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(54) **Title**: NUCLEIC ACIDS REGULATING OESTROGEN RECEPTOR (ER)-α SIGNALING IN BREAST CANCER

(57) **Abstract**: This invention relates to use of a nucleic acid or nucleic acids, preferably mi RNAs disclosed for the manufacture of a medicine for the treatment of breast cancer. This method also relates to a method of obtaining a marker value or values useful for differentiating patients with an oestrogen dependent breast cancer with an increased risk of progression into an oestrogen independent breast cancer by determining from a sample or samples of a tumour of breast cancer mi RNAs disclosed and establishing a marker value and/or values, which are a determined amount or amounts as such, or a function of the determined amount or amounts of disclosed mi RNAs. This invention also relates to a method of treatment and/or prevention of breast cancer.

NUCLEIC ACIDS REGULATING OESTROGEN RECEPTOR (ER)-α SIGNALING IN BREAST CANCER

FIELD OF THE INVENTION

This invention relates to the use of nucleic acids for the manufacture of a medicine for the treatment of breast cancer. This invention also relates to a method of obtaining marker values useful for differentiating patients at risk of progression of an oestrogen dependent breast cancer into an oestrogen independent breast cancer. This invention further relates to a method for the treatment of oestrogen dependent breast cancer and/or prevention of progression of oestrogen dependent breast cancer into oestrogen independent breast cancer. This invention relates to particular miRNAs essential for the different aspects of the invention.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

Oestrogen receptor (ER) status is important for the development, progression, and treatment of breast cancer, and it has become a major target for the treatment of this disease. The two human oestrogen receptors, ERα and ERβ, are encoded by separate *ESR1* and *ESR2* genes, respectively [Cheskis BJ et al. J Cell Physiol 2007 213(3):610-7; Hewitt SC et al. Reproduction 2003 125(2):143-9]. ERα is the primary mediator of the mitogenic activities of oestrogen in breast tissue, and its levels are tightly controlled in a tissue- and development-specific manner [Couse JF and Korach KS Endocr Rev 1999 20(3):358-417]. Understanding the molecular basis of ER expression is critically important as most breast cancers express high levels of ERα, and the inhibition of oestrogenic signals by antioestrogens and other endocrine therapies is part of the routine management of breast cancer patients. However, many patients relapse during endocrine therapies and develop

oestrogen-independent breast cancer [Milano A et al. Eur J Cancer 2006 42(16):2692-705].

Multiple mechanisms are known to regulate ERα levels in cells, including transcriptional control [Reid G et al. Cell Mol Life Sci 2002; 59(5):821-31], rate of mRNA degradation [Kenealy MR et al. Endocrinology 2000 141(8):2805-13] and, according to recent evidence, microRNAs (miRNAs) [Adams BD et al. Mol Endocrinol 2007 21(5):1132-47; Zhao JJ et al. J Biol Chem 2008 283(45): 31079–31086]. MiRNAs target messenger RNAs (mRNAs) for cleavage or translational repression by binding to the 3' untranslated region (3' UTR) of the target mRNAs [Esquela-Kerscher A and Slack FJ. Nat Rev Cancer 2006 6(4):259-69]. Genes encoding 721 miRNAs have so far been identified (miRBase v.14.0) [Griffiths-Jones S et al. Nucleic Acids Res 2008 36(Database issue):D154-8], and they are predicted to regulate the expression of at least 60 % of all human protein-encoding genes [Friedman RC et al. Genome Res 2009 19:92-105]. Several studies have linked miRNAs in regulating breast cancer progression and hormone receptor status [Verghese ET et al. J Pathol 2008 215(3):214-21]. A few miRNAs have been associated with breast cancer proliferation and invasion [Huang Q et al. Nat Cell Biol 2008 10(2):202-10; Ma L, Teruya-Feldstein J and Weinberg RA Nature 2008 455(7210):256; Tavazoie SF et al. Nature 2008 451(7175):147-52], and miR-7, miR-128a, miR-210, and miR-516-3p are involved with the aggressiveness of lymph node-negative, oestrogen receptor-positive breast cancer [Foekens JA et al. Proc Natl Acad Sci U S A 2008; 105(35):13021-6]. MiRNA expression profiles can distinguish different breast cancer subtypes and classify oestrogen receptor status [Mattie MD et al. Mol Cancer 2006; 5:24; Blenkiron C et al. Genome Biol 2007; 8(10):R214]. For instance, the expression of miR-142-5p, miR-200a, miR-205 and miR-25 positively correlates with the ERα expression [Mattie MD et al. (2006)]. Adams *et al.* (2007) showed how miR-206 binds to two sites in the 3'UTR region of ERα, and directly downregulates ERα expression [Adams BD et al. 2007]. In addition, low miR-206 expression has been linked to ERα-positive clinical breast cancers [Kondo N et al. Cancer Res 2008; 68(13):5004-8]. A recent study also described miR-221/222 as negatively regulating ERα and contributing to the Tamoxifen resistance in breast cancer [Zhao JJ et al. (2008)].

Liu et al. (Gastroenterology 2009 136: 683–693) identifies miR-18a to be differentially expressed between male and female hepatocellular carcinoma (HCC), and to target the ESR1 gene leading to downregulation of ERα protein. miR-18a overexpression had opposite effects on cell proliferation between the hepatoma and breast cancer cell lines: miR-18a-mediated downregulation of ERα increased cell proliferation in HCC cells but decreased the proliferation of MCF-7 breast cancer cells.

WO 2008/014008 discloses a method of reducing angiogenesis, the method comprising contacting a cell with an effective amount of an inhibitory nucleic acid molecule complementary to at least a portion of a microRNA nucleic acid molecule of the mir-17-92 cluster, thereby reducing angiogenesis. WO 2008/014008 identifies mir-18a as an alternative of six microRNAs as well as treating breast cancer as an alternative of seventeen cancers to be treated.

WO 2009/004632 discloses a method of treating a hyperproliferative or degenarative disease in a subject, the method comprising administering to the subject a therapeutically effective amount of an oligonucleotide agent capable of down-regulating at least one microRNA selected from the group wherein miR-18a and miR-18b are defined within the group comprising 19 alternatives altogether. WO 2009/004632 further discloses a pharmaceutical composition comprising at least one microRNA selected from the group wherein miR-18a and miR-18b are defined within the group comprising 16 alternatives altogether.

WO 2007/016548 discloses methods and composition for the diagnosis, prognosis and treatment of breast cancer and provides methods of identifying anti-breast cancer agents. Methods of diagnosis, prognosis of breast cancer as well as identification of anti-breast cancer agents relate to measurement of a marker miRNA or miRNAs, or miRNA gene product or products. The methods of treatment and pharmaceutical compositions relate to down-regulation or up-regulation of a miR gene product or products, or isolated miR gene products or miR expression inhibitor compounds respectively.

OBJECT AND SUMMARY OF THE INVENTION

One object of the present invention is to provide use of a nucleic acid or acids for the manufacture of a medicine for the treatment of breast cancer.

Another object of the present invention is to provide a method of obtaining a marker value or values useful for differentiating patients with an oestrogen dependent breast cancer with a increased risk of progression into an oestrogen independent breast cancer.

A further object of the present invention is to provide a method for the treatment of oestrogen dependent breast cancer and/or prevention of progression of oestrogen dependent breast cancer into oestrogen independent breast cancer.

The present invention provides a use of a nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

b) any one of said nucleic acids defined in a) or b) comprising a chemical modification or modifications and able to modulate the expression of ERα;

c) any nucleic acid having at least 85 % identity to said nucleic acids as defined in a) or b) and able to modulate the expression of ERα; and

d) any functional fragment, i.e. a fragment able to modulate the expression of ERα, of any one of said nucleic acids defined in a), b) or c);

e) any precursor, i.e. any nucleic acid that can be processed through natural processing or synthetic processing into any of said nucleic acids as defined in a), b), c) or d);

single-stranded or double-stranded, and any combination thereof for the manufacture of a medicine for the treatment of breast cancer.

The present invention also provides a method of obtaining a marker value or values useful for differentiating patients with an oestrogen dependent breast cancer with an increased risk of progression into an oestrogen independent breast

cancer. Characteristic for the method is that a nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

b) any precursor, i.e. any nucleic acid that can be processed through natural processing into any of the nucleic acids defined in a);

single-stranded or double-stranded, and any combination thereof are determined from a sample or samples of a tumour of the breast cancer and a marker value and/or values, which are a determined amount or amounts as such, or a function of the determined amount or amounts, are established.

The present invention further provides a method

i) for the treatment of oestrogen dependent breast cancer, and/or

ii) prevention of progression of oestrogen dependent breast cancer into oestrogen independent breast cancer

in human wherein treatment comprises introducing into a tumour of said breast cancer a nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

b) any one of said nucleic acids defined in a) or b) comprising a chemical modification and able to modulate the expression of ERα;

c) any nucleic acid having at least 85 % identity to nucleic acids as defined in a) or b) and able to modulate the expression of ERα; and

d) any functional fragment, i.e. a fragment able to modulate the expression of ERα, of any one of said nucleic acids defined in a), b) or c);

e) any precursor, i.e. any nucleic acid that can be processed *in vivo* through natural processing or synthetic processing into any of the nucleic acids defined in a), b), c) or d);

single-stranded or double-stranded, and any combination thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates an lysate microarray LMA screen for identifying miRNAs regulating ERα.

Figure 2A and 2B illustrate validation of ERα regulating miRNAs.

Figure 3 illustrates that miRNA-mediated downregulation of ERα suppresses oestrogen-stimulated growth of MCF-7 breast cancer cells.

Figure 4 shows that miR-18a, miR-18b, miR-193b, and miR-206 induce accumulation of MCF-7 cells in G_1/G_0 phase of the cell cycle.

Figure 5A and 5B show that ERα is a direct target for miR-18a/b, miR-193b, miR-206, and miR-302c.

Figure 6 demonstrates that expression of miR-18a and miR-18b shows inverse correlation with ERα

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to that the inventors have identified twenty or so miRNAs from hundreds of known miRNAs that clearly affect the level of ERα and its mRNA in breast cancer cells. Thus, a level of these miRNAs in a cell which deviates from that of normal cells suggests a deviating amount of ERα protein in the cell. By determining the level of these miRNAs in a tumour cell one can asses which particular miRNA-molecules can be involved in development and growth of this particular tumour and accordingly also predict whether this miRNA could be used for treatment of this particular tumour.

Definitions

As used herein a *functional fragment* of a nucleic acid is a fragment of a nucleic acid sequence able to modulate the expression of ERα.

As used herein, an *miRNA molecule* is a small RNA molecule, typically 21–23 nucleotides, encoded by the human genome or produced synthetically with a sequence which corresponds to one encoded by the human genome. miRNA molecules may be single-stranded or double- stranded, and contain chemical modifications, such as the substitution of phosphate bonds with phosphorothioate bonds, the methylation of the oxygen at position 2 in the ribose (2′-O-methyl), the addition of an extra bridge between the carbons of the ribose ring (locked nucleic acid or LNA), the substitution of the sugar backbone by a pseudopeptide backbone (peptide nucleic acid, i.e. PNA) or a morpholino ring (morpholino) and the attachment of a cholesterol at the 3′ OH end [Bumcrot et al. Nat Chem Biol. 2006, 12(2):711-719; Castanotto and Rossi, Nature 2009, 457: 426-433]. It should be emphasized that the modifications mentioned above are only non-limiting examples.

miRNA refers to the unprocessed (precursor) or processed (mature) RNA transcript from a miR gene. The unprocessed miR gene transcript is also called a *miR precursor* or *pre-miR* and typically comprises an RNA transcript of 70-100 nucleotides in length. The miR precursor can be processed by digestion with an RNAse into an active typically 21–23 nucleotide RNA molecule. This active typically 21–23 nucleotide RNA molecule is also called the *processed* miR gene transcript or *mature miRNA*. The mature miRNA molecule can be obtained from the miR precursor through natural processing or by synthetic processing. The mature miRNA molecule can also be produced directly by biological or chemical synthesis, without having been processed from the miR precursor.

miRNAs disclosed in table 2 contain a seed sequence of 6–7 nt in the 5'-end (nucleotides at position 2–8, disclosed in table 3) displaying Watson-Crick base pairing with the target mRNA [Lewis et al. Cell 2003, 115(7): 787-798, Friedman et al. Genome Res. 2009 19 (1):92-105]. Additional determinants for target binding by miRNA include non-Watson-Crick recognition of an A at nucleotide position 1 and of an A or U at nucleotide position 9 [tables 2 and 3; Lewis et al. Cell 2005, 120 (1):15-20; Nielsen et al. RNA 2007 13(11): 1894-1910].

When a microRNA (miR) is referred to herein by name, the name corresponds to both the precursor and mature forms, unless otherwise indicated. Table 1 and 2 depict the nucleotide sequences of particular precursor and mature human microRNAs, respectively. The seed sequence determinants of each miRNA are disclosed in table 3.

In addition to the full-length miRNA molecules, such as those shown in sequences SEQ ID NOS: 1–42, the term *miRNA molecules* also include fragments of miRNA molecules provided that the fragments are functional fragments. The term *fragment* of a miRNA molecule means a portion of the full-length molecule. The size of the fragment is limited only in that it must be a functional fragment, that is, able to modulate the expression of ERα.

Naming of miRNAs both precursor (see e.g. table 1), mature (see e.g. table 2) or miRNA seed sequences refer to sequences provided at Sanger Center miRBase with accession numbers as disclosed in tables 1 to 3.

By *modification* is meant any biochemical or other synthetic alteration of a nucleotide, amino acid, or other agent relative to a naturally occurring reference agent.

Preferred embodiments of the invention

The present invention involves applications of known miRNA sequences and sequences related to these known miRNA sequences. Known precursors to the miRNA sequences related to the invention are listed in table 1. The miRNA sequences are listed in table 2 and the seed sequences of these miRNA sequences are listed in table 3.

Table 1 Precursor miRNA (pre-miR) sequences

SEQ ID NO	miRBase accession	Sequence
1	hsa-mir-18a MI0000072	UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGAUUA GCAUCUACUGCCCUAAGUGCUCCUUCUGGCA
2	hsa-mir-18b MI0001518	UGUGUUAAGGUGCAUCUAGUGCAGUUAGUGAAGCAGCUUA GAAUCUACUGCCCUAAAUGCCCCUUCUGGCA
3	hsa-mir-193b MI0003137	GUGGUCUCAGAAUCGGGGUUUUGAGGGCGAGAUGAGUUU AUGUUUUAUCCAACUGGCCCUCAAAGUCCCGCUUUUGGGG UCAU
4	hsa-mir-302c MI0000773	CCUUUGCUUUAACAUGGGGGUACCUGCUGUGUGAAACAAA AGUAAGUGCUUCCAUGUUUCAGUGGAGG
5	hsa-mir-9-1 MI0000466	CGGGGUUGGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAG UGGUGUGGAGUCUUCAUAAAGCUAGAUAACCGAAAGUAAA AAUAACCCCA
6	hsa-mir-9-2 MI0000467	GGAAGCGAGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAG UGUAUUGGUCUUCAUAAAGCUAGAUAACCGAAAGUAAAAAC UCCUUCA

7	hsa-mir-9-3 MI0000468	GGAGGCCCGUUUCUCUCUUUGGUUAUCUAGCUGUAUGAG UGCCACAGAGCCGUCAUAAAGCUAGAUAACCGAAAGUAGA AAUGAUUCUCA
8	hsa-mir-22 MI0000078	GGCUGAGCCGCAGUAGUUCUUCAGUGGCAAGCUUUAUGU CCUGACCCAGCUAAAGCUGCCAGUUGAAGAACUGUUGCCC UCUGCC
9	hsa-mir-219-1 MI0000296	CCGCCCCGGGCCGCGGCUCCUGAUUGUCCAAACGCAAUU CUCGAGUCUAUGGCUCCGGCCGAGAGUUGAGUCUGGACG UCCCGAGCCGCCGCCCCCAAACCUCGAGCGGG
10	hsa-mir-219-2 MI0000740	ACUCAGGGGCUUCGCCACUGAUUGUCCAAACGCAAUUCUU GUACGAGUCUGCGGCCAACCGAGAAUUGUGGCUGGACAU CUGUGGCUGAGCUCCGGG
11	hsa-mir-93 MI0000095	CUGGGGGCUCCAAAGUGCUGUUCGUGCAGGUAGUGUGAU UACCCAACCUACUGCUGAGCUAGCACUUCCCGAGCCCCCG G
12	hsa-mir-301a MI0000745	ACUGCUAACGAAUGCUCUGACUUUAUUGCACUACUGUACU UUACAGCUAGCAGUGCAAUAGUAUUGUCAAAGCAUCUGAA AGCAGG
13	hsa-mir-130a MI0000448	UGCUGCUGGCCAGAGCUCUUUUCACAUUGUGCUACUGUC UGCACCUGUCACUAGCAGUGCAAUGUUAAAAGGGCAUUGG CCGUGUAGUG
14	hsa-mir-130b MI0000748	GGCCUGCCCGACACUCUUUCCCUGUUGCACUACUAUAGGC CGCUGGGAAGCAGUGCAAUGAUGAAAGGGCAUCGGUCAG GUC
15	hsa-mir-372 MI0000780	GUGGGCCUCAAAUGUGGAGCACUAUUCUGAUGUCCAAGUG GAAAGUGCUGCGACAUUUGAGCGUCAC
16	hsa-mir-373 MI0000781	GGGAUACUCAAAAUGGGGGCGCUUUCCUUUUUGUCUGUA CUGGGAAGUGCUUCGAUUUUGGGGUGUCCC
17	hsa-mir-181a-1 MI0000289	UGAGUUUUGAGGUUGCUUCAGUGAACAUUCAACGCUGUCG GUGAGUUUGGAAUUAAAAUCAAAACCAUCGACCGUUGAUU GUACCCUAUGGCUAACCAUCAUCUACUCCA
18	hsa-mir-181a-2 MI0000269	AGAAGGGCUAUCAGGCCAGCCUUCAGAGGACUCCAAGGAA CAUUCAACGCUGUCGGUGAGUUUGGGAUUUGAAAAAACCA CUGACCGUUGACUGUACCUUGGGGUCCUUA
19	hsa-mir-181c MI0000271	CGGAAAAUUUGCCAAGGGUUUGGGGGAACAUUCAACCUGU CGGUGAGUUUGGGCAGCUCAGGCAAACCAUCGACCGUUG AGUGGACCCUGAGGCCUGGAAUUGCCAUCCU
20	hsa-mir-181d MI0003139	GUCCCCUCCCCUAGGCCACAGCCGAGGUCACAAUCAACAU UCAUUGUUGUCGGUGGGUUGUGAGGACUGAGGCCAGACC CACCGGGGGAUGAAUGUCACUGUGGCUGGGCCAGACACG GCUUAAGGGGAAUGGGGAC
21	hsa-mir-517a MI0003161	UCUCAGGCAGUGACCCUCUAGAUGGAAGCACUGUCUGUUG UAUAAAAGAAAAGAUCGUGCAUCCCUUUAGAGUGUUACUG UUUGAGA
22	hsa-mir-517c MI0003174	GAAGAUCUCAGGCAGUGACCCUCUAGAUGGAAGCACUGUC UGUUGUCUAAGAAAAGAUCGUGCAUCCUUUUAGAGUGUUA CUGUUUGAGAAAAUC
23	hsa-mir-520d MI0003164	UCUCAAGCUGUGAGUCUACAAAGGGAAGCCCUUUCUGUUG UCUAAAAGAAAAGAAAGUGCUUCUCUUUGGUGGGUUACGG UUUGAGA

Table 2 Mature miRNA sequences.

SEQ ID NO	miRBase accession	Sequence
24	hsa-miR-18a MIMAT0000072	5'-UAAGGUGCAUCUAGUGCAGAUAG-3'
25	hsa-miR-18b MIMAT0001412	5'-UAAGGUGCAUCUAGUGCAGUUAG-3'
26	hsa-miR-193b MIMAT0002819	5'-AACUGGCCCUCAAAGUCCCGCU-3'
27	hsa-miR-302c MIMAT0000717	5'-UAAGUGCUUCCAUGUUUCAGUGG-3'
28	hsa-miR-9 MIMAT0000441	5'-UCUUUGGUUAUCUAGCUGUAUGA-3'
29	hsa-miR-22 MIMAT0000077	5'-AAGCUGCCAGUUGAAGAACUGU-3'
30	hsa-miR-219-5p MIMAT0000276	5'-UGAUUGUCCAAACGCAAUUCU-3'
31	hsa-miR-93 MIMAT0000093	5'-CAAAGUGCUGUUCGUGCAGGUAG-3'
32	hsa-miR-301a MIMAT0000688	5'-CAGUGCAAUAGUAUUGUCAAAGC-3'
33	hsa-miR-130a MIMAT0000425	5'-CAGUGCAAUGUUAAAAGGGCAU-3'
34	hsa-miR-130b MIMAT0000691	5'-CAGUGCAAUGAUGAAAGGGCAU-3'
35	hsa-miR-372 MIMAT0000724	5'-AAAGUGCUGCGACAUUUGAGCGU-3'
36	hsa-miR-373 MIMAT0000726	5'-GAAGUGCUUCGAUUUUGGGGUGU-3'
37	hsa-miR-181a MIMAT0000256	5'-AACAUUCAACGCUGUCGGUGAGU-3'
38	hsa-miR-181c MIMAT0000258	5'-AACAUUCAACCUGUCGGUGAGU-3'
39	hsa-miR-181d MIMAT0002821	5'-AACAUUCAUUGUUGUCGGUGGGU-3'
40	hsa-miR-517a MIMAT0002852	5'-AUCGUGCAUCCCUUUAGAGUGU-3'
41	hsa-miR-517c MIMAT0002866	5'-AUCGUGCAUCCUUUUAGAGUGU-3'
42	hsa-miR-520d-3p MIMAT0002856	5'-AAAGUGCUUCUCUUUGGUGGGU-3'

Table 3 miRNA seed sequences

SEQ ID NO	miRNA	Seed sequence
43	hsa-miR-18a hsa-miR-18b	5'-UAAGGUGC-3'
44	hsa-miR-193b	5'-AACUGGCC-3'
45	hsa-miR-302c	5'-UAAGUGCU-3'
46	hsa-miR-9	5'-UCUUUGGU-3'
47	hsa-miR-22	5'-AAGCUGCC-3'
48	hsa-miR-219-5p	5'-UGAUUGUC-3'
49	hsa-miR-93	5'-CAAAGUGC-3'
50	hsa-miR-301a hsa-miR-130a hsa-miR-130b	5'-CAGUGCAA-3'
51	hsa-miR-372 hsa-miR-520d-3p	5'-AAAGUGCU-3'
52	hsa-miR-373	5'-GAAGUGCU-3'
53	hsa-miR-181a hsa-miR-181c hsa-miR-181d	5'-AACAUUCA-3'
54	hsa-miR-517a hsa-miR-517c	5'-AUCGUGCA-3'

A typical use according to the invention of the sequences involved in the invention is use of a nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

b) any functional fragment, i.e. a fragment able to modulate the expression of ERα, of any one of said SEQ ID NOS: 24–42;

c) any one of said nucleic acids defined in a) or b) comprising a chemical modification or modifications;

d) any nucleic acid having at least 85 % identity to said nucleic acids as defined in a), b) or c); and

e) any precursor, i.e. any nucleic acid that can be processed through natural processing or synthetic processing into any of said nucleic acids as defined in a), b), c) or d);

single-stranded or double-stranded, and any combination thereof for the manufacture of a medicine for the treatment of breast cancer.

In preferred embodiments the nucleic acid is RNA or a derivative thereof, i.e. RNA comprising a chemical modification or modifications. Typically the chemical modification or modifications are selected from the group consisting of substitution of a phosphate bond with a phosphorothioate bond, methylation of the oxygen at position 2 in ribose, addition of an extra bridge between the carbons of the ribose ring, substitution of the sugar backbone by a pseudopeptide backbone or a morpholino ring, attachment of a cholesterol at the 3´OH end, and any combination thereof.

Typical preferred embodiments involve constructs which either comprise mature miRNAs or result in the target cell, through processing, in mature miRNAs, e.g. as defined in a), c) or d) above.

In many preferred embodiments the use of the nucleic acids referred to in a) of the use above are selected from the group consisting of SEQ ID NOS: 24–28 and 31–34, and preferably from the group consisting of SEQ ID NOS: 24–27.

If functional fragment or fragments as referred to in b) above are used said functional fragment or fragments preferably consists of 7 to 22, or 12 to 22, and most preferably of 12 to 17 nucleotides; each including a seed sequence, i.e. any one of SEQ ID NOS: 43 to 54.

If a precursor or precursors as referred to in e) above are used said precursor typically consists of 25 to 200, preferably 50 to 150, and most preferably 80 to 100 nucleotides. In some preferred embodiments the precursor used is selected from the group consisting of any one of SEQ ID NOS: 1–23.

In preferred embodiments of the invention the medicine is manufactured for the treatment of

a) oestrogen dependent breast cancer and/or

b) the prevention of progression of oestrogen dependent breast cancer into oestrogen independent breast cancer.

In many preferred embodiments the use of the nucleic acid or acids as defined above is combined with the use of an antioestrogen. Preferably the antioestrogen is selected from the group consisting of fulvestran, letrozole, raloxifene, tamoxifen and toremifene.

A typical method of the invention involves obtaining a marker value or values useful for differentiating patients with an oestrogen dependent breast cancer with an increased risk of progression into an oestrogen independent breast cancer wherein nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

b) any precursor, i.e. any nucleic acid that can be processed through natural processing into any of the nucleic acids defined in a) or b);

single-stranded or double-stranded, and any combination thereof are determined from a sample or samples of a tumour of the breast cancer and a marker value and/or values, which are a determined amount or amounts as such, or a function of the determined amount or amounts, are established. Depending on the marker or markers determined; and/or if the determined amount or amounts are used as a marker or markers as such, or if a function of the determined amount or amounts are used as a marker or markers; an increased or decreased marker value suggests an increased risk of progression of an oestrogen dependent breast cancer into an oestrogen independent breast cancer. E.g. increased miR-18a and/or miR18b levels suggest an increased risk of progression of an oestrogen dependent breast cancer into an oestrogen independent breast cancer.

Preferably the nucleic acid or acids are determined by any target polynucleotide recognizing detection method, preferably a method employing nucleic acid amplification.

Another method of the invention involves

i) treatment of oestrogen dependent breast cancer, and/or

ii) prevention of progression of oestrogen dependent breast cancer into oestrogen independent breast cancer

in human wherein treatment comprises introducing into a tumour of said breast cancer a nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

b) any functional fragment, i.e. a fragment able to modulate the expression of ERα, of any one of said SEQ ID NOS: 24–42;

c) any one of said nucleic acids defined in a) or b) comprising a chemical modification;

d) any nucleic acid having at least 85 % identity to nucleic acids as defined in a), b) or c); and

e) any precursor, i.e. any nucleic acid that can be processed through natural processing or synthetic processing into any of the nucleic acids defined in a), b) or c);

single-stranded or double-stranded, and any combination thereof.

The invention also involves a method for downregulating oestrogen receptor ERα expression levels in breast cancer cells comprising administering miRNAs as defined above to said breast cancer cells.

The invention further involves a method for inhibiting oestrogen dependent cell proliferation, comprising administering miRNAs to oestrogen receptor containing cells in an amount effective to inhibit proliferation in said cells.

The typical and preferred embodiments disclosed above relate to all aspects of the invention disclosed in this application.

miRNA precursors or miRNA mimics can be chemically synthesized or recombinantly produced. There are many commercial suppliers for synthetic

miRNA mimics which are designed to enter the miRNA pathway and act as mature miRNA species. The commercial suppliers include *e.g.* Dharmacon Inc. (Chicago, IL), Ambion Inc. (Austin, TX), Exiqon (Vedbaek, Denmark), and Qiagen Inc. (Valencia, CA). miRNA precursors can also be expressed from DNA plasmids containing miRNA precursor sequences using suitable promoters, such as Pol II, Pol III or the cytomegalovirus (CMV) promoters.

Normal, unmodified RNA has low stability under physiological conditions because of its degradation by ribonuclease enzymes present in the living cell. If the oligonucleotide shall be administered exogenously, it is highly desirable to modify the molecule according to known methods to enhance its stability against chemical and enzymatic degradation. Modifications of nucleotides to be administered exogenously *in vivo* are extensively described in the art [Bumcrot et al. 2006] [Castanotto and Rossi, 2009]. The affinity, average half-life, toxicity and/or delivery of miRNAs can be improved through chemical modifications, such as the substitution of phosphate bonds with phosphorothioate bonds, the methylation of the oxygen at position 2 in the ribose (2′-O-methyl), the addition of an extra bridge between the carbons of the ribose ring (locked nucleic acid or LNA), the substitution of the sugar backbone by a pseudopeptide backbone (peptide nucleic acid or PNA) or a morpholino ring (morpholino) and the attachment of a cholesterol at the 3′ OH end. It should be emphasized that the modifications mentioned above are only non-limiting examples.

A number of strategies for the delivery of miRNA precursors or plasmids encoding the miRNA precursors could be applied to the miRNA-based therapy. Successful administration of siRNAs has already been reported in the livers of mice and non-human primates using chemically modified duplexes with cholesterol conjugation or liposomal formulation [Soutschek et al. Nature 2004; 432:173-8, Zimmermann et al. Nature 2006; 441:111-4, Morrissey et al. Nat Biotechnol 2005; 23:1002-7], and these strategies could be applied for miRNA delivery as well. The activity of therapeutic miRNAs can be enhanced by chemical modifications and by an adjuvant that protects it from degradation and makes sure that the therapeutic miRNA reaches the diseased tissue. Chemically modified miRNA mimics can be

delivered to the cells by using suitable lipid-based transfection reagents or cholesterol-conjugates, which are well known in the art, and have been succesfully used in miRNA *in vivo* studies [Krützfeldt et al. Nature 2005, 685-689] [Elmen et al. Nature 2008, 452: 896-899]. In addition, ligands for specific cell surface receptors capable of being internalized can be conjugated to the miRNA-oligonucleotides, thereby facilitating both cellular uptake and cell type–specific delivery [Juliano et al. Nucleic Acids Res 2008, 36:4158–71]. The use of supramolecular nanocarriers, such as liposomes, and polymeric nanoparticles represents another potential strategy for delivering miRNAs [Juliano et al. Nucleic Acids Res 2008, 36:4158–71]. In addition, a prostate cancer xenograft model has revealed that atelocollagen can efficiently deliver synthetic miRNAs to tumor cells on bone tissues in mice when injected into tail veins [Takeshita et al. Mol Ther 2009, 18(1):181-7].

An alternative means of triggering miRNA overexpression is through promoter-driven *miRNA expression cassettes* encoding miRNA precursors, which are processed as mature, active miRNAs in the target cells. These expression cassettes can be inserted into the backbones of viral vectors under the control of Pol II, Pol III, or CMV promoters. A potential advantage of vector delivery is that a single administration triggers long-term expression of the therapeutic miRNA. Any viral vector which is capable for delivering miRNA precursors can be used. These include, but are not limited to, e.g. lentiviruses, adenoviruses, or adeno-associated viruses. Several proof of concept studies have been carried out, with one example showing that overexpression of let-7 by an adenovirus can reduce hyperplasia in a mouse model of lung cancer [Esquela-Kerscher et al. Cell Cycle 2008, 7:759-64]. Other examples of successful delivery of miRNAs via viral vectors are provided in the literature [McLaughlin et al. PNAS 2007, 104(51): 20501-20506] [Gentner et al. Nature Methods 2009, 6(1):63-66].

EXPERIMENTAL PART

We applied the protein lysate microarray (LMA) technology for rapid systematic profiling of miRNAs that directly impact ERα levels in breast cancer cells. We transiently transfected a library of 319 pre-miRNAs in MCF-7 and BT-474 cells and

studied their impact on ERα protein expression. We identified 21 novel miRNAs which potently modulated ERα protein and mRNA levels in MCF-7 cells, and showed that miR-18a/b, miR-193b, miR-206, and miR-302c directly targeted ERα by binding to its 3'UTR region and inhibited ERα target genes. Furthermore, miR-18a and miR-18b levels were significantly higher *in vivo* in ERα-negative breast cancer, suggesting a role for these miRNAs in the development of ERα negative breast cancers.

Materials and methods

Cell Culture and Reagents

MCF-7 cells were obtained from Interlab Cell Line Collection (ICLC, Italy) and BT-474 from American Type Culture Collection (ATCC). MCF-7 cells were cultured in DMEM (1 g/l glucose) supplemented with 10 % foetal bovine serum (FBS), 2 mM L-glutamine and 1 % penicillin/streptomycin. BT-474 cells were cultured in DMEM (4.5 g/l glucose) supplemented with 10 % FBS, 4 mM L-glutamine, 0.01 mg/ml insulin, 1 mM Na-pyruvate, and 1 % penicillin/streptomycin.

Human Pre-miR™ miRNA Precursors and an siRNA for human oestrogen receptor-α (GGCCAAAUUCAGAUAAUCGTT; SEQ ID NO:55) were purchased from Ambion (Austin, TX), and used at a final concentration of 20 nM. β-estradiol (oestrogen) and 4'OH-Tamoxifen were purchased form Sigma-Aldrich (St. Louis, MO).

Lysate Microarray Screening and Data Analysis

MCF-7 and BT-474 cells were transfected with 20 nM human Pre-miR™ miRNA Precursor library v2 (Ambion Inc., Austin, TX) containing 319 chemically modified double-stranded RNA molecules designed to mimic endogenous mature human miRNAs. Briefly, the pre-miRNAs were printed robotically to 384-well black, clear-bottom assay plates (Greiner Bio-One GmbH, Frickenhausen, Germany). SilentFect™ transfection agent (BioRad Laboratories, CA) diluted into OptiMEM (Gibco Invitrogen, CA) was aliquoted into each 384-plate well using Multidrop 384 Microplate Dispenser (Thermo Labsystems, Thermo Electron Corporation, MA),

and the plates were incubated for 1 h at room temperature. Subsequently, 35 µl of cell suspension (1500 MCF-7 or 2000 BT-474 cells) was added on top of the miRNA-lipid complexes and the plates incubated for 48 h or 72 h.

After transfections, the cells were lysed by adding 15 µl of lysis buffer (100 mM Tris, pH 8.0; 0.2% SDS; 25 mM DTT) directly onto the 384-well plates. Thereafter, the lysates were denatured in 95 °C for 15 min, and stored at -20 °C prior printing. Microarray printer QArray^2 with 32 split pins (Genetix) was used to print arrays on nitrocellulose-coated microarray slides (FAST slides, Whatman inc.). Four drops of the cell lysates were stamped to each spot position ending up with a total of 3072 spots on a slide (~300 µm in diameter). Each slide was blocked with near-infrared blocking buffer (Rockland Immunochemicals) for 1 h at room temperature. PAP pen was used to draw a hydrophobic barrier around the nitrocellulose membrane to keep 500 µl of the sample volume stably on the membrane during incubations. ERα antibody (Ab-15; Labvision Corp, Fremont, CA) incubation at dilution of 1:200 was done overnight at 4 °C in a humidified chamber to prevent the slides from drying. The slides were washed with TBS + 0.1% Tween-20 for three times 5 min, and exposed to Alexa Fluor 680 tagged secondary antibody (Invitrogen Inc.) at a dilution of 1:5000 for 45 min. For total protein measurement, the arrays were stained with Sypro Ruby Blot solution (Invitrogen Inc.). Sypro signal was detected using Tecan LS400 (Tecan inc.) microarray scanner at 488/670 nm, and Alexa Fluor 680 label at 700 nm using IR-scanner (Li-Cor Inc.). Array-Pro Analyzer Microarray Analysis Software (Median Cybernetics Inc.) was used to measure median pixel intensities of each spot and the slide background from each channel. After background subtraction, spot intensity was normalized to the median intensity of pin to correct pin-to-pin as well as spatial variation on the array. Net signal from antibody staining was acquired after normalization to the Sypro signal. Finally, normalized values were converted to Z-scores. Candidate miRNAs down-regulating ERα were picked for secondary assays from Z-score window determined by positive and negative control siRNAs (ESR1 siRNA, Qiagen) and miRNAs (Scrambled, Ambion) respectively.

Two target prediction methods were used to assess the specificity of ERα downregulation after miRNA transfection. The TargetScanS v.4.2 [Grimson A et al. Mol Cell 2007; 27(1):91-105] and mirDB v.2.0 [Wang X and El Naqa IM Bioinformatics 2008; 24(3):325-32] were used to obtain miRNA target predictions. Enrichment factor and p-value according to the hypergeometric distribution was calculated using the minimum Z-score value of the negative control scrambled miRNAs as a threshold. Additionally, a t-test was used to show that the miRNAs predicted to target ERα were more effective in reducing the ERα levels than the miRNAs not predicted to target ERα.

Immunoblotting

Aliquots of total cell lysates were fractionated on SDS-polyacrylamide gels and transferred to Whatman Protran nitrocellulose membrane (Schleicher & Schuell, Whatman Inc, Florham Park, NJ). The filters were blocked against non-specific binding using 5 % skim milk. Membranes were probed with a specific antibody for human ERα (Ab-15; Labvision Corp, Fremont, CA). Equal loading was confirmed by probing the same filter with a specific antibody for human β-actin (Sigma, St. Louis, MO). Signals were revealed by incubating the filters with secondary antibody Alexa Fluor 680 anti-mouse IgG (Invitrogen Corp, Carlsbad, CA) and scanning the filters with Odyssey Licor (LI-COR Biosciences, Lincoln, NE). The relative levels of ERα protein were quantitated with Odyssey LI-COR 2.1 software and normalized for β-actin.

Real-time quantitative PCR analysis

Total cellular RNAs were isolated with MiRVana™ Total RNA isolation kit (Ambion Inc., Austin, TX). For cDNA synthesis, 200 ng of total RNA was reverse transcribed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Thereafter, the cDNAs were diluted 1/10 and the Taqman quantitative real-time PCR (qRT-PCR) analysis performed with Applied Biosystems 7900HT instrument using specific primers for ERα (ESR1) and β-actin (ACTB) designed by the Universal Probe Library Assay Design Center (Roche Applied Biosciences, Basel, Switzerland). The sequences of the primers were as

follows: ESR1 forward 5'-TTACTGACCAACCTGGCAGA-3' (SEQ ID NO:56), ESR1 reverse 5'-ATCATGGAGGGTCAAATCCA-3' (SEQ ID NO:57), ACTB forward 5'-CCAACCGCGAGAAGATGA-3' (SEQ ID NO:58), ACTB reverse 5'-CCAGAGGCGTACAGGGATAG-3' (SEQ ID NO:59). The fluorescent Taqman probes were obtained from Roche Human Probe Library (#24 for ESR1 and #64 for ACTB). The results were analyzed with SDS 2.3 and RQ manager software (Applied Biosystems, Foster City, CA), and the expression of ESR1 mRNA determined by relative quantitation method using β-actin as an endogenous control. The data is from two separate biological experiments, which were both run twice with triplicate samples.

ER 3'UTR reporter constructs and luciferase assays

Four separate fragments covering the ERα 3'-UTR region were amplified from the genomic DNA of MCF-7 cells using the following primers: ER#1_13-961, sense 5'-ATATACTAGTCCCACACGGTTCAGATAATC-3' (SEQ ID NO:60) and antisense 5'-ATATACGCGTACCCTCATCATGCATCACCA-3' (SEQ ID NO:61); ER#2_911-2050, sense 5'-ATATACTAGTGCCTTACACAGGGGTGAACT-3' (SEQ ID NO:62) and antisense 5'-ATATACGCGTTTTGCATGTTAACCCAGTCA-3' (SEQ ID NO:63); ER#3_2032-3222, sense 5'-ATATACTAGTCTGGGTTAACAT-GCAAAAACC-3' (SEQ ID NO:64) and antisense 5'-ATATACGCGTTTGTTCAG-CCCTCAAAAGG-3' (SEQ ID NO:65); ER#4_3201-4243, sense 5'-ATATACTAG-TCCTTTTGAGGGCTGAACAAA-3' (SEQ ID NO:66) and antisense 5'-ATATAC-GCGTCCCGCTGGATTCTTTTTCAA-3' (SEQ ID NO:67). The resulting ERα 3'UTR fragments were cloned into the Spe/MluI sites of a pMIR-REPORT™ Luciferase vector (Ambion Inc, Austin, TX) downstream of a luciferase gene.

For luciferase assays, MCF-7 cells (15000 per well) were plated 24 h before transfection onto white, clear-bottom 96-well plates in normal culture medium without antibiotics. The cells were co-transfected with 50 ng of ER 3'UTR reported plasmid, 50 ng Renilla luciferase plasmid, and with 50 nM pre-miR construct with Lipofectamine 2000 (Invitrogen Corp, Carlsbad, CA) according to the manufacturer's protocol. The luciferase activity was assayed 24 h after

transfection with Dual-Glo Luciferase Assay System (Promega Corp, Madison, WI) and measured with Envision Plate-reader (Perkin Elmer Inc, Wellesley, MA).

Proliferation assays

In experiments for assaying the effect of miRNAs on the oestrogen-stimulated growth of MCF-7 cells, the cells were cultured in phenol-red-free DMEM (Sigma, St. Louis, MO) supplemented with 10 % charcoal-stripped FBS and 2 mM L-glutamine. The cells (10000 per well) growing on white, clear-bottom 96-well plates were transfected with 20 nM pre-miR constructs (Ambion) or with siERα using SiLentFect (Bio-Rad). After 24 h incubation, the cells were treated with 2 nM 17β-estradiol or DMSO (vehicle), as indicated, for 72 h. Cell proliferation was assayed with CellTiter-Glo cell proliferation assay (Promega Corp, Madison, WI).

Cell cycle analysis

The cell cycle analyses were done from the nuclei preparations of MCF-7 cells. Cells (30000 per well on 24-well plates) were transfected with 20 nM pre-miR constructs or with siERα and incubated for 48 h. Thereafter, the cells were trypsinized, processed for the cell cycle analysis with BD CycleTest Plus DNA reagent kit (BD Biosciences, San Jose, CA), and examined for the cell cycle distribution with FACSArray (BD Biosciences, San Jose, CA). The data was analyzed using Multicycle software (Phoenix FlowSystems, San Diego, CA).

Illumina gene expression analyses

MCF-7 cells (300 000 per well on 6-well plates) were transfected with an siRNA for ERα or with Ambion pre-miR™ constructs for miR-18a, miR-193b, miR-206, miR-302c, or pre-miR negative control #1 (scrambled pre-miR) at 20 nM, and incubated for 24h. Thereafter, the total cellular RNAs were isolated with MiRVana™ total RNA isolation kit (Ambion). The quality of the RNAs was assayed with Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA). For Illumina gene expression profiling, 400 ng of total RNA was amplified and labelled with Illumina Total Prep RNA Amplification kit (Ambion) and hybridized on to Illumina Sentrix Human-6 Expression BeadChips. Statistical analysis of differential gene expression after miRNA transfection was performed with R/Bioconductor

[Gentleman RC et al. Genome Biol 2004; 5(10):R80] using the limma [Smyth GK Stat Appl Genet Mol Biol 2004; 3:Article3] and lumi [Du P, Kibbe WA and Lin SM Bioinformatics 2008; 24(13):1547-8] packages. Gene expression after siRNA or miRNA transfections was compared to the scrambled miRNA negative control in pairwise fashion using the eBayes statistic. The threshold for differential expression was $q<0.05$ after the Benjamini-Hochberg multiple testing correction. In order to display the differentially expressed genes, hierarchical clustering was performed with R after dividing the data into similar groups using the package "cluster" and the partitioning around medoids (PAM) method [Kaufman L and Rousseeuw PJ. Finding groups in data: An introduction to cluster analysis. New York: J. Wiley and Sons, Inc.; 1990]. The Gene Set Analysis (GSA) R package [Efron B and Tibshirani R Ann Appl Stat 2007; 1(1):107-29] was used for defining enriched gene sets in the data. Gene sets were obtained from the Molecular Signatures Database (MSigDB, Broad Institute of MIT and Harvard, Cambridge, MA, USA) [Subramanian A et al. Proc Natl Acad Sci U S A 2005; 102(43):15545-50]. The MSigDB curated gene sets (C2, 1892 sets) and motif gene sets (C3, 837 sets) including the 3'-UTR miRNA binding motifs (222 sets) [Xie X et al. Nature 2005; 434(7031):338-45] were downloaded from the GSEA web site. A false discovery rate (FDR) cut-off of 0 was used in all analyses.

MicroRNA expression and immunohistochemistry analyses of breast cancer samples

RNA from 96 breast cancer samples was extracted as described before [Sørlie T et al. BMC Genomics 2006; 7:127]. The samples were hybridized in two replicates (except four samples that were run once) on Human miRNA Microarray (v2) from Agilent Technologies according to the manufacturer's protocol (G4170-90010 v1.5). We used the Agilent Microarray Scanner (G2565A) and scanned at 100 % and 5 % PMT. Feature Extraction version 9.5.3.1. was used to extract signals intensites and filter the detectable miRNAs. Log2 values were used. The tumours where graded by immunohistochemistry staining of ERα, depending on the percentage of cells nuclei that were stained [Naume B et al. Mol Oncol 2007; 1(2):160-71]. Twenty-eight tumours were ERα negative, and 68 tumours with

staining over 1 % were considered as ERα positive. Of these, 8 tumours had 1–10 %, 9 tumours had 10–50 %, 11 tumours 50–75 %, and 40 tumours have 75–100 % cells with ESR1 positive staining. T-test and non-parametric Kruskal Wallis test were performed for association between miRNA expression level and ER status/grading in SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Lysate microarray screens revealed several putative ERα regulating miRNAs

Protein lysate microarray (LMA) technology, or reverse-phase protein microarray, allows proteomic profiling of a large number of biological samples with specific antibodies. We applied LMA technology in order to identify ERα targeting miRNAs in breast cancer cell lines. MCF-7 and BT-474 cells were transfected with Pre-miR™ miRNA library containing 319 chemically modified, double-stranded pre-miR constructs mimicking endogenous miRNAs. After 48 h and 72 h incubation, the cells were lysed and the lysates printed on nitrocellulose slides to be stained with a specific antibody against ERα. The slides were also counterstained with Sypro Ruby in order to normalize the levels of ERα for the total protein. With LMA screening, we identified 60 and 93 miRNAs which reduced the expression of ERα in both cell lines at 48 h and 72 h time points, respectively (Figure 1). The siRNA for ERα (siERα) was used as a positive control. Among the miRNAs that downregulated ERα was miR-206, which has previously been shown to directly target ERα [Adams BD et al. 2007] (Figure 1). Furthermore, miRNAs predicted to target ERα according to various prediction algorithms were enriched among the ERα downregulating miRNAs. Two sample Wilcoxon test p-values for the enrichment were highly significant for MCF-7 cells at 48 h and 72 h timepoints ($p< 2.1e^{-06}$ and $p<1.2e^{-06}$, respectively) . The p-values for BT-474 were also <0.05 at both time points (not shown).

Figure 1, LMA screen for identifying miRNAs regulating ERα

MCF-7 and BT-474 breast cancer cells were transfected with Pre-miR miRNA precursor library containing 319 pre-miR constructs and incubated for 48 h or 72 h.

Thereafter, the cells were lysed, printed onto nitrocellulose coated slides, and stained with a specific antibody for ERα. Slides were counterstained with Sypro Ruby in order to normalize the total protein amount. Figure 1 shows representative LMA screens of MCF-7 and BT-474 cells plotted against each other.

Validation of the lysate microarray data

To validate the primary LMA screening data, we selected miRNAs which were identified in at least two replicate LMA screens as putative ERα downregulating miRNAs. As an additional selection criteria we used miRNA target predictions from TargetScan [Grimson A et al. (2007)], PicTar [Krek A et al. Nat Genet 2005; 37(5):495-500], and MiRanda [Betel D et al. Nucleic Acids Res 2008; 36(Database issue):D149-53]. We were then left with 21 miRNAs indicated in Table 4. In western blotting, all these miRNAs downregulated ERα protein levels in MCF-7 and BT-474 cells by up to 80 %, validating the LMA primary screening data (Figure 2A). Transfections of pre-miR constructs for miR-200a and miR-200b, not predicted to target ERα and not scoring positive in the LMA screens, served as negative controls (Figure 2A).

In addition to translational repression, miRNAs regulate the gene expression by targeting mRNAs for cleavage. We therefore examined the effect of pre-miR overexpression on the levels of ERα mRNA with real-time qRT-PCR. Transfection of MCF-7 cells with siERα reduced the levels of ERα mRNA by 2-fold (Figure 2B). The miRNAs miR-18a/b, miR-22, miR-93, miR-130a/b, miR-302c, miR-372, miR-373, and miR-520d also downregulated ERα mRNA levels, suggesting that these miRNAs target ERα mRNA to degradation (Figure 2B). In comparison, miR-181d, miR-193b and miR-206 had only minor effects on ERα mRNA levels, indicating that these miRNAs may regulate ERα mainly at the translational level. Negative control miRNAs, miR-200a/b, did not significantly affect the levels of ERα mRNA in MCF-7 cells (Figure 2B).

Table 4. LMA Z-scores and ERα predictions for the miRNAs selected for validation.
The data is from MCF-7 screens performed at 48 h and 72 h time points (sorted according to 72 h).

	ERα/sypro z-score		Predicted sites in the ERα 3'UTR			
Sample	48h	72h	TargetScan, conserved	TargetScan, non-conserved	PicTar[a]	MiRanda[a]
ERα siRNA	-1.27	-2.75				
hsa-miR-206	-0.94	-2.74		7mer-m8, 7mer-1A		1
hsa-miR-219	-2.43	-2.54	7mer-m8		1	1
hsa-miR-222	-2.78	-2.40	8mer	7mer-1A	1	1
hsa-miR-9	-1.70	-2.16	7mer-1A	7mer-m8	2	1
hsa-miR-517a	-1.68	-1.82				1
hsa-miR-18a	-0.47	-1.77	8mer	8mer, 7mer-1A	3	2
hsa-miR-373	-0.64	-1.69	7mer-m8	7mer-1A	2	
hsa-miR-181d	-1.16	-1.64	8mer			2
hsa-miR-302c	-1.68	-1.57	7mer-m8	7mer-1A	2	1
hsa-miR-301	-0.87	-1.56	7mer-m8	7mer-m8, 7mer-1A	2	2
hsa-miR-372	-1.69	-1.53	7mer-m8	7mer-1A	2	
hsa-miR-193b	-0.49	-1.42			1	
hsa-miR-130a	-0.96	-1.41	7mer-m8	7mer-m8, 7mer-1A	2	2
hsa-miR-130b	-0.71	-1.27	7mer-m8	7mer-m8, 7mer-1A	2	1
hsa-miR-181a	-1.32	-1.19	8mer		1	1
hsa-miR-517c	-0.80	-1.14				1
hsa-miR-18b	-1.13	-1.08	8mer	8mer, 7mer-1A	3	2
hsa-miR-22	-1.18	-0.89	8mer	7mer-m8, 7mer-1A	3	1
hsa-miR-93	-0.37	-0.84	7mer-m8	7mer-1A	2	2
hsa-miR-520d	-1.18	-0.25	7mer-m8	7mer-1A		
hsa-miR-181c	-0.95	-0.08	8mer		1	1
pre-mir neg.ctrl	0.39	0.06				
cells only ctrl	-0.10	0.10				

[a]For PicTar and MiRanda the number of predicted sites is shown.

Figure 2A, Validation of ERα regulating miRNAs

MCF-7 and BT-474 breast cancer cells were transfected with pre-miR constructs (20 nM) and incubated for 48 h. Thereafter, the cells were lysed and the lysates analyzed with western blotting for the expression of ERα.

Figure 2B, Validation of ERα regulating miRNAs

MCF-7 breast cancer cells were transfected with pre-miR constructs and incubated for 24 h. Thereafter, the total cellular RNAs were isolated and analyzed with Taqman qRT-PCR for the expression of ERα mRNA. The results normalized for β-actin are shown as relative expression using scrambled negative control miRNA (Scr) as a reference. Mean ± SEM (n=4).

MiRNA-mediated downregulation of ERα suppresses oestrogen-stimulated growth of MCF-7 cells

Oestrogen stimulates the proliferation of hormone-dependent breast cancer cells, and this is mediated primarily via ERα [Couse JF and Korach (1999)]. To study the functional significance of the ERα-downregulating miRNAs, we analyzed whether the miRNA-mediated downregulation of ERα could inhibit the growth stimulatory effects of oestrogen on MCF-7 cell growth. For that purpose, the MCF-7 cells were first cultured in hormone-free growth media for 24 h, and then transfected with the corresponding pre-miR constructs. After additional 24 h incubation, the cells were treated with oestrogen 17β-estradiol (2 nM) or with vehicle (DMSO) control for 72 h. Thereafter, the proliferation was assayed, and the fold-induction between oestrogen and vehicle treated samples measured. Oestrogen stimulation enhanced the growth of negative control pre-miR transfected MCF-7 cells by 2.4-fold, whereas pre-treatment of cells with 4'OH-Tamoxifen (OHT) totally suppressed the oestrogen-stimulated growth of MCF-7 cells (Figure 3). When ERα was knocked down with siERα, the oestrogen-induced growth was inhibited by 60 % (Figure 3). Most of the miRNAs showed similar or slightly lower inhibition as siERα. The most potent growth inhibitory miRNAs were miR-93, miR-193b, miR-222, miR-302c, miR-372, and miR-520d (Figure 3). In comparison, transfection of

cells with an siRNA for ERβ had no significant effect confirming that growth inhibition was specifically dependent on ERα.

Figure 3, miRNA-mediated downregulation of ERα suppresses oestrogen-stimulated growth of MCF-7 breast cancer cells

MCF-7 cells cultured in hormone-free growth media were transfected with pre-miR constructs or with an siRNA for ERα. After 24-h incubation, the cells were treated with oestrogen 17β-estradiol (2 nM) or with vehicle (DMSO) for 72 h. Cell growth was measured with CellTiter-Glo cell viability assay. Relative increase in cell number in response to oestrogen stimulation is shown in the graph. The data represent mean ± SEM from three independent experiments, each performed in triplicate (*p< 0.05; ***p=0.00003).

MiR-18a, miR-18b, miR-193b, and miR-206 induce accumulation of MCF-7 cells in G_1/G_0 phase of the cell cycle

In the absence of oestrogens or in the presence of antioestrogens, proliferation of hormone-dependent breast cancer cells is inhibited and they accumulate in the G_1 phase of the cell cycle [Doisneau-Sixou SF et al. Endocr Relat Cancer 2003; 10(2):179-86]. Therefore, we next studied how downregulation of ERα by miRNAs affects the cell cycle of MCF-7 cells. The cells were transfected with pre-miR constructs for the 21 ERα down-regulating miRNAs, and the cell cycle distribution was analyzed after 48 h by FACS. While many of the miRNAs did not change the proportion of MCF-7 cells in G_1, S, or G_2 phases of the cell cycle, miR-18a, miR-18b, miR-193b, and miR-206 significantly induced accumulation of the cells in the G_1/G_0 phase. In these experiments, the average percentage of cells in G_1/G_0 phase was 71 %, 68 %, 80 %, and 73 %, respectively, whereas in the controls this was 47 %. These data were again comparable with the results for siRNA-mediated ERα knockdown as well as with antioestrogen 4'OH-Tamoxifen (OHT; 1 μM) treated samples (Figure 4).

Figure 4, MiR-18a, miR-18b, miR-193b, and miR-206 induce accumulation of MCF-7 cells in G_1/G_0 phase of the cell cycle

MCF-7 cells were transfected with the pre-miR constructs (20 nM) for the indicated miRNAs or with siERα, and incubated for 48 h. Thereafter, the cell cycle distribution was analyzed from the nuclei preparations with FACS. Results are shown as percentage of cells in each phase of the cell cycle. 4'OH Tamoxifen (OHT; 1 μM) was used as a positive control for G_1 arrest. Mean±SD, n=3, $^{**}p< 0.01$.

MiR-18a, miR-193b, miR-206, and miR-302c repress ERα-responsive genes

Typically, miRNAs regulate the expression of hundreds of different genes, and often target several genes on the same pathways [Lewis BP et al. Cell 2005; 120(1):15-20]. To better understand the role for miR-18, miR-193b, miR-206, and miR-302c in oestrogen-mediated signal transduction, we examined their effects on the gene expression profile of MCF-7 breast cancer cells by microarray. The cells were transfected with the pre-miR constructs and siERα control and incubated for 24 h, followed by analysis using Illumina Sentrix Human-6 Expression BeadChips. A clustering analysis of all the differentially expressed genes revealed genes specifically downregulated by each miRNA. Also, a consensus set of genes downregulated by all four miRNAs (miR-18a, miR-193b, miR-206, and miR-302c) and siERα included many well-known ERα-responsive genes, such as *MYB, GREB1, CD44, PDZK1, IGFBP4* and *STC2.* When the expression profiles were compared against profiles from the Molecular Signatures Database (MSigDB) [Subramanian A et al. (2005)] using the Gene Set Analysis (GSA) software [Efron B and Tibshirani R (2007)], the most frequently enriched signature among miRNA downregulated genes was an MSigDB signature "FRASOR_UP" (Table 5). This signature was originally obtained after treating MCF-7 cells with oestrogen [Frasor J et al. Cancer Res 2004; 64(4):1522-33 (35)], and it contains ERα-dependent genes. The Frasor_UP signature was highly enriched with a FDR of 0 and was ranked the first among the curated genes sets in the MSigDB (C2, 1892 sets) after both miR-18a and miR-302c treatments (Table 5). Moreover, the Frasor_UP signature was ranked in the top ten also among the miR-193b, miR-206 and siERα

enriched signatures (FDR = 0). This indicates that in MCF-7 cells, and very likely in breast cancer in general, the ERα mRNA is an important target for these miRNAs.

Table 5. Expression profiles. Expression profiles obtained after overexpressing miR-18a (Table 5A), miR-193b (Table 5B), miR-206 (Table 5C), miR-302c (Table 5D), or siERα (Table 5E) in MCF-7 cells for 24 h were compared to the 1892 curated gene sets (C2) in the MSigDB v2.5. Twenty most significant signatures are displayed arranged according to descending score. Signatures present in all different microRNA transfections in bold. The most frequently enriched signature among miRNA downregulated genes was an MSigDB signature "Frasor_UP", which was originally obtained after treating MCF-7 cells with oestrogen, and which contains ERα-responsive genes.

Table 5A

Gene set number	Gene set name; miR-18a signatures	Score
215	**FRASOR_ER_UP**	**1.73**
1685	TSA_RKO_UP	1.28
1001	**XU_ATRA_PLUSNSC_DN**	**0.98**
1150	STRESS_IONIZING_SPECIFIC_UP	0.94
1365	ERM_KO_SERTOLI_DN	0.86
1556	CITED1_KO_WT_UP	0.82
1369	TSA_CD4_UP	0.81
1241	HBX_HEP_UP	0.81
1395	INSULIN_ADIP_INSENS_UP	0.78
284	GLUTAMATE_METABOLISM	0.78
591	LIZUKA_L1_GR_G1	0.77
595	POMEROY_MD_TREATMENT_GOOD_VS_POOR_DN	0.77
808	VERNELL_PRB_CLSTR2	0.76
1736	HSA00450_SELENOAMINO_ACID_METABOLISM	0.73
1310	BRCA1_SW480_UP	0.71
461	CELLCYCLEPATHWAY	0.69
688	RACCYCDPATHWAY	0.68
804	SCHURINGA_STAT5A_UP	0.68
526	SCHUMACHER_MYC_UP	0.68
198	MTA3PATHWAY	0.64

Table 5B

Gene set number	Gene set name; miR-193b signatures	Score
1385	P21_MIDDLE_DN	1.50
128	ONE_CARBON_POOL_BY_FOLATE	1.27
779	SA_G1_AND_S_PHASES	1.21
1779	HSA00670_ONE_CARBON_POOL_BY_FOLATE	1.03
400	P53PATHWAY	1.00
880	ZHAN_MM_CD138_PR_VS_REST	0.98
1397	IGFR_IR_UP	0.93
541	GOLDRATH_CELLCYCLE	0.92
215	**FRASOR_ER_UP**	**0.90**
1532	AD12_32HRS_DN	0.90
637	COLLER_MYC_UP	0.89
593	NUCLEOTIDE_METABOLISM	0.87
119	UNDERHILL_PROLIFERATION	0.85
1055	P21_ANY_DN	0.84
187	MENSSEN_MYC_UP	0.83
1162	BLEO_MOUSE_LYMPH_HIGH_24HRS_DN	0.79
1001	**XU_ATRA_PLUSNSC_DN**	**0.79**
216	TCA	0.77
1305	CANCER_UNDIFFERENTIATED_META_UP	0.74
1419	SERUM_FIBROBLAST_CELLCYCLE	0.73

Table 5C

Gene set number	Gene set name; miR-206 signatures	Score
1147	CANCERDRUGS_PROBCELL_DN	1.92
1571	UVC_LOW_C3_DN	1.30
1483	CAMPTOTHECIN_PROBCELL_DN	1.30
1397	IGFR_IR_UP	1.29
1001	**XU_ATRA_PLUSNSC_DN**	**1.28**
297	PENTOSE_PHOSPHATE_PATHWAY	1.23
1695	HSA00030_PENTOSE_PHOSPHATE_PATHWAY	1.02
591	LIZUKA_L1_GR_G1	0.98
644	GLUTATHIONE_METABOLISM	0.93
595	POMEROY_MD_TREATMENT_GOOD_VS_POOR_DN	0.88
1336	PITUITARY_FETAL_UP	0.86
1150	STRESS_IONIZING_SPECIFIC_UP	0.79
215	**FRASOR_ER_UP**	**0.79**
128	ONE_CARBON_POOL_BY_FOLATE	0.73

733	HINATA_NFKB_DN	0.69
779	SA_G1_AND_S_PHASES	0.68
1323	ADIP_VS_FIBRO_DN	0.65
1779	HSA00670_ONE_CARBON_POOL_BY_FOLATE	0.64
899	HASLINGER_B_CLL_12	0.59
720	TRNA_SYNTHETASES	0.57

Table 5D

Gene set number	Gene set name; miR-302c signatures	Score
215	**FRASOR_ER_UP**	**1.65**
1556	CITED1_KO_WT_UP	1.31
806	MA_ATRA_EMP_DN	1.26
1001	**XU_ATRA_PLUSNSC_DN**	**1.03**
1420	P21_P53_MIDDLE_DN	0.99
397	MAGRANGEAS_MULTIPLE_MYELOMA_IGG_VS_IGA_DN	0.91
549	MAGRANGEAS_MULTIPLE_MYELOMA_IGG_VS_IGA_UP	0.91
1375	HDACI_COLON_CLUSTER4	0.87
41	JISON_SICKLE_CELL	0.86
198	MTA3PATHWAY	0.85
1017	HYPOXIA_FIBRO_UP	0.83
308	WELCSH_BRCA_UP	0.82
400	P53PATHWAY	0.78
1310	BRCA1_SW480_UP	0.72
1241	HBX_HEP_UP	0.72
1276	P53GENES_ALL	0.72
162	ST_G_ALPHA_S_PATHWAY	0.71
526	SCHUMACHER_MYC_UP	0.71
1483	CAMPTOTHECIN_PROBCELL_DN	0.70
1571	UVC_LOW_C3_DN	0.69

Table 5E

Gene set number	Gene set name; siERα signatures	Score
128	ONE_CARBON_POOL_BY_FOLATE	1.67
1068	MMS_HUMAN_LYMPH_LOW_4HRS_DN	1.66
593	NUCLEOTIDE_METABOLISM	1.50
1779	HSA00670_ONE_CARBON_POOL_BY_FOLATE	1.44
198	MTA3PATHWAY	1.40

1385	P21_MIDDLE_DN	1.35
215	**FRASOR_ER_UP**	**1.24**
1527	BLEO_MOUSE_LYMPH_LOW_24HRS_DN	1.05
775	BHATTACHARYA_ESC_UP	1.04
526	SCHUMACHER_MYC_UP	0.96
1670	IRS_KO_ADIP_UP	0.83
1803	HSA00970_AMINOACYL_TRNA_BIOSYNTHESIS	0.81
1001	**XU_ATRA_PLUSNSC_DN**	**0.76**
637	COLLER_MYC_UP	0.76
1397	IGFR_IR_UP	0.74
189	LIZUKA_L0_SM_L1	0.73
566	SHIPP_FL_VS_DLBCL_DN	0.71
283	TELPATHWAY	0.68
430	HCC_SURVIVAL_GOOD_VS_POOR_DN	0.67
1697	HSA00040_PENTOSE_AND_GLUCURONATE_INTERCONVERSIONS	0.58

ERα is a direct target for miR-18a/b, miR-193b, miR-206 and miR-302c

Based on the TargetScan predictions [Grimson A et al. (2007)], miR-18/b has the highest scoring target sites in the 3'UTR region of ERα. A conserved 8mer site can be found at position 1938-1945 of the ESR1 3' UTR with a context score percentile of 94 %. In addition, the ER 3'UTR contains one non-conserved 8-mer site and one 7mer-1A site for miR-18a/b at positions 1917-1924 and 527-533, respectively. MiR-302c also has a favourable target site in the ERα 3'UTR with context score percentile of 86 %. MiR-193b is predicted to target ERα by another algorithm, PicTar [Krek A et al. (2005)], and it has a conserved, predicted 7mer site at position 3999-4005 of the ER 3'UTR. Since our studies suggested that miR-18a/b, miR-193b, miR-206, and miR-302c are the most potent modulators of oestrogen-receptor signalling, we analyzed whether these miRNAs directly target ERα. For that purpose, we cloned four separate fragments covering the ERα 3'UTR region into a luciferase reporter vector (Figure 5A; Table 6). The resulting ERα 3'UTR-luciferase reporter plasmids were transfected in MCF-7 cells together with the corresponding pre-miR constructs and with a Renilla luciferase control plasmid. Transfection of miR-18a, miR-18b, miR-193b, miR-206 and miR-302c significantly inhibited the reporter activity as compared to scrambled miRNA transfected negative control, indicating that these miRNAs directly target ERα (Figure 5B).

Figure 5A, ERα is a direct target for miR-18a/b, miR-193b, miR-206, and miR-302c

Figure 5A illustrates a schematic representation of the subfragments of ERα 3'UTR which were inserted into the pMIR-REPORT Luciferase reporter vector. The predicted miRNA target sites are indicated with arrows, and are listed in table 6.

Table 6. Predicted miRNA sites.

miRNA	Construct no #	Position in the ERα 3'UTR	Prediction algorithm
hsa-miR-18a/b	#1	527-533	TargetScan, PicTar
	#2	1917-1924 & 1938-1945	TargetScan, PicTar
hsa-miR-193b	#4	3999-4005	PicTar
hsa-miR-206	#1	86-109	Adams et al. 2007 (RNAHybrid)[1]
	#2	1152-1158 & 1532-1538	TargetScan
	#4	3938-3959	MiRanda
hsa-miR-302c	#1	379-385	TargetScan & PicTar
	#2	1832-1838	TargetScan & PicTar

[1]Predicted site in ERα 3'UTR as identified by RNAHybrid Adams et al. (2007).

Figure 5B, ERα is a direct target for miR-18a/b, miR-193b, miR-206, and miR-302c

Activity of luciferase reporters containing segments of the ERα 3'UTR. The reporters (100 ng) were cotransfected with pre-miR constructs (50 nM) for the indicated miRNAs, and luciferase activity measured after 24 h incubation. Firefly luciferase activity was normalized to that of Renilla luciferase. The data shown are the mean ±SEM of three independent experiments, each performed in triplicate. *p<0.05, **p<0.01

MiR-18a and miR-18b have lower expression in ERα-positive tumours

To study the clinical relevance of the miRNAs directly targeting ERα, we analyzed the miRNA microarray expression data for miR-18a, miR-18b, miR-193b, miR-206, and miR-302c in 96 primary breast cancer specimens. The analysis was done both by dichotomizing the tumors into ERα-positive and ERα-negative as well as by correlating the miRNA expression levels with the semiquantitative assessment of ERα immunostaining. The expression of miR-302c was not detected in any of

the tumours, and the expression of miR-206 was detected in only 26/96 tumours. With miR-206 and miR-193b, there was no association with ERα grading (data not shown). However, the results provided a statistically significant inverse association between the expression of miR-18a and miR-18b and that of the ERα immunostaining (p=0.0001, p=0.0004 respectively) (Figure 6). This suggests that the expression of miR-18a and miR-18b is an important *in vivo* regulatory mechanism of ERα in breast cancer patients.

Figure 6, The expression of miR-18a and miR-18b shows inverse correlation with ERα

Ninety-six (96) primary breast cancer specimens were analyzed for miRNA expression using Agilent miRNA microarray. The analysis was done both by dichotomizing the tumours into ERα-positive and ERα-negative as well as by correlating the miRNA expression levels with the semiquantitative assessment of ERα immunostaining. The tumours where graded (0-4) by immunohistochemistry (IHC) staining of ERα, depending on the percentage of cells nuclei that were stained (0=0 %; 1=1–10 %; 2=10–50 %; 3=50–75 %; 4=75–100 %). The boxplots show the expression of hsa-miR-18a (left panel) and hsa-miR-18b (right panel) in relation to the ERα grading. The open dots indicate outliers.

Other preferred embodiments

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the expert skilled in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive

CLAIMS

1. Use of a nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

b) any one of said nucleic acids defined in a) comprising a chemical modification or modifications and able to modulate the expression of ERα;

c) any nucleic acid having at least 85 % identity to said nucleic acids as defined in a) or b) and able to modulate the expression of ERα; and

d) any functional fragment, i.e. a fragment able to modulate the expression of ERα, of any one of said nucleic acids defined in a), b) or c);

e) any precursor, i.e. any nucleic acid that can be processed through natural processing or synthetic processing into any of said nucleic acids as defined in a), b), c) or d);

single-stranded or double-stranded, and any combination thereof for the manufacture of a medicine for the treatment of breast cancer.

2. The use according to claim 1 **characterized** in that the nucleic acid is RNA or a derivative thereof, i.e. RNA comprising a chemical modification or modifications.

3. The use according to claim 2 **characterized** in that a chemical modification or modifications selected from the group consisting of substitution of a phosphate bond with a phosphorothioate bond, methylation of the oxygen at position 2 in ribose, addition of an extra bridge between the carbons of the ribose ring, substitution of the sugar backbone by a pseudopeptide backbone or a morpholino ring, attachment of a cholesterol at the 3´OH end, and any combination thereof are used.

4. The use according to claim 1, 2 or 3 **characterized** in that the nucleic acids referred to in a) of claim 1 are selected from the group consisting of SEQ ID NOS: 24–28 and 31–34, preferably from the group consisting of SEQ ID NOS: 24–27.

5. The use according to any of the preceding claims **characterized** in that a functional fragment as referred to in b) of claim 1 is used, said fragment consisting of 7 to 22, preferably 7 to 17, or 12 to 22, and most preferably 12 to 17 nucleotides including a seed sequence, i.e. any one of SEQ ID NOS: 43–54.

6. The use according to any of the preceding claims **characterized** in that a precursor is used consisting of 25 to 200, preferably 50 to 150, and most preferably 80 to 100 nucleotides.

7. The use according to claim 6 **characterized** in that the precursor used is selected from the group consisting of any one of SEQ ID NOS: 1–23.

8. The use of any of the preceding claims **characterized** in that the medicine is manufactured for the treatment of

a) oestrogen dependent breast cancer and/or

b) the prevention of progression of oestrogen dependent breast cancer into oestrogen independent breast cancer.

9. The use of any of the preceding claims **characterized** in that the use is combined with the use of an antioestrogen.

10. The use according to claim 9 characterized in that the antioestrogen is selected from the group consisting of fulvestran, letrozole, raloxifene, tamoxifen and toremifene.

11. A method of obtaining a marker value or values useful for differentiating patients with an oestrogen dependent breast cancer with an increased risk of progression into an oestrogen independent breast cancer **characterized** in that a nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

b) any precursor, i.e. any nucleic acid that can be processed through natural processing into any of the nucleic acids defined in a);

single-stranded or double-stranded, and any combination thereof are determined from a sample or samples of a tumour of the breast cancer and a marker value and/or values, which are a determined amount or amounts as such, or a function of the determined amount or amounts, are established.

12. The method of claim 11 **characterized** in that the nucleic acid or acids are determined by any target polynucleotide recognizing detection method, preferably a method employing nucleic acid amplification.

13. A method

i) for the treatment of oestrogen dependent breast cancer, and/or

ii) prevention of progression of oestrogen dependent breast cancer into oestrogen independent breast cancer

in human wherein treatment comprises introducing into a tumour of said breast cancer a nucleic acid or nucleic acids selected from the group consisting of

a) any one of SEQ ID NOS: 24–42;

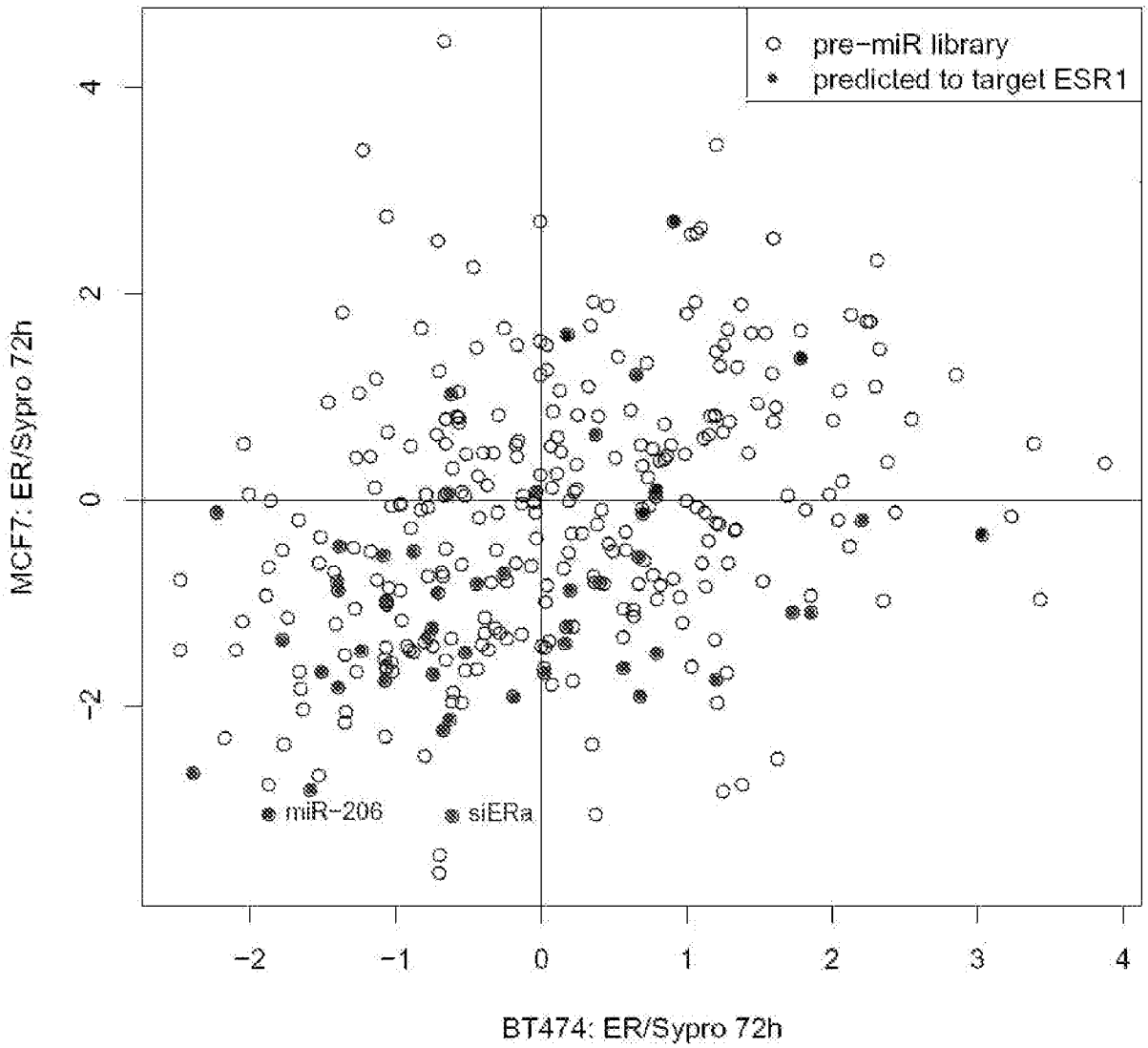
b) any one of said nucleic acids defined in a) or b) comprising a chemical modification and able to modulate the expression of ERα;

c) any nucleic acid having at least 85 % identity to nucleic acids as defined in a) or b) and able to modulate the expression of ERα; and

d) any functional fragment, i.e. a fragment able to modulate the expression of ERα, of any one of said nucleic acids defined in a), b) or c);

e) any precursor, i.e. any nucleic acid that can *in vivo* be processed through natural processing or synthetic processing into any of the nucleic acids defined in a), b), c) or d);

single-stranded or double-stranded, and any combination thereof.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predicted to target ESR1
miR−206
siERa
MCF7: ER/Sypro 72h
BT474: ER/Sypro 72h
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2
4
−2
−1
0
1
2
3
4

Figure 1

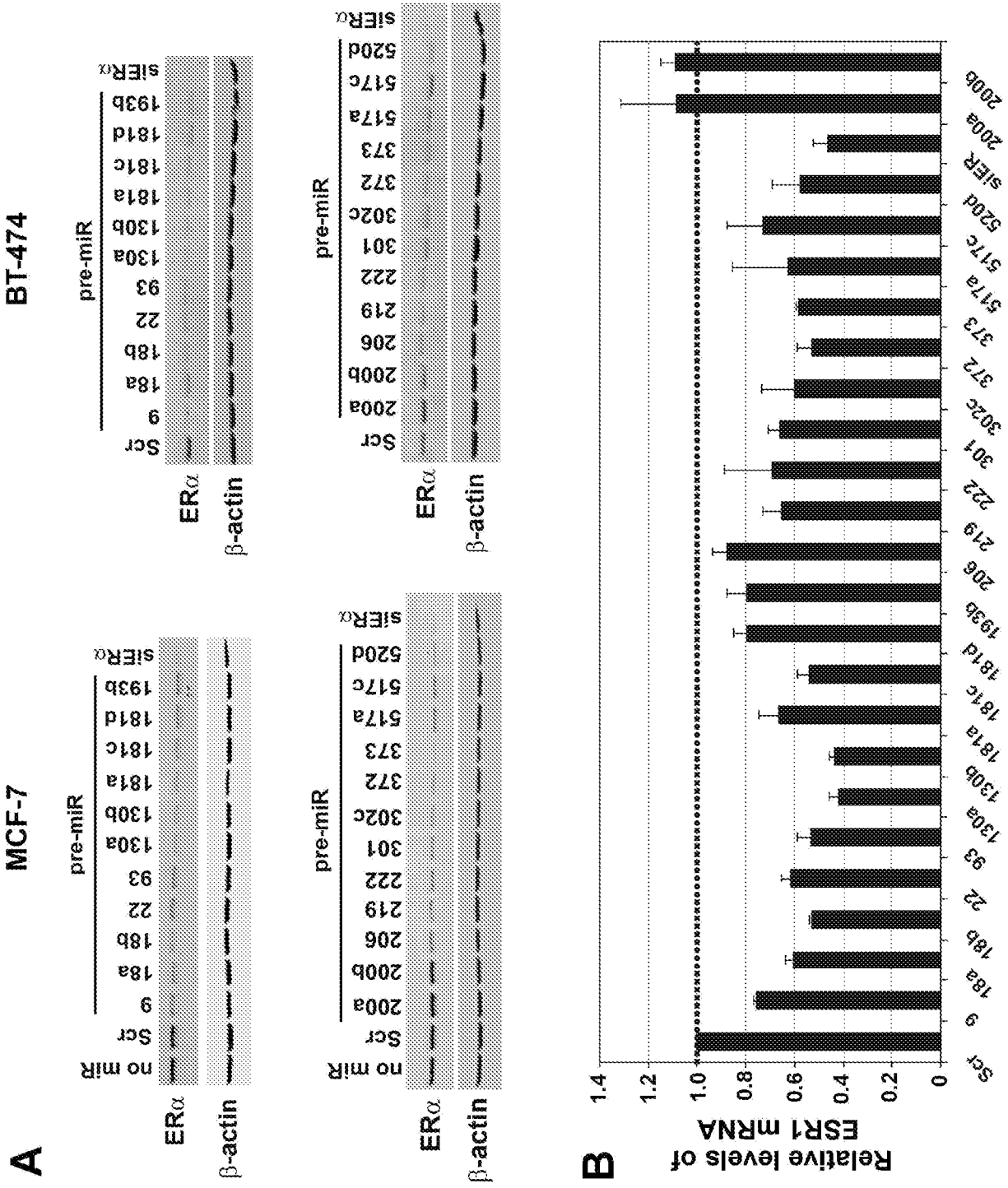
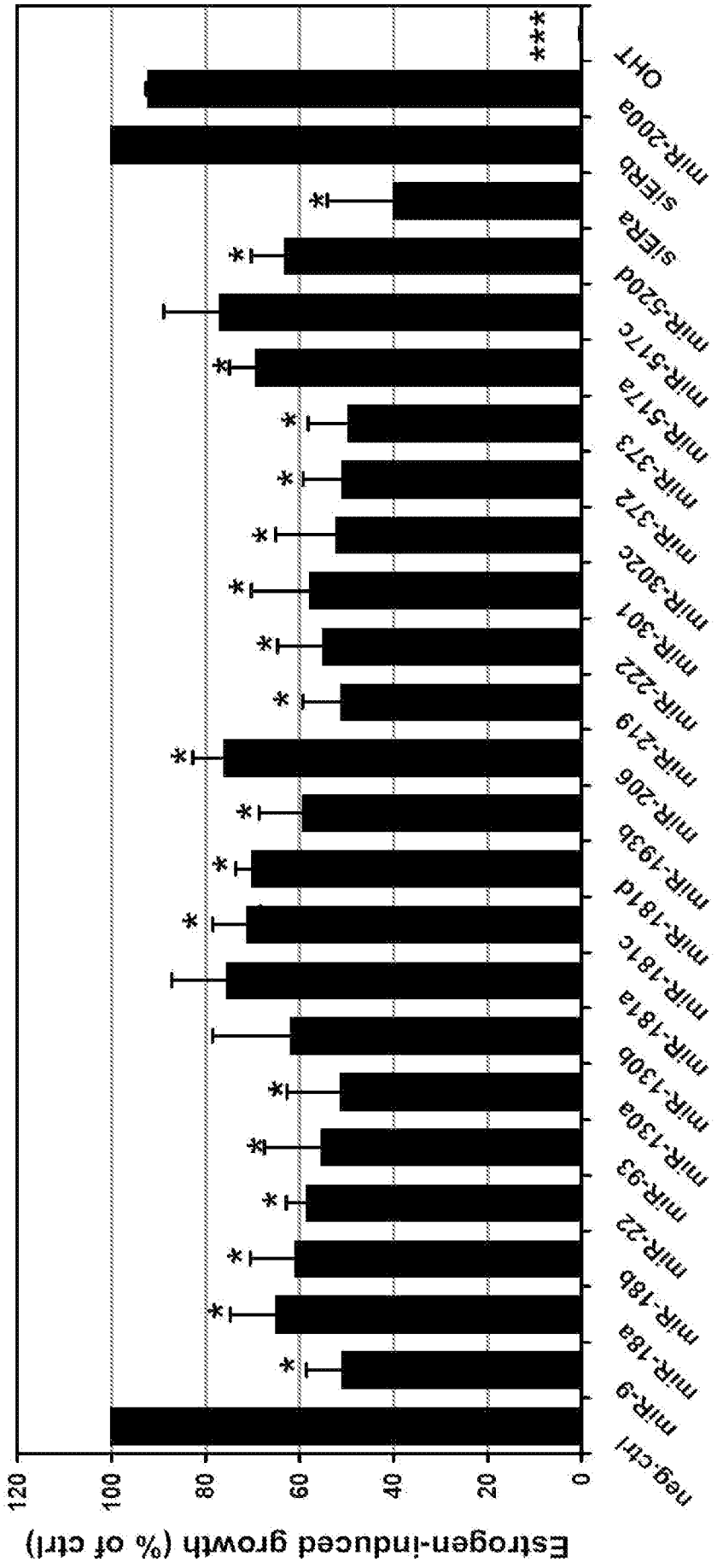

A
MCF-7
BT-474
pre-miR
no miR
Scr
9
18a
18b
22
93
130a
130b
181a
181c
181d
193b
siER$_\alpha$
ERα
β-actin
B
Relative levels of ESR1 mRNA
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Scr
9
18a
18b
22
93
130a
130b
181a
181c
181d
193b
206
219
222
301
302c
372
373
517a
517c
520d
siER
200a
200b

Figure 2


Estrogen-induced growth (% of ctrl)
120
100
80
60
40
20
0
neg.ctrl
miR-9
miR-18a
miR-18b
miR-22
miR-93
miR-130a
miR-130b
miR-181a
miR-181c
miR-181d
miR-193b
miR-206
miR-219
miR-222
miR-301
miR-302c
miR-372
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miR-517c
miR-520d
siERa
siERb
miR-200a
OHT
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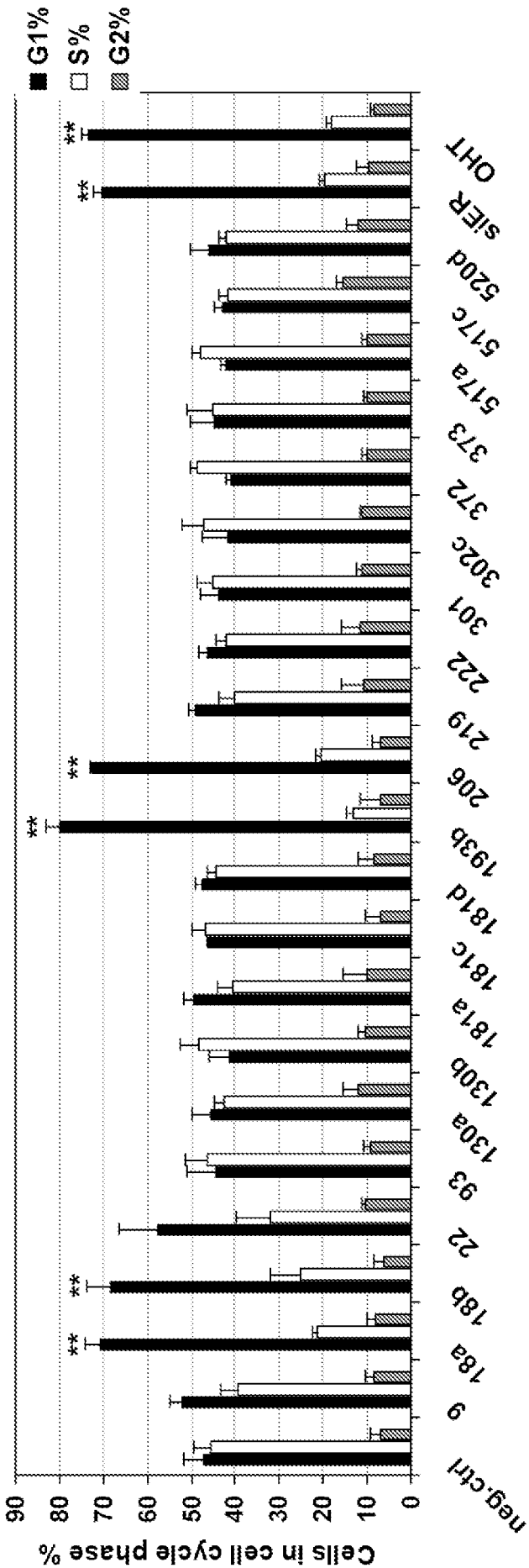
Figure 3


G1%
S%
G2%
Cells in cell cycle phase %
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10
20
30
40
50
60
70
80
90
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18b
22
93
130a
130b
181a
181c
181d
193b
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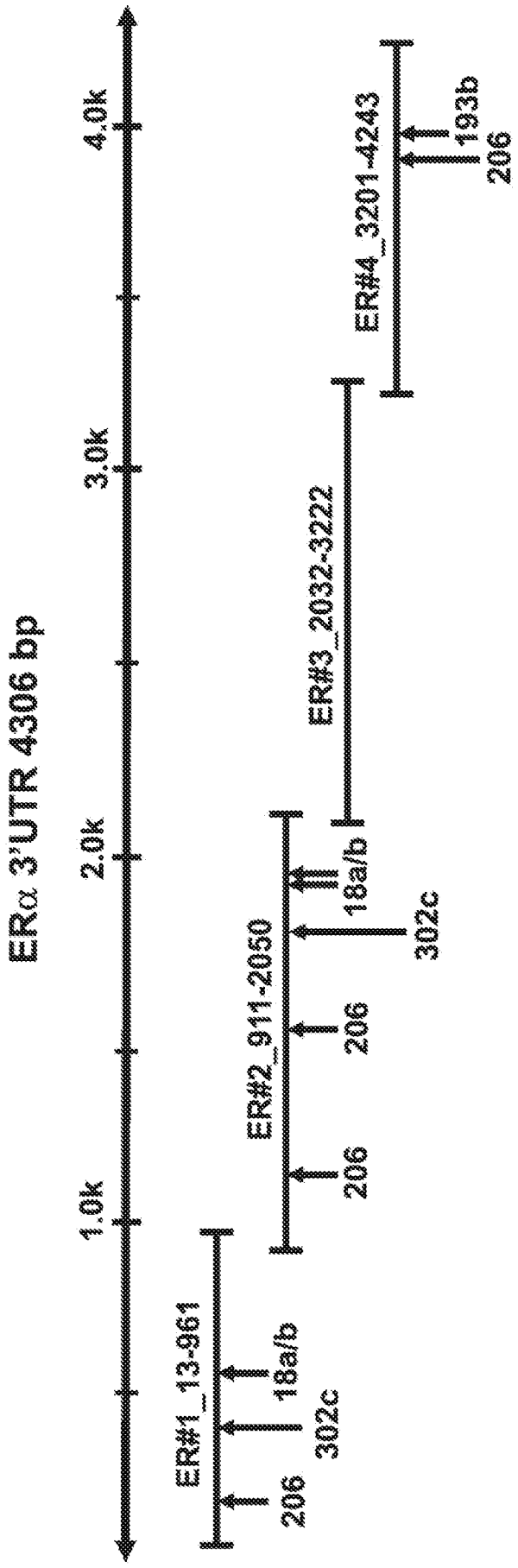
222
301
302c
372
373
517a
517c
520d
siER
OHT
**

Figure 4


ERα 3'UTR 4306 bp
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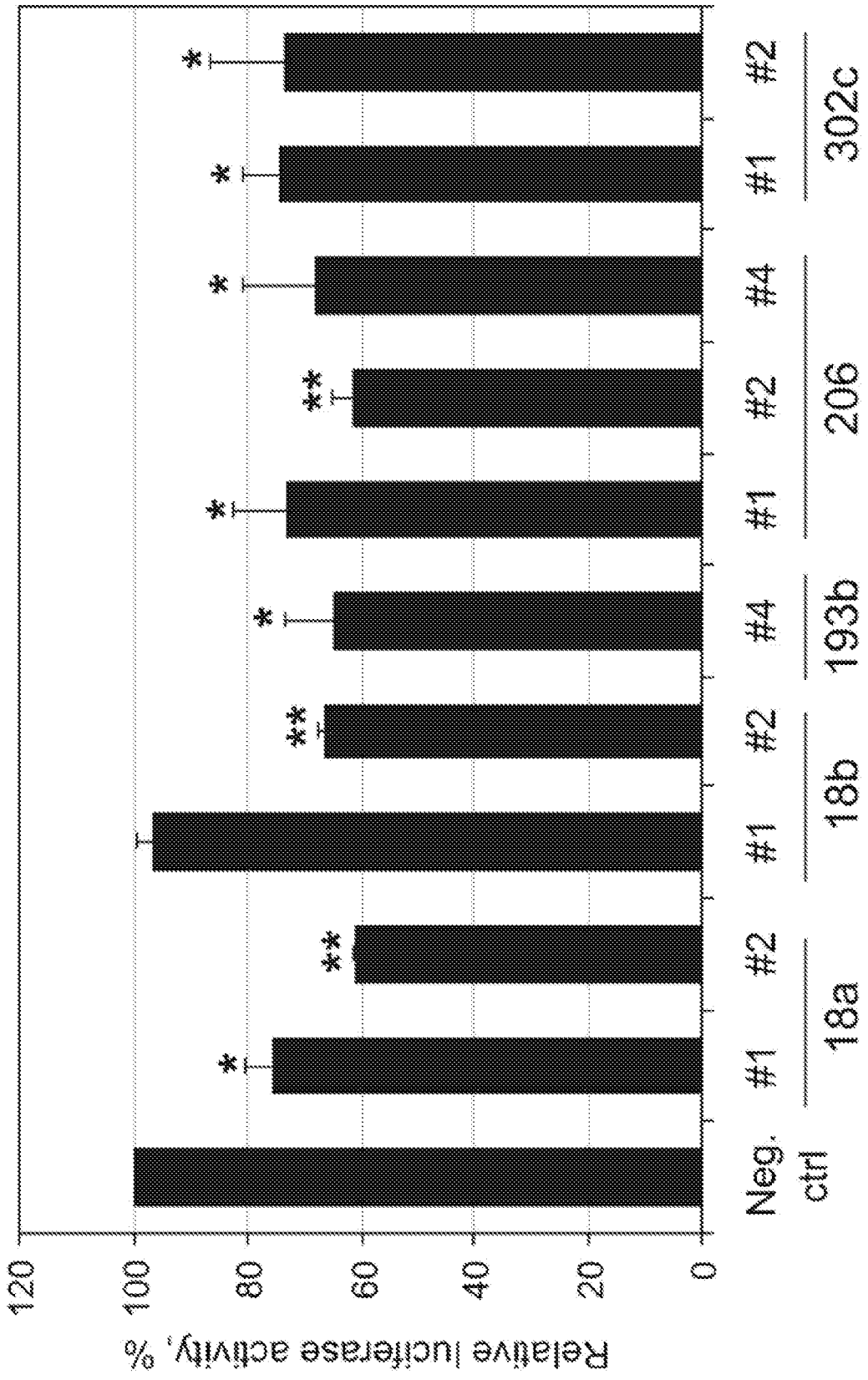
2.0k
3.0k
4.0k
ER#1_13-961
206
18a/b
302c
ER#2_911-2050
206
206
18a/b
302c
ER#3_2032-3222
ER#4_3201-4243
206
193b

Figure 5A


Relative luciferase activity, %
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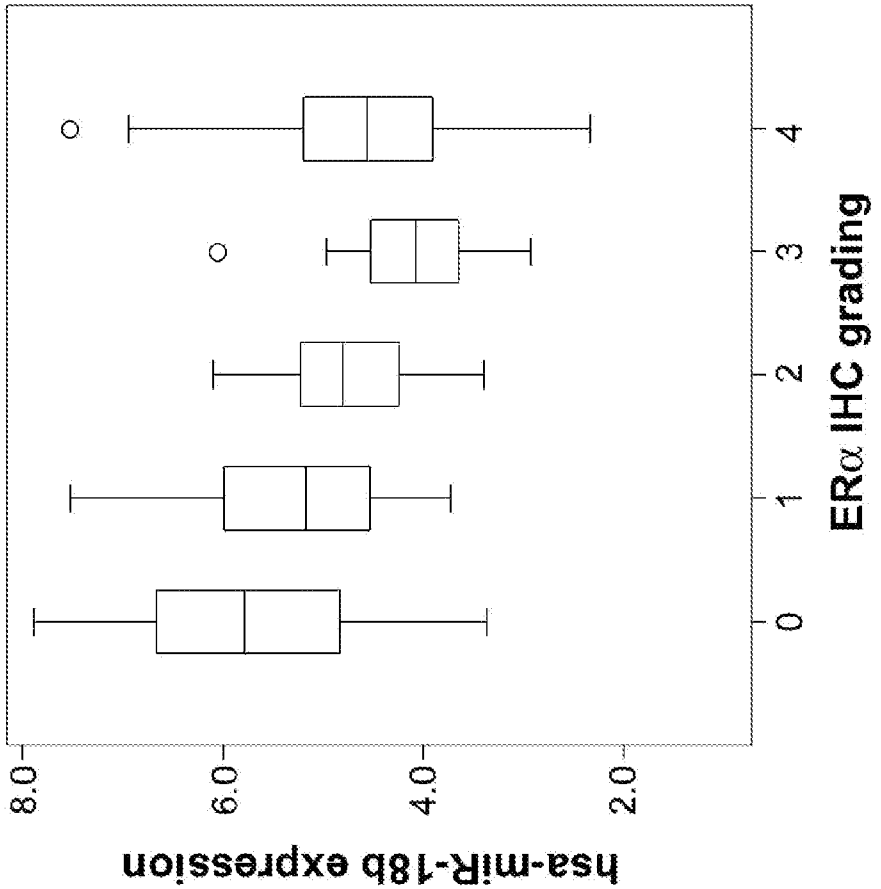
100
80
60
40
20
0
Neg. ctrl
18a
#1
#2
18b
#1
#2
193b
#4
206
#1
#2
#4
302c
#1
#2
*
**
*
**
*
**
*
*
*
*

Figure 5B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6.0
4.0
2.0
0
1
2
3
4
ERα IHC grading

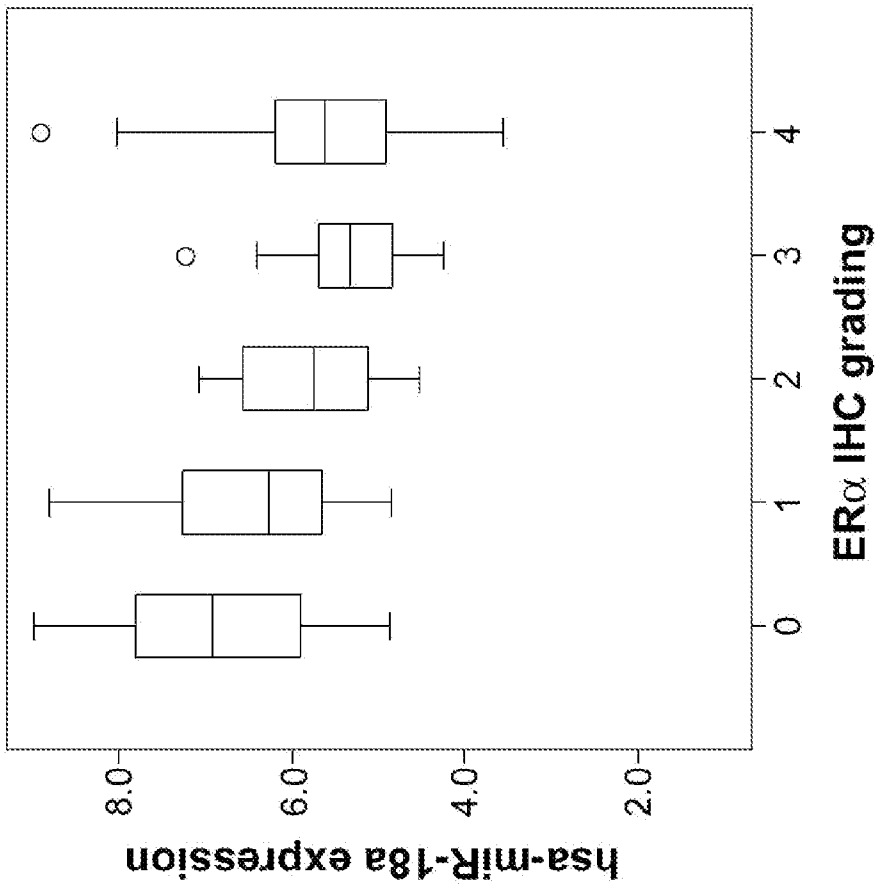
hsa-miR-18a expression
8.0
6.0
4.0
2.0
0
1
2
3
4
ERα IHC grading

Figure 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2010/050137

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113
ADD. A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/014008 A2 (UNIV JOHNS HOPKINS [US]; MENDELL JOSHUA T [US]; WENTZEL ERIK A [US]; T) 31 January 2008 (2008-01-31) claims 6,36 page 31, lines 16-22 examples 1-5	1-13
A	WO 2009/004632 A2 (YEDA RES & DEV [IL]; PILPEL YITZHAK [IL]; SHALGI REUT [IL]; OREN MOSHE) 8 January 2009 (2009-01-08) page 4, line 25 - line 28 page 19 - line 31 claim 11 page 230, line 19 - line 20	1-13

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2010	15/07/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Rodrigo-Simón, Ana

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2010/050137

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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