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A Regulatory "miRcircuitry" Involving miR221&222 and ERα Determines ERα Status of Breast Cancer Cells

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ABSTRACT (English Version).

Estrogen Receptor α (ERα) is the most important diagnostic and prognostic factor in breast cancer. Several lines of evidence have suggested that ERα-negative breast tumors, highly aggressive and non-responsive to hormonal therapy, arise from ERα-positive precursors through different molecular pathways. microRNAs (miRs) are small non-coding RNAs that regulate gene expression at post-transcriptional level and are aberrantly expressed in breast cancer. We hypothesized that microRNAs may have a pivotal role in ERα suppression and ERα-negative tumors formation. MicroRNAs microarray, quantitative real-time PCR, immunohistochemistry and in-situ hybridization analyses of breast cancer cell lines and primary tumors with different ERα-status indicated that miR221&222 are exclusively expressed in ERα-negative breast tumors. Overexpression of both microRNAs in ERα-positive cell lines strongly reduces the levels of ERα protein and, using luciferase reporter assays, we clearly demonstrated that ERα is a bona fide target of miR221&222. Gene expression profiles were analyzed after miR221 or miR222 over-expression in ERα-positive cells: up-regulation of anti-apoptotic genes, growth factors and down modulation of adhesion molecules indicated that miR221&222 may not only increase the ability to suppress apoptosis and accelerate tumor formation but also promote remodeling of the tumor microenvironment and escape of tumor cells through the basement membrane, all characteristics of ERα-negative cells. To gain more insights into the inverse correlation between miR221&222 and ERα in breast cancer, by computational analyses and reporter assay, we characterized the transcriptional unit of miR221&222. We identified several estrogen-responsive elements (ERE) at the genomic locus of miR221&222 and, by using different approaches, we demonstrated that ERα suppresses miR221&222 expression. Overexpression of exogenous or endogenous ERα protein in ERα-negative cells markedly repressed miR221&222; conversely, knockdown of ERα in ERα-positive cells increased their expression. Finally, chromatin immunoprecipitation (ChIP) on ERα-positive cells showed that ligand-bound ERα and its co-repressor proteins, NcoR and SMRT, were highly enriched at miR221&222 genomic locus. Notably, ChIP experiments after estradiol (E2) stimulation or ERα-knockdown revealed that ERα recruitment is essential for the co-repressor enrichment and miR221&222 repression. These findings suggest that the negative

regulatory loop involving miR221&222 and ERα may confer proliferative advantage and migratory activity to breast cancer cells and promote the transition from ERα-positive to ERα-negative tumors. Therefore, the elucidation of this pathway could be an important step in the development of the next generation of breast cancer therapeutics agents that may prevent hormone-resistance that frequently occurs during treatment.

ABSTRACT (Italian Version).

Il recettore degli estrogeni (ERα) e` il piu` importante fattore diagnostico e prognostico nel cancro della mammella. Numerosi studi hanno proposto che i tumori ERα negativi, altamente aggressivi e non-responsivi alla terapia ormonale, hanno origine da precursori ERα positivi. I microRNA (miRs) sono piccoli RNA non codificanti che regolano l`espressione genica a livello post-trascrizionale e sono espressi in maniera aberrante nel cancro della mammella. In questo studio abbiamo ipotizzato e dimostrato che i microRNA possano esercitare un ruolo importante nella soppressione dell` ERα ed indurre la formazione dei tumori ERα-negativi. A tale scopo, analisi di microarray, qRT-PCR, immunoistochimica e in situ-ibridazione sono state impiegate per analizzare l`espressione dei microRNA in linee cellulari e tumori primitivi di mammella. Tali analisi hanno evidenziato che il miR221 ed il miR222 sono esclusivamente espressi nei tumori della mammella ERα negativi. L`over-espressione di miR221 o miR222 in linee cellulari ERα-positive riduce fortemente i livelli proteici di ERα e, usando saggi reporter di luciferasi, abbiamo chiaramente dimostrato che ERα e` target diretto di miR221&222. Analisi di microarray sono state realizzate dopo l`over-espressione di miR221 o mir222 in linee cellulari ERα-positive: up-regolazione di geni anti-apoptotici, fattori di crescita e down-modulazione di molecole di adesione cellulare hanno indicato che miR221 o miR222 possono non solo ridurre l`apoptosi ed accelerare la crescita tumorale ma anche promuovere il rimodellamento dello stroma circostante la regione tumorale e facilitare il movimento delle cellule tumorali, tutte caratteristiche delle cellule ERα-negative.

Successivamente, utilizzando analisi computazionali e saggi reporter di luciferasi, abbiamo identificato l`unita` trascrizionale dei miR221&222 e valutato la presenza di numerosi elementi responsivi agli estrogeni (ERE) nel locus genomico di miR221&222. Attraverso l`utilizzo di differenti approcci molecolari, abbiamo dimostrato che ERα sopprime l`espressione di miR221&222. In particolare, l`over-espressione di ERα in cellule ERα-negative sopprime i livelli dei miR221&222; viceversa, la down-regolazione di ERa mediante RNAi in cellule ERα-positive aumenta la loro espressione. Infine, analisi di ChIP (Chromatin Immunoprecipitation) realizzata su linee cellulari ERα-positive hanno evidenziato che il recettore degli estrogeni e i suoi co-repressori, NcoR e SMRT, sono altamente arricchiti nella regione genomica di miR221&222. Di nota, esperimenti di ChIP

dopo stimolazione con estradiolo o di knockdown contro ERα hanno evidenziato che il recrutamento di ERα e` essenziale per il recrutamento dei co-repressori e repressione di miR221&222.

Tali risultati suggeriscono che il circuito regolativo coinvolgente miR221&222 e ERα, conferendo alle cellule tumorali un vantaggio proliferativo e forte attivita` migratoria, potrebbe promuovere la transizione da tumori positivi a tumori negativi per l`espressione del recettore degli estrogeni. Quindi l`elucidazione di questo pathway potrebbe rappresentare un importante passo avanti nello sviluppo di nuove terapie anti-tumorali per il cancro al seno.

INTRODUCTION

1. The discovery of microRNAs

The discovery of microRNAs began in the early 1981 when Martin Chalfie, during a loss-of-function study in C. elegans, discovered that mutations in lin-4 gene lead to continued synthesis of larval-specific cuticle [1]. At that time, together with lin-14, lin-29, and lin-28, these genes were classified as heterochronic genes, capable to control the timing of specific post-embryonic developmental events in C. elegans. Seven year later, Victor Ambros described, in hypodermal cells, a hierarchy of interaction between the heterochronic regulatory genes to coordinate the "larva-to-adult switch": the experiments have shown that, in the early stages of development, lin-14 and lin-28 inhibit lin-29, preventing the switch; next, lin-4 inhibits lin-14 and lin-28, triggering the activation of lin-29 and the following switch in L4 larval stage [2].

Lin-14 has been shown to encode a nuclear protein that is normally present in most somatic cells of late embryos and L1 larvae but not in the later larval stages or adults. Gary Ruvkun found that two lin-14 gain-of-function mutations lead to an abnormal protein accumulation in the later larval stages; these mutations delete the 3` untranslated region of lin-14 mRNA, highlighting a regulatory element in the 3`UTR of lin-14 mRNA that controls the temporal gradient of the protein [3]. Since lin-4 was described to down-regulate the lin-14 protein temporal levels, the authors proposed that lin-4 gene product could be the trans-acting factor capable to bind the 3`UTR of lin-14 and negatively regulate it. Finally in 1993, two independent studies, published in the same issue of "Cell" by Ambros and Ruvkun [4] [5], presented the real nature of lin-4 gene and its ability to regulate the heterochronic gene expression. After cloning the lin-4 gene, they demonstrated that the potential open-reading frame of lin-4 does not encode for a protein; they identified two small lin-4 transcripts of approximately 22 and 61 nt and found that the 3` UTR of lin-14 mRNA contains sequences complementary to lin-4 small RNA products. According to these data, the authors suggested that the temporal regulation of lin-14 is operated at the protein translation level driven by lin4 RNA via antisense RNA-RNA interactions, involving the small RNA lin-4 and the 3`UTR of lin-14. Seven years latter, Reinhart et al. showed that let-7 gene, another heterochronic switch gene, is coding for a small 21 nt RNA with complementary sequence to the 3`UTR of lin-14, lin-28, lin-41, lin-42 and daf-12; they proposed that the sequential stage-specific

expression of let-7 and lin-4 RNAs was capable through an RNA-RNA interaction with the 3`UTR of the target genes to trigger the temporal cascade of regulatory heterochronic genes specifying the timing of C. elegans developmental events [6].

At that time, these discoveries were considered as a new piece in the complicated gene expression regulation puzzle restricted to the small temporal RNA let-7 and lin-4 (stRNA) in worms. This idea was completely changed when independent groups tried to investigate whether RNAs similar to stRNA could play a more general role in gene regulation [7,8 9]. Isolating and cloning the small RNA from different organisms and cellular systems, by using the same strategy applied to clone siRNA processed from exogenous dsRNAs in an embryo lysate, researchers in three laboratories were able to isolate a group of RNAs with the same characteristics of lin-4 and let-7, providing evidence for the existence of a large class of small RNAs with potential regulatory roles. Because with their small size, the authors referred to these novel RNAs as microRNA (abbreviated miRNAs), a new abundant class of riboregulators that could regulate the gene expression at post-translational level by base-pairing the 3`UTR of mRNA targets. After this discovery, a new challenge for the researchers was to define the biological function and the potential target genes of these new genes.

2 MiRNAs: GENOMICS, BIOGENESIS AND MECHANISM OF ACTION

2a. How many miRNAs are enough?

At the beginning, the majority of microRNAs was identified by direct cloning of small RNAs[10]; this approach was very reductive because it permitted just the detection of abundant expressed miRNAs. Three observations suggested that miRNAs genes could be identified using computational approaches. First, miRNAs are produced from a precursor transcript of 70-100 nt with extended stem-loop structure. Second, miRNAs are usually highly conserved between the genomes of related species. Third, miRNAs display a characteristic pattern of evolutionary divergence. In according with these criteria, many computational procedures have been developed to predict miRNAs genes in the genome of different organisms, like MiRNAscan in humans, miRNAseeker in Drosophila or others in C. elegans (for review see [11,12]). The sensitivity of these bioinformatic approaches was demonstrated by the presence of a high percentage of previously experimentally identified miRNAs within the top predicted candidates and by confirmation using Northern blotting analysis and a more sensitive RT-

PCR method. The estimate of miRNA genes in the metazoan genome represents nearly 1% of the predicted genes, a fraction similar to that seen for other very large gene families with regulatory roles, such as those encoding transcription-factor proteins.

In 2003, the rapid growth of the miRNA genes number has led Sam Grifftihs-Jones from Wellcome Trust Sanger Institute to create a comprehensive and searchable database of published miRNAs sequences via a web interface, "The microRNA Registry" (http://www.sanger.ac.uk/software/Rfam/mirna/) [13]. The primary aims of this registry are two-fold. The first is to avoid inadvertent overlap by assigning unique names to distinct miRNAs. The microRNAs are annotated with numerical identifiers based on sequence similarity: a standard name is like "miR-15" and the next miR without similarity will receive the name "miR-16". For homologue miRNAs in different organisms, it is usual to assign the same name on the similarity of the 22 nt mature sequence. Identical mature forms are assigned the same name and if they are produced from different genomic loci they are differentiate by suffixes as, for example, "miR-15-1" and "miR-15-2". Differences in one or two bases are identified by suffixes, such as "miR-181a" and "miR-181b". If a microRNA hairpin precursor give rise to two mature miRNAs, one from each arms, the rule is to denote miRNAs in the form "miR-142-5p" (5` arm) and "miR-142-3p" (3` arm) until the expression data will confirm which form is predominantly expressed; in such cases, the specie less expressed will be identified by using an asterisk (such as "miR-191*"). The second aim of miRNA registry is to provide an all miRNAs sequences database including the stem-loop structure, with the highlighted microRNA in red, genomic location, homologous sequences and possible target predictions.

At the beginning of 2005, a phylogenetic shadowing study on microRNAs in primate species revealed a characteristic conservation profile of miRNAs genes that the authors have used to efficiently detect the 83% of the known miRNAs and predict an extensive set of novel miRNAs, opening the possibility that as many as 1000 microRNAs may exist in the human genome [14]. The MicroRNA Registry contained in the version 12.0 (September 2008) 8619 entries from in primates, rodents, birds, fish, worms, flies, plants and viruses; actually, the human miRNAs are 695 and the number may still increase.

2b. Genomics of microRNAs.

Almost 50% of mammalian microRNAs are located in introns of protein coding genes or long ncRNAs transcripts, whereas the remaining part is considerable as indipendent transcription units with specific promoter core elements and polyadenilation signals (for review see [15-17]). Among the intragenic miRNAs, 40% are found in introns of protein coding genes, whereas ~10% are located in introns of long ncRNA transcripts. The vast majority of miRNA clusters are single transcription units or overlapped in the same host transcripts, within exons or introns, and in some cases depending on alternative splicing of the host gene, implying that they are transcribed as polycistronic transcripts. Additionally, many miRNAs overlap with two or more transcription units transcribed on opposite DNA strands.

The analysis of miRNAs genomic loci evidences that host genes encoding proteins are involved in a broad spectrum of biological function ranging from embryonic development to the cell cycle and physiology. When the miRNAs host genes are classified for Gene Ontology (GO) "biological process", the two most commonly identified biological process are "metabolism" and "cellular physiological process" whereas the classification for GO "molecular function" identifies "purine nucleotide binding" and "DNA binding" proteins.

In addition to the miRNAs located in protein coding genes, a large group of microRNAs resides in transcripts that lack a significant protein-coding potential, classified as long ncRNAs. These types of ncRNA transcripts are sometimes referred to as mRNA-like ncRNAs (mlncRNA) because they are spliced, polyadenilated and also spatio-temporally expressed. Deleted in Leukemia 2 (DLEU2) and BIC are host-genes mlncRNAs respectively for miR-15/miR-16 cluster and miR-155 [18, 19].

The maturation of miRNAs is a very complex process and in the following sections we will try to illustrate the machinery that the cell needs to activate the intricate multi-step processing from nucleus to cytoplasm, required for the production of microRNAs.

2c. MicroRNAs Transcription and Maturation.

Initially, the researchers believed that microRNAs were transcribed by RNA polymerase III like other small RNAs, as some as tRNAs. However, numerous evidences supported the possibility of a transcription mediated by RNA polymerase II. In 2004, three direct evidences have been reported to evaluate the strict correlation between microRNAs and pol II: (i) the miRNAs transcripts are capped and polyadenilated; (ii) the transcription of miRNAs

transcripts is sensitive to alpha-amanitine at the specific concentration for pol II inhibition; (iii) the promoter region, responsible for miRNA transcription, is associated to pol II complex [15].

Animal microRNAs are identified as part of 80 nt RNA with stem-loop structure (pre-miRNA) that are included in several hundreds/thousands nucleotide long miRNAs precursors, named primary miRNAs precursor (pri-miRNA) (**Figure 1**). Until now, few different pri-miRNA precursors have been isolated and characterized. They are all capped, polyadenilated and apparently non-coding: the human cluster miR-23a~27a~24-2 primary precursor is an unspliced ~2.2 kb RNA; in contrast, the pri-miRNA for human miR-155 (BIC) includes two introns and two alternative poly-A sites capable to produce two spliced pri-miRNAs of 0.6 and 1.4 kb [16].

The production of microRNAs from pri-miRNA to mature miR is a complex and coordinated process where different groups of enzymes and associated proteins, located in the nucleus or cytoplasm, operate the multistep maturation of these tiny RNAs. Principally, the maturation process of microRNAs can be resumed in three important steps: cropping, export and dicing (**Figure 1**).

In the cropping, the pri-miRNA is converted in pre-miRNA trough the cleavage activity of Drosha enzyme, a nuclear Ribonuclease III endonuclease capable to crop the flank regions of pri-miRNA in turn to liberate the 60-70 nt pre-miRNA [10]. Different pri-miRNA requisitions are necessary to obtain an efficient precursor maturation by Drosha: first, a large terminal loop (> 10 nucleotides) in the hairpin and a stem region one turn bigger than the pre-miRNA; second, a 5` and 3` single stranded RNA extensions at the base of the future microRNAs [20, 21]. It has been proposed that Drosha may recognize the primary precursor through the stem-loop structure and then cleave the stem at a fixed distance from the loop to liberate the pre-miRNA. How the enzyme is capable to discriminate the pri-miRNA stem-loop structure in respect to the others stem-loop cellular RNAs is not clear, but probably proteins associated with Drosha confer specificity to this process. In fact, Drosha has been found as a part of large protein complex of ~650kDa, which is known as the “Microprocessor”, where Drosha interacts with its cofactor, the Di George syndrome critical region gene 8 (DGCR8) protein in human and Pasha in Drosophila melanogaster [22]. The Microprocessor appears to represent a heterotetramer consisting of two Drosha and two DGCR8 molecules; because DGCR8

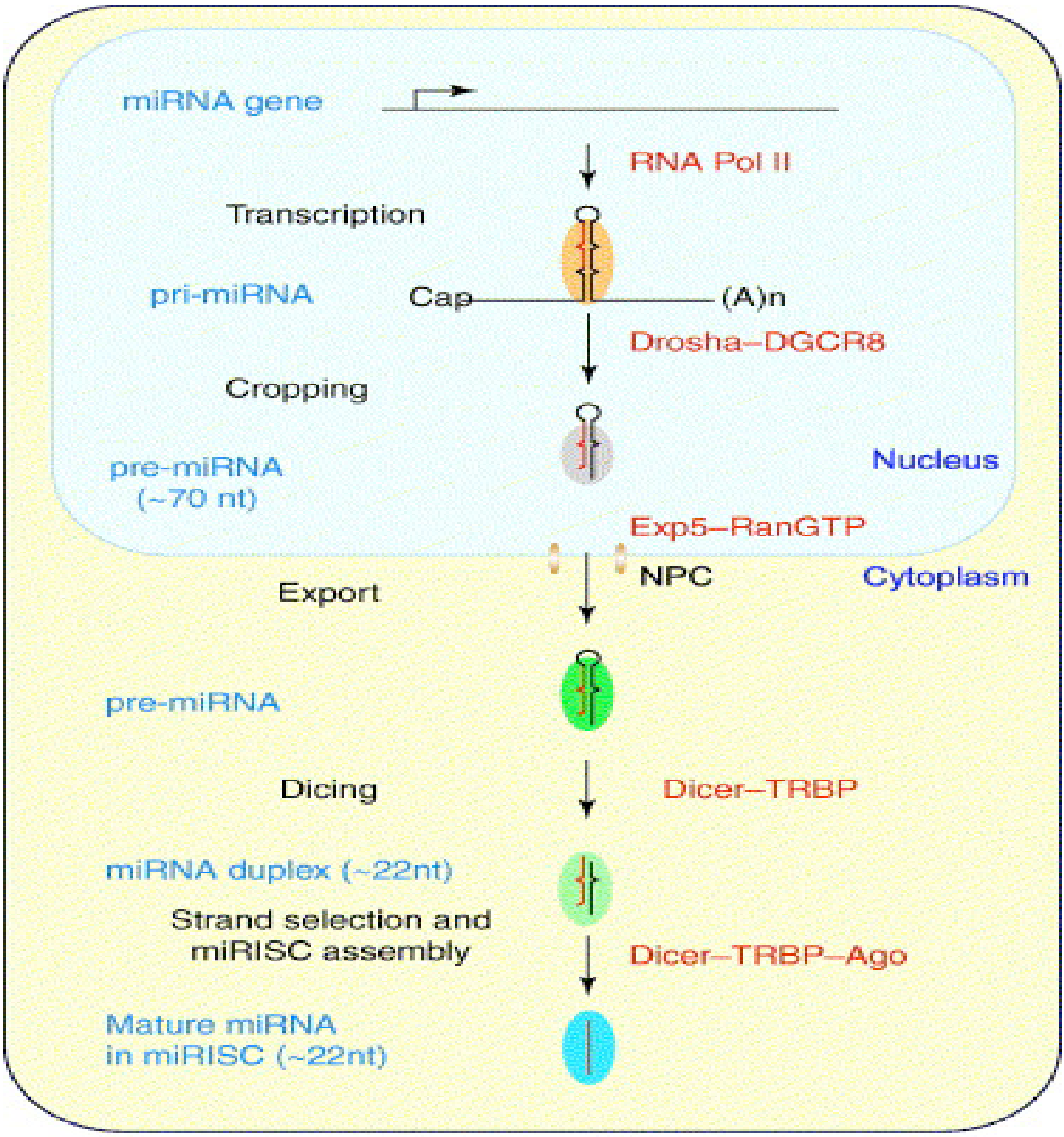


FIGURE 1. MicroRNA (miRNA) genes are transcribed by RNA polymerase II (pol II) to generate the primary transcripts (pri-miRNAs). The initiation step ('cropping') is mediated by the DroshaΞDGCR8 complex (also known as the Microprocessor complex). Drosha and DGCR8 are both located mainly in the nucleus. The product of this nuclear processing step is a 70-nucleotide (nt) pre-miRNA, which possesses a short stem plus a 2-nucleotide 3' overhang. This structure might serve as a signature motif that is recognized by the nuclear export factor exportin-5. Pre-miRNA constitutes a transport complex together with exportin-5 and its cofactor Ran (the GTP-bound form). Following export, the cytoplasmic RNase III Dicer participates in the second processing step ('dicing') to produce miRNA duplexes. The duplex is separated and usually one strand is selected as the mature miRNA, whereas the other strand is degraded.

contains two consensus dsRNA binding domain, this protein may play an important role in the substrate discrimination and binding.

The resulting product of cropping, the pre-miRNA, presents a 5` phosphate and 3` hydroxy termini, and two or three nucleotides single-stranded overhanging ends, classic characteristics of Rnase III cleavage of dsRNAs. After the Microprocessor nuclear activity, the produced pre-miRNA is exported to the cytoplasm by Exportin-5/RnaGTP[23]. Exp-5 forms a nuclear heterotrimer with RanGTP and pre-miRNA, resulted from Drosha processing. This interaction, which is dependent on RNA structure but independent of sequence, stabilizes the nuclear pre-miRNA and promotes the export to the cytoplasm. In any export, once the Exp5-RanGTP-pre-miRNA complex has reached the cytoplasm through the nuclear pore, the RnaGTP is hydrolyzed to RanGDP and the pre-miRNA is released.

Arrived into the cytoplasm, the pre-miRNA is processed in 18~22 nucleotides miR duplexes by the cytoplasmic Rnase III Dicer and, in humans, its partner TRBP. The PAZ domain of Dicer is thought to interact with the nucleotides 3` overhang present in the pre-miRNA hairpin while the dsRNA binding domain binds the stem and defines the distance of cleavage from the base of pre-miRNA. The cleavage 22nt-long miRNA duplexes have a reduced half-life. Normally, one strand of this duplex is degraded whereas the other strand accumulates as a mature miRNA. Studies on siRNAs have highlighted that the selection of the right strand is related to the thermodynamic stability of the duplex and the strand with relatively unstable base pairs at the 5` end usually represents the mature miR.

2d. MicroRNA in action: RISC and gene target inhibition

In the RNA duplex produced from the Dicer activity, the mature miRNA is only partially paired to the miRNA*, the small RNA that resides on the opposite pre-miRNA stem. From the miRNA-miRNA* duplex, only the miRNA enters preferentially in the protein effector complex, the RNA Induced Silencing Complex (RISC) or miRNAsC or miRgonaute, which mediates the degradation or translation inhibition of mRNAs target gene[24]. Several proteins have identified as essential components of RISC, but only a few have been functionally characterized in the post-translational regulation. The core component of every RISC is a member of the Argonaute (Ago) protein family, whose members present a central PAZ domain like Dicer and a carboxy terminal PIWI domain. This domain binds the miR/miR* duplex to the 5` end whereas the PAZ domain binds to the 3` end of singled-strand RNAs;

moreover, structural and biochemical studies have suggested that the Ago proteins are the target-cleaving endonuclease of RISC and in this activity the complex is helped and coordinated by other proteins whose function is not really understood like RNA-binding protein VIG, the Fragile-X related protein in Drosophila, the exonuclease Tudor-SN and many other putative helicases[25]. In the human cells, after the microRNAs transfection by miR-expressing vectors or miRNA precursors, and the following activation of RISC activity, the core component of RISC, together with the triggering miRNA target mRNA, is concentrated in cytoplasmic foci known as Processing bodies (P-bodies) or GW-bodies. According with this triggered RISC localization, the researchers thought that the microRNAs, in association with AGO proteins, might be capable to repress the translation at ribosomal level and to re-localize the mRNA targets to the P-bodies[26].

The majority of the animal miRNAs are imperfectly paired to the 3` UTR of the target mRNA and inhibit the protein production by an unknown or very controversial mechanism (**Figure 2**); in some cases, the microRNAs show nearly precise complementarity to their target and trigger mRNA degradation as siRNA in the RNA interference process[24].

The most important characterization of the function of microRNAs is the identification of mRNA target. Because the animal miRNAs have a 5`end restricted complementarity to the mRNA target (only 5-8 nucleotides perfectly complementary), RNA sequence named "seed region", the miRNAs are predicted to regulate a large number of animal genes. Different algorithms have been developed to predict the animal miR targets; they are based on different criteria, resulted from the analysis of the in vivo demonstrated targets: (i) perfect or nearly perfect base-paring at the seed region and thermodynamically stability of the duplex miR-mRNA; (ii) phylogenetic conservation of the seed region; (iii) multiple target sites in a single target by the same or different miRNAs; (iiii) absence of strong secondary structures at the miR-binding site on the target. Several computational procedures are available to predict microRNA targets such as DianaMicroT (http://www.diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi), TargetScan (http://genes.mit.edu/targetscan/), and miRanda (http://genes.mit.edu/targetscan/) [27-29].

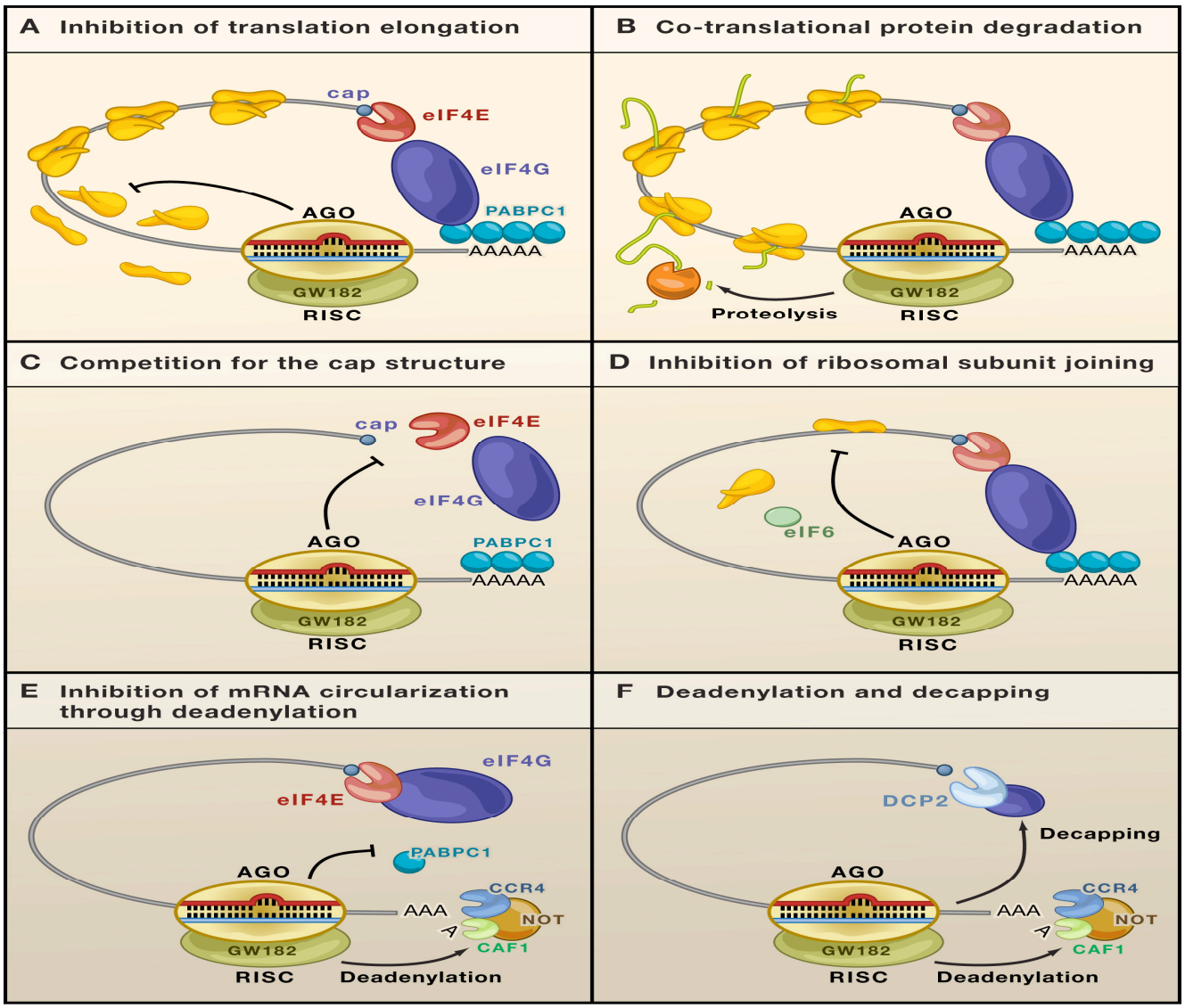


FIGURE2. Mechanisms of miRNA-Mediated Gene Silencing. **(A)** Postinitiation mechanisms. MicroRNAs (miRNAs; red) repress translation of target mRNAs by blocking translation elongation or by promoting premature dissociation of ribosomes (ribosome drop-off). **(B)** Cotranslational protein degradation. This model proposes that translation is not inhibited, but rather the nascent polypeptide chain is degraded cotranslationally. The putative protease is unknown. **(C–E)** Initiation mechanisms. MicroRNAs interfere with a very early step of translation, prior to elongation. **(C)** Argonaute proteins compete with eIF4E for binding to the cap structure (cyan dot). **(D)** Argonaute proteins recruit eIF6, which prevents the large ribosomal subunit from joining the small subunit. **(E)** Argonaute proteins prevent the formation of the closed loop mRNA configuration by an ill-defined mechanism that includes deadenylation. **(F)** MicroRNA-mediated mRNA decay. MicroRNAs trigger deadenylation and subsequent decapping of the mRNA target. Proteins required for this process are shown including components of the major deadenylase complex (CAF1, CCR4, and the NOT complex), the decapping enzyme DCP2, and several decapping activators (dark blue circles). (Note that mRNA decay could be an independent mechanism of silencing, or a consequence of translational repression, irrespective of whether repression occurs at the initiation or postinitiation levels of translation.) RISC is shown as a minimal complex including an Argonaute protein (yellow) and GW182 (green). The mRNA is represented in a closed loop configuration achieved through interactions between the cytoplasmic poly(A) binding protein (PABPC1; bound to the 3′ poly(A) tail) and eIF4G (bound to the cytoplasmic cap-binding protein eIF.

3. microRNAs: function in normal and disease states.

With the discovery on a "daily - basis" of new members of the miRNA family, it becomes evident that these small genes are involved in normal cells homeostasis [10, 30]. Furthermore, with the development of new techniques of genome-wide screening of microRNAs expression, abnormal levels of microRNAs were identified in tumor cells in respect with normal counterparts [31- 33]. The functions of microRNAs, initially a "shadow" area of research, revealed a general participation in every functional aspect of normal cells in organisms with different degrees of complexity. For example, in *Drosophila, miR-14* suppresses cell death and is required for normal fat metabolism[34] , while *bantam* encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid*[35]. As Shown in **Table 1**, miRNAs participation in essential biological processes have consistently been proven, such as cell proliferation control (*miR-125b* and *let-7*), hematopoietic B-cell lineage fate (*miR-181*), B-cell survival (*miR-15a* and *miR-16-1*), brain patterning (*miR-430*), pancreatic cell insulin secretion (*miR-375*) and adipocyte development (*miR-143*) (for reviews see [36-38]).

As a consequence of extensive participation in normal functions, it is quite logical to ask the question if microRNAs abnormalities should have importance in human diseases. As shown in **Table 2**, microRNAs and/or proteins involved in the processing of miRNAs are involved in various types of human diseases, the most studies example being human cancers.

MiRNAs can act both as tumor suppressors and oncogenes [31-33].

Homozygous deletions (as is the case for *miR-15a/miR-16a* cluster), the combination mutation + promoter hypermethylation or gene amplification (as is the case of miR-155 or the cluster miR-17-92) seems to be the main mechanisms of inactivation or activation, respectively [39-41]. Because of the small size, the loss-of-function or gain-of-function point mutations represent rare events [42]. MiRNAs activity can be influenced either by the reposition of other genes close to miRNAs promoters/regulatory regions (as is the case of *miR-142s* – c-MYC translocation) or by the relocalization of a microRNA near other regulatory elements. The overall effects in the case of miRNAs inactivation is the overexpression of target mRNAs, while the miRNAs activation will lead to downregulation of target mRNAs involved in apoptosis, cell cycle, invasion or angiogenesis.

To date, it was elegantly demonstrated that *let-7* microRNA family regulates RAS oncogenes and that *let-7* expression is lower in lung tumors than in normal lung tissue, while RAS protein has an inverse variation [43]. Furthermore, enforced expression of the *miR-17-92* cluster from chromosome 13q32-33 in conjunction with c-myc accelerates tumor development in a mouse B-cell lymphoma model [40]. Two microRNAs from the same cluster, *miR-17-5p* and *miR-20a* negatively regulates the E2F1 transcription factor, a gene proved to function as a tumor suppressor in some experimental systems [39].Recently, an unexpected mechanism of involvement of miRNAs in human disease was identified. Tourette syndrome is a neurologic disorder manifested particularly by motor and vocal tics and associated with behavioral abnormalities. Sequence variants of a candidate gene on chromosome 13q31.1 named SLITRK1 (Slit and Trk-like 1) were identified in patients with TS. One of them, named var321, found in two unrelated patients, was located in the 3'UTR binding site for the miR-189 and might affect SLITRK1 expression [44]. This mechanism of abnormal miRNA::mRNA interaction seems to be a general one, as it was proved also in the case of oncogene c-KIT. Three of the highly overexpressed miRNAs in thyroid cancers, *miR-221*, *miR-222* and *miR-146*, are predicted to interact with KIT oncogene mRNA at two different sites. Tumors in which the up-regulation of these miRNA was the strongest showed dramatic loss of KIT and in half of the cases the down-regulation was associated with germline single nucleotide polymorphysms (SNP) in the two recognition sites in KIT for these three miRNAs [45]. One important proof for the functional importance of such abnormalities is represented by the reproduction of similar diseases in mouse models with abnormal microRNAs expression. Recently, the first example of transgenic microRNA mouse was published: as expected by the overexpression of miR-155 in human lymphomas [19] [46], the miR-155 transgenic mice overexpressing the gene only in B cells, exhibit a preleukemic pre-B cell proliferation in spleen and bone marrow, followed by frank B cell malignancy [47]. These findings indicate that the role of miR155 is to induce polyclonal expansion, favoring the capture of secondary genetic changes for full transformation. This is the final proof that deciphering miRNA alterations is important and that miRNAs, as small as they are, represent big culprits in human diseases.

AIM OF THIS STUDY

Breast cancer is the second leading cause of death among women with cancer in western world and its molecular pathogenesis is still not completely understood. Breast cancers arise from the mammary gland epithelium and its complex cellular hierarchy is becoming evident during breast tumorigenesis. For example, several intensive studies to determine gene-expression profiles of breast tumors enabled classification of, at least, five reproducible subtypes including luminal A, luminal B, basal-like, Her2+, and normal basal-like[48,49], which partially correlate with the traditional histological classification of the tumor[50]. Overall, such studies pointed out that Estrogen Receptor α (ERα) expression status is one of the strongest factors governing the gene expression profile in breast cancer[51] and thus, determines the biopathological phenotype of the tumor.

ERα is a nuclear hormone receptor that mediates the mitogenic action of 17β-estradiol on the breast ductal epithelium. E2-bound ERα is involved not only in the epithelial cells development but also in progression of breast cancer[52]. About 75% of diagnosed breast tumors are ERα positive and the expression of ERα is correlated with a better prognosis mainly because of the likelihood of response to hormonal treatment[53]. Different studies suggest that a fraction of ERα-negative tumors arise from ERα-positive precursors[54]. Several molecular events, such as estrogen withdrawal[55], hypoxia[56], overexpression of epidermal growth factor receptor (EGFR) or ErbB2, which brings hyperactivation of mitogen activated protein kinase (MAPK)[57] and DNA methylation occurring at the promoter of *ERα* gene[58], have been reported to suppress ERα expression.

miRNAs play a pivotal role in tumorigenesis of breast cancer. Iorio et al. have previously determined miRNA expression profiles of breast cancer tissues[59]. This study demonstrated that miRNAs were aberrantly expressed in breast cancer as compared to miRNAs expressed in normal tissue and, more interestingly, miRNA expression pattern could discriminate between breast tumors with different bio-pathologic phenothypes such as ERα status. A recent study further showed that miR-206, identified as an upregulated miRNA in ERα-negative tumors[59], targets ERα mRNA[60].

Here, we have determined microRNA expression profiles of a set of breast cancer cell lines with different ERα expression status and found a remarkable and consistent up-regulation of miR221&222, exclusively in ERα-negative cell lines. Demonstration of the suppressive

effect of miR221&222 on ERα protein and the identification of miR221&222 responsive target sites in the 3'UTR of ERα mRNA led us to conclude that ERα is a *bonafide* target of miR221&222. We showed that overexpression of miR221&222 in ERα-positive cells induces a global change in gene expression that differs from miR206 signature and may account for the generation of a more invasive and deadly tumor phenotype. Furthermore, we showed that ERα directly binds to the promoter of *miR221/222* gene and recruits co-repressors NCoR and SMRT, thereby functioning as a transcriptional repressor for *miR221/222* gene. Taken together, our results uncovered the presence of a regulatory loop composed of miR221&222 and ERα in human breast cancer cells, which may provide a new insight into understanding how breast cancer cells determine ERα-positive and-negative status.

METHODS

Cell Culture and Tumor Samples

Human breast cancer cell lines (T47D, MCF7, BT-474, BT483, MDA-MB-231, MDA-MB-436, MDA-MB-468) were purchased from ATCC and grown in DMEM containing 10% heat-inactivated FBS, 2mM L-glutamine and 100U/ml penicillin/streptomycin. All transfections were carried out with Lipofectamine 2000 (Invitrogen) according to the manifacturer's instructions. For estradiol treatments, cells were grown to 70% confluency in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 5% charcoal-dextran-stripped fetal bovine serum for at least 5 days. Meg01 cells were purchased from ATCC and grown in RPMI 1640 containing 10% heat-inactivated FBS, 2mM L-glutamine, 100U/ml penicillin/streptomycin. For proliferation assay, MCF7 were plated in 96-well plates and grown for 96hrs after transfection (miR final concentration 100nM) in normal culture condition. Cell growth was measured using Celltiter 96 AQ_{ueous} Non-Radicative Cell Proliferation Assay (Promega), according to manifacturer`s instructions. Cell Cycle was performed on propidium iodide stained, ethanol-fixed cells. All tumors samples were obtained with patient consent from the Department of Experimental Oncology, Fondazione IRCCS "Istituto Nazionale dei Tumori", Milano. All samples were histologically confirmed.

Plasmid Constructions

To generate the ERα luciferase reporter constructs, two potential binding sites for miR221&222 within the 3` UTR of ERα mRNA were amplified by PCR and cloned downstream of the luciferase coding sequence in the pGL3-control vector (Promega). Mutations were introduced into the miRNA binding sites by using QuikChange mutagenesis kit (Stratagene). For mapping *miR221/222* gene promoter, upstream genomic sequence of miR222 hairpin was amplified by PCR and cloned at the NheI and XhoI sites of pGL3-basic vector (Promega). To construct the pCRUZ-HA-ERα, ERα cDNA from ATG to TGA was synthesized from MCF7 mRNA by RT-PCR and was cloned in frame at the KpnI and EcoRV sites of pCRUZ-HA (Santa Cruz Inc). All constructs were sequenced to verify their integrities.

Microarray Analysis

For microRNAs microarray analysis[61], total RNAs from 6 breast cancer cell lines were hybridized to the Ohio State University custom miRNA microarray chip (OSU_CCC version 3.0), which contains 1150 miRNA probes, including 326 human and 249 mouse miRNA genes, spotted in duplicates. Differentially expressed miRNAs were identified using the Class Comparison Analysis of BRB tools version 3.6.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html). The criteria for inclusion of a gene in miRs list is a p-value lower than a specified threshold value (0.05). A tree cluster was generated by the hierarchical cluster analysis; for this analysis, we used average linkage metrics and centered Pearson correlation (Cluster 3.0). Java Treeview 1.1 was used for tree visualization. The labeling of mRNA and the hybridization of the labeled cRNA onto the Affymetrix GeneChip HG-U133A 2.0 arrays were performed according to Affymetrix standard protocols (Santa Clara, CA). BRB Arraytools was used for Affymetrix HU133Plus2 genechips. Normalization was performed by GC-RMA and genes showing minimal variation across the set of arrays were excluded from the analysis. Genes whose expression differed by at least 1.5 fold from the median in at least 20% of the arrays were retained. Genes were considered statistically significant if their p-value was lower than 0.001 (False detection rate<0.01). All differentially expressed genes, either up- or down-regulated, along with their corresponding fold-change values, were input into Pathway-Express, a program that automatically determines the most related pathways based on pathway topology, gene expression level (fold-change), and relative positions of

genes within a pathway(s). Returned pathways were considered significant if the corrected gamma-p-value was less than 0.01. Pathways were then ranked according to impact factor.

Real Time PCR

For quantitative detection of mature miRNA, real-time PCR was performed using the TaqMan PCR Kit, followed by the detection with Applied Biosystems 7900HT Sequence Detection System (P/N: 4329002, Applied Biosystems). PCR was carried out in 10 μl reaction containing 0.67 μl RT product, 1 μl TaqMan Universal PCR Master Mix (P/N: 4324018, Applied Biosystems), 0.2 mM TaqMan probe, 1.5 mM forward primer and 0.7 mM reverse primer. The reaction mixture was incubated in a 96-well plate at 95 ˚C for 10 min, followed by 40 cycles of denaturation (95 ˚C for 15 s) and extension (60 ˚C for 1 min). All reactions were run in triplicate. The comparative CT method for relative quantization of gene expression (User Bulletin #2, Applied Biosystems) was used to determine miRNA expression level. Simultaneously determined U6 CT was used to normalize the data. For quantification of ERα, pS2 and GAPDH mRNAs, the appropriate TaqMan probes were purchased from Applied Biosystems.

Luciferase Assays

For identification of miR221&222 responsible elements, Meg01 cells were cotransfected in 24 well plates with 250ng of pGL3 reporter vector carrying the miR221 &222 binding site (see plasmid construct), 25ng of the phRL-SV40 control vector (Promega), and 100nM miR precursors or scramble control (Ambion) using Lipofectamine 2000. For mapping *miR221/222* gene promoter, Meg01 cells were cotransfected in 24 well plates with 250ng of pGL3 reporter vector carrying upstream genomic sequence of miR222 hairpin (see plasmid construct) and 25ng of the phRL-SV40 control vector. Firefly and Renilla luciferase activities were measured consecutively by using Dual Luciferase Assay (Promega) 24h post-transfection. Reporter assay were carried out in triplicate.

Acrylamide and Agarose Northern Blot Detection.

For mature miRNA detection, acrilamide Northern blotting was performed as previously described [16]. Briefly, 10 μg aliquots of total RNAs from cell lines were resolved in a 15% denaturing polyacrylamide gel (Bio-Rad) and were electrophoretically transferred to bright-

Star blotting membrane (Ambion). The oligonucleotide encoding the complementary sequence of the mature miRNA annotated in miRNA Registry was end-labeled with [γ^{32} P]-ATP by T4 polynucleotide kinase (USB,Cleveland). RNA blotted membrane was prehybridized in Ultrahyb Oligo solution (Ambion) and subsequently hybridized in the same solution containing probe at a concentration of 10^6 cpm/ml at 37˚C, overnight. Membrane was washed at high stringency in the solution containing 2X SSC and 1% SDS at 37˚C. Northern hybridization signals was captured and converted to digital image by using Typhoon Scanner. For the detection of primary transcript of miR221&222, total RNA (10 μg per sample) was size-fractionated through 1.2% agarose-formaldehyde gels and transferred to Amersham nylon filters. Northern blot was hybridized with random-primed and α-^{32}P-labeled genomic DNA fragment spanning either miR221 or miR222 hairpin, as a probe (Roche).

ChIP Assay

ChIP assays were performed by using the ChIP assay kit from Upstate Biotechnology (Lake Placid, NY) with minor modifications. Briefly, cells were grown to 70% confluency in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. The cross-linking was performed with 1% formaldehyde at 37°C for 10 min. Cells were then rinsed with ice-cold PBS and resuspended in 0.4 ml of lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1xprotease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and sonicated. A 30μl aliquot of the preparation was treated to reverse the cross-linking, deproteinized with proteinase K, extracted with phenol chloroform, and determined for DNA concentration. An aliquot of chromatin preparation containing 25 μg DNA was used per ChIP. DNase-free RNase (Roche) was added at a concentration of 200 μg/ml during reverse cross-linking. After deproteinization with proteinase K, DNA was purified in 50 μl of Tris-EDTA with a PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A 2-μl aliquot was used for PCR. Primer sequences are listed in Supplementary Experimental Procedures. The primary antibodies used for immunoprecipitation were ERα (Bethyl laboratories A300-498A), SMRT (Santa Cruz Inc. sc-20778), NCoR (Santa Cruz Inc. sc-8994), rabbit IgG control (Zymed). In some experiments, ChiP-enriched DNA was also subjected to qPCR by using SYBR reagent

(Applied Biosystem). In this case, results were expressed as relative enrichment according to the following formula: $2^{-[(ct_{ChIP}-ct_{input})-(ct_{IgG}-ct_{input})]}$.

Results

Up-Modulation of miR221&222 in ERα-negative Cells and Primary Tumors.

First, we determined miRNA expression profiles of a small set of human breast cancer cell lines with different ERα expression status. MicroRNA microarray analysis of ERα-positive cell lines (T47D, BT474 and BT483) and ERα-negative cell lines (MB-468, MB-436 and MB-231) was performed by using our custom miRNA microarray platform[61]. Hierarchical clustering analysis based on the miRNA expression data distinguished sharply ERα-negative from ERα-positive cell lines (**Figure 3a**). MiRNAs displaying more than 4-fold change of difference are listed in **Table 3**. As shown previously[59], we also identified miR191 and miR26a as up-regulated miRNAs in ER**α**-positive breast cancer cells (**Figure 3a** and **Table 3**). We also noted that the expression level of miR206, up-regulated in ER**α**-negative breast cancer[59] and later shown to target ERα transcript[60], was up-regulated in ERα-negative cells (**Figure 3b**) with statistical significance, although it was excluded from the list due to the current cut-off setting. Among up-regulated miRNAs in the ERα-negative cells, miR221&222 were identified as the miRNAs displaying the highest differential expression against the ERα-positive counterpart (**Figure 3a,b** and **Table 3**). Validation of the microarray results was performed by northern blot analysis and, further, by qRT-PCR for the most differentially and biologically relevant microRNAs, miR191, miR203, miR221 and miR222 (**Figure 3c,d**). Both analyses confirmed that miR221&222 were detectable only in the ERα-negative cells. Then, we wished to determine whether the observation was also extended to breast cancer tissues. To this end, qRT-PCR of miR221&222 was performed on 66 breast cancer tissues, including 44 ERα-positive and 22 ERα-negative tumors (**Table 4**). Results showed that the expression level of miR221&222 differentiates the two cohorts at statistical significance, according to the ERα status (**Figure 3e**). No other statistically significant correlations with the other patient`s or tumor`s characteristics were found. These results indicated that the up-regulation of miR221&222 in ERα-negative cells is a common feature both in established breast cancer cell lines and primary breast cancer tissues.

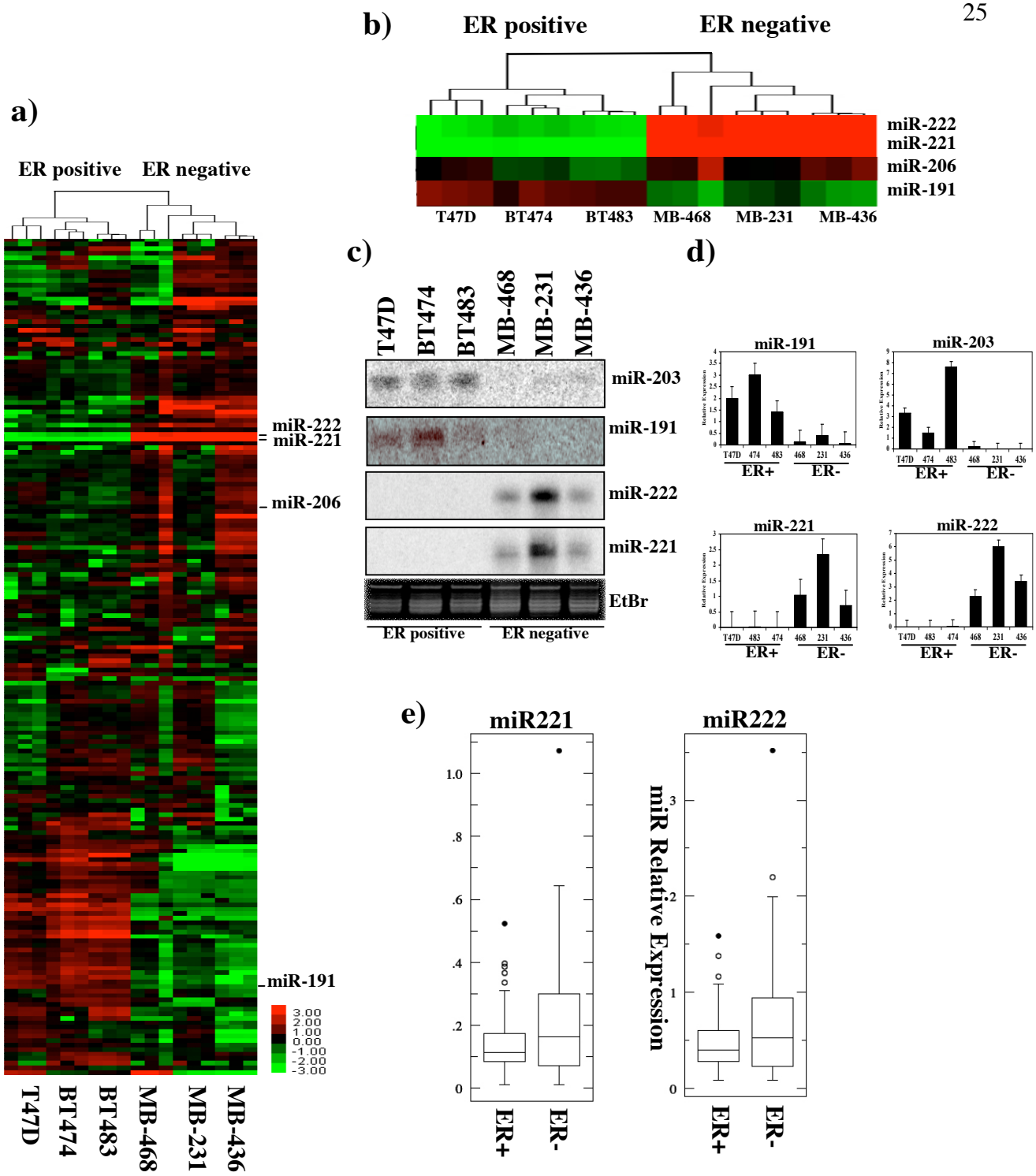


Figure 3. miR221&222 Expression Distinguishes ERα-positive from ERα-negative Breast Cancer Cells and Primary Tumors. a,b, tree generated by the hierarchical cluster analysis **(a)** of ERα-positive (T47D, BT-474, BT-483) and ERα-negative (MDA-MB-231, MDA-MD-436, MDA-MB-468) breast cancer cells on the basis of miRNA expression data **(a)**. The tree displays their average absolute expression values after log2 transformation. Zoomed results of the clustering analysis of miR221,miR222, miR206 and miR191 expression are shown in **(b)**. **c,d,** Detection of miR203, miR191, miR221 and miR222 in the breast cancer cells by Northern blot **(c)** or quantitative RT-PCR (qRT-PCR) **(d)**. **e,** Detection of miR221 and miR222 by qRT-PCR in 66 breast cancer tissues (42 ER+ and 24 ER-); two data sets obtained from the two different tumor subtypes were compared by student *t*-test.

ERα Is Target of miR221&222.

The consistent and highly differentiated expression of miR221&222 in ERα-negative cells raised the possibility of a role for miR221&222 in the loss of ERα in ERα-negative cells. To this end, two ERα-positive cell lines, T47D and BT474, lacking miR221&222 expression, were transfected with either pre-miR221, pre-miR222 or scrambled control. qRT-PCR analyses showed highly efficient production of mature miR221 or miR222 in both transfected cell lines (**Figure 4a**). Subsequent western blot analysis demonstrated that enforced expression of miR221 or miR222 caused remarkable reduction of ERα protein (**Figure 4b**). Furthermore, an additional ERα-positive cell line, MCF7, was treated by using the same procedures as described above. Cells were collected at different time points (24, 48, 72, and 96hrs) and the levels of ERα protein, assayed by western blot (**Figure 4c**), were undetectable 96hrs after transfection. qRT-PCR was also carried out on the same samples in order to evaluate the effect of miR221&222 overexpression on ERα mRNA (**Figure 4d** and data not shown). Since ERα mRNA levels were slightly reduced only after 96hrs of transfection (about 20% of reduction), the effect of miR221&222 on ERα protein reduction was due primary to a translational block and not to an ERα mRNA degradation.

MiR221 and miR222 share an identical seed sequence of 8 nucleotides and are predicted by bioinformatic algorithms (PicTar and TargetScan4.2) to bind to the ERα-3'UTR at two different sites, referred to as cER3'UTR and ncER3'UTR, respectively (**Figure 4e**). cER3'UTR is highly conserved across several species while ncER3'UTR is poorly conserved (**Figure S1**). Subsequent luciferase reporter assays showed that the insertion of both sites resulted in miR221- and miR222- dependent inhibition of luciferase activity (**Figure S1**, **Figure 4f, g** light and dark green columns). Importantly, mutation of the seed sequences against miR221 and miR222 markedly reduced their inhibitory effect (**Figure S1**, **Figure 4f, g** light and dark yellow columns). We concluded that miR221&222 target ERα through the two elements localized within the 3' UTR of ERα mRNA and that up-regulation of miR221&222 in ERα-negative cells may play a role in loss of ERα expression.

Differential Effect of miR221&222 and miR206 on Gene Expression in Breast Cancer

As previously described, ERα is also targeted by miR206[60]. To determine the differential effect of miR221&222 and miR206 on ERα production and their impact on breast tumorigenesis, MCF7 were transfected with miR221, miR222, miR206 and scrambled

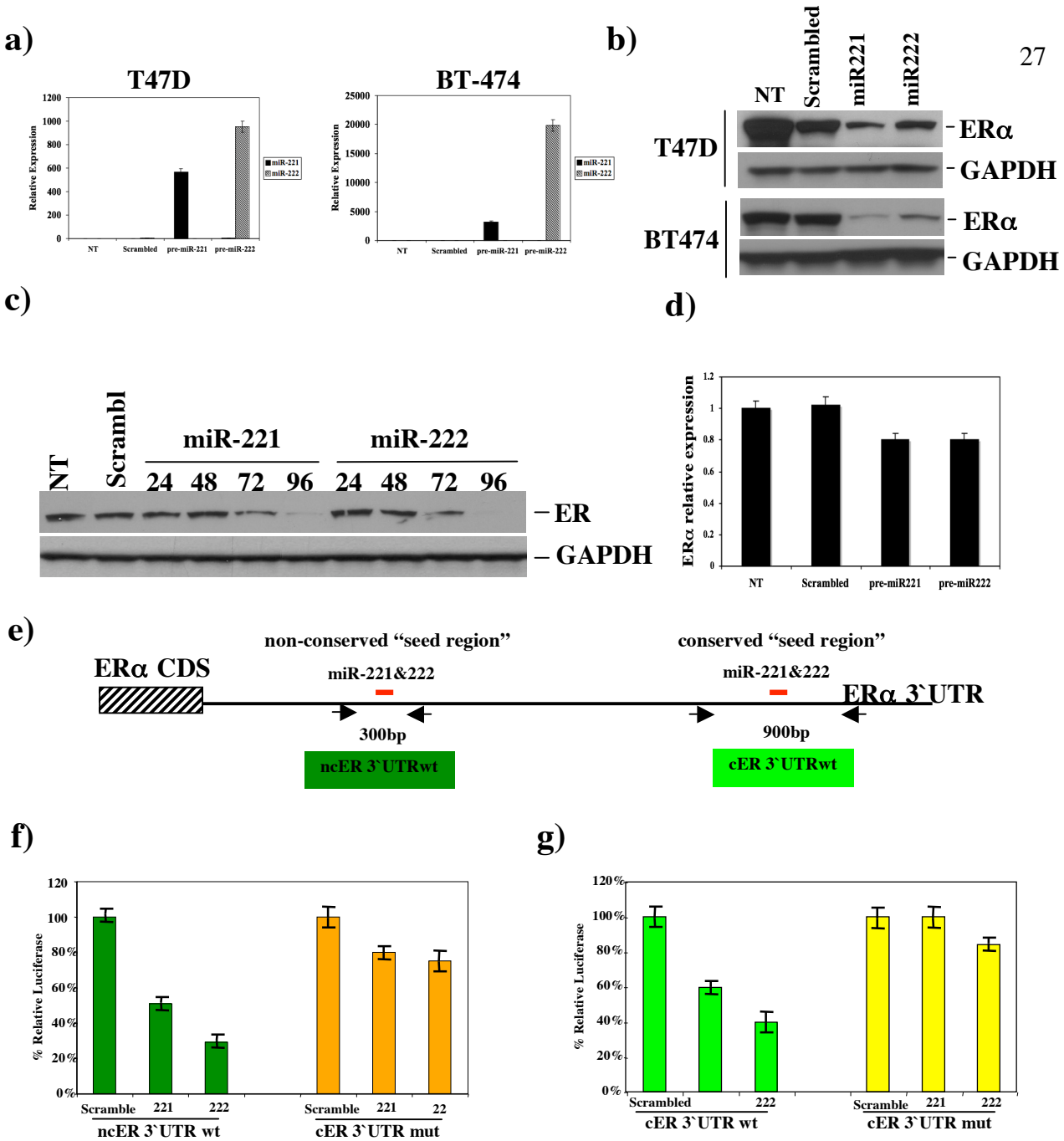
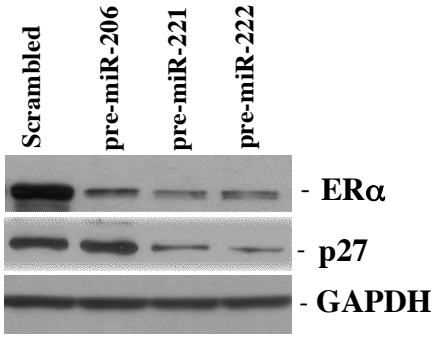


Figure 4. ERα Is a *bonafide* Target of miR221&222. a,b, two ERα-positive breast cancer cells, T47D (upper panel) and BT-474 (lower panel), were transfected with 100nM each of scrambled control, pre-miR-221 or pre-miR-222. 72hr post-transfection, total RNAs were isolated and subjected to qRT-PCR analyses to determine the levels of mature miR221 and miR222 **(a)**. Total proteins from the transfected cells were used to detect ERα by western blot. The levels of GAPDH were shown as a loading control **(b)**. **c,d**, MCF7 were treated by the same procedure as described in **a.** Total proteins were collected at different time points and levels of ERα were measured by western blot analysis **(c)**. Total RNAs were prepared from the transfected cells at 96h post-transfection. Levels of *ERα* mRNA were mesuasured by qRT-PCR and normalized for the amount of *gapdh* mRNA **(d)**. **e**, schematic diagram depicts two potential binding sites for miR221&222, predicted by TargetScan database, in the ERα 3`UTR; two pairs of the arrows indicate the fragments spanning the potential binding sites that were cloned into the pGL3 reporter vector. Non-conserved (dark green) and conserve (light green) seed regions were mapped at 549-555nt and 2253-2260nt from the translation stop codon, respectively. Luciferase activities were determined after transfection of Meg01 cells. See the text for the detail.

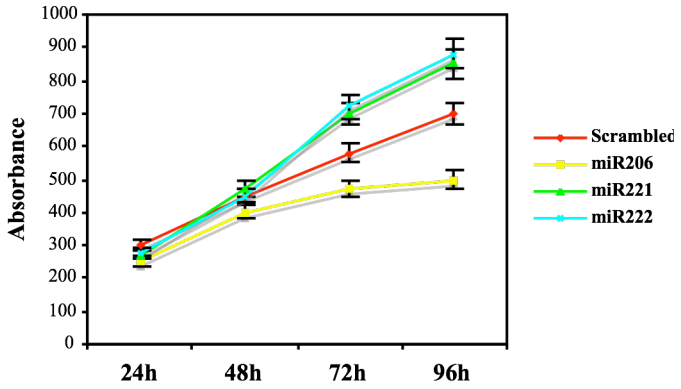
control. This study clearly showed that all these miRNAs suppress ERα protein at the same levels (**Figure 5a**). Recently, several studies have shown that miR221&222 target as well the tumor suppressor protein $p27^{Kip1\ 62\text{-}65}$. These studies pointed out that a high level of miR221&222 are required to maintain low levels of $p27^{Kip1}$ protein, allowing for cancer cell proliferation and tumor growth. We found that $p27^{Kip1}$ protein was repressed only in cells expressing miR221&222 but not miR206 (**Figure 5a**). Moreover, it was recently shown that miR206 inhibits cell growth of ER+ cells[66]. To assess the differential role of miR221&222 and miR206 in ER+ cells proliferation, MCF-7 were transfected either with miR206, miR221, miR222 and scrambled control and incubated in normal growth condition for up to 96hrs. MTT-based proliferation assay revealed that miR221 and miR222 increase cells proliferation although, as previously reported, miR206 has an inhibitory effect (**Figure 5b**). According to the miR221&222 and miR206 differential effect on p27 protein levels, FACS analyses showed that only miR221 and miR222 induce a significant increase in the transition from G1 to S phase when compared to the scrambled control (**Figure 5c**); indeed, a significant block in G1 phase was found for miR206 transfected cells and may be responsible for the decreased proliferation (**Figure 5c**). Finally, miR-transfected-MCF7 were subjected to gene expression profiles. First, SVD analyses showed that miR-transfected cells werdifferent from scrambled-transfected cells (**data not shown**); notably, miR221- and miR222-overexpressing MCF7 were clustering together and were separated from miR-206. Statistical analyses identified 1966, 1091, 1079 upregulated and 2390, 1014, 936 downregulated genes in miR-206, miR221, and miR222 overexpressing cells, respectively. According to these gene lists, SVD analyses were further confirmed by two-tailed Fisher's exact test; we showed that almost 75% of the genes regulated by miR221 and miR222 were overlapping while only the 30% was in common with miR206.

Finally, by using Pathway Express on all gene data set, we evaluated also that miR221&222 modulated-processes are overlapping and different from those of miR206 (**Figure 5e**). These co-regulated-processes are represented by apoptosis, MAPK and TGF-beta signaling pathway, focal adhesion and colorectal cancer (**Figure 5e**). By using only the genes up-regulated or down-modulated by miR221&222 compared to miR206, we found that main activated processes are cell cycle and apoptosis, respectively (**data not shown**).

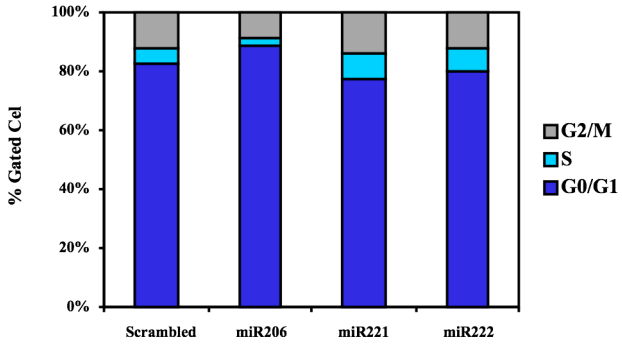
a)



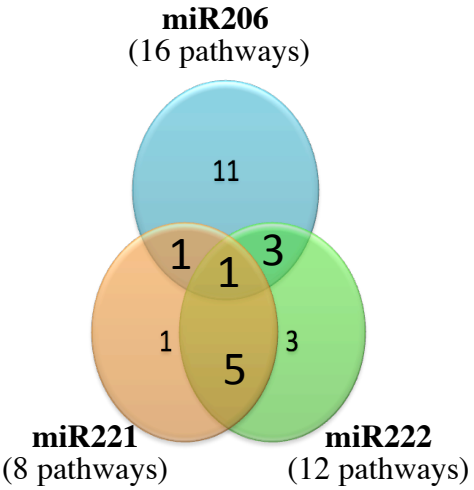
b)



c)



d)



e)

Rank	miR206	miR221	miR222
1	Cell adhesion molecules (CAMs)	TGF-beta signaling pathway	Adherens junction
2	DNA replication	MAPK signaling pathway	Colorectal cancer
3	Cell cycle	Adherens junction	TGF-beta signaling pathway
4	Homologous recombination	Apoptosis	Apoptosis
5	Adherens junction	Colorectal cancer	Cell cycle
6	p53 signaling pathway	Natural killer cell mediated cytotoxicity	Pancreatic cancer
7	Base excision repair	Antigen processing and presentation	MAPK signaling pathway
8	Mismatch repair	Focal adhesion	p53 signaling pathway
9	Nucleotide excision repair		Axon guidance
10	Circadian rhythm		Focal adhesion
11	Ubiquitin mediated proteolysis		ErbB signaling pathway
12	Pancreatic cancer		Chronic myeloid leukemia
13	Antigen processing and presentation		
14	Non-small cell lung cancer		
15	Glioma		
16	Prostate cancer		

Figure 5. miR221&222 induce a change in gene expression. a, MCF7 were transfected with scrambled control, miR206, miR-221 and miR-222 (100nM). ERα and p27 levels were analyzed by western blot analysis. **b,** Cell growth was measured by an MTT-based cell proliferation assay. **c**, miR-transfected MCF7 were also subjected to FACS analysis and the relative G1,S and G2/M compartments calculated. Data are representative of three independent experiments. **d**, Venn diagram of biological processes modulated by overexpression of miR-206, miR221 and miR222 in MCF7 cells. **e,** List of biological processes modulated by the overexpression of miR-206, miR221 and miR222. In red are reported all processes commonly shared by all three miRs, in green by miR206 and miR222, in orange by miR221 and miR222.

ERα Negatively Regulates miR221&222 Expression

Loss of miR221&222 expression in ERα-positive cells also raised the possibility that ERα negatively regulates transcription of these miRNAs. To test this hypothesis, first we overexpressed ERα in hormone-starved ERα-negative MDA-MB-436 cells (**Figure 6a**). After E2 stimulation, a strong reduction of miR221&222 expression was detected, indicating that ERα inhibits miR221&222 expression (**Figure 6b**). To corroborate this finding, another ERα-negative cell line, MDA-MB-231, previously reported to carry the promoter hypermethylation of *ERα* gene[67], was treated for 10 days with 5-aza-2'-deoxycytidine (5'AZA). This treatment resulted in induction of ERα expression (**Figure 6c**) that was accompanied by reduced expression of miR221&222 (**Figure 6d**). Next, we investigated whether endogenous ERα is involved in miR221&222 down-regulation observed in ERα-positive cells. siRNA knockdown of ERα was carried out in MCF7 cells (**Figure 6e**). Highly efficient suppression of ERα was accompanied by p27 downregulation and induction of miR221&222 expression (**Figure 6f**). To further confirm the inhibitory effect of ERα on miR221&222 expression, T47D cells were treated with the anti-estrogen Fulvestrant, which promotes ERα protein degradation through cellular proteosome activation[68]. Fulvestrant-treated T47D showed a strong reduction of ERα protein (**Figure 6g**) and a time-dependent induction of miR221&222 expression (**Figure 6h**). In summary, the enforced expression of ERα in ERα-negative cells and the elimination of ERα from ERα-positive cells caused inhibition and induction of miR221&222 expression, respectively, demonstrating a novel function of ERα as a negative regulator for the expression of these miRNAs.

Genomic Structure and Transcriptional Regulation of miR221&222

miR221 and miR222 genes, located on human chromosome X, are tandemly aligned with 720 nt spacer sequence (**Figure 7a**). Since the genomic region lacks any protein-coding gene, both miRNAs appear to represent a single transcriptional unit. MDA-MB-231 cells, which express high levels of miR221&222, were transfected with siRNA against Drosha to produce global accumulation of pri-miRNAs[69]. Western blot analysis demonstrated highly efficient Drosha knockdown by siRNA (**Figure 7b**). Northern blot analysis of Drosha-knockdown cells, probed with genomic DNA fragment spanning pre-miR221, enabled detection of ~2.1

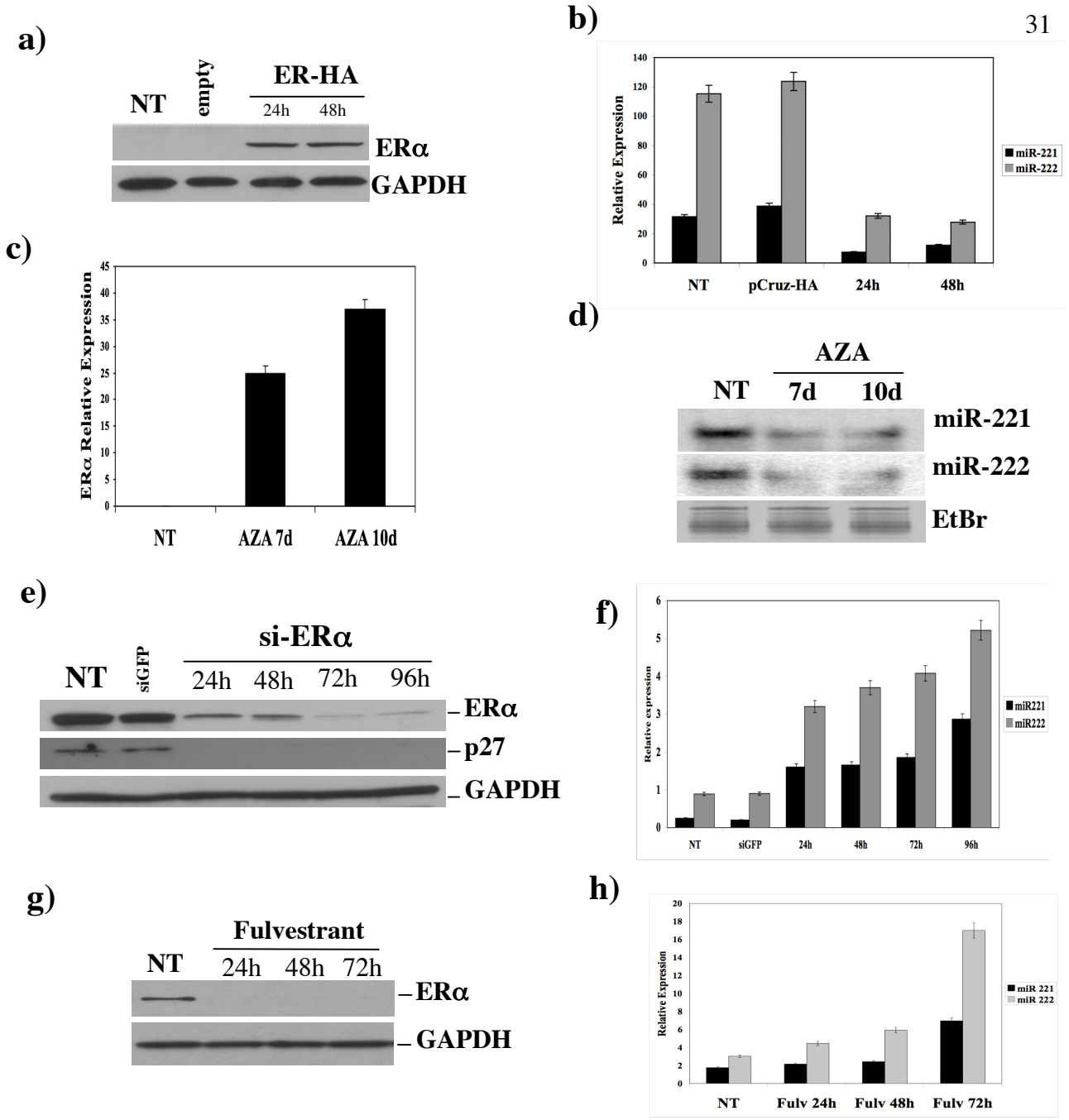


Figure 6. ERα Negatively Regulates miR221&222 Expression. a,b, ERα-negative MDA-MB-436 cells were cultured for 5 days in hormone-deprived media and were transfected with pCruz-HA-ERα construct. 24hrs after transfection, cells were stimulated by adding E2 at a concentration of 10nM and were kept in culture for additional 48hrs. Transfected cells were subjected to western blotting analysis **(a)** for the detection of HA-ERα and to qRT-PCR analysis **(b)** for the quantitative detection of mature miR221&222. **c,d,** MDA-MB-231 cells were treated with 5-aza-2`-deoxycytidine (5`AZA) at concentration of 10μM for 10 days. 5'AZA-treated cells were subjected to qRT-PCR analysis for the detection of ERα transcript **(c)**. miR221&222 were detected by Northern blot **(d)**. **e,f,** ERα-positive MCF7 cells were transfected with siRNA against ERα or with a control siRNA targeting GFP. 24, 48, 72 and 96 hrs after transfection, transfected cells were subjected to the same analytical procedures as used in **a** and **b**. Levels of p27 protein were also measured by western blot. **g,h,** T47D cells were treated with pure ERα antagonist, fulvestrant, after three days of hormone deprivation and were kept in culture for additional 72hrs. Fulvestrant-treated cells were subjected to the same analytical procedures as used in **a** and **b**.

kb transcript that is hardly detectable in control cells (**Figure 7c, left panel**). Detection of the ~2.1 kb transcript, by reprobing the blot with DNA fragment spanning pre-miR222 (**Figure 7c, right panel**), indicated that miR221&222 are transcribed into a single species of pri-miRNA. To specifically define the 5' and 3' termini of the transcriptional unit of pri-miR221&222, we carried out 5' and 3' RACE analyses and found 5' extension of ~230nt from the 3' end of pre-miR222 and 3' extension of ~1000nt from the 5' end of pre-miR221, respectively (data not shown). Based on these findings, we assumed that pri-miR221&222 of ~2.1kb starts around 120nt upstream from the 5' terminus of miR222 hairpin structure (**Figure 7a**).

Analysis of *miR221/222* gene by using "Promoter.2" and polyA signals database predicted two canonical TATA boxes located ~550nt and ~190nt upstream from the 5` end of pre-miR222 and reiterated polyadenylation sites close to the 3' end of the primary transcript (blue arrow heads and green arrows, respectively in **Figure 7a**). To determine whether the upstream genomic region from the transcription start site functions as a regulator element for miR221&222 transcription, we constructed reporter plasmids by inserting the fragments spanning -1600 ~ -3000 and +3 ~ -1600 (+1 position corresponds to the 5' terminus of pre-miR222) into the promoter-less vector pGL3basic (**Figure 7d**). Subsequent luciferase assay showed that only -1600 pGL3b gave rise to ~9-fold induction of luciferase activity as compared to the empty vector (**Figure 7d**). Next, 5' end deletion mutants of -1600 pGL3b enabled us to map the minimal promoter of miR221&222 gene at -150bp ~ -50bp, where the proximal TATA box is excluded (**Figure 4d**). In summary, both miR221&222 are transcribed into a single species of 2.1kb RNA and the transcription is regulated by the upstream sequence located at -150bp/-50bp from the 5' end of miR222 hairpin structure.

Co-occupancy of ERα and Co-repressor Proteins, NCoR and SMRT, at miR221&222 Genomic Region.

ERα is a nuclear receptor whose activity is regulated by E2. This ligand-dependent activation confers two important functions: 1) localization of ERα at the target genes by binding specific DNA sequences (Estrogen Responsive Element, ERE) and then 2) recruitment of additional transcriptional regulator, termed cofactors, that have either activator or repressor function on target genes[70]. To determine the effect of E2-bound activated ERα on *miR221/222* gene transcription, hormone-starved MCF7 cells were treated with10nM E2.

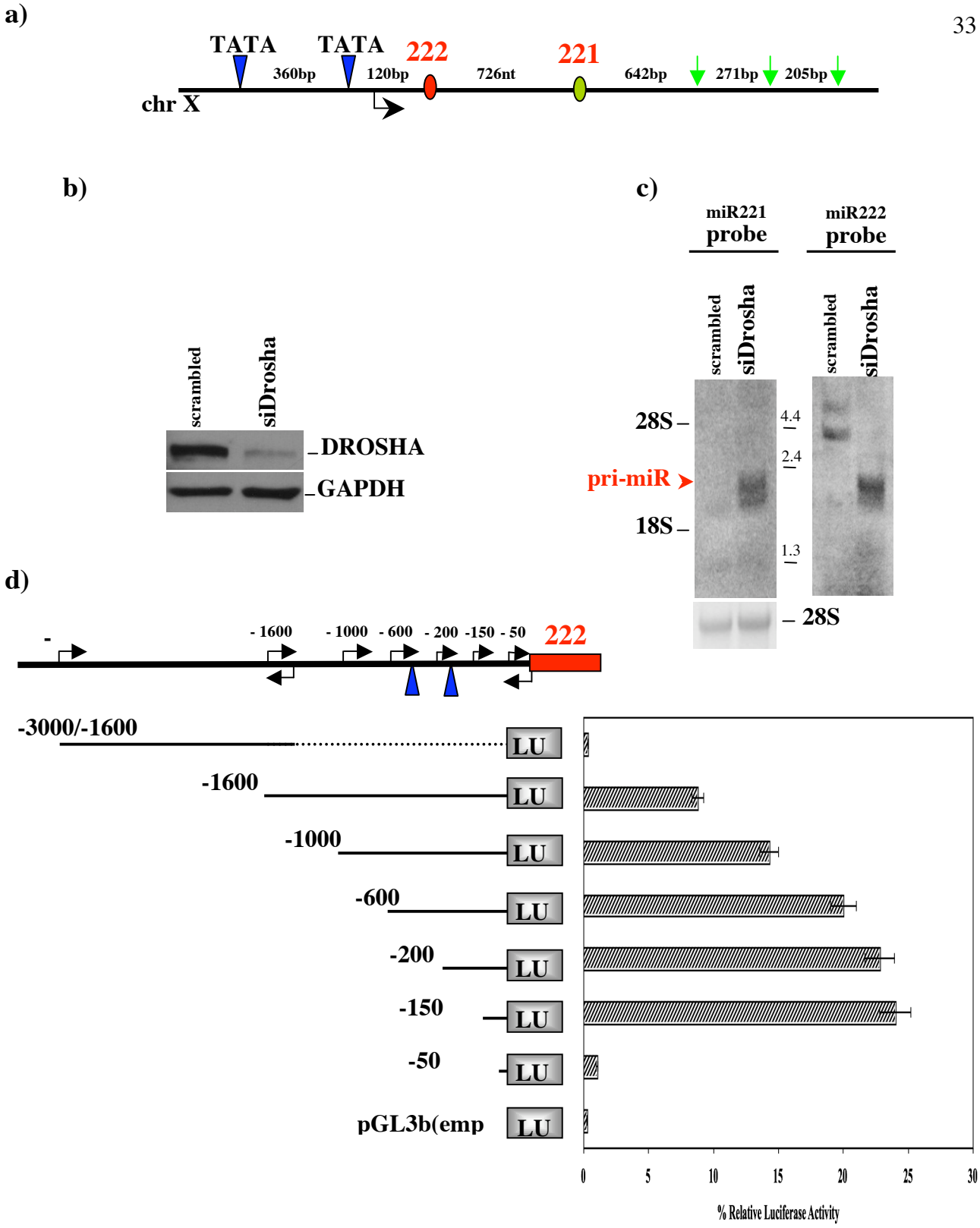


Figure 7. Identification of miR221&222 Transcriptional Unit. a, 11kb genomic region spanning miR221&222 was analyzed by using Promoter.2 (http///www.cbs.dtu.dk/services/Promoter/) and human polyA signals (http://rulai.cshl.org/tools/polyadq/polyadq_form.html) database to search for promoter and polyA signal sequences. The schematic diagram represents two canonical TATA box (blue triangle) located 550bp and 190bp upstream of pre-miR222 and three polyA signals (green arrows) located downstream of pre-miR221. **b**,**c**, Overexpressing miR221&222 MDA-MB-231 cells were transfected with siRNA against DROSHA or control siRNA against GFP (100nM). 72hrs post-transfection, siRNA-treated cells were subjected to western blot analysis for the detection of DROSHA protein **(b)** and to Northern blot analysis for the detection of miR221&222 primary transcript **(c)**. Positions of the RNA marker (Invitrogen) are shown on the right side of the panel. EtBr staining of 28S rRNA are shown as a loading control. **d,** Luciferase assay was carried out to identify *miR221/222* gene promoter. Genomic fragments located upstream of pre-miR222 and cloned into the pGL3 basic vector are shown on left. 24hrs post-transfection, luciferase activity of Meg01 cells transfected with respective reporter construct were performed.

First, the time-dependent induction of pS2 transcript, a known target of ERα, was evaluated after E2 treatment (**Figure 8a**). In sharp contrast, miR221&222 expression was markedly inhibited 24h after the treatment, suggesting that E2-bound ERα functions as a transcriptional repressor of *miR221/222* gene (**Figure 8b**). Another ERα-positive cell line, T47D, showed the same ligand-dependent repression of miR221&222 (**data not shown**). In order to investigate whether ERα was bound directly to *miR221/222* gene and how it was responsible for miR221&222 repression, chromatin immunoprecipitation (ChIP) experiments were carried out. First, we searched for ERE as well as AP1 and SP1 binding sites by using TESS database (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and found 5 potential EREs within the genomic region of 5kb, spanning miR221&222 (**Figure 8c**). Taking into account the predicted ERα binding sites, a total of 3 chromatin regions were analyzed (**Figure 8c**). ChIP assay of ERα-positive MCF7 cells showed remarkable ERα binding at ChIP analyzed region 2, which span the promoter of miR221&222 (**Figure 8d**). No ERα binding was observed in ERα-negative MDA-MB-231. Next, we performed ChIP assay against NcoR and SMRT and found co-occupancy of these corepressors with ERα in the region 2 (**Figure 8d**). Since no apparent bindings of NcoR and SMRT were detected in the region 1 and 3 of MCF7 and in all ChIP-analyzed regions of MDA-MB-231, we suggested that ERα is responsible for the co-occupancy of the corepressors.

We next examined the occupancy of E2-activated ERα and the corepressors in MCF7 cells. In hormone-starved MCF7 neither ERα nor co-repressors were recruited to *miR221/222* gene (**Figure 8e**). Following E2 stimulation, according to the previously reported miR221&222 repression, all three protein were located to the promoter of miR221&222 (**Figure 8e, upper panel)**. Quantitave PCR analyses of the same chromatin showed a strong enrichment of all three proteins, ERα, NCoR and SMRT (**Figure 8e, lower panel)**. Finally, applying siRNA-mediated ERα knockdown to MCF7 cells, we showed that elimination of ERα resulted in loss of occupancy of the co-repressors from *miR221/222* gene (**Figure 8f, upper panel**); also in this case, qPCR was used to confirm the loss of the three proteins (**Figure 8f, lower panel**). These results leaded us to conclude that ERα, engaged to *miR221/222* gene, recruits NcoR and SMRT to suppress *miR221/222* gene expression.

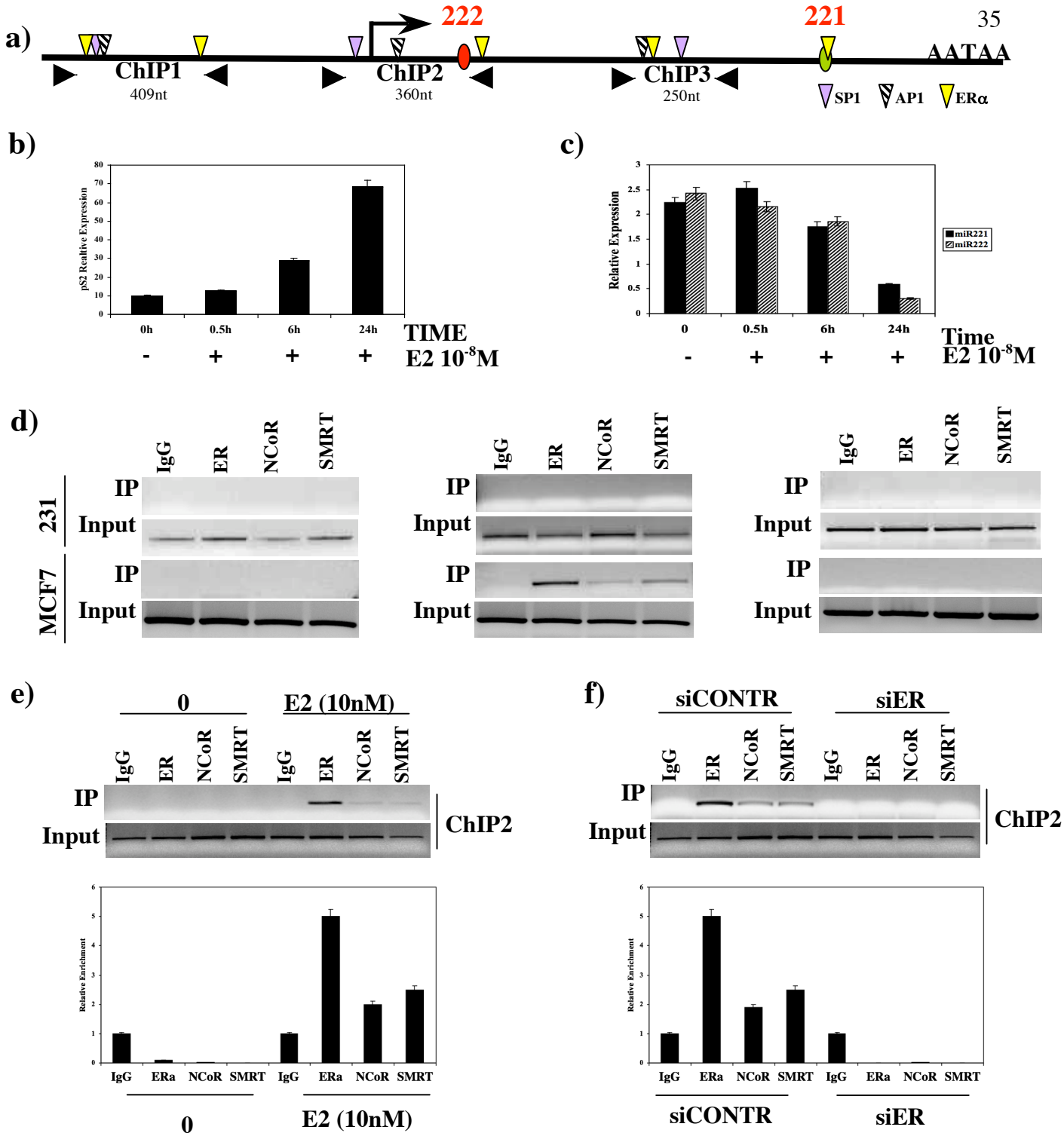


FIGURE8. ERα Binds to *miR221/222* Gene and Recruits Corepressor Proteins NCoR and SMRT. **a**, schematic diagram shows miR221&222 transcription unit defined with transcription start site and polyA signal, canonical ERα binding sites (yellow triangles), and binding sites for AP1 (black triangle) and SP1 (violet triangle). ChIP-analyzed regions are indicated with 3 pairs of the black arrowheads. **b, c,** MCF7 cells were hormone-starved for 5 days and were then stimulated by adding estradiol (E2) at a concentration of 10nM. Total RNAs from E2-treated cells were subjected to qRT-PCR for detection of pS2 transcipt (**b**) and mature miR221&222 (**c**). **d**, Cross-linked chromatins were prepared from MDA-MB-231 (ERα-negative) and from MCF7 (ERα-positive) cells and were subjected to ChIP assay. Input indicates 5% portion of ChIP input. **e,** MCF7 cells were hormone-starved for 5 days and were then stimulated by adding estradiol (E2) at a concentration of 10nM. 24hr after stimulation, cells were cross-linked and were subjected to ChIP assay. **f,** MCF7 cells were tranfected with siRNA against ERα or control siRNA. 72hr after transfection, siRNA-treated cells were cross-linked and were subjected to ChIP assay. In e and f, the amount of ChIP-enriched DNA was quantified by real time-PCR and the results were shown as relative enrichment.

DISCUSSION

In this study, we have shown that the bicistronic miR221&222 are consistently up-regulated in ERα-negative cell lines and primary breast tumors. Subsequent identification of ERα as a target of miR221&222 highlighted the importance of these miRNAs in the regulation of ERα expression in breast tumor. A recent study showed that miR206, originally identified as an upregulated miRNA in ERα-negative breast tumors[59], targets ERα transcript as well[60]. Therefore, it seems like that multiple miRNAs regulate ERα expression in a combinatorial manner. Several studies[62-65] describe that miR221&222 target the tumor suppressor protein $p27^{kip1}$. In fact, we found that $p27^{kip1}$ protein was repressed only in cells overexpressing miR-221&222 but not in cells overexpressing miR-206. Inhibition of miR221&222 induces apoptosis in ERα-negative cells[64]; moreover, as recently published, over-expression of miR206 in estrogen-dependent MCF7 cells inhibits cell growth in a dose- and time-dependent manner[66]. Furthermore, microarray analyses highlighted that overexpression of miR221&222 compared to miR206 in ERα-positive cells induces a specific activation of cell cycle and inhibition of apoptosis. Between the other biological pathway modulated by miR221&222, we found also the TGF-beta signaling pathway. Although, TGF-beta promotes cell growth inhibition, apoptosis and differentiation, and therefore, is considered a potent tumor-suppressor[71], recent studies indicated that TGF-beta plays a dual role in breast tumorigenesis; in fact, during advanced stages of breast cancer, TGF-beta factually stimulates cancer cell invasion and metastasis[72]. Notebly, also the focal adhesion pathway were strongly modulated by miR221 and miR222 overexpression; modulation of this process may confer to the cancer cells the ability to survive the apoptotic pressure of anchorage indipendent-conditions[73]. According with these data, the induction of miR-221&222 could confer a more effective proliferation advantage to the ERα-positive cells compared to miR206: notably, the suppression of ERα associated with low levels of $p27^{kip1}$ and modulation of other players not still defined will allow into the ERα-positive cells an estrogen-independent proliferation, an increase in cell cycle progression, tumor invasion and metastasis.

The loss of miR221&222 expression in ERα-positive cells and tumors, evidenced by Northern blot and qRT-PCR analyses, led us to investigate the involvement of ERα in

miR221&222 expression. Both gain and loss of function studies revealed that ERα inhibits miR221&222 expression. For example, we demonstrated that over-expression of ERα in ERα-negative cells reduces miR221&222 levels, explaining also the p27 induced-apoptosis after ERα restoration in ERα-negative cells[74].

Since accumulation of either primary transcript or precursor species encoding these miRNAs was not evident in Northern blot analysis of ERα-positive cells (data not shown), the suppression of miR221&222 mediated by ERα does not appear related to a block in microRNA processing; rather, our results revealed that ERα functions as a transcriptional repressor for *miR221/222* gene expression. While much is known about the mechanisms by which estrogen-bound ERα stimulates gene expression, the molecular mechanism(s) underlying gene repression caused by the hormone-ERα complex is largely unknown. Accumulating data concerning ERα-mediated transcriptional regulation suggest that, in general, estrogen-bound ERα recruits activator proteins, while in condition of hormone depletion, ERα recruits corepressor proteins thereby allowing the receptor to function as a transcriptional activator or repressor of its target genes[75]. We showed that in ERα-positive cells, either under regular culture condition or after E2-stimulation, ERα caused downregulation of miR221&222. Interestingly, we found that ERα is strongly bound to the *miR221/222* gene promoter and, in turn, it is responsible for the recruitment of co-repressor proteins NCoR and SMRT. Regarding other examples of corepressor recruitment by E2 activated ERα, transcriptional repression of cyclin G2 gene[76] and of vascular endothelial growth factor receptor 2 genes[77] have been recently reported. It is intriguing that both studies showed an involvement of Sp proteins for the recruitment of corepressor proteins at respective promoters. GC-rich stretch, a favorable site for SP1 protein binding, was also found in the region of miR221&222 promoter. Further study is needed to determine whether SP protein is involved as well in transcriptional repression of *miR221/222* gene mediated by ERα.

In summary, we showed that miR221&222 target ERα mRNA and that ERα functions as a transcriptional repressor of miR221/222 gene. Hence (**Figure 9**), the three components build a molecular circuitry whose deterioration may trigger an alteration of ERα expression status in breast cancer cells. Such molecular network would provide a foundation to understand

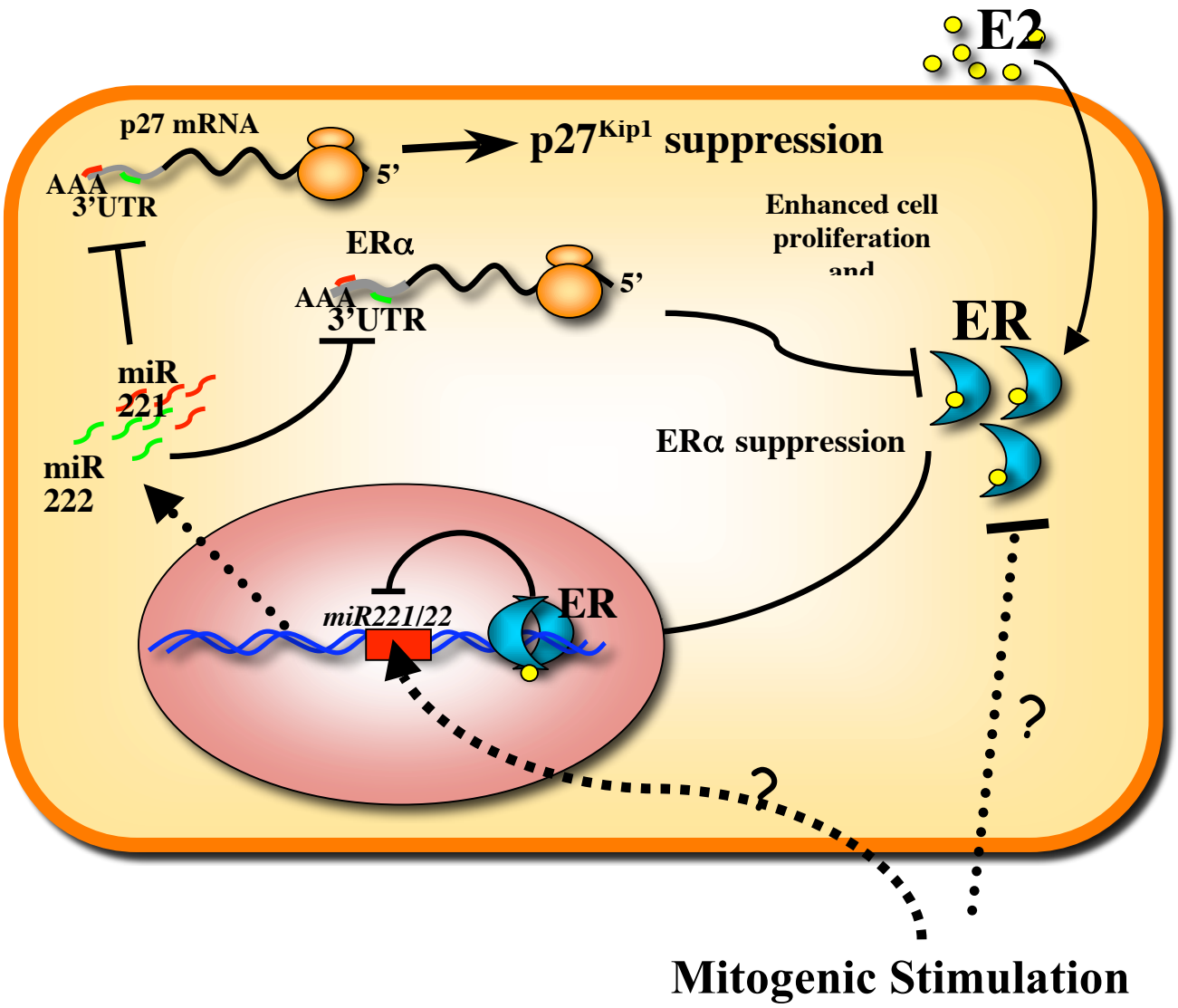


Figure 9. ERα constitutes a regulatory circuitry with miR221&222. Our results suggest a model in which estrogen bound-ERα represses miR221&222 expression. Mitogenic stimuli, as over-expression and continuous activation of ErbB2, induce through a molecular mechanism still unclear loss of ERα which in turn mediates *miR221&222* gene activation. The endpoint of gain of miR221&222 expression is suppression of ERα as well as of p27(Kip), which may be critical for establishing ER-negative status and producing de-regulated cell proliferation.

how ERα-positive breast tumors become ERα-negative, a frequent observation with profound impact on clinical outcome in recurrent breast cancer.

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REFERENCES

1 Chalfie, M., Horvitz, H.R. and Sulston, J.E. (1981). Mutations that lead to reiterations in the cell lineages of C. elegans. Cell. 24: 59-69.

2 Ambros, V. (1989). A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell. 57: 49-57.

3 Ruvkun, G., Wightman, B., Burglin, T. and Arasu, P. (1991). Dominant gain-of-function mutations that lead to misregulation of the C. elegans heterochronic gene lin-14, and the evolutionary implications of dominant mutations in pattern-formation genes. Dev Suppl. 1: 47-54.

4 Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 75: 843-54.

5 Wightman, B., Ha, I. and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 75: 855-62.

6 Reinhart, B.J. et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 403: 901-6.

7 Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science 294: 853-858.

8 Lau, N.C., Lim, L.P., Weinstein, E.G. and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science. 294: 858-862.

9 Lee, R.C. and Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science. 294: 862-864.

10 Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 116: 281-97.

11 Bentwich, I. (2005). A postulated role for microRNA in cellular differentiation. FEBS Lett. 579: 5904-10.

12 Brown, J.R. and Sanseau, P. (2005). A computational view of microRNAs and their targets. Drug Discov Today. 10: 595-601.

13 Griffiths-Jones, S. (2004). The microRNA Registry. NAR. 32: D109-D111.

14 Berezikov, E. et al. (2005). Phylogenetic shadowing and computational identification of human microRNA genes. Cell 120: 21-24.

15 Kim, V.N. and Nam, J.W. (2006). Genomics of microRNA. Trends Genet. 22: 165-73.

16 Cullen, B.R. (2004). Transcription and processing of human microRNA precursors.

17 Molecular Cell. 16: 861-865.

18 Pasquinelli, A.E. (2002). MicroRNAs: deviants no longer. Trends Genet 18: 171-173.

19 Calin, G.A. et al. (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sc U S A. 99: 15524-15529.

20 Eis, P.S. et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. (2005) Proc Natl Acad Sci U S A 102, 3627-32.

21 Tomari, Y. and Zamore, P.D. (2005). MicroRNA biogenesis: drosha can't cut it without a partner. Curr Biol. 15: R61-4.

22 Filipowicz, W., Jaskiewicz, L., Kolb, F.A. and Pillai, R.S. (2005). Post-transcriptional gene silencing by siRNAs and miRNAs. Curr Opin Struct Biol. 15: 331-41.

23 Landthaler, M., Yalcin, A. and Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Curr Biol. 14: 2162-7.

24 Kim, V.N. (2004). MicroRNA precursors in motion: exportin-5 mediates their nuclear export. Trends Cell Biol. 14: 156-9.

25 Eulalio A. (2008). Getting to the root of miRNA-mediated gene silencing. Cell. 132: 9-13.

26 Nelson, P. et al. (2003). The microRNA world: small is mighty. Trends Biochem Sci. 28: 534-540.

27 Liu, J., Valencia-Sanchez, M.A., Hannon, G.J. and Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol. 7, 719-723.

28 Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20.

29 Kiriakidou, M. et al. (2004). A combined computational-experimental approach predicts human microRNA targets. Genes Dev. 18: 1165-78.

30 Enright, A.J. et al. (2003). MicroRNA targets in Drosophila. Genome Biol. 5, R1.

31 Ambros, V. (2004). The functions of animal microRNAs. Nature 431: 350-355.

32 Calin, G.A. and Croce, C.M. (2006). MicroRNA-cancer connection: the beginning of a new tale. Cancer Res. 66: 7390-4.

33 Esquela-Kerscher, A. and Slack, F.J. (2006). Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 6: 259-69.

34 Hammond, S.M. (2006). MicroRNAs as oncogenes. Curr Opin Genet Dev. 16: 4-9.

35 Xu, P., Vernooy, S.Y., Guo, M. and Hay, B.A. (2003). The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol. 13: 790-5.

36 Brennecke, J. et al. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell. 113: 25-36.

37 Harfe, B.D. MicroRNAs in vertebrate development. (2005) Curr Opin Genet Dev. 15, 410-415

38 Miska, E.A. (2005). How microRNAs control cell division, differentiation and death. Curr Opin Genet Dev. 15: 563-568.

39 Hwang, H.W. and Mendell, J.T. (2006). MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer. 94: 776-80.

40 O'Donnell, K.A. et al. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435. 839-843.

41 He, L. et al. (2005). A microRNA polycistron as a potential human oncogene. Nature. 435: 828-833.

42 Lu, J. et al. (2005). MicroRNA expression profiles classify human cancers. Nature 435: 834-838.

43 Calin, G.A. et al. (2005). A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med. 352: 1667-1676.

44 Johnson, S.M. et al. (2005). RAS is regulated by the let-7 microRNA family. Cell. 120: 635-47.

45 Abelson, J.F. et al. (2005). Sequence variants in SLITRK1 are associated with Tourette's syndrome. Science. 310: 317-20.

46 He, H. et al. (2005). The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci U S A. 102: 19075-80.

47 Jiang, J., Lee, E.J. and Schmittgen, T.D. (2006). Increased expression of microRNA-155 in Epstein-Barr virus transformed lymphoblastoid cell lines. Genes Chromosomes Cancer. 45: 103-6.

48 Costinean, S. (2006). Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. PNAS. 103: 7024-9.

49 Perou CM, et al. (2000). Molecular portraits of human breast tumours. Nature. 406: 747-752.

50 Sørlie T, et al. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 98: 10869-10874.

51 Bertucci F, et al. (2005). Gene expression profiling identifies molecular subtypes of inflammatory breast cancer. Cancer Res. 65: 2170-2178.

52 Sims AH, Howell A, Howell SJ, and Clarke RB (2007). Origins of breast cancer subtypes and therapeutic implications. Nat Clin Pract Oncol. 4: 516-525.

53 Visvader G, and Lindeman H. (2003). Transcriptional regulators in mammary gland development and cancer. Int J Biochem Cell Biol. 35: 1034-1051.

54 Elledge RM, and Allred DC. (2004). Clinical aspect of estrogen and progesterone receptors. In: Harris JR, LippmanME, M. Morrow, C.K. Osborne, editors. Disease of the Breast. Philadelphia, PA: Lippincott Williams & Wilkins. pp. 601-617

55 Allred DC, Brown P, and Medina D. (2004). The origin of estrogen receptor alpha-positive and estrogen receptor alpha-negative human breast cancer. Breast Cancer Res. 6: 240-245.

56 Santen RJ, et al. (2002). The role of mitogen-activated protein (MAP) kinase in breast cancer. J Steroid Biochem Mol Biol. 80: 239-256.

57 Stoner M, et al. (2002). Hypoxia induces proteasome-dependent degradation of estrogen receptor alpha in ZR-75 breast cancer cells. Mol Endocrinol. 16: 2231-2242.

58 Oh AS, et al. (2001). Hyperactivation of MAPK induces loss of ERalpha expression in breast cancer cells. Mol Endocrinol. 15: 1344-1359.

59 Yan L, Yang X, and Davidson NE. (2001). Role of DNA methylation and histone acetylation in steroid receptor expression in breast cancer. J Mammary Gland Biol Neoplasia. 6: 183-192.

60 Iorio MV, et al. (2005). MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 65: 7065-7070.

61 Adams BD, Furneaux H, and White BA. (2007). The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha messenger RNA and protein expression in breast cancer cell lines. Mol Endocrinol. 21: 1132-1147.

62 Liu CG, Calin GA, Volinia S, and Croce CM. (2008). MicroRNA expression profiling using microarrays. Nat Protoc. 3: 563-578.

63 Galardi S, et al. (2007). miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem. 282: 23716-23724.

64 Gillies JK, and Lorimer IA. (2007). Regulation of p27Kip1 by miRNA 221/222 in glioblastoma. Cell Cycle. 16: 2005-2009.

65 le Sage et al. (2007). Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. EMBO J. 26: 3699-3708.

66 Visone R, et al. (2007). MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle. Endocr Relat Cancer. 14: 791-798.

67 Kondo N, Toyama T, Sugiura H, Fujii Y, Yamashita H. (2008). miR-206 Expression is down-regulated in estrogen receptor alpha-positive human breast cancer. Cancer Res. 68: 5004-5008.

68 Ferguson AT, Lapidus RG, Baylin SB, and Davidson NE. (1995). Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res. 55: 2279-2283.

69 Wijayaratne AL, and McDonnell DP. (2001). The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem. 276: 35684-35692.

70 Gregory, G. et al. (2004). The Microprocessor complex mediates the genesis of microRNAs. Nature. 432: 235-240.

71 Lonard DM, and O'malley BW. (2007). Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. Mol Cell. 27: 691-700.

72 Bachman KE, and Park BH. (2005). Dual nature of TGF-beta: tumor suppressor versus tumor promoter. Curr Opin Oncol. 17: 49-54.

73 Derynck R, Akhurst RJ, and Balmain A. (2001). TGF-beta signaling in tumor suppression and cancer progression. Nat Genet. 29: 117-29.

74 Kornberg LJ. (1998) Focal adhesion and its potential involvment in tumor invasion and metastasis. Head Neck. 20: 745-52.

75 Licznar A. Caporali S, Lucas A, Weisz A, Vignon F, et al. (2003). Identification of genes involved in growth inhibition of breast cancer cells transduced with estrogen receptor. Febs Lett. 553: 445-50.

76 Pearce ST, and Jordan VC. (2004). The biological role of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol. 50: 3-22.

77 Stossi F, Likhite VS, Katzenellenbogen JA. and Katzenellenbogen BS. (2006). Estrogen-occupied estrogen receptor represses cyclin G2 gene expression and recruits a repressor complex at the cyclin G2 promoter. J Biol Chem. 281: 16272-16278.

78 Higgins K.J, et al. (2008). Vascular endothelial growth factor receptor-2 expression is down-regulated by 17beta-estradiol in MCF-7 breast cancer cells by estrogen receptor alpha/Sp proteins. Mol Endocrinol. 22: 388-402.

APPENDIX

Table 1. Examples of functions of microRNAs in animals.

miRNA	Species	Function
lin-4	C.elegans	Regulation of life span (proportionally with expression)
miR-273	C. elegans	Controls laterality of the chemosensory system
miR-1	Drosophila	Post-mitotic growth of larval muscle
miR-2, miR-6, miR-11, miR-13, miR-308	Drosophila	Suppress embryonic apoptosis by posttranscriptional repression of proapoptotic factors
miR-2, miR-13	Drosophila	Normal development of head and posterior abdominal segments
miR-7	Drosophila	Promotes photoreceptor differentiation
miR-14	Drosophila	Suppression of cell death; normal fat metabolism
miR-278	Drosophila	Control of energy homeostasis by influencing insulin production and adipose-tissue glycogen stores.
Bantam	Drosophila	Controls cell proliferation and prevents apoptosis
miR-1-1	Mouse	Control of balance between differentiation and proliferation during cardiogenesis
miR-122	Mouse	Regulator of cholesterol and fatty-acid metabolism
miR-142s	Mouse	B lymphocyte differentiation; myeloid differentiation
miR-181	Mouse	B lymphocyte differentiation Myoblast differentiation by targeting Hox-11
miR-196	Mouse	Limb development, acting upstream of Hoxb8 and Sonic hedgehog
miR-200a, miR-141, miR-429, miR-199a	Mouse	Skin morphogenesis
miR-223	Mouse, Human	Myeloid differentiation Regulation of granulopoiesis
miR-15a, miR-16-1	Human	Regulation of B lymphocytes survival
miR-375	Human	Regulation of insulin secretion

Table 2. Examples of microRNAs involvement in human diseases.

Cancer types	miRNA involvement
Human leukemias and carcinomas	~ 50% of miRNAs located in minimal LOH/Amplified regions; microRNAs expression profiles classify human cancers
B cell CLL	Deletions and down regulation of miR-15a and miR-16-1 MiRNA profiles associates with survival and clinical parameters; Germline and somatic mutations in miRNA genes; miR-15 and miR-16 target BCL2
Pediatric Burkitt's Lymphoma	High expression of precursor for miR-155/BIC
B cell lymphomas	Accumulation of miR-155 and BIC RNA; BIC and miR-155 are highly expressed
Human lymphomas	Overexpression of pri-miRNA-17-92 cistron
Lung cancers	miRNA profiles associates with survival and clinical parameters
Breast cancers	miRNA profiles associates with survival and clinical parameters
Thyroid cancers	Germline polymorphisms with possible functional consequences in c-KIT oncogene interactions sites with miRNAs.
Tourette's syndrome	Polymorphisms with possible functional consequence in SLITRK1 interaction site with a miRNAs.
Fragile X syndrome	Loss of expression of the FMPR protein, that interact with members of the miRNA pathway
Spinal muscular atrophy (SMA)	Reduced expression of SMN protein, involved in RNP complexes containing miRNAs
Di George syndrome	Loss of expression of DGCR8 protein, a Drosha interactor in human cells

* NR – not reported; LOH – loss of heterozygosity; BIC gene - noncoding RNA transcript located at chromosome 21q21.3. pre-miRNA155 is likely processed from a transient spliced or unspliced nuclear BIC transcript; FMPR – fragile X mental retardation protein; SMN – Survival of Motor Neurons protein; RNP – ribo-nucleo-proteins; DGCR8 – Digeorge syndrome critical region gene 8.

TABLE 3: miRNAs differentially expressed in ER-positive versus ER-negative breast cancer cells.

miRNAs differentially expressed in ER-positive versus ER-negative breast cancer cells		
microRNA genes UP-REGULATED in ER positive-breast cancer cells		
microRNA genes	**p-value**	**Fold Change**
hsa-mir-203	1.07E-05	77.779
hsa-mir-375	4.56E-05	45.61
hsa-mir-200c	0.0003528	23.207
hsa-mir-10b	1.24E-05	22.78
hsa-mir-425	8.00E-06	15.739
hsa-mir-370	7.00E-07	13.553
hsa-mir-148b	< 1e-07	13.026
hsa-mir-200a	0.0004639	12.657
hsa-mir-200b	0.002323	9.733
hsa-mir-15b	3.61E-05	8.863
hsa-mir-423	3.22E-05	8.114
hsa-mir-365-2	< 1e-07	7.94
hsa-mir-148a	3.00E-07	7.926
hsa-mir-345	4.70E-06	7.603
hsa-mir-342	4.60E-06	7.03
hsa-mir-34b	< 1e-07	6.929
hsa-mir-15a	0.0001047	5.548
hsa-mir-141	0.0046452	5.487
hsa-mir-183	0.0010602	5.169
hsa-mir-196a-1	0.0002206	5.112
hsa-mir-26a-2	2.94E-05	5.039
hsa-mir-191	2.00E-07	4.993
hsa-mir-190	< 1e-07	4.848
hsa-mir-1-1	0.0022543	4.811
hsa-mir-10a	0.0187181	4.775
hsa-mir-26a-1	7.92E-05	4.478
hsa-mir-181b-1	0.0004517	4.117
microRNA genes DOWN-REGULATED in ER positive-breast cancer cells		
hsa-mir-155	0.0012452	0.235
hsa-mir-494	0.004397	0.185
hsa-mir-218-1	7.74E-05	0.182
hsa-mir-31	1.00E-07	0.111
hsa-mir-146	0.0199231	0.105
hsa-mir-138-2	7.85E-05	0.064
hsa-mir-222	< 1e-07	0.011
hsa-mir-221	< 1e-07	0.002

Table 4: Characteristics of breast tumors and patients

	ER+	ER-
Total number of patients	44	22
Tumor size (cm)		
≤2	16	8
>2	28	14
Menopause		
Yes	33	6
No	11	16
No. of posistive lymph nodes		
0	10	7
1-3	20	5
>3	14	10
p53 status		
negative	37	8
positive	7	14

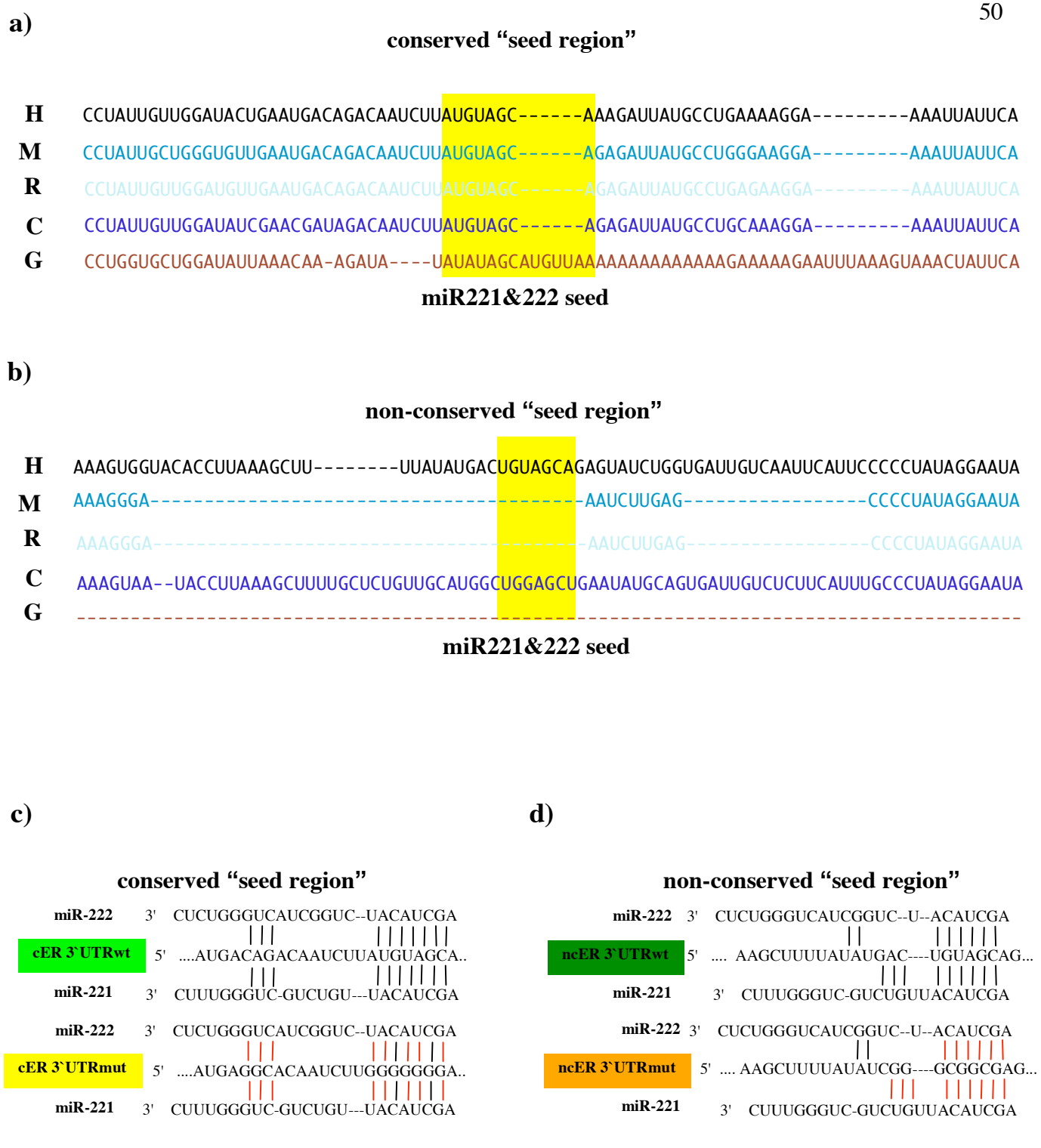

**Figure S1. miR221&222 Binding Sites in the ERa 3`UTR a,b** schematic representation of the conserved **(a)** and non-conserved **(b)** miR221&222 binding sites in the ERa 3`UTR; because of the perfect homology between the seed regions of miR221&222, both miRNAs are able to target the same sequence in the 3`UTR. Lower part of each panel shows mutations introduced into the binding site and the resultant disruption of homology against miR221&222 (indicated by red lines).

Primers

a) Luciferase assay cloning:

conserved miR221&222 seed region:

cERα3UTR wt Forw: 5` tctagaatgtgcacttaaatttggggaca 3`

cERα 3UTR wt Rev: 5`tctagacccagattcctgaatccacttcc 3`

non-conserved miR221&222 seed region:

ncERα3UTR wt Forw: 5`tctagaggctctaagaataagccacagc 3`

ncERα3UTR wt Rev: 5` tctagactgccttccctgtgtgcccctt 3`

miR221&222 Promoter:

-3000/-1600 pGL3b Forw: 5`gctagcacttccacatatagtccctagggtc 3`

-3000/-1600 pGL3b Rev: 5` ctcgagaccagtgtggttagctcttggtggg 3`

-1600 pGL3b Forw: 5`gctagccccaccaagagctaaccacactggt 3`

-1000pGL3b Forw: 5` gctagccctagccaccttatcgaaaatagcattcc 3`

-600 pGL3b Forw: 5` gctagcctgacatgctagtgagcacctgc 3`

-200 pGL3b Forw: 5`gctagcgtttgctgctggatctccagcacc 3`

-150 pGL3b Forw: 5`gctagcccagaggttgtttaaaattacgta 3`

-50 pGL3b Forw: 5`

ctagctagctagctttttttcttccacagagccctccccagaaggcaaaggatcacccagctccgctcgagcgg 3`

-50 pGL3b Rev: 5`

ccgctcgagcggagctgggtgatcctttgccttctggggagggctctgtggaagaaaaaagctagctagctag 3`

miR222 pGL3b Rev: 5` ctcgagagctgggtgatcctttgccttctg 3`

b) miR221 probes for Agarose Northern blot:

miR221 UP forward: 5`cttgaatgcagtaggcagttgtgt 3`

miR221 Down reverse: 5` ctccactggtttatacctcctgga 3`

miR222 UP forward: 5`gtcactcagtcagtatctgttgga 3`

miR222 Down reverse: 5` gtgtgtaattcaaggtaaagttttc 3`

c) ERα expressing vectors:

forward primers: 5` ggtaccatgaccatgaccctccacaccaaagc 3`

reverse primers: 5` gatatcccgtgtgggagccagggagctctcag 3`

d) Chromatin Immunoprecipitation primers:

1F: 5`ggattctaagacgtgtacttggag 3`

1R: 5` cttgcactcacggaagtttaagtc 3`

2F: 5` gtcactcagtcagtatctgttgga 3`

2R: 5` gtgtgtaattcaaggtaaagttttc 3`

3F: 5` ctacctacctacctatctacccct 3 `

3R: 5` caacagtcagaaatgctgggactt 3`

4F: 5`cttgaatgcagtaggcagttgtgt 3`

4R: 5`ctccactggtttatacctcctgga 3`