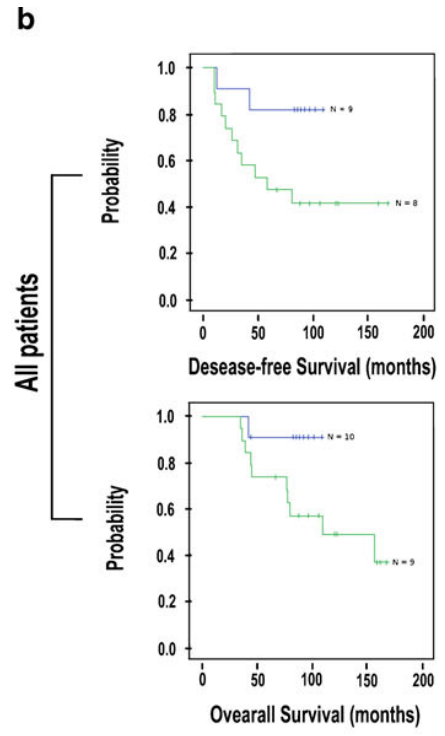
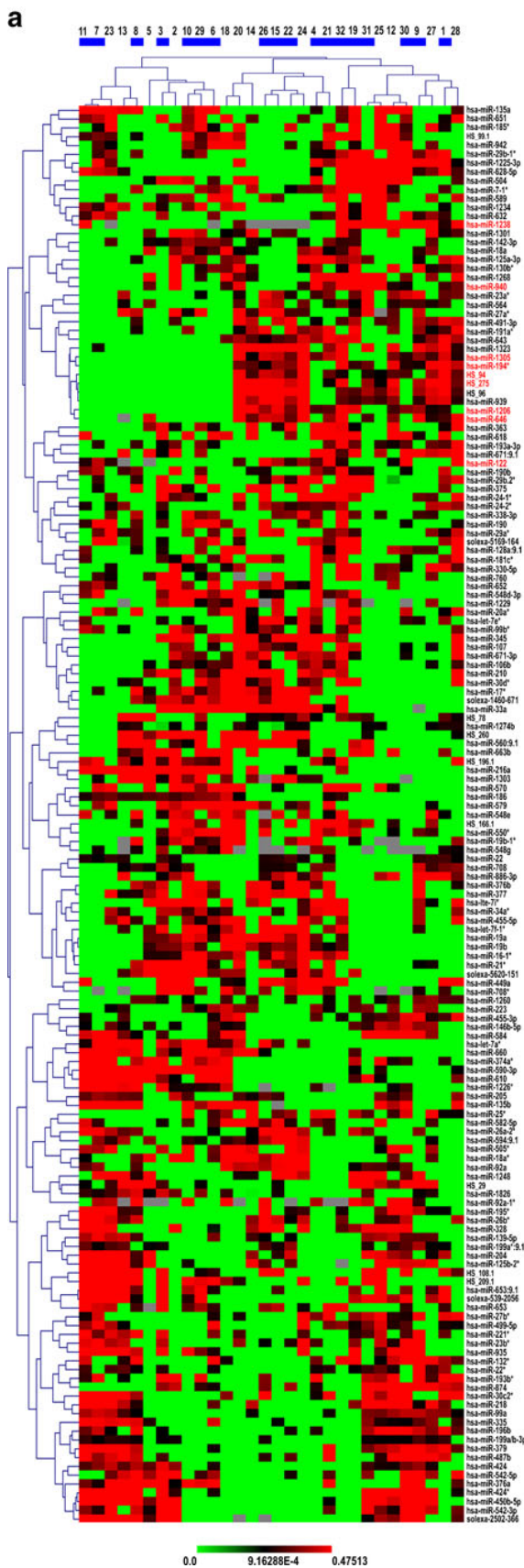
Considering the biological significance of the results obtained, we focused our attention on E2-regulated miRNAs in common between the two BC cell lines analysed as they represent a good starting point to investigate the role of miRNAs in modulating the final responses to oestrogen of hormone-responsive genes in BC cells and to understand in depth the spectrum of molecular mechanisms dependent upon oestrogen in BC. Among the many observations made, we noted that, together with miRNAs transcribed as independent transcripts, several miRNA gene clusters show the same changes in response to E2 in both cell lines, suggesting a global effect of ligand-activated receptor on the entire gene locus. Moreover, we observed that most regulated miRNAs exhibit a significant variation in expression 24–72 h after E2 stimulation. This may be due to the fact that increase/decrease of pri- and pre-miR expression level occurs early after E2 stimulation, while mature miRNA expression level starts to vary later, as shown by Castellano et al. [58], who focused their attention on the cluster 17–92 and demonstrated that if the pri-miR levels are quickly increased during the first 3 h of oestrogen stimulation, while the mature forms result mainly increased only after 24–72 h of oestrogen treatment.

As partial confirmation of the results described here, we observed that a number of miRNAs found oestrogen-responsive in our expression profiling assays were identified also in other studies performed in BC cells. E2, for example, was found to increase expression of some members of the miR-17-92 cluster, as well as of miR-424*, miR-450b-3p/5p and miR-542-3p and of miR-25* [28, 59]. Conversely, it was shown to decrease expression of miR-34a*, let-7e*, miR-125a-3p, miR-181a*, miR-181c*, miR-26b*, miR-618 and star members of the miR-23a/27a/24-2 cluster [28, 60].

Fig. 4 Analysis of miRNA expression in 30 breast cancer samples. **a** Hierarchical clustering of 161 miRNAs oestrogen-regulated in vitro in at least one cell line. The blue bars denote ER+ samples. Numbers at the top indicate individual tumour number (see Supplemental Table S3 for details). The two main branches were used in survival analysis: **b** Kaplan–Meier survival analysis and log rank test concerning all 30 samples ($p < 0.05$ for DFS, top; $p < 0.08$ for OS, bottom) or **c** limited to patients receiving adjuvant Tamoxifen ($p < 0.09$ for DFS, top; NS for OS, bottom). ‘N’ on the curves denotes the censored events in each group. The miRNAs marked in red are in common between the miRNAs classifying breast tumours and those E2-regulated in BC cell lines

Since miRNAs exert their actions on target mRNAs at the post-transcriptional level, a variation of their cellular levels upon E2 stimulation could have important functional roles. As a consequence, identification of miRNA targets is crucial to understand the functional significance of oestrogen-mediated miRNA expression changes in BC cells. For this reason, we performed an in silico target analysis on E2-regulated miRNAs and found how almost 33% of E2-responsive mRNAs represent putative targets of regulated miRNAs. To identify inverse relationships between changes in oestrogen-responsive miRNA levels and those of the corresponding target mRNAs, we took in account all mRNAs targeted by a single miRNA. Results reveal 125 mRNAs that show a correlation respect to the matched miRNA (Supplementary Table S1), with a significant prevalence ($p < 0.003$) for down-regulated mRNAs (14%, ranging from 6% to 23% for each of the E2-responsive gene clusters described in these same cell lines [48]), respect to up-regulated ones (8%, ranging between 3% and 13% in the activated gene clusters). These results indicate a deep impact of E2/ER α on BC cell transcriptome regulation via E2-responsive miRNAs. Considering the biological significance of this finding, we searched biological functions reflecting the activity of mRNAs that are significantly over-represented among all predicted target transcripts by Gene Ontology analysis. The results reported in Supplementary Fig. S2 show that several biological processes are indeed controlled via the miRNA-dependent pathway described here. Indeed, the presence of target genes involved in the cell proliferation, gene transcription, signal transduction and apoptosis indicates that ER α affects in this way a number of key cellular processes through this post-transcriptional regulatory mechanism where ERs appear to influence the activity of target genes through miRNA-mediated post-transcriptional regulation of the activity of gene networks.

Concerning the mechanism for miRNA regulation by the oestrogen receptor, starting from expression profiling and ER α ChIP-Seq and ChIA-Pet data, we propose at least two different mechanisms for hormone-mediated regulation of miRNA gene activity. One is represented by ER α binding in proximity of miRNA-encoding genes that suggests a direct



involvement of the receptor in transcriptional regulation of pri-miR synthesis by transcription. As shown in Tables 1 and S2, six E2-regulated miRNAs have an ER α binding site within 10 kb from the miRNA gene at a major distance (50 kb), but still compatible with the mechanism cited above. The presence of binding sites for the receptor upstream and/or downstream of E2-downregulated miR-27a, one of the components of the miR-23a-27a/24-2 cluster, is particularly interesting as targets of this miRNA identified based upon changes in protein levels after treatment with a 27a antagomiR include the transcriptional co-factor ZBTB10/RINZF. ZBTB10 is a repressor of the SP1 transcription factor, which is found overexpressed in a variety of cancers and is thought to play a role in the G0–G1 to S phase progression in BC cells [63]. Similarly, direct up-regulation of miR-135a of is interest, considering that this miRNA has been found to be oncogenic, able to regulate APC and Jak2 gene transcripts, which both encode proteins involved in cell survival and proliferation, angiogenesis and immune evasion [64, 65]. The second E2-mediated miRNA regulation mechanism suggested by the results reported here is represented by the involvement of E2-inducible expression of mRNA-encoding genes that harbour microRNA genes in their intronic regions. In order to investigate this mechanism, we controlled first which E2-regulated miRNAs were located in host mRNA genes and, subsequently, which of these genes were E2-regulated. In this way, we identified 6 E2-regulated miRNAs that are encoded by a host gene that, in all but one case, is itself regulated by the hormone with the same kinetics (Table 2). These considerations do not exclude, of course, other potential mechanisms by which ligand-activated ER α might affect miRNA expression levels. For example, several studies have demonstrated the presence of a crosstalk of the miRNA maturation pathways with intracellular signaling molecules as p53 [66], Smad proteins [67] and ER itself. Recent findings, in fact, suggest a role for E2/ER α action in the biogenesis of miRNAs, where ER α is able to down-regulate miRNA expression blocking Drosha-mediated processing of a subset of miRNAs by binding to Drosha in a p68/p72-dependent manner and inducing the dissociation of the microprocessor complex from pri-miRNA [68].

Analysis of miRNA expression in clinical samples confirmed the general finding that these noncoding RNAs show significant associations with clinical outcome more than protein coding genes [69, 70]. In fact, significant survival curve separations were observed in terms of both disease-free and overall survival, despite the fact that the cohort of patients examined here is small ($N=30$). Notably, the group of miRNAs regulated by oestrogen in vitro was found comparably effective in discriminating survival, although the small number of samples does not allow to evidence specific effects in the group of ER-positive, Tamoxifen-

treated samples. In contrast, we could not find any association of regulated miRNAs with ER status. One likely explanation for this is that miRNA expression levels in tumours respond to many different stimuli aside from oestrogen receptor-mediated regulation. The same finding was in part true for protein coding RNAs [71]. For the reason stated above, we are not surprised by the fact that there is no direct correlation between hormone-regulated miRNAs and the miRNA profile that correlates with clinical follow-up. Indeed, the number of RNAs found is not significant, as any other set of corresponding size will contain similar numbers of responsive probes.

Overall, our data indicate that miRNA expression play a key role in oestrogen-dependent functions in BC and possibly other cell types, suggesting that miRNA modulation by ER α represents a novel genetic pathway controlled by these steroid hormones that could impact oestrogen-dependent breast tumour biology and, thereby, influence the clinical and pharmacological profile of the disease.

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Conflict of interest The authors declare that they have no conflict of interest.

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