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ORIGINAL ARTICLE

Direct regulation of microRNA biogenesis and expression by estrogen receptor beta in hormone-responsive breast cancer

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Estrogen effects on mammary epithelial and breast cancer (BC) cells are mediated by the nuclear receptors ERα and ERβ, transcription factors that display functional antagonism with each other, with ERβ acting as oncosuppressor and interfering with the effects of ERα on cell proliferation, tumor promotion and progression. Indeed, hormone-responsive, ERα+ BC cells often lack ERβ, which when present associates with a less aggressive clinical phenotype of the disease. Recent evidences point to a significant role of microRNAs (miRNAs) in BC, where specific miRNA expression profiles associate with distinct clinical and biological phenotypes of the lesion. Considering the possibility that ERβ might influence BC cell behavior via miRNAs, we compared miRNome expression in ERβ+ vs ERβ− hormone-responsive BC cells and found a widespread effect of this ER subtype on the expression pattern of these non-coding RNAs. More importantly, the expression pattern of 67 miRNAs, including 10 regulated by ERβ in BC cells, clearly distinguishes ERβ+, node-negative, from ERβ−, metastatic, mammary tumors. Molecular dissection of miRNA biogenesis revealed multiple mechanisms for direct regulation of this process by ERβ+ in BC cell nuclei. In particular, ERβ downregulates miR-30a by binding to two specific sites proximal to the gene and thereby inhibiting pri-miR synthesis. On the other hand, the receptor promotes miR-23b, -27b and 24-1 accumulation in the cell by binding in close proximity of the corresponding gene cluster and preventing *in situ* the inhibitory effects of ERα on pri-miR maturation by the p68/DDX5-Drosha microprocessor complex. These results indicate that cell autonomous regulation of miRNA expression is part of the mechanism of action of ERβ in BC cells and could contribute to establishment or maintenance of a less aggressive tumor phenotype mediated by this nuclear receptor.

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Introduction

Estrogens have a role in breast cancer (BC) pathogenesis and progression by controlling mammary cell proliferation and key cellular functions via the estrogen receptors (ERα and ERβ: Heldring *et al*., 2007). ERs are members of the nuclear receptors superfamily of ligand-dependent transcription factors that both regulate gene expression controlling the estrogen signal transduction cascade with distinct and even antagonistic roles. In hormone-responsive, ERα-positive BC cells ERβ inhibits estrogen-mediated cell proliferation by increasing the expression of growth-inhibitory genes and by interfering with activation of cell cycle and anti-apoptotic genes by ERα in response to 17β-estradiol (E2: Chang *et al*., 2006; Grober *et al*., 2011). ERβ is frequently lost in BC, where its presence generally correlates with a better prognosis of the disease (Sugiura *et al*., 2007), is a biomarker of a less aggressive clinical phenotype (Novelli *et al*., 2008; Shaaban *et al*., 2008) and its downregulation has been postulated to represent a critical stage in estrogen-dependent tumor progression (Roger *et al*., 2001; Bardin *et al*., 2004). Despite the direct relationships between estrogen and breast carcinogenesis, the divergent roles of the two ER subtypes in BC are not fully understood, mostly because they are complex, involving genomic and non-genomic actions, regulation of gene transcription and control of mRNA stability and translation efficiency.

MicroRNAs (miRNAs) are small (20–25 nt) non-coding RNAs that can regulate gene activity in a posttranscriptional manner. These molecules, frequently transcribed as polycistronic RNAs, are synthesized in the nucleus by RNA polymerase II or III as long primary transcripts (pri-miRNAs), that are then

processed by the class-2 RNase-III Drosha (Han *et al*., 2004) in ~70-nucleotide stem-loop RNAs (pre-miRNAs), that in turn are exported from nucleus to cytoplasm by exportin 5 and Ran-GTP (Kim *et al*., 2009) and cleaved by Dicer/TRBP endoribonuclease into an imperfect miRNA/miRNA* duplex (Chendrimada *et al*., 2005). Only one strand of the duplex is finally selected to function as a mature miRNA, whereas the other (passenger) strand is typically degraded (Okamura *et al*., 2008; Newman and Hammond, 2010). Mature miRNAs are then incorporated into an RNA-induced silencing complex, which binds to target mRNAs, determining gene silencing by either inhibition of translation or mRNA degradation (Newman and Hammond, 2010). miRNAs have been shown to regulate a wide variety of cellular phenotypes, including neoplastic transformation, cell proliferation, differentiation and homeostasis (Garzon *et al*., 2009) and altered expression of these small RNAs contributes to tumorigenesis, as some of them can function as either tumor suppressors or oncogenes (Zhang *et al*., 2007; Croce, 2009). Interestingly, in solid tumors, such as prostate, colon, stomach, pancreas, lung and breast, the spectrum of miRNAs expressed (miRNome) is different from that of the corresponding normal tissues (Volinia *et al*., 2006), suggesting the involvement of miRNAs in transformed cell biology. Differential expression of miRNA genes was found associated with specific pathological features of BC, where distinct miRNA expression profiles in normal vs cancer tissue or between different molecular and clinical tumor subtypes appears to be the rule (Iorio *et al*., 2005; Lu *et al*., 2005; Blenkiron *et al*., 2007; Tavazoie *et al*., 2008). There is increasing evidence, in fact, that specific miRNAs may be responsible at large for disease heterogeneity, functioning as regulators of tumorigenicity, invasion and metastasis (Tavazoie *et al*., 2008). Moreover, genetic defects in key components of the miRNA biosynthetic pathway have been described in tumors (Hill *et al*., 2009; Melo *et al*., 2009, 2010), and several genes involved in BC progression have been identified as targets of miRNAs that, in turn, are found deregulated in BC cells (Garzon *et al*., 2009).

Several evidences indicate that ERα is among the transcription factors regulating miRNA biogenesis in hormone-responsive BC cells (Bhat-Nakshatri *et al*., 2009; Castellano *et al*., 2009; Maillot *et al*., 2009; Yamagata *et al*., 2009; Cicatiello *et al*., 2010; Ferraro *et al*., 2010, 2011). More recently, global mapping of ERβ binding to ERα-positive, hormone-responsive BC cells chromatin *in vivo* showed ERβ interaction with several miRNA genes, suggesting the possible involvement of this receptor in hormonal control of small non-coding RNA biogenesis in this cell type (Grober *et al*., 2011). Starting from this observation, we investigated here miRNA expression pattern in estrogen-responsive BC cell lines engineered to express full-length ERβ and in primary-tumor samples selected according to the presence or absence of this nuclear receptor. Results indicate a role of ERβ in the control of miRNA biogenesis and expression pattern in BC cells.

Results

ERβ induces widespread changes in miRNA expression in hormone-responsive cells

In order to investigate the role of ERβ in BC, we generated MCF-7 cells stably expressing full-length human ERβ (ERβ-1) fused at the N- (N-TAP-ERβ) or C- (C-TAP-ERβ) terminus to a TAP tag in pTRE2pur-HA expression vector (Puig *et al*., 2001). As shown in Figure 1a, the expression levels of C-TAP-ERβ (two independent clones: lanes 2–3), N-TAP-ERβ (lane 4) or C-TAP-ERα (used as control: lane 5) are comparable to those relative to endogenous ERα, as detected by WB under comparable test conditions, to avoid toxic and artifactual events consequent to overexpression of the exogenous protein. The functional integrity of tagged ERβ was assessed by measuring their ability to counteract induction of ERE-TK-luciferase reporter-gene transcription by ligand-activated endogenous ERα. As shown in Figure 1b, cell expressing TAP-ERβ show a marked reduction in E2-mediated activation of reporter-gene transcription compared with *wt* cells, a phenotype that could be almost completely recovered by stimulation with the ERα-selective ligand 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT). TAP-ERβ effects on E2-induced MCF-7 cell proliferation and cell cycle progression were also investigated and the results, reported in Figures 1c and d, show that cells expressing exogenous ERβ grow much slower in response to estrogen than *wt* or C-TAP-ERα cells, consequent to reduced G1–S transition (Figure 1d). It is worth mentioning that the cell cycle inhibitory effects of ERβ are well known (Heldring *et al*., 2007; Grober *et al*., 2011, and references therein) and are more evident at relatively higher concentrations of E2 ($\geqslant 10^{-10}$), compatible with the lower affinity of this ER subtype for the hormone (compare, for each cell clone, the S + G2 fraction in hormone-stimulated vs -starved cells). The efficiency of PPT in promoting cell cycle progression (Figure 1d) relates to its ability to promote ERα-mediated gene transcription (Figure 1b), confirming the direct link between transcriptional activity of this receptor subtype and the mitogenic effects of estrogen (Cicatiello *et al*., 2010). Gene-expression profiling of asynchronously growing cells showed no major differences between N- and C-TAP-ERβ cells, whereas their transcriptomes were significantly different from that of C-TAP-ERα cells (Supplementary Figure S1), confirming previous results obtained in E2-stimulated cells (Grober *et al*., 2011). Based on these results, expression of the TAP-ERβ fusion proteins appears to significantly affect ERα-mediated estrogen signal transduction to target genes and the cell cycle, confirming previous observations indicating that they are fully functional *in vivo* (Grober *et al*., 2011; Nassa *et al*., 2011).

Multiple roles have been proposed for miRNAs in hormone-responsive BC, where the presence of ERβ has been shown to associate with less aggressive disease forms. We decided to use our ERβ-expressing cells to investigate potential links between ERβ and miRNA activity in hormone-responsive BC cells, as these

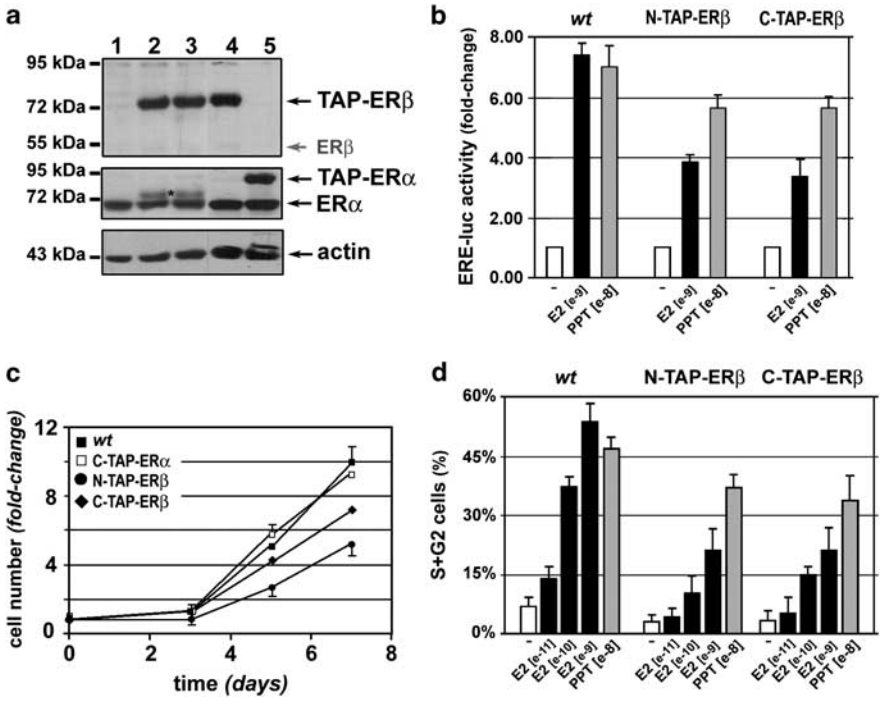

Figure 1 Functional characterization of ERβ-expressing MCF-7 cell clones. (**a**) Western blotting analysis of protein extracts from control (*wt*, lane 1) and TAP-ERs (N-TAP-ERβ: lanes 2–3, C-TAP-ERβ: lane 4, C-TAP-ERα: lane 5) expressing cells. Asterisks mark non-specific bands. (**b**) The ability of tagged ERβ to interfere with ERα activity was assessed by comparing estrogen effects in *wt*, N-TAP-ERβ and C-TAP-ERβ cells by ERE-TK-luciferase reporter-gene activation mediated by E2 or PPT (selective ERα agonist). (**c**) Proliferation rate of *wt*, N-TAP-ERβ and C-TAP-ERβ cells was measured in hormone-starved cells stimulated with 10^{-8} M E2, respect to untreated cells. Cell counting was performed with a colorimetric assay at the indicated times. (**d**) Analysis of cell cycle progression after estrogen stimulation of *wt* or TAP-ERβ-expressing cells. The percent of S + G2 phase cells was determined by flow cytometry in estrogen-starved cultures 27 h after treatment with either vehicle alone (EtOH) or the indicated concentrations of E2 or PPT.

represent a useful *in vitro* model to investigate the molecular mechanisms underlying the biological effects of this receptor subtype in hormone-responsive tumors. To this aim, total RNA was extracted from *wt* MCF-7, N-TAP-ERβ, C-TAP-ERβ (2 independent clones) and C-TAP-ERα cells. Global analysis of miRNome expression was peformed with microarrays detecting the vast majority of known and characterized miRNAs (Illumina MicroRNA Expression Beadchip, Illumina Italia, Milano, Italy) as described in Material and methods. Results indicates that expression of ERβ has a deep impact on BC cell miRNome, as 84 miRNAs were found differentially expressed in three ERβ+ vs two ERβ− cell lines, whereas no significant differences could be detected among cells expressing the different tagged forms of ERβ, or between C-TAP-ERα, *wt* and MCF7-TAP cells (not shown), that express only the TAP peptide and show no differences in ERα signaling with respect to *wt* cells (Ambrosino *et al*., 2010; Grober *et al*., 2011). To validate this result, we performed miRNA expression profiling with a different microarray platform (Agilent Human microRNA Microarrays 18 × 15 K v3, Agilent Technologies Italia, Milano, Italy) and compared the results obtained in the two experimental settings. As expected, we observed some differences between the two data sets, likely due to technical differences between the two microarrays platforms (in particular sensitivity and quality of the probes) and the two probe sets (Supplementary Materials and methods and data not shown). Nevertheless, 73 among the differentially expressed miRNAs identified with the Illumina platform were either fully confirmed with the Agilent array or, in some instances, could not be detected here due to a lower sensitivity of this platform. For this reason, we performed a further validation of the results obtained with the Illumina arrays analyzing by real-time RT–PCR (reverse transcriptase–PCR) the expression levels of 10 miRNAs selected according to their relative expression level, ranging from very low to high, and including also miRNA undetectable with Agilent arrays or differentially expressed between cell lines (except for miR-181c, that was not differentially expressed and is included as negative control). Results (reported in Supplementary Figure S2) show a very high correlation between rtPCR and Illumina array data (correlation coefficient: 0.76), indicating reliability of this microarray platform. The 73 differentially expressed miRNAs listed in Figure 2a and Table 1 were thus considered validated. To gather insights on the molecular mechanisms for ERβ effects on miRNAs, expression profiling was carried out in both cell types after estrogen starvation. Under these conditions, no differences could be detected between ERβ+ and ERβ− cells (left panel in Figure 2b, referring to average values measured in ERβ− vs ERβ+ cell lines), indicating a key role of the liganded in determining the observed differences. For this reason, we next investigated whether expression of the 73 miRNAs identified in the

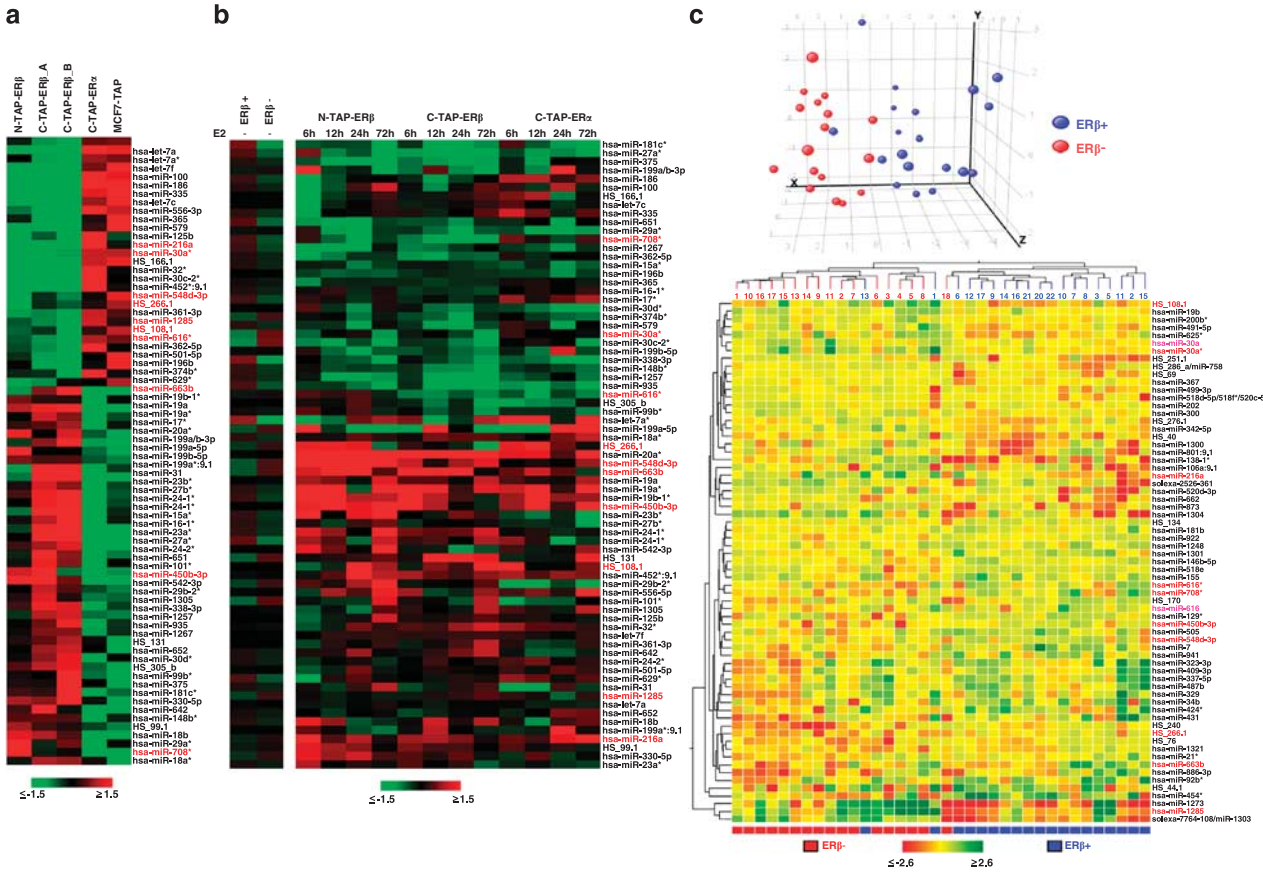

Figure 2 Correlations between ERβ and miRNome expression in hormone-responsive BC cells and primary breast carcinomas. (**a**) Heatmap showing 73 miRNAs differentially expressed between ERβ+ and ERβ− cells maintained in standard culture conditions. Data displayed represent the ratio between the fluorescence intensity value of each miRNA in a given array (cell line) vs the average of the fluorescence intensity value of the same miRNA in all arrays. (**b**) Heatmaps showing relative expression of 73 ERβ-responsive miRNAs in ERβ+ and ERβ− cells treated with vehicle alone (EtOH; left panel) or with E2 for the indicated times (right panel). Data displayed represent the ratio between the fluorescence intensity value of each miRNA at the indicated time after E2 stimulation (+E2) vs the same in hormone-starved cells (−E2, control). (**c**) Top: Principal component analysis (PCA) relative to differential miRNA expression in 17 ERβ+ and 19 ERβ− primary BC samples. Bottom: Cluster analysis of 67 miRNAs discriminating between ERβ+ and ERβ− BC samples. Data displayed represent the ratio between the fluorescence intensity value of each miRNA in a given array (tumor sample) vs the average of the fluorescence intensity value of the same miRNA in all arrays. miRNA marked in red were differentially expressed both in ERβ+ cell lines and BC samples, whereas those marked in purple in C derive from the same pre-miR of those differentially expressed in ERβ+ cell lines and tumors.

previous experiment was affected by estrogen. To this aim, N-TAP-ERβ, C-TAP-ERβ and C-TAP-ERα were E2-deprived and subsequently stimulated with 10^{-8} M E2 for 6–72 hrs before miRNA analysis. Results displayed in Figure 2b (right panel) show that all investigated miRNAs respond to the hormone in a time-dependent manner. Although kinetics and extent of miRNA response to the stimulus were comparable between the two ERβ+ cell lines, they were significantly different in ERβ+ vs ERβ− (C-TAP-ERα) cells. Direct comparison of the data from the two cell types indicates that the differences in steady-state miRNA levels consequent to ERβ expression are due to ERβ antagonism upon ERα activity or to a specific effect of ligand-activated ERβ. As shown in Supplementary Figure S3, for example, expression of hsa-miR30a* and, to a lesser extent, hsa-miR30a shows a time-dependent decrease following E2 stimulation only in ERβ+ cells, whereas it is unaffected by the stimulus in the absence of ERβ. On the contrary, hsa-miR-23b and -23b*, hsa-miR-27b and -27b* and hsa-miR-24 and -24-1* levels decrease in the presence of E2 in ERβ− whereas they increase in ERβ+ cells. The putative mRNA targets of the miRNAs regulated by ERβ were searched with TargetScan and, subsequently, analyzed for Gene Ontology term over-representation, in order to identify biological processes likely to be influenced by this ER subtype via miRNAs. In this way, several cellular processes were found downstream of ERβ-responsive miRNAs, including those known to be affected by ERβ, such as response to hormonal stimuli, regulation of transcription and cell proliferation and others that represent key cellular processes in malignant cells, including cell motility, migration, adhesion, differentiation and fate determination, and are targeted by regulatory cascades in cancer cells (Supplementary Figure S4).

The data described above were obtained *in vitro* in a BC cell model that, although it has been shown to reflect

Table 1 Seventy-three miRNAs differentially expressed following ERβ expression in hormone-responsive human breast cancer cells

miRNA	*Fold-change (ERβ+/ERβ−)*	P-*value*	*miRNA*	*Fold-change (ERβ+/ERβ−)*	P-*value*
HS_108.1	−1.58	*0.03780*	hsa-miR-24-2*	1.73	*0.00002*
HS_131	1.46	*0.00782*	hsa-miR-23b*	2.07	*0.00016*
HS_166.1	−2.00	*0.00001*	hsa-miR-27b*	1.60	*0.00024*
HS_266.1	−1.62	*0.01176*	hsa-miR-24-1*	2.06	*0.00000*
HS_305_b	1.84	*0.00000*	hsa-miR-24-1*(miR-189:9.1)	2.51	*0.00000*
HS_99.1	1.39	*0.00505*	hsa-miR-29a*	1.50	*0.00857*
hsa-let-7a	−1.50	*0.00648*	hsa-miR-29b-2*	1.41	*0.00509*
hsa-let-7a*	−13.48	*0.00000*	**hsa-miR-30a***	−2.97	*0.00012*
hsa-let-7c	−4.02	*0.00000*	hsa-miR-30c-2*	−2.05	*0.00003*
hsa-let-7f	−1.62	*0.00510*	hsa-miR-30d*	1.62	*0.00046*
hsa-miR-100	−4.57	*0.00000*	hsa-miR-31	1.65	*0.00435*
hsa-miR-101*	1.53	*0.00495*	hsa-miR-32*	−2.29	*0.00000*
hsa-miR-1257	1.46	*0.00331*	hsa-miR-330-5p	2.04	*0.00003*
hsa-miR-125b	−2.37	*0.00000*	hsa-miR-335	−4.61	*0.00000*
hsa-miR-1267	1.40	*0.00514*	hsa-miR-338-3p	1.94	*0.00000*
hsa-miR-1285	−1.64	*0.00090*	hsa-miR-361-3p	−1.44	*0.00998*
hsa-miR-1305	1.44	*0.00254*	hsa-miR-362-5p	−1.67	*0.00074*
hsa-miR-148b*	1.41	*0.00600*	hsa-miR-365	−2.92	*0.00000*
hsa-miR-15a*	2.05	*0.00097*	hsa-miR-374b*	−1.53	*0.00680*
hsa-miR-16-1*	1.98	*0.00004*	hsa-miR-375	1.65	*0.00274*
hsa-miR-17*	1.51	*0.00113*	**hsa-miR-450b-3p**	1.69	*0.00645*
hsa-miR-181c*	1.70	*0.00005*	hsa-miR-452*:9.1	−1.98	*0.00024*
hsa-miR-186	−7.14	*0.00000*	hsa-miR-501-5p	−1.79	*0.00033*
hsa-miR-18a*	1.40	*0.01173*	hsa-miR-542-3p	3.22	*0.00000*
hsa-miR-18b	1.78	*0.00000*	**hsa-miR-548d-3p**	−1.97	*0.00001*
hsa-miR-196b	−1.65	*0.00101*	hsa-miR-556-5p	−4.07	*0.00000*
hsa-miR-199a-5p	1.78	*0.00412*	hsa-miR-579	−2.31	*0.00079*
hsa-miR-199b-5p	1.52	*0.00574*	**hsa-miR-616***	−1.56	*0.00366*
hsa-miR-199a*:9.1	1.43	*0.00283*	hsa-miR-629*	−1.67	*0.00746*
hsa-miR-199a-3p/199b-3p	1.72	*0.00001*	hsa-miR-642	1.79	*0.00144*
hsa-miR-19a	1.29	*0.04889*	hsa-miR-651	1.56	*0.00148*
hsa-miR-19a*	2.86	*0.00000*	hsa-miR-652	1.54	*0.00179*
hsa-miR-19b-1*	1.95	*0.00800*	**hsa-miR-663b**	−1.37	*0.02954*
hsa-miR-20a*	1.46	*0.00291*	**hsa-miR-708***	2.05	*0.00478*
hsa-miR-216a	−2.47	*0.02759*	hsa-miR-935	1.53	*0.00068*
hsa-miR-23a*	1.93	*0.00007*	hsa-miR-99b*	1.56	*0.00327*
hsa-miR-27a*	2.10	*0.00000*			

Bold entries denote miRNAs differentially expressed also in ERβ-positive vs ERβ-negative breast tumor biopsies.

only in part the complexity of the hormone-responsive phenotype, in several cases provided molecular insights that could be validated and find application in the clinical setting. For this reason, we considered these evidences as an indication that ERβ might indeed influence miRNome activity also in primary breast tumors. To this end, BC samples were selected, among those originally included in the study reported by Novelli *et al*. (2008), for presence or absence of ERβ expression according to immuno-histochemistry (Supplementary Figure S5). Tumors were divided in two groups of 22 ERβ+ and 18 ERβ− tumors, respectively, that did not show significant differences from each other with respect to key clinical and molecular parameters, summarized in Supplementary Table S1, with the notable exception of the presence of lymphnodal metastases and a worst tumor grading for ERβ− tumors. RNA was extracted from formalin-fixed, paraffin-embedded tissues and that from 17 ERβ+ and 19 ERβ− tumors was of quality and concentration apt to perform miRNA expression profiling as described (Ravo *et al*., 2008). This led to the identification of 67 miRNAs, whose expression level discriminates ERβ+ from the ERβ− breast tumors, including 10 miRNAs that were found differentially expressed also in ERβ+ vs ERβ− BC cells *in vitro* (Figure 2c and Supplementary Table S2). These results confirm those obtained in cell lines (Figures 2a and b), pointing to a role of ERβ in the control of BC miRNome and thereby indicating that miRNAs are integral components of the gene regulation cascade mediating the effects of this nuclear receptor in tumor cells.

Direct regulation of miRNA biogenesis by hormone-activated ERβ in BC cells

Mature miRNA expression can be regulated through control of either transcription or one of the key steps of primary transcript (pri-miR) maturation. We analyzed by chromatin immunoprecipitation sequencing (ChIP-Seq) the entire ERα and ERβ cistromes in the ERβ+ (Grober *et al*., 2011) and ERβ− cells (Cicatiello *et al*., 2010) upon E2 stimulation. Aligning ER-binding sites and miRNA gene positioning in the genome we observed that several miRNA-encoding genes differentially expressed in ERβ+ vs ERβ− cell lines (Supplementary Table S3A) and/or mammary tumors

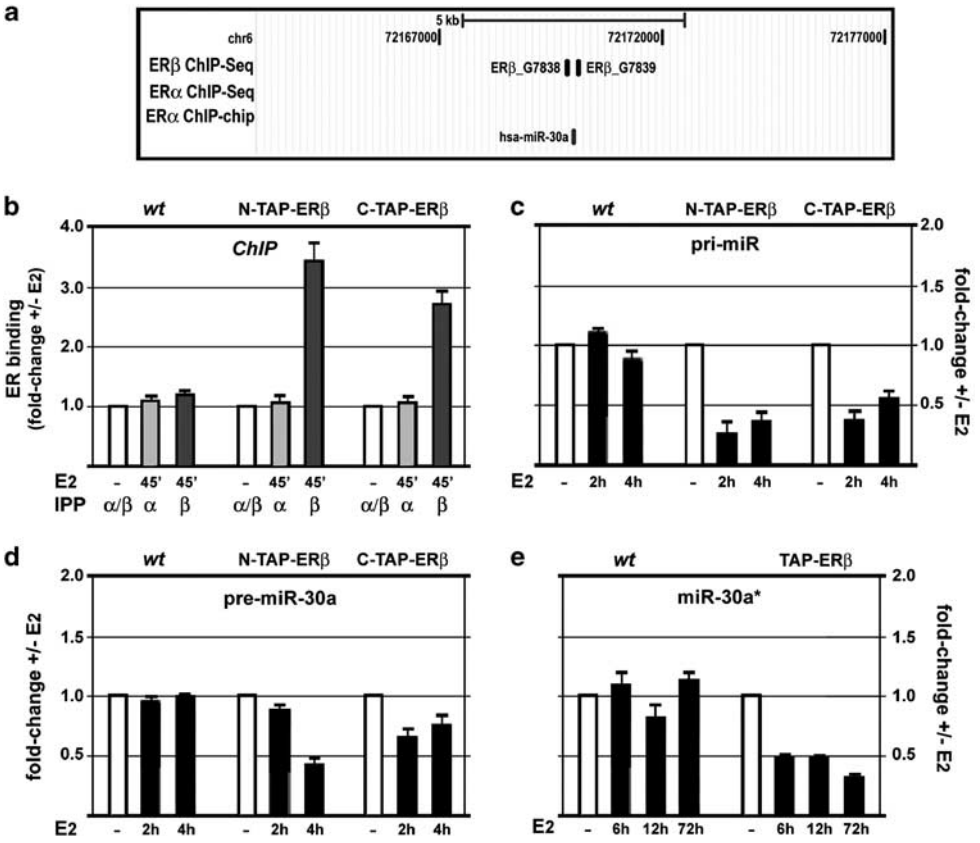

Figure 3 Analysis of ERβ regulation of miR-30a and precursor biogenesis. (**a**) Genome browser view of the two ERβ-binding sites within 10 kb upstream or downstream from hsa-miR-30a locus on chromosome 6. (**b**) Validation of ERβ-binding site by ChIP and real-time PCR in *wt* or ERβ+ cells before (-) and after stimulation with E2 for 45 min. (**c**, **d**) Real-time rtPCR analysis of pri-miR-30a (**c**) and pre-miR-30a (**d**) in *wt* or ERβ+ cells before (-) and after stimulation with E2 for the indicated times. (**e**) Real-time rtPCR analysis of mature hsa-miR-30a* in *wt* or ERβ+ cells (C- and N-TAP-ERβ cell RNA combined) before (-) and after stimulation with E2 for the indicated times.

(Supplementary Table S3B) display ER-binding sites within 10 kb of the transcription unit, including sites where both ERs can be found together, likely associated in heterodimers. This finding suggested us the possibility that miRNA gene activity could be modulated in BC cells by an interplay of the two ER subtypes bound to chromatin, with ERβ antagonizing ERα-mediated regulation of pri-miR biosynthesis and/or maturation rate. To verify this possibility, we choose to investigate in detail differences in miRNA precursor levels in ERβ+ vs ERβ− cells following stimulation with E2, focusing on miR-30a gene and the miR-23b/27b/24-1 chromosomal cluster. The first was selected as it encodes two miRNAs (miR-30a and -30a*) that are downregulated by estrogen in ERβ+ cells only (Supplementary Figure S3) and it shows two binding sites for ERβ in close proximity —one upstream and one downstream— of the transcription unit, but no ERα sites (Figures 3a and b). The second caught our attention, instead, as it shows sites for both receptors (Figure 4a and Supplementary Figure S6) and it encodes three distinct couples of miRNAs, all accumulating in ERβ+ cells and decreasing in ERβ− cells in response to the hormone (Supplementary Figure S3). Interestingly, in both cases the effect of the hormone was more evident on the 'star' strand that, for this reason, led us first to their identification (Figure 2a) and was routinely used here to monitor ERβ effects.

The results relative to the miR-30a locus are reported in Figure 3 and show that ERβ binding results in a significant reduction of pri-, pre- and mature miR-30a levels following E2 stimulation, detectable already after 2 h (Figures 3c–e), to indicate that the predominant effect of ligand-activated ERβ is to *trans*-repress basal gene transcription by direct binding to this transcription unit. Noteworthy, activation of ERα alone (*wt* cells) did not affect miR-30a biogenesis, in agreement with the lack of binding of this receptor to the locus (Figure 3a). When combined, these results indicate a specific and direct role of ERβ in repression of miR-30a expression in BC cells, possibly mediated by promoter *trans*-repression. This could be due to direct transcriptional repression, via recruitment of a repressor complex to the chromatin by ligand-activated ERβ, or, alternatively, to inhibition of gene *trans*-activation caused by tethering of ERβ to a transcription factor constitutively bound to the locus, resulting in displacement or inhibition of an activator complex. The latter possibility, that could explain also lack of ERα binding to such regulatory site, is worth investigating further, extending the analysis

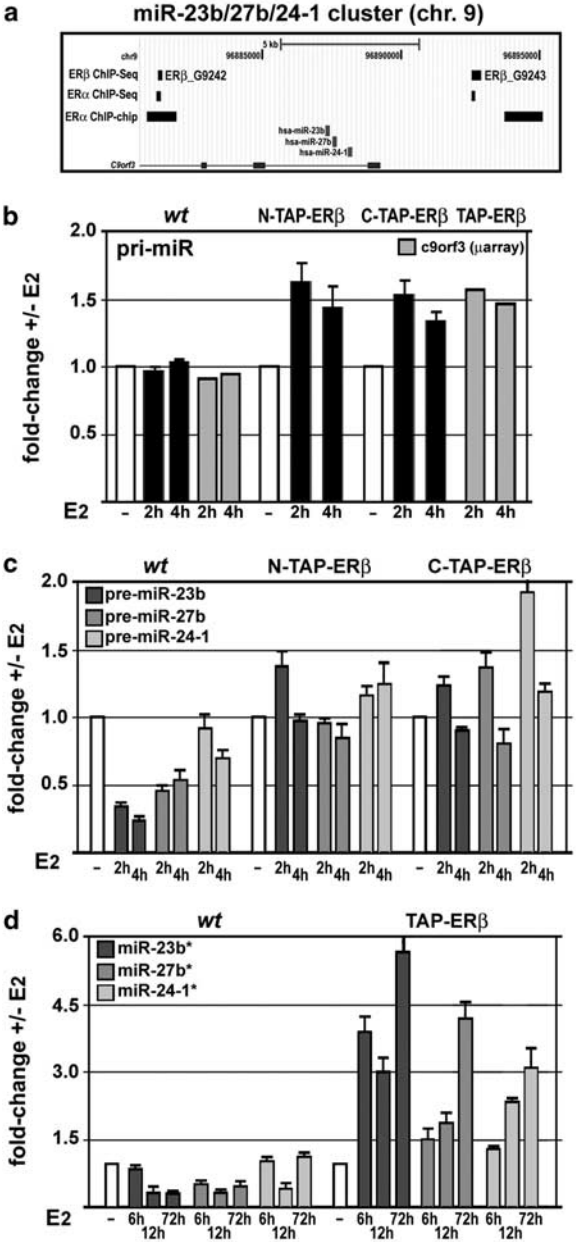

Figure 4 Analysis of ERβ regulation of miR-23b/27b/24-1 and precursor biogenesis. (**a**) Genome browser view of ERβ- and ERα-binding sites within 10 kb upstream or downstream from miR-23b/27b/24-1 cluster within the c9orf3 locus on chromosome 9. (**b–d**) Real-time rtPCR analysis of the 23b/27B724-1 pri-miR (**b**), pre-miR (**c**) and mature miRNA* (**d**) in *wt* or ERβ+ cells (C- and N-TAP-ERβ cell RNA combined) before (-) and after stimulation with E2 for the indicated times. The right columns in (**b**) show the relative expression of c9orf3 RNA in ERβ+ cells following E2 stimulation, measured by mRNA expression profiling (Grober *et al*., 2011).

also to other genetic loci selectively regulated by ERβ in BC cells under the same conditions.

Our attention focused next on the miR-23b/27b/24-1 cluster on chromosome 9, whose organization is showed in Figure 4a. In this case, both ERβ- and ERα-binding sites are detected. Noteworthy, the two ERα-binding sites identified by ChIP-Seq were also found by ChIP-on-chip in an independent study (Hurtado *et al*., 2008) and binding of the two ERs to both sites identified here in ERβ+ cells was confirmed by ChIP (Supplementary Figure S6). The effects of ERβ in regulation of the first step in miRNA biogenesis were investigated by measuring changes in pri-miR expression in control (*wt*), N-TAP- and C-TAP-ERβ cells before and after E2 stimulation. Results show that in the absence of ERβ, estrogen stimulation did not influence primary-transcript levels, assessed by both quantitative real-time rtPCR and RNA-expression profiling (*c9orf3* RNA; Figure 4b and Cicatiello *et al*., 2010). On the other hand, a slight but reproducible accumulation of pri-miR-23b/-27b/-24-1 was detectable in ERβ+ cells already 2 h after E2 (Figure 4b). We next measured the intracellular concentration of the individual pre-miR deriving from this primary transcript (pre-miR-23b, -27b and -24-1) in both cell types and the results obtained were surprisingly very different. Indeed, as shown in Figure 4c, whereas stimulation with E2 of ERβ− cells caused a substantial loss of pre-miR (ranging from −20 to −75%), the same treatment caused instead accumulation of these pre-miRs in ERβ+ cells. This was reflected in comparable changes in expression of the corresponding mature miRNAs for up to 72 h after E2 stimulation (Figure 4d and Supplementary Figure S3). These results indicate that the presence of ERβ in ERα-expressing, estrogen-responsive BC cells can modify substantially the response of miRNA genes to hormonal stimulus. In the case of the miR-23b/27b/24-1 gene cluster, this results from changes in pri-miR maturation, rather than synthesis, leading to increase in pre-miR biosynthesis in the presence of chromatin-bound ERβ.

ERβ interferes with ERα-mediated recruitment of Drosha in inactive chromatin-bound complexes

Yamagata *et al*. (2009) reported ERα-mediated regulation of miRNA maturation by direct interaction in the nucleus of ERα with a protein complex comprising Drosha and the DEAD box RNA helicase p68/DDX5, resulting in inhibition of pri- to pre-miRNA conversion by Drosha. We thus considered the possibility that the enhancing effect of ERβ on pri-miR-23b/-27b/-24-1 maturation shown in Figure 4 could result from competition for binding of the ERα-p68-Drosha complex to this locus by ERβ, as recently described for other target genes (Grober *et al*., 2011), thereby preventing the inhibitory effect of ERα on nascent pri-miR maturation. In both cases, we should expect inhibition of ERα-mediated p68/DDX5-Drosha recruitment to miR-23b/-27b/-24-1 chromatin by ERβ. Indeed, this appears to be the case, as E2-induced p68/DDX5 and Drosha binding to ERβ-G9242 and ERβ-G9242 chromatin sites was strongly reduced in C-TAP-ERβ compared with *wt* cells, concomitant with a reduction of ERα and appearance of ERβ (upper panel of Figure 5a and data not shown). It is worth mentioning that binding of ERα to chromatin in ERβ+ cells occurs mainly via heterodimerization with ERβ (Grober *et al*., 2011). ERβ-mediated inhibition of p68/DDX5 binding could be observed also at the ERβ-G5984 site of the TFF1/pS2 gene promoter,

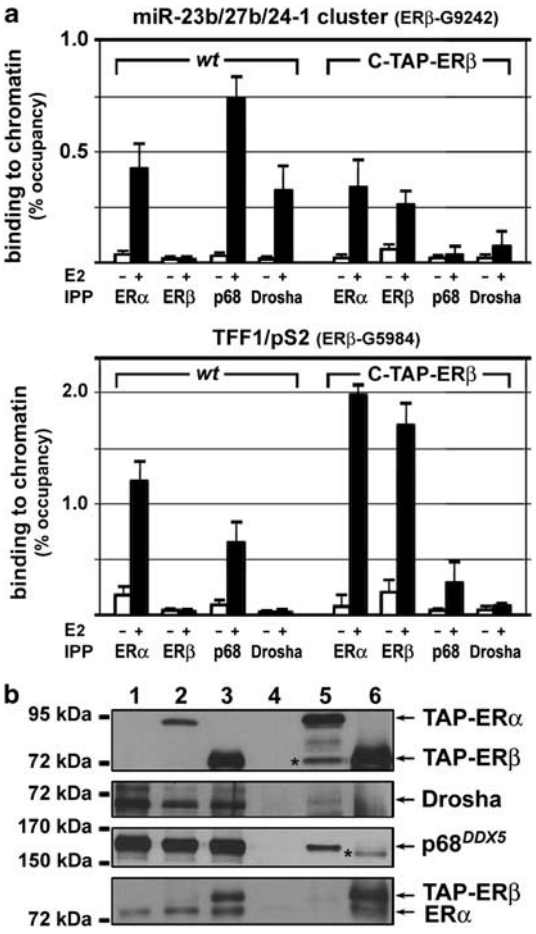

Figure 5 ER/Drosha interaction in MCF-7 cell nuclei. ChIP real-time PCR results showing binding of ERα, ERβ, p68 and Drosha to miRNA 23b/27b/24-1 cluster and the TFF1/pS2 loci (**a**) in the TFF1/pS2 loci (**b**) in *wt* and C-TAP-ERβ cells; data are expressed as % occupancy respect to input chromatin. (**b**) Western blot analysis of whole nuclear extracts (lanes 1–3) and IgG-Sepharose-affinity-purified nuclear extracts (lanes 4–6) from *wt* (lanes 1 and 4), C-TAP-ERα (lanes 2 and 5) or C-TAP-ERβ (lanes 3 and 6) cells, probed with the indicated antibodies. Asterisks mark non-specific bands.

although Drosha could not be detected tethered to this site under any condition (lower panel of Figure 5b), suggesting that association of this enzyme to chromatin may be promoted by ERα only at sites of pri-miR synthesis, where Drosha could be 'locked' in an inactive complex comprising ERα and the hairpin structure of the nascent pri-miR. Concerning the nature of the physical interaction between ERα and Drosha, it was suggested that this is mediated by p68/p72 RNA helicases (Yamagata *et al*., 2009). Interestingly, a systematic analysis of the ERβ interactome of MCF-7 cell nuclei (Nassa *et al*., 2011) failed to identify p68/DDX5 binding to this receptor subtype as well as to ERα/ERβ heterodimers, suggesting that the presence of ERβ could determine inhibition of p68/DDX5-mediated sequestering of Drosha to the chromatin in an inhibitory complex. This possibility would provide a rationale for the ChIP results obtained in ERβ+ cells, where we failed to detect these two proteins in the presence of both ERs (Figure 5a). To verify this possibility, we performed co-purification analysis of all these proteins in nuclear extracts from *wt*, C-TAP-ERα or C-TAP-ERβ cells. The two ERs were adsorbed to Sepharose-bound IgG via their TAP tag, as described (Ambrosino *et al*., 2010; Nassa *et al*., 2011). As shown in Figure 5b, Drosha and p68/DDX5 could be co-purified with ERα but not with ERβ, demonstrating that ERβ is unable to bind these proteins. It is worth mentioning that as under these experimental conditions ERα co-purifies with C-TAP-ERβ (Nassa *et al*., 2011 and lower section of Figure 5b), ERα/ERβ heterodimers do not bind Drosha and p68/DDX5.

Discussion

The results described here demonstrates that ERβ controls synthesis, maturation and steady-state levels of a significant number of miRNAs in BC cells by interfering with ERα activity or acting autonomously, as demonstrated here for the miR-23b/-27b/-24-1 cluster and the miR-30a gene, respectively. This, in turn, determines a profound effect on miRNome expression and activity in tumors expressing ERβ, which could help explain their less aggressive clinical phenotype (Novelli *et al*., 2008; Shaaban *et al*., 2008). Identification of the intracellular targets of these ERβ-regulated miRNAs, and the effects they exert on key cellular functions of BC cells, will now provide a new venue to understand the pleiotropic role of this oncosuppressive factor in breast carcinogenesis and tumor progression. Furthermore, it is reasonable to conceive that proteins encoded by the mRNAs targeted by these miRNA may represent molecular markers exploitable for prognostic evaluation of primary breast tumors or for prediction of the disease responsiveness to hormonal therapy.

Materials and methods

Cell Culture, transient transfection and cell cycle analyses
Human hormone-responsive BC cells MCF-7 Tet-Off (Clontech-Takara) expressing TAP (control cells), C-TAP-ERα, C-TAP-ERβ or N-TAP-ERβ were described previously (Ambrosino *et al*., 2010; Nassa *et al*., 2011). They were propagated, hormone starved and analyzed for estrogen signaling, cell cycle progression and cell proliferation as described earlier (Cicatiello *et al*., 2000; Grober *et al*., 2011).

RNA purification
Total RNA was extracted from hormone-starved (+EtOH, −E2) or stimulated (+E_2) cell cultures as described previously (Cicatiello *et al*., 2004). FFPE tumor samples were cut in 5-μm-thick sections on a microtome with a disposable blade. RNA was extracted from three and eight sequential sections as described (Ravo *et al*., 2008). RNA concentration in each sample was determined with a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific Italy, Cinisello Balsamo, Italia) and quality assessed with the Agilent 2100 Bioanalyzer and Agilent RNA 6000 cartridges (Agilent Technologies). For microarray analysis, RNAs extracted from replicate samples of the same tumor were pooled.

Microarray analyses
See Supplementary Materials and methods.

Protein-complex immunoprecipitations and analysis
Cells were hormone starved for 5 days and following stimulation with 10^{-8} M E2 for 2 h, nuclear proteins were extracted and incubated with IgG-Sepharose beads (GE Healthcare, Milano, Italy) for 4 h at 4 °C, as described earlier (Ambrosino *et al*., 2010). Affinity-purified complexes were resuspended in SDS sample buffer (Invitrogen Life Technologies Italia, Milano, Italy) and analyzed by SDS–PAGE and western blotting by using anti-TAP (CAB1001, Open Biosystems, Euroclone Spa, Milano, Italy), anti-ERα (sc-543, Santa Cruz Biotechnology), anti-Drosha (ab12286, Abcam, Cambridge, UK) and anti-DDX5 (ab21696, Abcam) antibodies. The primary antibodies were detected with a horseradish peroxidase-conjugated anti-rabbit antibody (GE Healthcare) and revealed by chemiluminescence and autoradiography.

Chromatin immunoprecipitation
Cells were hormone deprived for 4 days and chromatin was extracted from replicate samples before (−E2) or 45 min after stimulation with E2 as described previously (Cicatiello *et al*., 2010; Grober *et al*., 2011). Chromatin samples were incubated at 4 °C overnight with Abs against the C- (HC-20, from Santa Cruz Biotechnology, Europe) or N- (18–32, Sigma Aldrich Italia, Milano, Italy) terminus of human ERα, anti-Drosha (ab12286, Abcam, used as described by Nakamura *et al*. (2007), anti-DDX5 (ab21696, Abcam) or, for TAP-ERβ, with IgG Sepharose 6 fast Flow (GE Healthcare) as described earlier (Grober *et al*., 2011). As control, aliquots of the same chromatin were processed in the same way but Abs were omitted from the incubation mixtures (+E2/-Abs) or, where required, underivatized Sepahrose was used.

Quantitative real-time rtPCR
Total RNA was extracted from cell lines (as described before) after stimulation for 2 h and 4 h with10^{-8} M E2. For miRNA analysis, mature miRNA was reverse transcribed using a miRNA-specific stem-loop reverse transcriptase and real-time PCR was performed using Taqman microRNA assays (Assay ID: 2822, 416, 2439, 2445, 2441, 2126, 2174, 2440, 2333, 482; Applied Biosystems Italia, Monza, Italy) according to the manufacturer's instruction. RNU49 was used as an internal control to normalize all data using the Taqman RNU49 assay (Applied Biosystems Italia). RNU49 was unaffected by hormone treatment. For pre-miRNA and pri-miRNA analysis, RNA was reverse transcribed using Quantitect Rev. Transcription kit (Qiagen Italy, Milano, Italia) and real-time PCR was performed in triplicates in three independent experiments using Power Syber Green PCR Master Mix (Applied Biosystems Italia) and normalized to U6 snRNA. All the real-time PCR were performed on a MJ Research PTC-200 Opticon Instrument (MJ Research, Waltham, MA, USA). Primers used are listed in Supplementary Table 4.

ChIP-Seq data analysis
For ER-binding-site mapping in genome, ChIP-Seq data relative to ERβ (Grober *et al*., 2011; accession number E-MTAB-345) and ERα (Cicatiello *et al*., 2010; accession number E-MTAB-131) were analyzed as follows. Enriched ChIP-Seq peaks were identified using FindPeaks (Fejes *et al*., 2008), with a subpeaks value of 0.5. To select only highly relevant sites, the statistical cut-off of the first quartile was applied. The binding sites supported by a number of tags lower than 25% of the range of the values was discarded. This led to re-mapping of ERβ-binding sites (renumbered here from ERβ_G1 to ERβ_G12430); for ERα-binding sites, numbering was the same as previously described (Cicatiello *et al*., 2010).

miRNA target prediction and functional analysis of their putative mRNA targets
For comprehensive prediction of miRNA-target genes, we used TargetScan, release 5.1 (http://www.targetscan.org). To identify statistically overrepresented 'biological process' gene ontology terms among sets of selected mRNA target, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) functional annotation tool (Dennis *et al*., 2003; Huang *et al*., 2009). To this aim, we used as background data coming from gene expression-profiling experiments performed on the same cell line and under the same experimental conditions used in this study.

Immunohistochemistry
See: Supplementary Materials and methods.

BC samples clinical hallmarks
For the purpose of this study, 40 breast carcinomas were selected from a series of 936 cases with a median follow up (FU) of 50 months (min 1–max 108) subjected to breast surgery at the Regina Elena Cancer Institute between 2001 and 2005 (Novelli *et al*., 2008). Of these, 22 were ERβ+ without any recurrence, whereas 18 were ERβ− and presented local or distant metastasis. In these patients, ERβ expression was routinely determined at the time of surgical treatment along with other conventional biological factors namely ERα and progesterone receptors (PgR), HER2 and Ki-67, before any adjuvant therapy was planned. As showed in Supplementary Table S1, the group included 37 (92.5%) invasive ductal carcinomas and 3 (7.5%) invasive lobular carcinomas. Among these, 28 (70%) were pT1, 9 (22.5%) pT2 and 3 (7.5%) pT3-4, 27 (67.5%) were node negative and 13 (32.5%) were node positive, 29 (72.5%) G1-2 and 11 (27.5%) G3. ERα was positive in 37 tumors (92.5%) and negative in 3 (7.5%), PgR was positive in 31 tumors (77.5%) and negative in 9 (22.5), HER2 was positive in 12 tumors (30%) and negative in 28 (70%) and Ki-67 was positive in 16 tumors (40%) and negative in 24 (60%). Tumors were graded according to Bloom and Richardson and staged according to the Unione Internationale Contre le Cancer tumor-node-metastasis system criteria, and histologically classified according to the World Health Organization (Tavassoli and Devilee, 2003). In the selected group, ERβ+ was significantly associated to negative lymphnodes ($P<0.0001$) and low tumor grade (G1-2) ($P=0.03$) whereas, as already described on a large series of BC patients, (Novelli *et al*., 2008) no significant correlation was observed between ERβ expression and the other parameters analyzed. Follow-up data were obtained from hospital charts and by corresponding with the referring physicians.

Conflict of interest

The authors declare no conflict of interest.

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