University of Siena

PhD in Oncology and Genetics

Retinoic acid and breast cancer: how to improve the therapeutic effect on the basis of molecular knowledge

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SUMMARY

INTRODUCTION

Breast cancer

Breast cancer is the second leading cause of cancer deaths in women and is the most common cancer among women. Extensive efforts have been made to characterize the disease and both molecular and histopathological features have been identified and associated with the clinical outcome (Sims *et al.*, 2007). Unfortunately, despite the improvement introduced by target therapy to the more generic chemotherapy in the adjuvant setting, still different subsets of patients are excluded from any benefit and recurrence represents a significant trait even in those patients that initially respond to therapies (KaKarala and Wicha, 2008). Breast cancer, for this reason, is considered a highly heterogeneous group of cancers arising from different cell types and each having its own clinical implications.

Retinoids (vitamin A and related molecules) are biologic agents that have demonstrated, in preclinical and clinical models, potent activity in the prevention and treatment of a variety of malignancies. These agents have been proposed in the adjuvant treatment of breast carcinoma for the ability to inhibit growth and induce morphological or phenotypic differentiation of the breast carcinoma cell lines (Paik *et al.*, 2003, Yang *et al.*, 2002).

Breast cancer classification

A better understanding of breast tumor heterogeneity and the nature of tumor-propagating cells requires delineation of the mammary epithelial subtypes that reside within normal human breast tissue. Analogous to the paradigm established by the hematopoietic compartment, there is increasing evidence for the existence of a differentiation hierarchy in the adult mammary gland. Mammary stem cells (MaSCs) are presumed to be important for both organ development and maintaining tissue homeostasis. These cells give rise to mature epithelium of either the luminal or myoepithelial lineage via a series of lineage-restricted intermediates. The luminal lineage can be further subdivided into ductal and alveolar luminal cells that line the ducts and constitute the alveolar units that arise during pregnancy, respectively. In contrast, myoepithelial cells are specialized,

contractile cells located at the basal surface of the epithelium adjacent to the basement membrane (Fig.1). The profound expansion of mammary epithelium that occurs during puberty and pregnancy further implicates a stem-like cell with remarkable regenerative capability (Visvader, 2009).

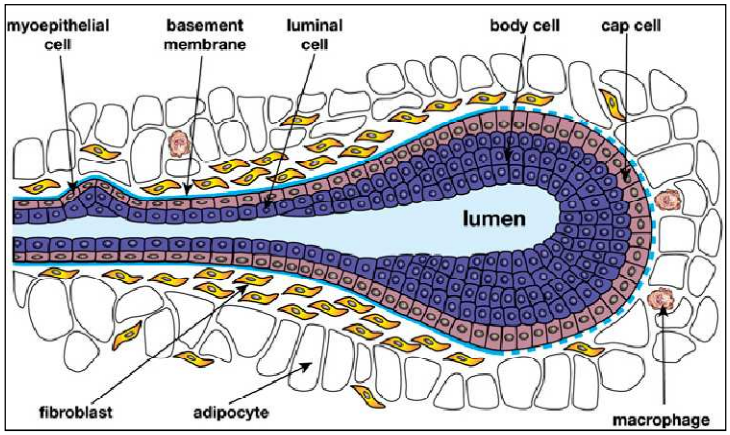

Figure 1: Schematic representations of a terminal end bud (TEB) in the mammary gland during puberty TEB. (Visvader, Genes Dev, 2009).

In recent years, microarray analyses of protein-encoding gene expression among different breast cancers have demonstrated the existence of fundamentally different types of cancer (Hu *et al.*, 2006 and Peppercorn *et al.*, 2008). The differences in tumor subtypes are hypothesized to reflect different mutation profiles, as well as differences in the cell of origin (Perou *et al*., 2000; Gusterson *et al*., 2005). These molecular analyses have revealed the presence of at least five subtypes, based on luminal or basal markers (e.g. cytokeratins and cell-cell and cell-matrix adhesion proteins) and on expression of HER2.

The most important determinants of these subtypes being the presence or absence of the Estrogen Receptor α (ERα) or the Progesteron Receptor (PR), or the amplification/over expression of the Her2/ERBB2 locus.

The *luminal A* subtype, which is the most commonly diagnosed form of breast cancer, is typically of low grade (grades 1 or 2), is ERα positive, PR positive, HER2 negative, and responds well to selective estrogen receptor modulators (SERMs) (e.g. tamoxifen) and aromatase inhibitors and has a good prognosis.

A subset of luminal A cancers can be more precisely categorized as *luminal B*, which respond to SERMs and aromatase inhibitors but have higher levels of proliferative indices, are of higher grade (grades 2 and 3), and have a significantly lower frequency of relapse-free survival than do luminal A cancers.

The *basal-like* subtype is very heterogeneous and comprises 15%–20% of breast cancers (Gusterson, 2009). This subset expresses basal-specific markers, and is typically a high-grade tumor (grade 3) that does not express ERα, PR, or HER2, although a significant percentage over-expresses the epidermal growth factor receptor (EGFR) (Hoadley *et al.*, 2007). Basal-like tumors are responsive to chemotherapy, as opposed to current "hormonal" therapies. This subset is associated with breast cancers that develop in women harboring a BRCA1 mutation (Peppercorn *et al.*, 2008) and, in general, has a higher risk of recurrence than do luminal A cancers.

The fourth subset of breast cancer, the HER2 subset, displays a robust expression of HER2, usually due to gene amplification. These cancers are of high grade (grade 3), occur at an earlier age than do other subtypes, do not express ERα or PR, and have a lower probability of relapse-free survival than do luminal A cancers. This subset of patients can respond to anti-HER2 therapies (e.g. trastuzumab) in conjunction with chemotherapy.

The *claudin-low* subtype of receptor-negative cancers expresses low levels of genes involved in tight junctions, cell–cell adhesion, and luminal genes, including potential Gata-3 target genes (Herschkowitz *et al*., 2007). Interestingly, this subclass is also characterized by expression of endothelial and lymphocytic markers and has mesenchymal features. Metaplastic breast cancers, a subtype of the basal-like group that is largely chemoresistant, were reported recently to have a distinct molecular profile that most closely resembles that of the claudin-low subgroup and is enriched for epithelial - mesenchymal - transition (EMT) signature genes (Hennessy *et al*., 2009).

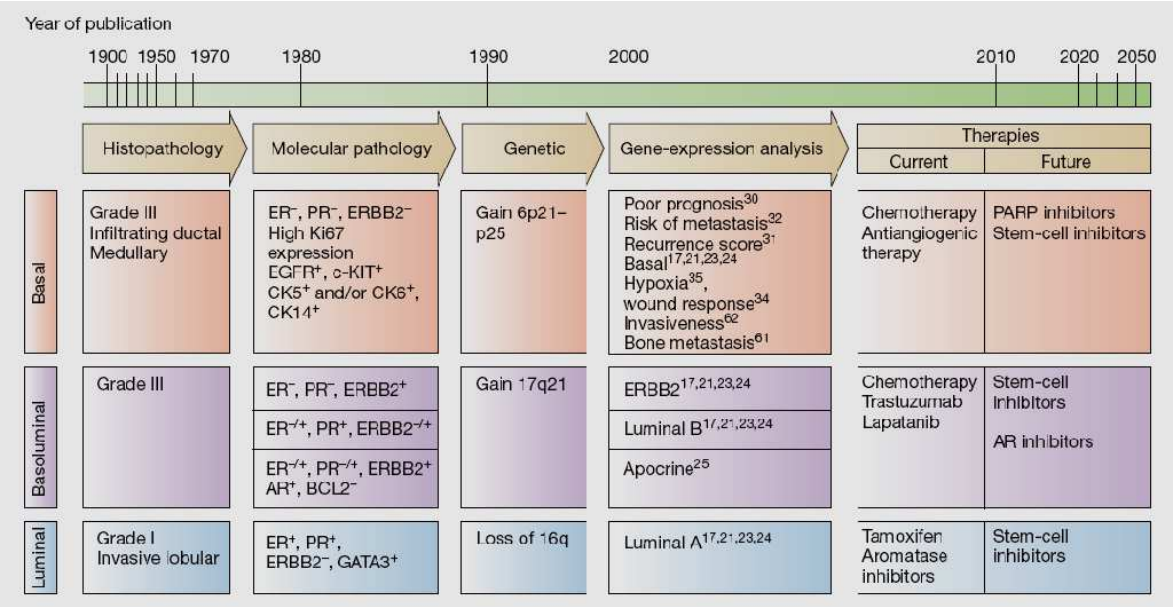

Figure 2: Comparison of the histopathology, molecular pathology, genetic and gene-expression analysis methods used to delineate breast cancer tumor subtypes and suggested current and future therapies in a historical context. Abbreviations: AR, androgen receptor; BCL2, B-cell CLL/lymphoma 2; CK, cytokeratin; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERRB2 (HER2), human epidermal growth factor Receptor 2PARP, poly (ADP-ribose) polymerase; PR, progesterone receptor. (Sims *et al*., 2007).

The recent derivation of specific gene signatures for human MaSC-enriched, luminal progenitor, mature luminal, and stromal populations has provided insight into potential target cells for the different breast tumor subtypes (Fig.3). Interrogation of the breast cancer subtype gene sets with the different mammary epithelial signatures unexpectedly revealed that the basal-like group shares a striking similarity with the luminal progenitor gene signature. This finding has profound implications for the basal subtype of cancer, as the stem cell has been presumed to be the cell of origin for these breast cancers (Visvader, 2009) (Fig.3).

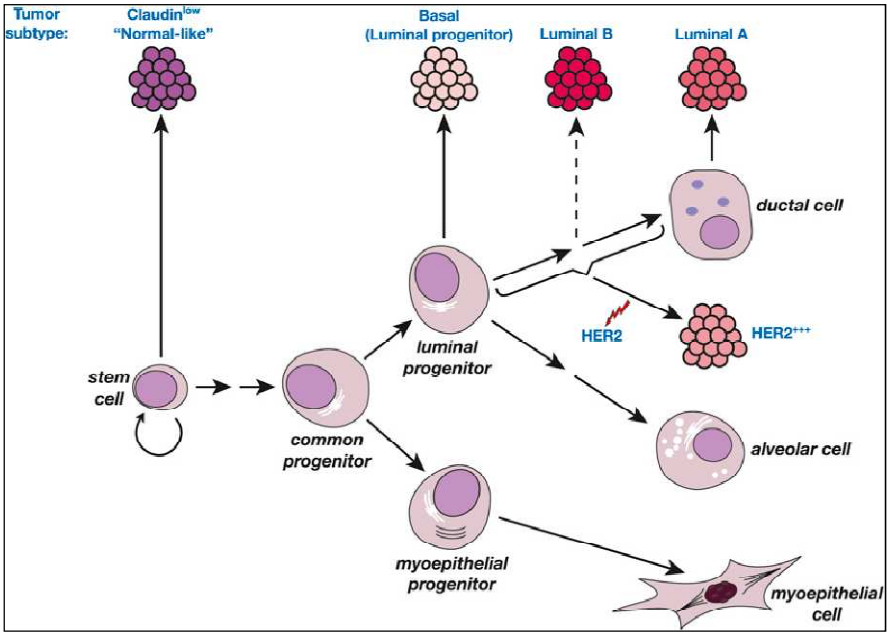

Figure 3: Schematic model of the human breast epithelial hierarchy and potential relationships with breast tumor subtypes. The different tumor types are shown together with their closest normal epithelial counterpart based on gene expression analyses. The luminal progenitor subtype may be a more appropriate name for basal tumors. The HER2 subtype could originate through amplification of the HER2 locus in a target cell restricted to the luminal cell lineage. (Visvader, 2009).

As described above, the presence or not of ERα or PR, or the amplification/over expression of the Her2/ERBB2 locus seem to be the most important molecular factors of these subtypes.

Estrogen Receptor

Estrogen Receptor (ER) is a member of the nuclear steroid hormone family of intracellular receptors which is activated by the hormone 17β-estradiol (E_2) (Dahlman-Wright *et al.*, 2006). The main function of ER is as a DNA-binding transcription factor which regulates gene expression (Levin, 2005).

There are two different forms of ER, referred as α and β, each encoded by a separate gene. The α isoform is encoded by the ESR1 and the β isoform is encoded by the ESR2 gene (Cowley *et al.*, 1997). There is significant overall sequence homology among the two isoforms (Hall, Couse and Korach; 2001). ESR1 is encoded on chromosome 6 (6q25.1) and ESR2 is encoded on chromosome 14 (14q) (Menasce *et al.*, 1993; Sluyser *et al.*, 1988). Both ERs are widely expressed in different tissue types, however, there are some differences in their expression patterns (Couse *et al.*, 1997). ERα is expressed in endometrial, breast cancer cells, ovarian stromal cells and in the hypothalamus. ERβ is

expressed in kidney, brain, bone, heart, lungs, intestinal mucosa, prostate, and endothelial cells. The ERα proteins are regarded as being cytoplasmic receptors in their unliganded state, but it has shown that a fraction of the ERα resides in the nucleus of ER-negative breast cancer epithelial cells (Htun *et al.,* 1999). A specific domain, the helix 12 domain, determines the interactions with co-activators and co-repressors thereby affecting the respective agonist or antagonist effect of the ligand (Ascenzi, *et al.*, 2006, Bourguet, *et al.*, 2000).

Two main pathways of estrogen receptor alpha activation by estrogens have been described: the genomic and the non genomic pathway (Pietras and Márquez-Garbán, 2007) (Fig.4).

In the genomic pathway, estrogen binds ER to promote dimerization and phosphorylation of the receptor (Weigel, 1996). This allows the translocation of ligand-activated ERs to the nuclei for regulating gene transcription. This transcriptional control is carried out by binding to DNA at a sequence containing either full estrogen response element (ERE) site or an ERE half site adjacent to the binding site for another transcription factor like Sp1. An additional mechanism involves gene regulation at alternate response element through protein-protein interactions with other transcription factors (AP-1, CREB, NF-B) (Fig.4A, Kalra *et al*., 2008).

Activated ERs control also mitochondrial gene transcription by binding to ERE like sequences (mtERE) leading to modulation of mitochondrial functions including metabolism, oxidative stress and apoptosis. (Fig.4B, Kalra *et al*., 2008).

The non-genomic pathways of estrogen action involves protein-protein interactions and do not require direct ER binding to DNA (Björnström and Sjöberg, 2005). Membrane localized ERs are G-protein coupled receptors that activate various protein kinase pathways interacting with transmembrane growth factor receptors such as EGFR, HER2, and insulin-like growth factor receptor I (IGFR1) and other signaling molecules, including components of the ras-MAPK and phosphatidylinositol 3-kinase (PI3K)/AKT pathways, Shc, Src kinases, Janus-activated kinase/signal transducer and activator of transcription signaling, nitric oxide synthase (NOS), and G-proteins (Pietra *et al*., 2001; Pedram *et al*., 2007; Osborne and Schiff, 2005). (Fig. 4C, Kalra *et al*., 2008).

These signal transduction cascades in turn regulate functions of many transcription factors resulting in modulation of expression of a number of genes involved in cell proliferation, survival, apoptosis and inflammation (Kalra *et al*., 2008).

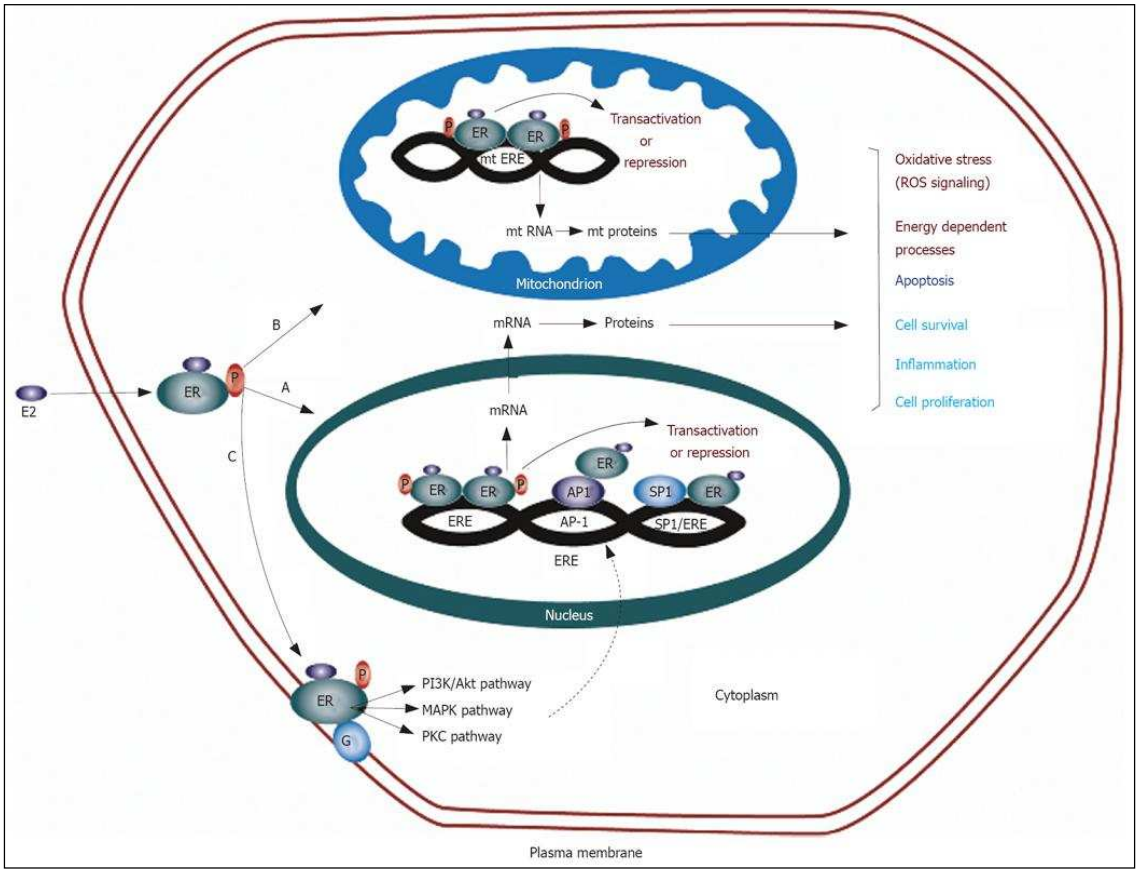

Figure 4: Genomic and non-genomic functions of estrogen mediated by estrogen receptors (ERs) localized in different sub-cellular organelles of a cell, (Kalra et al., 2008).

ERα is expressed in around 70% of breast cancer cases, that are referred to as "ER-positive" (ERα +) tumors. Binding of estrogen to ER stimulates proliferation of mammary cells, with the resulting increase in cell division and DNA replication and increases mutation rate. This causes disruption of the cell cycle, apoptosis and DNA repair processes eventually leading to tumor formation. Additionally, estrogen metabolism leads to the production of genotoxic by-products that could directly damage DNA, resulting in point mutations (Deroo and Korach, 2006). ERα expression is associated with more differentiated tumors, while evidence that ERβ is involved is controversial (Herynk and Fuqua, 2004). Different versions of the ESR1 gene have been identified (with single-nucleotide polymorphisms) and are associated with different risks of developing breast cancer (Deroo and Korach, 2006).

Patients with high levels of ER are treated with endocrine therapy (Normanno *et al*., 2005). Endocrine therapy for breast cancer involves Selective ER Modulators (SERMS), which act as ER antagonists in breast tissue or aromatase inhibitors, which work by inhibiting the action of the enzyme aromatase, which converts androgens into estrogens (Osborne, 1999).

Modulators of ER activity represent the first example of targeted anti-neoplastic therapy. Tamoxifen, a non-steroidal serum estrogen receptor modulator (SERM), was the first ER-targeted agent to be approved for clinical use. Tamoxifen is a competitive inhibitor of estrogen binding to the ER, and induces a receptor conformational change such that ER transcriptional activity is repressed. Raloxifene, which has anti-estrogenic behavior, has been used as a preventative chemotherapy for women judged to have a high risk of developing breast cancer (Oseni *et al*., 2008). Other ER modulators used clinically for the treatment of ER-positive breast cancers include aromatase inhibitors (Fabian and Kimler, 2005) and steroidal anti-estrogens such as ICI 182,780 (Faslodex) (Buzdar *et al.*, 1998).
The breast cancers that show low or absent ER expression display primary resistance to ER-targeted therapies. A subset of ER-positive breast cancers also demonstrates primary resistance, while the majority of tumors that initially respond to ER modulators eventually acquire resistance. Hence, endocrine resistance is a major clinical problem in the treatment of breast cancer. ER expression and function are generally intact in endocrine-resistant breast tumors, as supported by data showing full DNA binding capability of ER with subsequent transcriptional activity in resistant cells (Johnston *et al.*, 1997). Mechanisms leading to the development of endocrine resistance are not completely understood, but current data strongly supports a role for growth factor receptor cross talk to the estrogen receptor.

Progesterone Receptor

The progesterone receptor (PR) also known as NR3C3 (nuclear receptor subfamily 3, group C, member 3), is an intracellular steroid receptor that binds progesterone. PR is encoded by the PGR gene which lies on chromosome 11 (11q22) (Law *et al.*, 1987). This gene has two main forms, A and B that differ in their molecular weight (A: 94kD and B: 114kD) (Horwitz and Alexander 1983). These two isoforms are transcribed from distinct, estrogen-inducible promoters within a single-copy PR gene; the only difference between them is that the first 164 amino acids of B are absent in A (Giangrande and McDonnell, 1999). PR is expressed in reproductive tissue and has important roles in folliculogenesis, ovulation, implantation and pregnancy (Gadkar-Sable *et al*., 2005). Estrogen is necessary to induce the progesterone receptors (PRs) activity (Horwitz, Koseki and McGuire, 1978). PRs become hyperphosphorylated upon binding of the steroid ligand. PR phosphorylation is complex, occurring in different cellular compartments and perhaps requiring multiple serine kinases (Takimoto and Horwitz, 1993). After progesterone binds to the receptor,

restructuring with dimerization follows and the complex enters the nucleus and binds to DNA. There, transcription takes place, resulting in formation of messenger RNA that is translated by ribosomes to produce specific proteins (Edwards *et al*., 1995, Li and O'Malley, 2003).

About 65% of ER-positive breast cancers are also PR-positive and about 5% of breast cancers are ER-negative and PR-positive. If cells have receptors for both hormones or receptors for one of the two hormones, the cancer is considered hormone-receptor positive.

Co-regulators of PR either enhance or suppress transcription activity and thereby modulate the function of the PR. Nuclear receptor co-repressor, BRCA1 and Ubiquitinactivating enzyme 3 suppress PR transcription activity (Fan *et al*., 2002; Gao and Nawaz 2002). A mutation or change in expression of the co-regulators affects the normal function of the PR and may disrupt the normal development of the mammary gland, thereby leading to breast cancer (Gao and Nawaz 2002).

HER2/Neu

HER2/neu (also known as ErbB-2, ERBB2) stands for "Human Epidermal growth factor Receptor 2" and is a member of the ErbB protein family, more commonly known as the epidermal growth factor receptor (EGFR) family and encodes for a 185 Kd glycoprotein. The EGFR family consists of four trans-membrane receptor tyrosine kinases, EGFR (HER1 or ErbB-1), HER2/*neu* (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4), involved in signal transduction pathways that regulate cell growth and proliferation (Zhou and Hung 2003).

In general, each receptor protein is composed of an extracellular binding domain, a transmembrane lipophilic segment and an intracellular tyrosine kinase domain with a regulatory carboxyl terminal segment. While HER1, HER2, and HER4 are all intact receptors, HER3 has an inactive tyrosine kinase domain. A soluble ligand has been identified for all members of the HER family except for HER2. (Mendelsohn and Baselga, 2000; Hynes and Lane, 2005). All of these receptors are activated by dimerization, either with an identical receptor (homodimerization) or with a different receptor of the same family (heterodimerization). Normally, HER2 activation occurs when a ligand binds to another HER family member heterodimerized with HER2. Ligand-binding activates the intracellular tyrosine kinase domain, which results in activation of downstream targets through phosphorylation. As illustrated in figure 5, these targets include pathways involved

in cell proliferation (via Ras/Raf/MAP-kinase) and survival (via PI3-Kinase/Akt) (Lin and Winer, 2007).

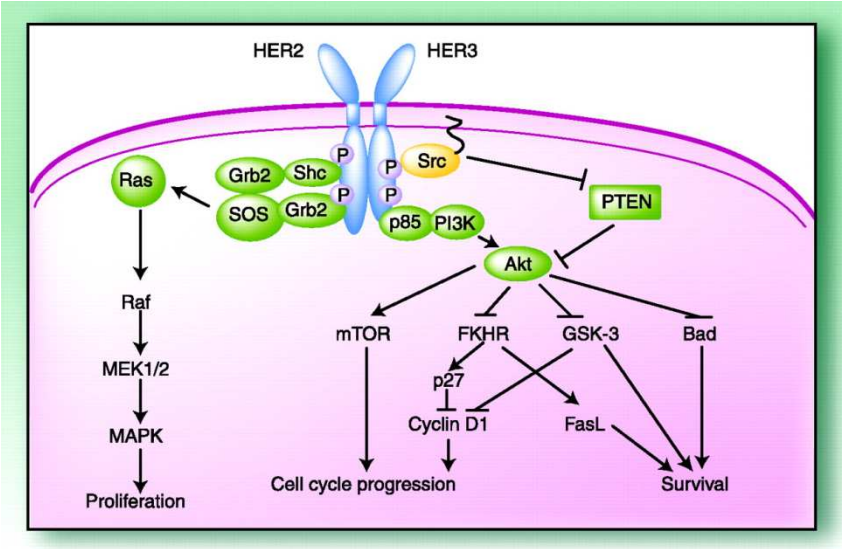

Figure 5: The HER2 signaling pathway. Ligand binding induces dimerization, leading to activation of the intracellular tyrosine kinase. On auto- and cross-phosphorylation of the receptor complex, key downstream effectors are recruited. This figure illustrates a HER2-HER3 heterodimer, but HER2 can also form homodimers or heterodimerize with other members of the HER2 family. FKHR, forkhead in rhabdomyosarcoma; Grb2, growth factor receptor-bound protein 2; GSK-3, glycogen kinase synthase-3; MAPK, mitogen-activated protein kinase; mTOR, molecular target of rapamycin; PI3K, phospatidyl-inositol 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SOS, son-of-sevenless guanine nucleotide exchange factor (Lin and Winer, 2007).

Her2 is an attractive therapeutic target in breast cancers because of the tight correlation between over-expression and poor prognosis, and because normal cells express relatively low levels of HER2. Indeed, approximately 20–30% of newly diagnosed breast cancers are found to over-express HER2/*neu*. Amplification of this gene, located on chromosome 17, with concomitant over-expression, is associated with a more aggressive form of malignancy and poorer outcome (Slamon *et al.*, 1987). Since this relationship with outcome was first elucidated in 1987, interest in HER2/*neu* has largely been focused on the development of targeted therapy for HER2/*neu*-positive patients. This interest culminated in the development of the anti-HER2/*neu* monoclonal antibody trastuzumab (Herceptin). It was approved by the United States Food and Drug Administration (U.S.F.D.A.) in 1998 for clinical use against metastatic breast cancers that overexpress HER2. Trastuzumab targets the extracellular domain of the HER2/*neu* molecule, (Gschwind *et al.*, 2004) and has been demonstrated to significantly improve disease-free survival when given in conjunction with chemotherapy (Piccart-Gebhart *et al.*, 2005 and Romond *et al.*, 2005)

The Retinoids

Retinoids are formed from dietary vitamin A, particularly from eggs, milk, butter and fish-liver oils, and the provitamin β-carotene of plant origin. Dietary-derived all-*trans* RA (ATRA) is the main signaling retinoid in the body, and mediates its action through RAR–RXR heterodimers (retinoic acid receptors - retinoid X receptors). After uptake by the intestinal mucosa cells, retinol is esterified to retinyl esters before passing into the lymphatics and being transported through the blood in chylomycrons, from where they are taken up by the liver and stored. After mobilization, by cleavage of retinyl esters, retinol is oxidized to retinal and retinoic acid (RA). A very small proportion of plasma and tissue retinol (0.2-5%) is converted to all-*trans* RA (ATRA), the main activator of RA receptors (RARs), but not rexinoid receptors (RXRs). Other metabolites with signaling activity include 9-*cis* RA and 13-*cis* RA. 13-*cis* RA is a metabolite of ATRA that has weaker activity than all-*trans* and 9-*cis* RA in transcription assays. The precise origin of 9-*cis* RA, which binds to and activates transcription from RARs and RXRs, is less clear. It is still not proven that 9-*cis* RA acts as a physiological ligand of RXRs. Other endogenous ligands have also been described (De Urquiza *et al.*, 2000). The occurrence and physiological significance of other retinoids, such as the 4-oxo- and didehydro- derivatives, and 1,4-hydroxy- 4,14-*retro*retinol, are largely unknown (Buck *et al.*, 1991). Numerous synthetic retinoids are now available for experimental exploitation. So, for nearly every receptor isotype, selective agonists or antagonists exist and several three-dimensional structures of RAR and RXR ligand-binding domain (LBD) monomers and heterodimers, some bound to agonists and antagonists, have been solved (Bourguet *et al.*, 2000). Furthermore, ligands can be synthesized that selectively activate a subset of the functions of the natural ligands.

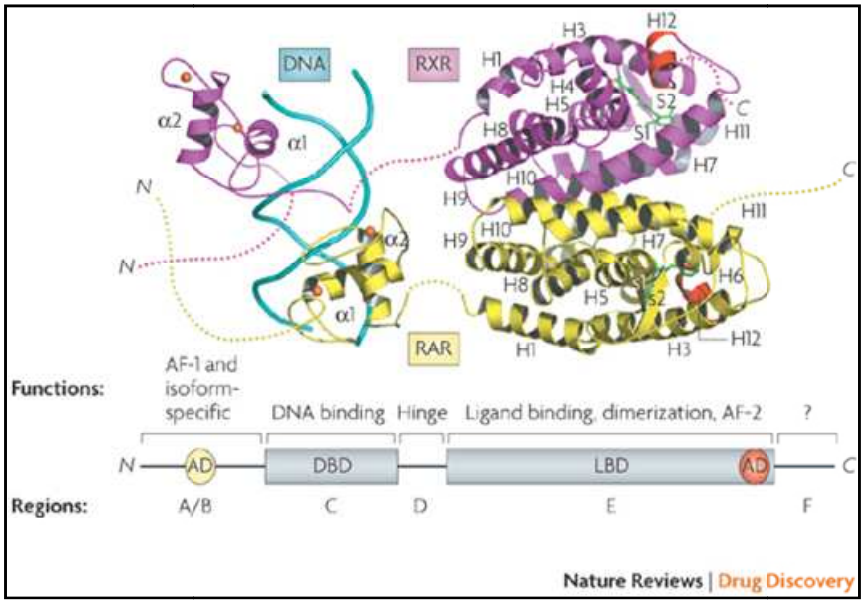

Figure 6: Like other nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) consist of five to six structural and functional domains denoted A to F. The evolutionarily conserved DNA-binding domains (DBDs) and ligand-binding domains (LBDs) are schematically represented by cylinders. The DBD is composed of two zinc-finger motifs and mediates sequence-specific DNA recognition. Transcriptional regulation is mainly mediated by the multifunctional LBD, which contains a ligand-binding pocket, a dimerization surface and a ligand-dependent transcriptional activation function (AF-2). The divergent regions are represented as thin tubes. The N-terminal A/B region contains a ligand-independent activation function (AF-1), the short D region corresponds to a linker allowing the proper orientation of DBDs and LBDs within DNA-bound dimers and finally the C-terminal F domain, which is present in RARs but not in RXRs and for which no clear function has been assigned. The activation domains (ADs) contain transcriptional activation functions that can activate transcription when fused to a heterologous DBD. In the LBD, this domain corresponds to critical residues of the most C-terminal helix H12. The structure of the full-length RXR–RAR heterodimer has been virtually reconstituted from the crystal structures of the DNA-bound and 9-*cis*-retinoic acid-bound RXR–RAR DBD (Protein Data Bank code 1dsz) and LBD (Protein Data Bank code 1xdk) heterodimers, respectively. Dotted lines denote domains with unresolved structures. Helices are represented as ribbons and labelled from H1 to H12 (LBD) or α1 and α2 (DBD). Helix H12 (AF-2 AD) is shown in red in each subunit. The short LBD β-strands are labelled S1 and S2. 9-*cis*-retinoic acid in RAR and RXR LBDs is represented by green sticks lines. The orange spheres in the DBD indicate Zn^{2+} ions. (de Lera *et al*., 2007).

Retinoids Receptors

Retinoid receptors are nuclear, ligand-regulated transcription factors of the steroid/thyroid hormone receptor superfamily, that are activated *in vivo* by binding vitamin A, derived retinoids including ATRA, and 9-*cis*-RA [Germain *et al*, 2006 (A) and Germain *et al*, 2006 (B)]. Essential roles for retinoid receptors in growth, reproduction, retinal development, and vision have been inferred from the consequences of vitamin A deficiency, and from the analysis of retinoid receptor–deficient mouse strains. As described above there are two distinct classes of retinoid receptor, retinoic acid receptors

(RAR) and retinoid X receptors (RXR), each of which comprises three isotypes encoded by separate genes, designated α, β and γ, as well as several isoforms generated by alternative splicing or promoter usage [Germain *et al*, 2006 (A) and Germain *et al*, 2006 (B)] RARs and RXRs are transcription factors that act mainly, if not exclusively, as RAR–RXR heterodimers (Kastner *et al.*, 1997). These heterodimers have two distinct functions: first, they modulate the frequency of transcription initiation of target genes after binding to RA response elements (RAREs) in their promoters; and second, they affect the efficiency of other signaling pathways ('crosstalk') by mechanisms that remain elusive. RARs and RXRs are distinguished by their differential affinities for naturally occurring ligands. Although 9-*cis*-RA is a ligand for both groups, only RARs bind ATRA. Furthermore, RXRs and RARs differ in the spectrum of proteins with which they interact, both at the level of nuclear receptors, and in terms of transcriptional co-repressors. Functional activity of both RARs and RXRs requires dimerization with a member of the nuclear nonsteroidal receptor family. However, although RARs dimerize predominantly with RXRs, in marked contrast, RXRs can interact with many different factors, indicative of their role as the master heterodimerizing member of the nuclear receptor superfamily. Thus, in addition to RARs, RXRs also dimerize with thyroid hormone receptors (TRs), vitamin D receptor (VDR), peroxisome proliferator–activated receptors (PPARs), liver X receptors (LXRs), farnesoid X receptor (FXR), pregnane X receptor (PXR), and constitutively activated receptors. All of these nuclear receptors require RXR as a heterodimerization partner in order to regulate gene transcription (Howe, 2007).

Retinoid receptors regulate transcription via interaction with RAR elements and RXR elements in target gene promoters, and subsequent recruitment of transcriptional coactivators. Because RXRs are obligate heterodimerization partners for a multitude of nuclear receptors, retinoids have the potential to regulate the activity of entire regulatory networks. Response specificity is achieved through several levels of control. Firstly, receptor responsiveness is defined by the nature of the ligand: each retinoid receptor has a unique affinity for individual retinoids. As mentioned above, natural ligands for retinoid receptors are derived from vitamin A and include 13-*cis*-RA, 9-*cis*-RA, and ATRA. Of these, ATRA selectively binds to RARs, but 9-*cis*-RA is a pan-retinoid that binds to both RARs and RXRs, albeit with differing affinities. 13-*cis*-RA gets isomerized to ATRA, and thus, is functionally equivalent.

In the absence of ligands for RAR–RXR dimers, or in the presence of some antagonists, the receptors' target genes are repressed. This is due to the possibly gene-specific (Jepsen

et al., 2000) recruitment of histone deacetylase (HDAC)-containing complexes that are tethered through corepressors (CoRs), such as nuclear receptor (NR) corepressor (NCoR) or silencing mediator for retinoid and thyroid hormone receptors (SMRT), to the non-liganded (apo-) RAR–RXR dimer. Retinoic acids do not act solely through the two subunits of the RAR–RXR heterodimer. RXR is a promiscuous heterodimerization partner for various NRs.

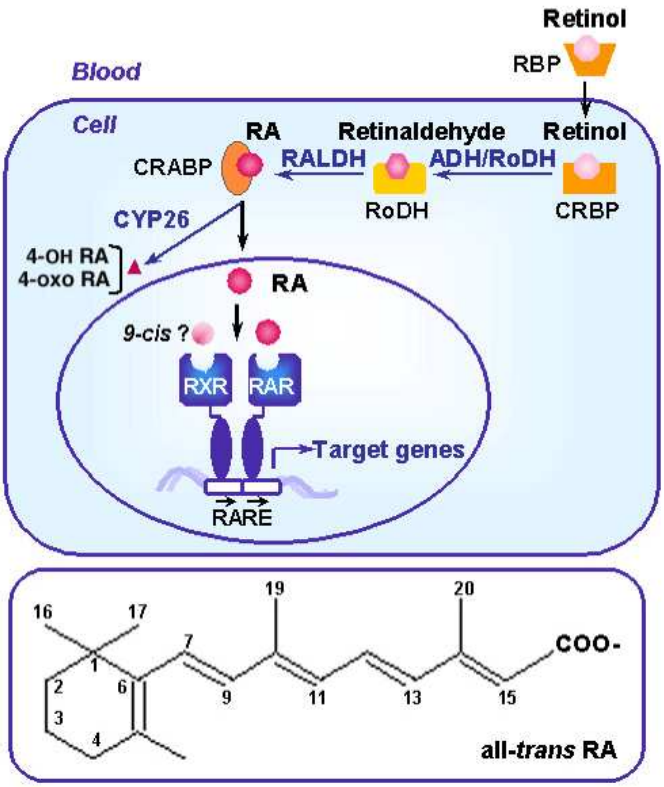

Figure 7: Scheme of the intracellular pathways involving retinoids and chemical formula of all-*trans* retinoic acid (below). CRBP: cellular retinol binding protein; CRABP: cellular retinoic acid binding protein; RARE: retinoic acid response element; RBP: retinol binding protein; (http://www.igbmc.fr/recherche/Dep_BCD/Eq_PDoll/DP3.html).

The Retinoids and cancer prevention mechanisms

Carcinogenesis is a chronic and multistep process, resulting from mutagenic damage to growth-regulating genes and their products, that ultimately leads to development of invasive or metastatic cancers (Weston, 1997). This transformation from normal through pre-neoplasia to overt malignancy results from defined steps including: A) initiation, where DNA damage occurs; B) promotion, where additional genetic and epigenetic changes augment prior genomic damage, and C) progression to locally invasive or distant metastatic

disease. Carcinogen exposure is hypothesized to form "fields" of altered cells long before invasive malignant disease is detected clinically (Slaughter *et al.*, 1953). Perhaps intermediate markers of carcinogenic changes at affected tissue sites will identify pre-neoplastic lesions that are likely to progress to a fully transformed phenotype. Alternatively, absence of these markers might indicate epithelial lesions that are unlikely to become malignantly transformed.

It is not yet known which individual or cassette of carcinogenic changes are rate-limiting in the maintenance or progression of pre-neoplastic lesions. Conceivably, these changes are distinct for each epithelial site or carcinogenic agent. Frequent genetic gains or losses are reported to occur at diverse sites, including the head and neck (Califano *et al.*, 1996), bladder (Sidransky *et al.*, 1992), colon (Kinzler *et al.*, 1996 and Fearon *et al.*, 1990), lung (Lonardo *et al.*, 1999; Rusch *et al.*, 1995; Albanell *et al.*, 1997; Wistuba *et al.*, 1999 and Park *et al.*, 1999), and others. Those carcinogenic pathways required for development or maintenance of the transformed phenotype at a tissue site may represent attractive therapeutic targets for cancer prevention. Changes in these pathways or of affected dominant oncogenes or recessive tumor-suppressor genes may prove useful to monitor response to clinical cancer prevention agents. The chronic and multistep nature of carcinogenesis provides a strong rationale for cancer prevention as an attractive therapeutic strategy to arrest or reverse one or more of these carcinogenic changes.

Strong clinical validation for clinical cancer prevention was provided through a randomized trial using the selective estrogen receptor modulator (SERM) tamoxifen in women at high risk for breast cancer development (Fisher *et al.*, 1998). In those women randomized to receive tamoxifen compared to controls, there was a highly statistically significant reduction in the risk of invasive and noninvasive breast cancers (Fisher *et al.*, 1998). This reduction was seen for hormone-sensitive breast cancer. Clinical benefits were not seen for hormone-resistant breast cancers. Based on these clinical findings, tamoxifen is now approved by the U. S. F. D. A. for breast cancer risk reduction in high-risk women. This breast cancer prevention trial will be built upon by analysis of other candidate prevention agents, including other SERMs that may have more favorable therapeutic or toxicity profiles than tamoxifen. Effective breast cancer prevention strategies are needed for hormone-resistant breast cancers. One approach taken to address this need is examination of the retinoid N-(4-hydroxyphenyl) retinamide (fenretinide, 4HPR) for prevention of a second breast malignancy in women with early breast cancer (Veronesi *et*

al., 1999). This 4HPR randomized trial reported a potential benefit in premenopausal women for reducing second breast cancers.

The carcinogenic steps of initiation, promotion, progression, and invasion or metastasis can be targeted by antiproliferative, differentiation-inducing, or pro-apoptotic agents, as reviewed (Hong *et al.*, 1997). Extensive epidemiological, preclinical, and clinical data point to an important role for retinoids in cancer chemoprevention.

These and other findings provided a basis for use of retinoids in clinical cancer prevention trials. Added support for a retinoid-based clinical chemopreventive approach stemmed from the successful retinoid treatment of premalignant lesions, inhibiting the progression of cancer from the premalignant to the malignant stage, such as oral leukoplakia (Hong *et al.*, 1986), cervical dysplasia (Meyskens *et al.*, 1994) and xeroderma pigmentosum (Kraemer *et al.*, 1988). Clinical trials reveal that retinoids are active in reducing some second primary cancers. For example, 13-*cis*-retinoic acid (13-cRA) reduces second aerodigestive tract tumors in patients with resected head and neck cancers (Hong *et al.*, 1990). Second primary lung cancers are reduced by retinol palmitate treatment of patients following resection of stage I lung cancer (Pastorino *et al.*, 1993). The acyclic retinoid, polyprenoic acid, inhibits second hepatocellular carcinomas after resection or ablation of primary liver cancer (Muto *et al.*, 1996).

Retinoids in oncology

Many factors can contribute to tumorigenesis, including inherited and acquired genetic changes, chromosomal rearrangements, epigenetic phenomena and chemical carcinogenesis. Retinoic acids can interfere with these events at several levels (Fig.8), their principal known actions being induction of differentiation and/or apoptosis of tumor cells, and inhibition of tumor promotion in chemically induced cancers. In keeping with their ability to regulate growth and induce differentiation throughout life, retinoids affect the growth of many tumor cell lines in culture.

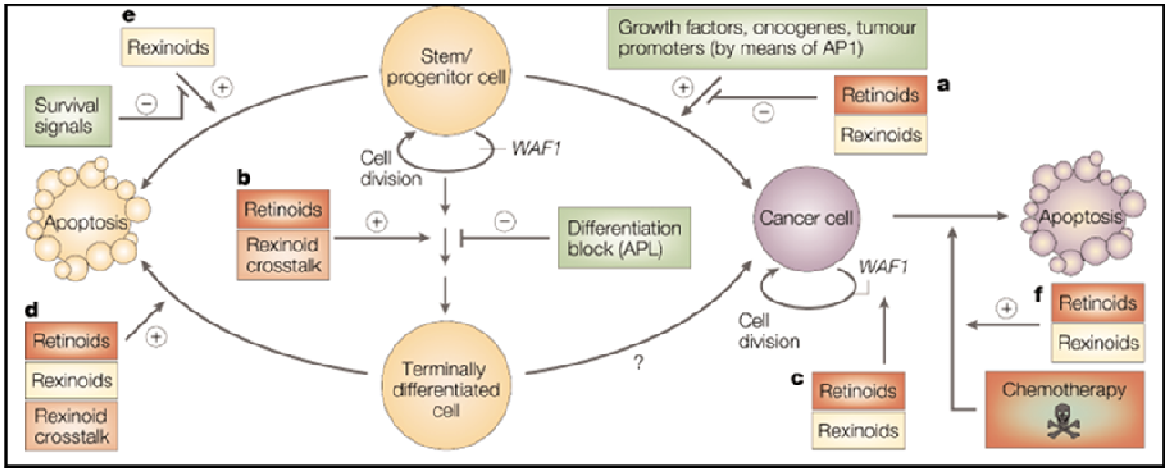

Figure 8: Retinoids and rexinoids can interfere with events leading to tumorigenesis at several levels. **a**: At least in skin, chemical carcinogenesis is blocked at the promotion step, probably because of the anti-AP1 activity of retinoids and rexinoids. Whether it is this activity that blocks chemical carcinogenesis in other systems remains to be established. **b**: Retinoids and rexinoids (through 'crosstalk' with other signaling pathways) are strong inducers of cell differentiation and might overcome a block in a differentiation pathway, such as the one induced by the action of fusion proteins in acute promyelocytic leukaemia (APL). **c**: Retinoid and rexinoid action is usually associated with a cell-cycle block in G1, frequently because of the increased expression of *WAF1*, a direct target gene of retinoid and vitamin D3 receptors. **d**: In myeloid cells, retinoid and rexinoid action or crosstalk between rexinoids and protein kinase A leads to post-maturation apoptosis. In some cell systems, the differentiation effect of retinoids has been dissociated from the apoptogenic effect of rexinoids on the differentiated cells. **e**: Under certain conditions, rexinoids can autonomously induce premature cell death; survival and differentiation signals rescue the cells from rexinoid apoptosis. **f**: Perhaps owing to activation of death receptors and/or their cognate ligands, retinoids and rexinoids, alone or in combination with chemotherapy ('sensitization'), can synergistically induce apoptosis. (Altucci and Gronemeyer, 2001).

Because of the above described properties, *ATRA* and other natural or synthetic derivatives, are promising agents for the treatment and prevention of cancer (Altucci and Gronemeyer, 2001; Altucci *et al.*, 2007; Garattini *et al.*, 2007). The most striking example of retinoic acid anticancer activity is the use of *ATRA* for the treatment of patients with acute promyelocytic leukemia (*APL*). More than 85% of *APL* patients can be induced into complete remission by *ATRA* in combination with anthracyclins (Degos and Wang, 2001). In this disease context, *ATRA* exerts its beneficial effect predominantly by a cyto-differentiating action inducing the leukemic blast to undergo terminal differentiation along the granulocytic pathway. Currently *ATRA* is the only example of clinically useful differentiating drug.

ATRA and derivatives are interesting agents not only for the treatment of certain types of leukemia but also in primary or secondary chemoprevention of solid tumors. Indeed, there is a wealth of data demonstrating that retinoids inhibit the progression of cancer from the premalignant to the malignant stage. Studies on chemically-induced skin carcinogenesis in

mouse demonstrating that the promotion step is Activating Protein 1 (*AP-1*)-repression-dependent suggest that retinoid-mediated interference with the latest pathway could account for their demonstrated chemopreventive activity (Huang *et al*., 1997). Furthermore *RARβ* has been demonstrated to be a tumor suppressor, which is frequently lost or epigenetically silenced in various cancers, and its expression inversely correlates with tumor grade (Xu *et al*., 2007). Recently a new generation of retinoids known as atypical retinoids have been developed showing promising properties as anticancer drugs. Although these compounds are able to bind to and transactivate *RARs*, their activity does not fully explain their growth inhibitory and apoptogenic effects offering new and alternative pathways to counteract cancer development (Garattini *et al*., 2004). Collectively, this body of evidence supports the concept that retinoids are useful chemopreventive and chemotherapeutic agents directing the neoplastic cell to differentiate, arrest its growth, or undergo apoptosis. Unfortunately, promising preclinical studies were not consistently translated into clinical practice (Altucci *et al*., 2007; Fields *et al*., 2007).

Retinoids and Breast Cancer

One of the main problems associated with the clinical use of retinoids in oncology, is represented by the variability of the responses observed in different tumors and different subtypes within a single tumor (Fan *et al*., 2006; Perreard *et al*., 2006; Sørlie *et al*., 2006). The molecular determinants responsible for the sensitivity/resistance of the neoplastic cell to retinoids are generally unknown, although they are likely to include the complement of RARs, RXRs and accessory factors expressed (Rochette-Egly *et al*., 2009). Breast carcinoma is a paradigmatic example of heterogeneity in the responses to retinoids. Clinical data indicate that only pre-menopausal women benefit from the use of the synthetic retinoid, fenretinide, when the drug is used in an adjuvant setting (Veronesi *et al*., 1996; Veronesi *et al*., 2006; Decensi *et al*., 2007). It is well established that steroid hormones and their receptors, peptide growth factors, oncogenes and tumor suppressor genes play a crucial role in the neoplastic transformation of the breast (Keen and Davidson, 2004). Two of the main players are the Estrogen Receptor α (Yager and Davidson, 2006) and HER2/*neu*.

ERα as a retinoid target

Approximately 70-80% of all breast tumors express ERα. ERα-positive tumors tend to grow more slowly, are more differentiated, and are associated with better overall prognosis. Pre-clinical observations indicate that ERα + breast carcinoma cells are generally sensitive, while ERα - are generally resistant to the anti-proliferative activity of ATRA and derivatives (Rousseau *et al*., 2003; Rousseau *et al.*, 2004).

RARα is an estrogen-induced gene (Laganiere *et al*. 2005), and its expression in breast tumors has been shown to correlate with ER expression (Roman *et al*., 1993). Recently, the binding events of a tagged version of RARα were mapped, and RARα and ER were shown to share a subset of binding regions within the genome (Hua *et al*., 2009). Hua *et al*., concluded that RARα in the presence of its ligand can antagonize estrogen–ER function, and *viceversa*. Their hypothesis is that RARα and ER can, in some cases, share common *cis*-regulatory elements, and that the two nuclear receptors compete for transcriptional activity.

Targeting of *ERα* with *SERM* is currently one of the most successful type of preventive therapy for secondary breast malignancy. However the efficacy of this approach is limited by *de novo* and acquired resistance in treated patients (Yager and Davidson, 2006). Retinoids are of therapeutic interest because of their efficacy in preventing carcinogen-induced rat mammary cancer (Anzano *et al*., 1994; Gottardis *et al*., 1996) and their anti-proliferative effect towards breast cancer cells *in vitro* (Fontana, 1987; van der Burg *et al.*, 1993; van der Leede *et al*., 1995; del Rincon *et al.*, 2003). Moreover, as mentioned above, there is also clinical evidence that retinoids may be beneficial in breast cancer chemoprevention (Veronesi *et al.*, 2006; Lawrence *et al.*, 2001).

Her2 as a retinoid target

In breast cancer one of the most clearly defined biologic abnormalities with clinical relevance is HER2 overexpression, which is seen in 20%–30% of breast cancer cases. Amplification of this gene, located on chromosome 17, with concomitant over-expression, is associated with a more aggressive form of malignancy and poorer outcome (Slamon *et al*., 1987). As already mentioned, target therapy using the monoclonal antibody against Her2, trastuzumab, have proved to be successful. However, despite its efficiency in inducing high complete or partial remission rates, Trastuzumab treatment is associated

with induction of resistance in a large proportion of patients. Resistant patients benefit from second-line treatment with the tyrosine kinase inhibitor, Lapatinib, which targets Her2/Neu as well as others members of the receptor family (Frampton, 2009).

The HER2-targeted agent, lapatinib (Tykerb, GW572016, GlaxoSmithKline), was approved by the U. S. F. D. A. in 2007 for use against HER2-overexpressing breast cancers in combination with the chemotherapeutic agent capecitabine (Geyer *et al*., 2007). Lapatinib is a dual inhibitor of the EGFR and HER2 tyrosine kinase domains, and has shown efficacy in preclinical models of HER2-overexpressing breast cancer including trastuzumab-refractory cells (Ritter *et al*., 2007, Konecny *et al*., 2006, Nahta *et al*., 2007).

Identification of HER2/neu, and the subsequent development of targeted therapy for patients who over-express it, has revolutionized their management. Therefore, many research studies have focused on the area of chromosome 17 in which HER2/neu is located in order to identify other genes in the vicinity that could be helpful in the treatment of breast cancer. A minimal common region of amplification around HER2/*neu,* which spans between 280 and 746 Kb, and may contain more than 20 genes, has been identified (Johnston *et al.*, 1997 and Fuqua *et al.*, 2000). Furthermore, at least 40 genes have been located either within this minimal region or adjacent to it (Gee *et al.*, 2001). Among them there is also *RARA*, the gene coding for RARα that maps to chromosome 17 at a short distance (0.65 Mb) from the *ERBB2* locus (Fig.9). This evidence provides the rationale for testing retinoids in the specific genetic background of ERBB2/RARA amplification even in the absence of estrogen receptor, a classical determinant of breast cancer sensitivity. Indeed the lack of estrogen receptor expression found in many Her2/neu breast cancers could be compensated by RARA amplification in the supply for RAR alpha protein expression.

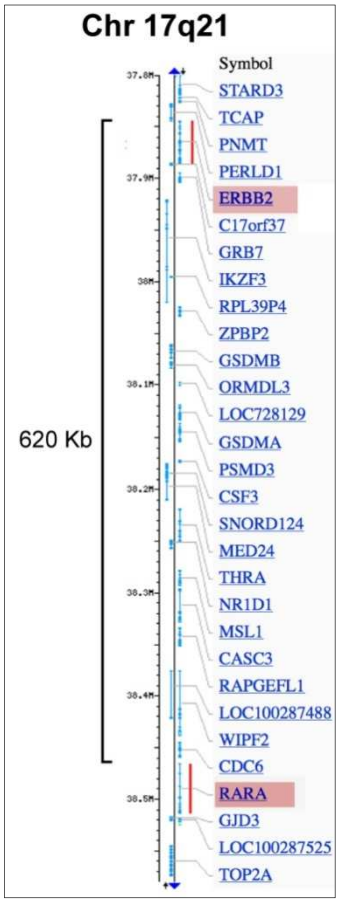

Figure 9: RARA, the gene coding for RARα maps at a short distance, 0.65 Mb, from the ERBB2 locus gene coding Her2, to chromosome 17.

AIM OF PROJECT

One of the main problems associated with the clinical use of retinoids in oncology, is represented by the variability of the responses observed in different tumors and different subtypes within a single tumor. The molecular determinants responsible for the sensitivity/resistance of the neoplastic cell to retinoids are generally unknown, although they are likely to include the complement of RARs, RXRs and accessory factors expressed. Clarification of the molecular and cellular mechanisms could give insight for a better and more efficient use of retinoic acid and derivates in the clinical of breast carcinoma.

The major aim of the research program of my PhD is to characterize the retinoid sensitivity/resistance in breast carcinoma and to define its molecular mechanism across the ERα and Her2 pathway. In particular we plan to:

1. Define the molecular mechanism at the basis of the observed association between ERα and retinoids in breast carcinoma. Indeed, ERα is considered a determinant of retinoids sensitivity.

2. Establish whether retinoids can be eligible as therapeutics in the management of Her2/neu-positive breast carcinomas alone or in combination with HER2-targeted therapeutics (lapatinib).

MATERIALS AND METHODS

Cell lines and chemicals

MCF-7, MDA-MB-231, MDA-MB-453, MDA-MB-361 , T47D, SKBR3, AU565, UACC-812, HCC-1569 cell lines were from the American Type Culture. Cells were grown in DMEM F12 medium (Invitrogen) containing 5% fetal calf serum (Lonza). Only during the experiments the cells were grown in DMEM F12 medium containing 5% charcoal stripped fetal calf serum (Lonza). The cells were grown without antibiotics at 37°C in 5% CO_2 atmosphere.

ATRA, Estradiol, Tetracycline, Puromycin were from Sigma Chemicals, Geneticin, Zeocin, Blasticidin were from Invitrogen. AM580, a RARα agonist, and CD437, a RARγ agonist, are been already described (Garattini *et al*., 2004 and Garattini *et al.*, 2007). Lapatinib (GW572016) is a tyrosine kinase inhibitor from LC Laboratories.

Cell growth, senescence and single-cell-motility

Cell growth was evaluated using MTT (Plumb, 2004) and senescence was determined with a β-galactosidase kit (Cell Signaling Technology). Single-cell-motility assays were performed on BSA coated substrate (Hirano *et al.*, 2008) using the Imaging Station Cell^R (Olympus) and the software IMAGEJ.

Generation of stable trasfectants

Generation of stable transfectants conditionally expressing ERα

For the production of MDA-MB-231clones conditionally expressing ERα we have used the T-REx™ System (Invitrogen). The system is a tetracycline-regulated mammalian expression system that uses tetracycline (Tet) resistance operon. Tetracycline regulation in the T-REx™ System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest. The system is based on a double transfection of a regulatory plasmid, pcDNA6/TR (Invitrogen) (Fig.10), which encodes the Tet repressor (*Tet*R) under the control of the human CMV and an

inducible expression plasmid for expression of the gene of interest under the control of the strong human cytomegalovirus immediate-early (CMV) promoter and two tetracycline operator 2 ($TetO_2$) sites promoter. For our purpose, we used, as the inducible expression plasmid, pcDNA4/TO/ERα (Fig.10) obtained from a sub-cloning. The ERα cds (*Estrogen receptor 1, ESR1,* NM_000125.2) was excised from pT-REx-DEST30 – Erα (ImaGenes GmbH, X, Germany) (Fig.10) in XhoI-ApaI position and sub-cloned in pcDNA4/TO (Invitrogen) (Fig.10). As a control expression plasmid we have used pcDNA4/TO LacZ (Invitrogen) (Fig.10), a construct containing the *LacZ* gene, which when co-transfected with pcDNA6/TR expresses the LacZ protein upon induction with tetracycline. The MDA-MB-231 cell line was transfected with pcDNA6/TR and the clonal transfectants were selected by addition of blasticidin (10µg/ml) in the culture medium for about two weeks. The cell line MDA-MB-231 pcDNA6/TR was used for the transfection of pcDNA4/TO/ERα and the clonal transfectants were selected by addition of geneticin (800µg/ml) in the culture medium for about one week.

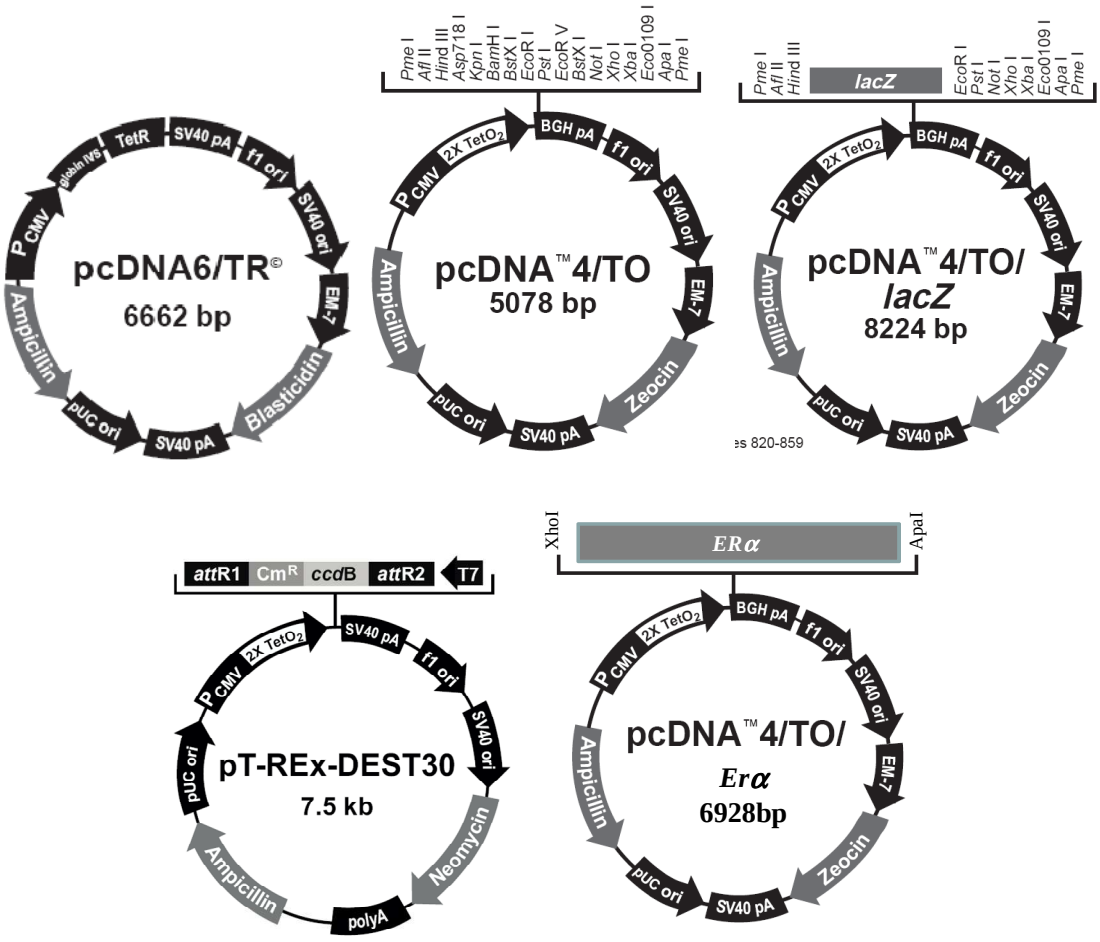

Figure 10: constructs used to generate the stable transfectants conditionally expressing ERα.

Generation of stable transfectants conditionally expressing shRNA against ERα

To obtain cellular clones inducibly expressing the shRNAs against ERα (MCF7 pcDNA6TR shERα) the pSuperior RNAi System (Oligoengine) was used. The system is based on the co-transfection of a plasmid constitutively expressing the Tet repressor (pcDNA6/TR, Fig.10) and a plasmid carrying the coding sequence for the transcription of the shRNA of interest (pSuperior Puro, Fig.11). The expression of the target shRNA depends upon an H1 promoter that is responsive to the Tet repressor. In the absence of tetracycline in the culture media, the Tet repressor binds to the promoter avoiding the transcription of the shRNA. In the presence of tetracycline the Tet repressor dissociates from the promoter and the H1 promoter gets activated expressing the shRNA of interest. The hairpin structure of the shRNA is then processed by cellular enzyme to produce the siRNA active in inhibiting the target protein expression (Fig.12).

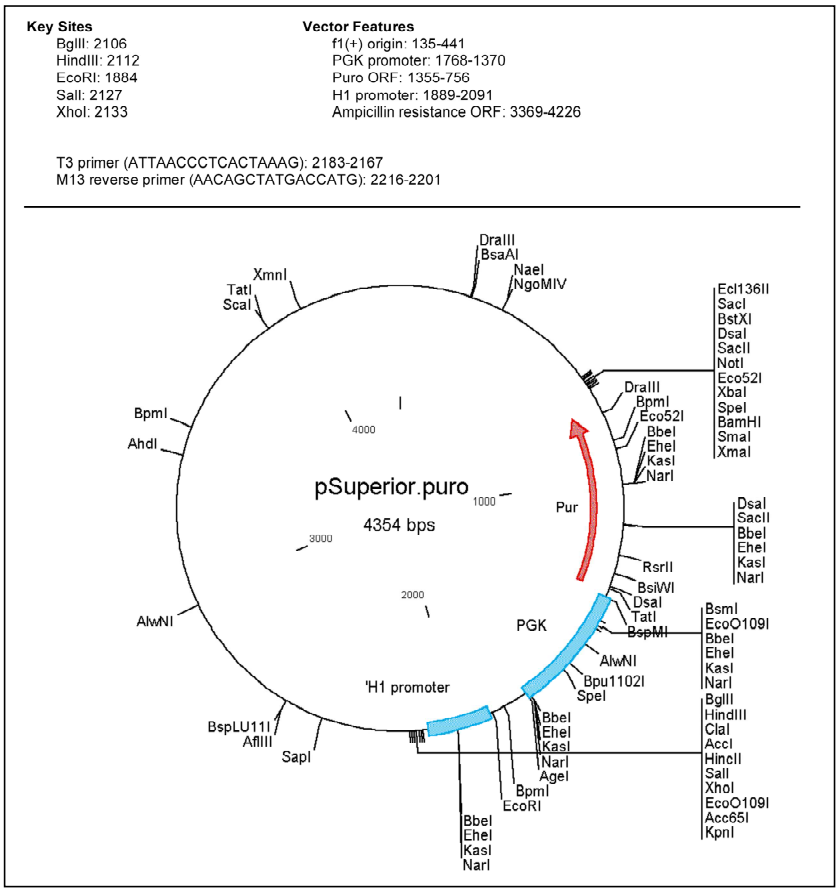

Figure 11: map of pSuperior.puro.

MCF7 were transfected with pcDNA6 TR and the clonal transfectants were selected by addition of blasticidin (10µg/ml) in the culture medium for about two weeks.

To clone the single cDNA target for the siRNA of interest in the pSuperior plasmid 59-nt forward and reverse oligonucleotides were designed with the following structure shown in table 1.

	Sequence of the oligos used to produced the siRNA against ERα
Forward	5'- gatccC GGCATGGAGCATCTGTACA TTCAAGAGA TGTACAGATGCTCCATGCC TTTTT GAATTC a –3'
Reverse	5'- agctt GAATTC AAAAA GGCATGGAGCATCTGTACA TCTCTTGAA TGTACAGATGCTCCATGCC Gg –3'
	Sequence of the oligos used to produced the non silencing shRNA (NS shRNA)
Forward	5'-gatccCTTCTCCGAACGTGTCACGT TTCAAGAGA ACGTGACACGTTCGGAGAA TTTTT a- 3'
Reverse	5'-agctt AAAAA TTCTCCGAACGTGTCACGT TCTCTTGAA ACGTGACACGTTCGGAGAA Gg-3'

Table 1: BglII (forward) and HindIII (reverse) restriction site (lowercase); Target sense of 19-nt (red); Hairpin of 9-nt (underlined); Target antisense of 19-nt (blu); Termination signal consisting of five thymidines.

The forward and reverse oligonucleotides were then annealed in Universal Buffer (100 mM NaCl, 50 mM Hepes pH 7.4) by incubating at 94°C for 4 minutes and then at 70 °C for 10 minutes followed by a progressive cooling step to room temperature. The pSuperior vector was digested with BglII and HindIII (New England's laboratory), gel purified (Eppendorf systems for nucleic acid purification) and ligated to the annealed oligos by incubation with T4 DNA ligase (New England's Laboratories). After transformation of *E.Coli* JM109 with the product of the ligation reaction, ampicillin resistant bacteria colonies were selected and screened for carrying the construct of interest (pSuperior Puro shERα).

The cell line MCF7 pcDNA6TR was used for the transfection of pSuperior Puro sh ERα. Cells were transfected with pSuperior Puro shERα and the clonal transfectants were selected by addition of puromycin (0,5µg/ml) in the culture medium for about one week. Puromycin has shown to be extremely toxic for MCF-7 thus selection was carried-out with a relative low concentration of the antibiotic as determined by the minimum concentration that kill all the control cells while allowing clones growth. As a control, a stable transfectant expressing a non silencing shRNA MCF7 TR6 (NSshRNA) was obtained by the same strategy used in the case of MCF7 pcDNA6TR shERα. The sequence of the NS shRNA is shown in table 1.

The resulting transcript of the recombinant vector is predicted to fold back on itself to form a 19–base pair stem-loop structure. The stem-loop precursor transcript is quickly cleaved in the cell to produce a functional siRNA or the NSshRNA. The figure 12 provides an overview of the insert design, and how the oligos are transcribed and process to functional siRNA.

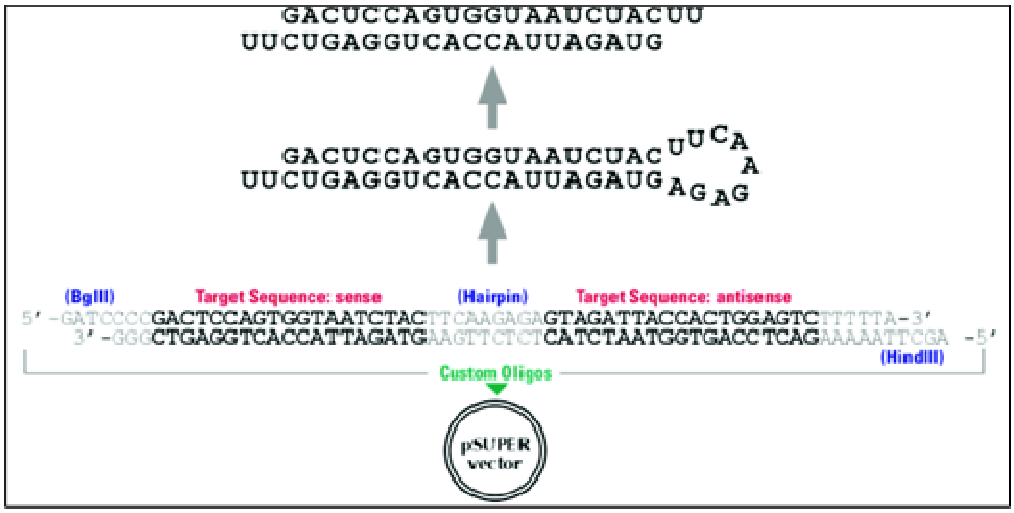

Figure 12: Transcription model of 60-nt oligo to hairpin RNA, processed to functional siRNA.

Chromatin immuno-precipitation, oligonucleotides, plasmid constructs and transfections in miRNA experiments

Chromatin immuno-precipitation assays (ChIP) (Gianni *et al.*, 2009) were performed with anti-RARα and anti-CYP1A1 irrelevant antibodies. The list of the oligonucleotides used in the study is described in table 2.

Experiment	Gene	Oligonucleotide	Sequence	Accession Number
Cloning of MIR21 promoter	MIR 21	MIR21 promoter S	5′-acaacttcacccctcactgatctc-3′	
		MIR21 promoter AS	5′-tagtcctcagagtaaggtcagctc -3′	
Real Time PCR	Pri-miR-21	Pri-miR-21 1S	5′-aatggccttgcactcttcttatg-3′	AY699265
		Pri-miR-21 1AS	5′-gttgaccaaacatgacatcagaaac-3′	
		Pri-miR-21 2S	5′-tggtggcacttagagtcttttgtg-3′	AY699265
		Pri-miR-21 2AS	5′-aggattttatggagaaatgggga-3′	
ChIP	MIR21	ChIP 1S	5′-ccagaagttagggatatgttagca-3′	
		ChIP 1AS	5′-tacctccagggttcaagtgattct-3′	
		ChIP 2S	5′-actgtctaccataaaccatgaaagga-3′	
		ChIP 2AS	5′-cccactagtcagaagtcagtgattaaca-3′	
Deletion of MIR21 promoter	MIR21	ΔRARE1 S-StuI	5′-ctaggcctccagcctggcaaagatggtgaa-3′	
		ΔRARE1 AS-StuI	5′-ctaggcctgatccccttcctcggcctcc-3′	
Point mutation of MIR21 promoter	MIR21	Mut1	5′-gaaaggggatcacgaggacaggagttcaagaccag-3′	
		Mut2	5′-accatgaaaggattcaaagtgcatagttccttctttgttcc-3′	
Cloning of 3′UTR	CCR1	CCR1 S	5′-ctcagaccataggaggccaaccca-3′	NM_001295
		CCR1 AS	5′-cacctgggaaagtgatcacaacttg-3′	
	PLAT	PLAT S	5′-ccaggaacacccgactcctcaaaa-3′	NM_000930
		PLAT AS	5′-cagaagtcaattaagtccaaactcag-3′	
	PTX3	PTX3 S	5′-atgttgtgaaactccacttgaagc-3′	NM_002852
		PTX3 AS	5′-aatgacgtgagctagttttataaaat-3′	
	TNFAIP3	TNFAIP3 S	5′-taaccggaaacaggtgggtcacct-3′	NM_006290
		TNFAIP3 AS	5′-gaaatccaacaaagaataggtggctttc-3′	
	ICAM1	ICAM1 S	5′-tgaaaccgaacacacaagccacgc-3′	NM_000201
		ICAM1 AS	5′-tttggcagttgagaaagctttattaac-3′	
	ITGB3	ITGB3 S	5′-tgataagcagtcatcctcagatca-3′	NM_000212
		ITGB3 AS	5′-cttcaagacctgtgaacttcattc-3	
	IL1B	IL1B S	5′-agagagctgtacccagagagtcct-3′	NM_000576
		IL1B AS	5′-cagtgaagtttatttcagaaccattg-3′	
Deletion of 3′-UTR	PLAT	PLAT delS-SalI	5′-atgtcgacaatttagattatgggggctctg-3′	NM_00093
		PLAT delAS-Sal I	5′-atgtcgactcctcttcctgaagttcacttc-3′	
	ICAM1	ICAM1 delS-SalI	5′-atgtcgacaacaccacacctggcaaattt-3′	NM_000201
		ICAM1 delAS-SalI	5′-atgtcgactcccagctactcaggaggctga-3′	
	IL 1B	IL 1B delS-SalI	5′-atgtcgacgattatttaaatgggaatattt-3′	NM_000576
		IL 1B del AS-SalI	5′-atgtcgacattttcagtcttaattaaagga-3′	

Table 2: The list of the oligonucleotides used in miRNA experiments.

In vitro mutagenesis was performed with the QuikChange sitedirected mutagenesis kit (Stratagene, Cedar Creek, TX). The ERE-tk-Luc, RARE-tk-Luc, the RARα and RARγ expression plasmids were described (Ciana *et al.*, 2001; Gianni *et al.*, 2009). The anti-miR-21 (ID AM10206), pre-miR-21 (ID PM10206) oligonucleotides and negative controls were from Ambion Inc. The green-fluorescent-protein plasmid (pEGFP-N1) was from Clontech Laboratories. Cells were transfected with 50 nM of pre-miR-21, anti-miR-21 or control oligonucleotides using the siPORT NeoFX reagent (Ambion). Transactivation experiments with the miR-21 promoter constructs and 3'UTR-luciferase assays were performed using *MCF-7* and *293T* cells (Gianni *et al.*, 2009; Gregory *et al.*, 2008).

The two predicted RAREs (retinoic acid responsive elements, RARE-1 and RARE-2) are indicated by blue boxes (Fig.13). The point mutations (Mut1 and Mut2) introduced in RARE1 and RARE2 are indicated in red underneath the sequence. The putative TATA box (Fujita et al., 2008) is shown in yellow. The putative transcription initiation site (Ribas et al., 2010) is indicated in red. The sequence boxed in grey represents exon 11 of the TMEM-49 coding gene, which overlaps the MIR21 gene. The sequence in bold red characters corresponds to the miR-21 stem-loop. Residue +1 corresponds to the first nucleotide of the miR-21 stem-loop sequence. The two red arrows below the sequence pointing in opposite directions indicate the position of the two oligonuceotides used for the amplification of the 5'-flanking region of the miR-21 gene. The amplified 1.5 Kb fragment was inserted in the corresponding firefly luciferase reporter plasmid both in the sense (miR-21 S) and antisense (miR-21 AS) orientations as follows. The fragment was first sub-cloned in the plasmid pCR2.1 (AT Cloning Kit, Invitrogen), and subsequently digested either by XhoI and HindIII (for the sense orientation), or by XhoI and KpnI (for the anti sense orientation). These fragments were inserted in the plasmid pGL-3 Basic Vector (Promega Italy) to obtain the plasmid DNA constructs in the appropriate orientations.

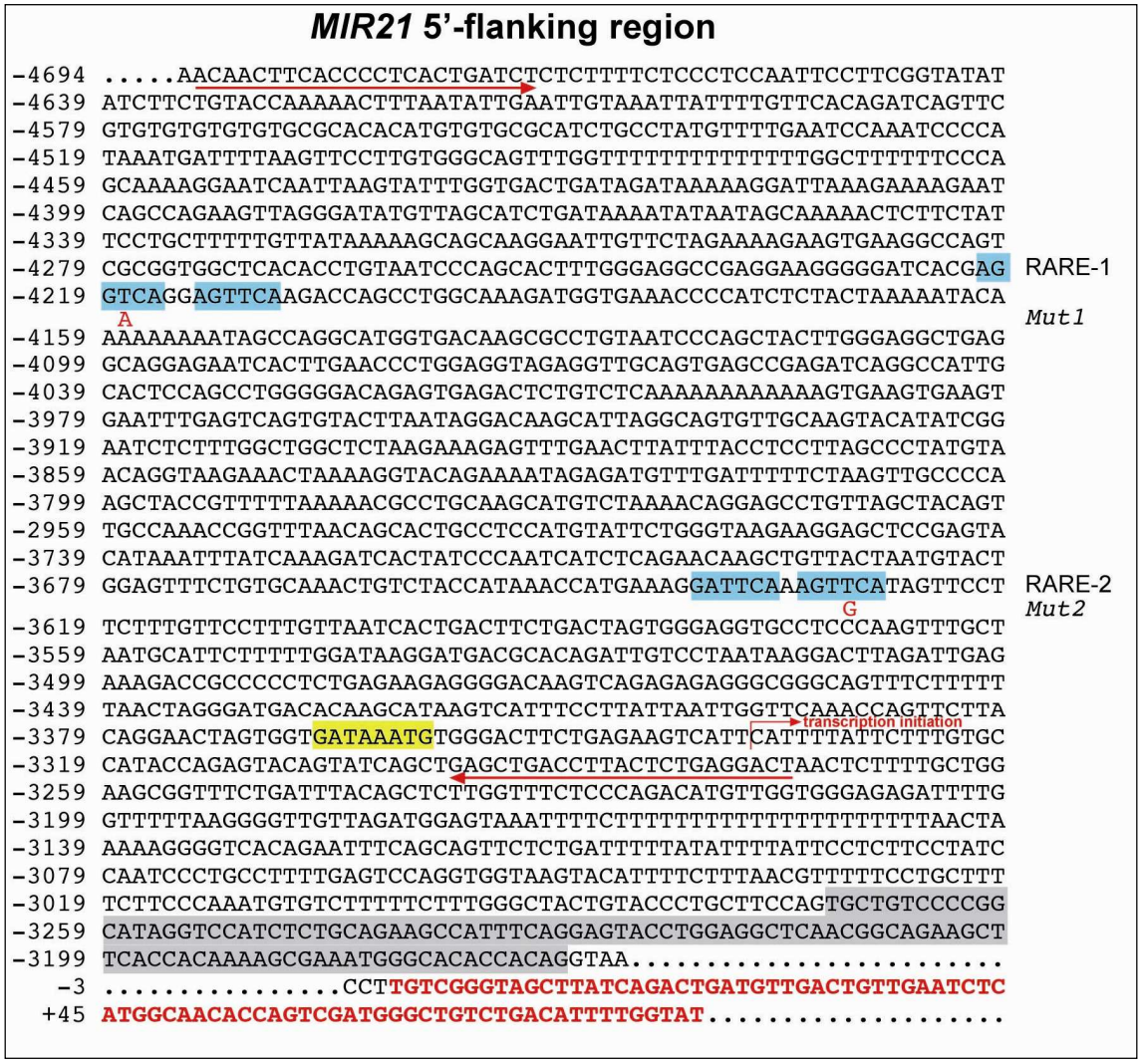

Figure 13

Western blot

Cell were lysed in Laemmli sample buffer or in a lysis buffer: 20mM Tris-HCl - 150mM NaCl - 1mM Na_2EDTA - 1mM EGTA - 1% Triton - 2.5mM Sodium Pyrophosphate - 1mM β/glycerophsphate - 1mM Na_3VO_4 - one tablet ROCHE complete - 1mM Sodium fluorure, supplemented with Protease Inhibitor Cocktails (Complete, Roche). Cell lysates were subjected to SDS-PAGE and protein transferred to a 0.2-µm pore-sized nitrocellulose (Whatman) by a semidry apparatus. For Western Immunoblot analysis the following primary antibody were used: Actin, α-tubulin, RARα, RARβ, RARγ (Santa Cruz Biotechnology) and by Caspase 3, Her2 (Cell signaling), PARP (Millipore). As secondary antibodies we have used the ECL Plex IgG cyanine 3 (anti-mouse) and the ECL Plex IgG cyanine 5 (anti-rabbit), GE Healthcare.

Transfections

For transfection experiments, cells were plated in 6-well plates (50%-70% confluence), seeded 48h before and transfected with Lipofectamine 2000 (Invitrogen,) according to the manufacture's instruction.

Reporter gene assays

For luciferase assays cell lines grown in 3 cm diameter-culture dishes were transfected at 30-40% of confluence with the indicated mammalian expression plasmids. Cells were collected and luciferase activity was measured and normalized for Renilla luciferase activity using the Dual Luciferase Reporter assay system, according to the manual instructions (Promega).

Time course

Cell growth of and viability has been scored automatically using the Vi-cell XR apparatus (Becton Dickinson).

Microarrays and real-time PCR for miRNA experiments

MiRNA microarrays were generated by spotting 1,450 miRNAs, (Exiqon miRNA probe set v8.1) in quadruplicate onto Corning epoxide-coated slides. Samples from Trizol extracted RNA (20 μg) were enriched for microRNA using the flash PAGE fractionator system (Ambion) and subsequently labeled for hybridization using the mirVana miRNA labelling kit (Ambion). Three competitive hybridization experiments were performed in duplicate using microRNA fractions pooled from three independent cell cultures (Powell *et al.*, 2009). Arrays were scanned using a GenePix 4000B Scanner driven by GenePix Pro 4.0 (Molecular Devices). All the analyses were performed using the statistical programming and graphics environment R (http://cran.r-project.org). Differentially expressed miRNAs were identified using the empirical Bayes approach which ranks genes on a combination of magnitude and consistency of differential expression (Powell *et al.*, 2009; Smyth, 2004). Gene-expression microarray (G4112F; Agilent, Palo Alto) experiments were performed as detailed (Terao *et al.*, 2009). MiRNA and gene-expression microarray results were deposited in the GEO database (GSE18693).

Mature miR-21 miRNA was measured using Taqman assays (Applied Biosystems). The primary transcript, pri-miR-21, and potential miR-21 target-transcripts were determined by PCR with SYBR green or custom-designed Taqman assays (Gianni *et al.*, 2009). For the detection of pre-miR21, miRNAs were fractionated on polyacrylamide gels and Northern blot analysis performed with a 212-bp probe (nucleotides 2,385- 2,596 of AY699265).

Real time PCR and Microarrays gene expression profiling experiments

The RNA samples were been extract from cultured cells by Qiagen miRNeasy mini kit and then evaluated by the Agilent 2100 Bioanalyzer for quality control and qRT-PCR were performed with cDNA generated from total RNA. Relative expression levels for specific genes were determined using SYBR Green PCR Master mix (Applied Bio-systems) or custom-designed Taqman assays (Gianni *et al.*, 2009) by 7300 Real time PCR system (Applied Bio-systems).

For expression profiling total RNA samples were retro-transcripted by the Amino Allyl MessageAmp II aRNA kit (Ambion) and subsequent were labeled by direct incorporation of Cyanine 3 or Cyanine 5 and hybridized to Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (Agilent Technologies) to measure the expression levels by Agilent 2-color arrays. Hybridized microarrays were scanned using a GenePix 4000B scanner (Agilent Technologies) at 5µm resolution. For the Microarray analysis the image data were analyzed using the Feature extraction software (Agilent, version 9.1) Subsequent data preprocessing all the analyses were performed using the statistical programming and graphics environment R (http://cran.r-project.org). Differentially expressed miRNAs were identified using the empirical Bayes approach which ranks genes on a combination of magnitude and consistency of differential expression (Powell *et al.*, 2009; Smyth, 2004). Gene-expression microarray experiments were performed as detailed (Terao *et al.*, 2009). MiRNA and gene-expression microarray results were deposited in the GEO database (GSE18693).

Gene expression microarray on MCF7 and MDA-MB-231cell lines

MCF7 and MDA-MB-231 cell-lines were either treated with 1 µM RA or untreated for 48 hours. The cells were harvested after 6 hours and after 48 hours, in each condition, and RNA was extracted. RNA expression levels were measured by Agilent 2-color arrays. Each rectangle in fig.14 represents an array, such that in each array there is one channel

(dye) for the untreated cells and another channel for the treated cells (in the same time point and cell type). Each array was done twice as a biological repeat, with a dye swap [i.e. in one replicate the treated cells were labeled with green (Cy3), and in the second replicate they were labeled with red (Cy5)].

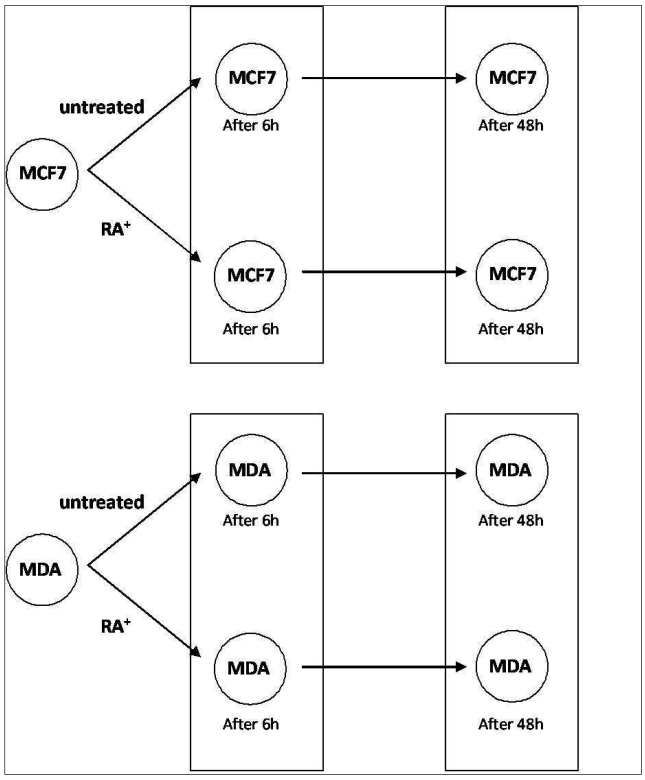

Figure 14

The microarrays were custom synthesized by spotting of complementary Locked Nucleic Acid probes of 1,450 miRNAs, (Exiqon miRNA probe set v8.1). RNA extraction, hybridization and analyses were performed as detailed above. Gene-expression microarray results were deposited in the GEO database (GSE18693). Mature miR-21 and the normalizing Z30 miRNAs were measured using Taqman assays (Applied Biosystems). The primary transcript, pri-miR-21, and potential miR-21 target-transcripts were determined by PCR with SYBR green or custom-designed Taqman assays (Gianni *et al.*, 2009). For the detection of pre-miR21, miRNAs were fractionated on polyacrylamide gels and Northern blot analysis performed with a 212-bp probe (nucleotides 2,385-2,596 of AY699265).

Gene expression microarray on SKBR3 cell line

SKBR3 cell line was treated with 100 mM RA, with 100mM Lapatinib and with the combination of both drugs or left untreated. The cells were harvested after 12 hours and after 48 hours from chemical addition, and RNA was extracted as described above. Four biological replicates from four independent experiments were used, with a square, balanced dye design, [i.e. of two technical replicates in one replicate the treated cells were labeled with green (Cy3), and in the second replicate they were labeled with red (Cy5)] (Fig.15).

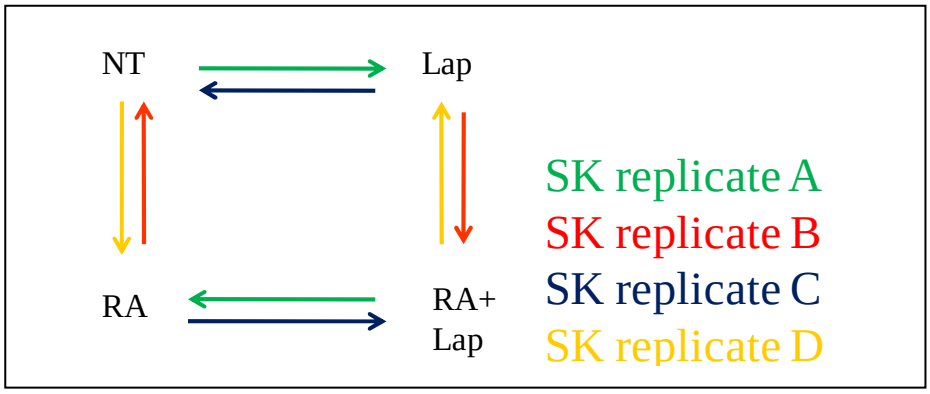

Figure 15

ERBB2 and RARA copy number assessment by quantitative PCR

For cell lines, DNA was prepared using the DNeasy Blood&Tissue Kit (Qiagen, Valencia). Cells were grown to 70–80% confluence, then harvested for genomic DNA extraction. DNA from paraffin-embedded patient samples was obtained by QuickExtract FFPE DNA Extraction Kit (Epicentre Biotechnologies, Madison, Wisconsin).

ERBB2 and RARA copy number was quantified by real-time quantitative PCR (Q-OCR), using the 7300 (SDS software v 1.3) System (Applied Biosystem, Foster City). Pre-designed TaqMan Copy Number Assays Hs01079964_cn (RARA) and Hs05520410_cn (ERBB2) were purchased by Applied

Biosystem.Ribonuclease P RNA component H1 (H1RNA) gene (RPPH1), located at 17q11.2 was used as a reference control. Reactions were carried out in a final volume of 20 µl containing 25ng genomic DNA and 16 µl TaqMan Genotyping Master Mix (Applied Biosystems, Foster City). PCR conditions were as follows: one cycle at 95°C for 10 minutes, followed by 40 cycles each at 95°C for 15 seconds and 60°C for 1 minute. Samples were analyzed in triplicate. ERBB2 copy number calculation was carried out using the comparative Ct method (Livak *et al.*, 2001). Human Genomic DNA (Cat.G1471)

(Promega, Madison), from multiple anonymous male donors, was run in every assay as a calibrator sample. ERBB2 gene copy number in normal human genomic DNA was set as 2 and copy number more than 4 in cell lines was considered to be increased.

Fluorescence Activated Cell Sorting

SKBR3 cell-lines were treated with 100 mM RA or with 100mM Lapatinib, with the combination of both drugs or left untreated. The cells were harvested after 12-24-48-72-96 hours from chemical addition. After harvesting, cells were counted, fixed in 70% ethanol and prepared for cytometric DNA analysis. Briefly samples were treated with RNAse, stained with Propidium Iodide and analyzed using a FACSCalibur flow cytometer (Becton– Dickinson Immunocytometry Systems, Inc., San Jose).

Sulphorodamine assay

The sulphorodamine assay was performed as described by Skehan *et al*. (Skehan *et al*., 1990). Briefly, cell cultures fixed with trichloroacetic acid were stained for 30 minutes with 0.4% (wt/vol) sul-forhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base [tris (hydroxymethyl)aminomethane] for determination of optical density in a computer-interfaced, 96-well microtiter plate reader (Tecan).

RESULTS

All-*trans* retinoic acid (ATRA) and derivatives (retinoids) have shown potential for the therapy and chemo-prevention of breast cancer. One of the main problems for the clinical use of retinoids in oncology is represented by the variability of the responses observed in different tumors and different subsets of patients within a single type of tumor. The principal molecular traits defining different breast cancer subtypes have been identified to be the presence or absence of ERα or the amplification/over expression of the Her2/ERBB2 locus. In order to characterize the response of the main breast cancer subtypes to retinoids we decided to decipher the cross-talk between the pivotal mediators of retinoids signaling (RARs receptors) and the molecular determinants of breast cancer (ERα and Her2).

1. Characterization of the retinoid sensitivity/resistance in breast carcinoma across the ERα pathway.

ERα influences the sensitivity of malignant cells to the anti-neoplastic activity of retinoids and pre-clinical observations indicate that estrogen-receptor-α-positive (ERα+) breast carcinoma cells are sensitive, while estrogen-receptor-α-negative (ERα-) are generally resistant to the anti-proliferative activity of ATRA and derivatives (Fontana, 1987; van der Burg *et al.*, 1993; del Rincon *et al.*, 2003). This phenomenon is potentially associated with the fact that ERα+ tumors contain higher levels of RARα than the ERα- counterparts (Schneider *et al.*, 2000).

To further validate this hypothesis and understand the molecular underlying mechanism, we have taken advantage of MCF7 and MDA-MB-231 breast cancer cell lines, considered paradigms of ERα+ and ERα- tumors type respectively, as an established model for the association between ERα presence/absence and retinoid sensitivity/resistance in breast cancer.

1.1 MDA-MB-231 and MCF7 respond differentially to the challenge with retinoic acid

MCF-7 breast carcinoma cells are ERα+, whereas the MDA-MB-231 counterparts are ERα- (Fig. 16A). MCF-7 express higher levels of the ERα transcript than MDA-MB-231 and express low but similar levels of the ERβ transcript. MCF-7 have a luminal phenotype (Mandal and Davie, 2007) and are sensitive, while MDA-MB-231 cells are basal-like (Jönsson *et al*, 2007) and are refractory to the transcriptional (Fig. 16B) effects of 17β-estradiol (E_2).

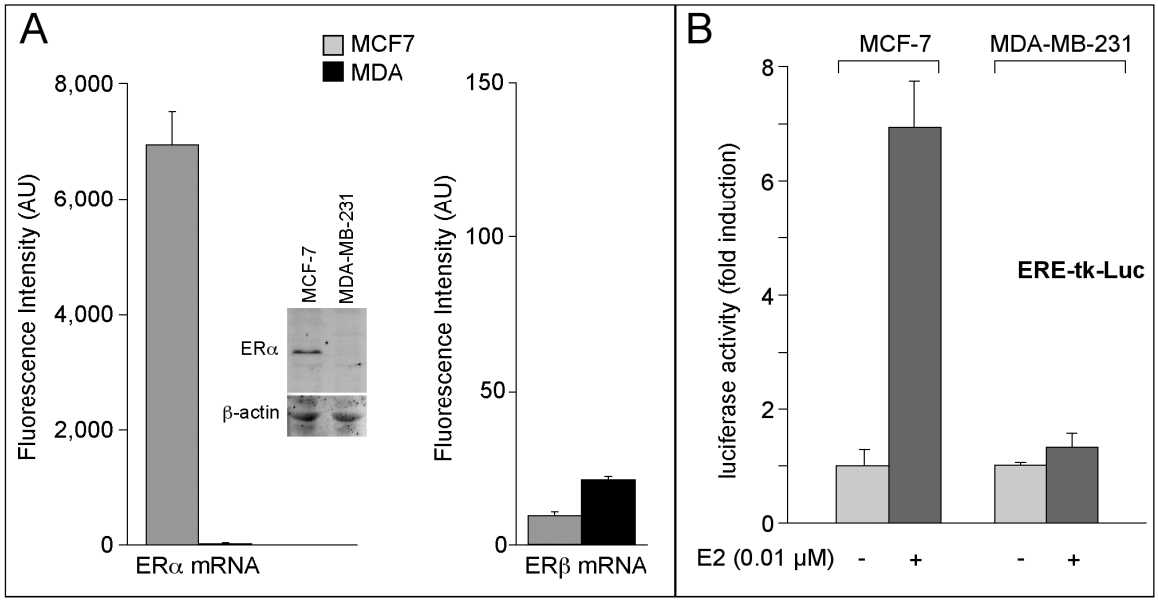

Figure 16: (A) The graphs indicate the relative basal levels of the ERα and ERβ transcripts in MCF-7 and MDA-MB-231 cells. The results were obtained from the whole-genome gene expression microarray dataset and represent the mean + S.D. of 6 separate slides. The inset shows the relative levels of the ERα protein, as determined by Western blot analysis with a specific anti-ERα antibody. Similar amounts of proteins were present in the two lanes, as indicated by the signal obtained with a specific anti-β-actin antibody. **(B)** MCF-7 and MDA-MB-231 cells were transfected with an estrogen responsive plasmid containing firefly luciferase, as the reporter gene (ERE-tk-Luc) along with the renilla luciferase normalization plasmid. 24 hours after transfection, cells were treated with the indicated concentration of E_2 or vehicle (DMSO) for a further 24 hours. Renilla and firefly luciferase enzymatic activities were measured in cell extracts. Luciferase activity is expressed in fold induction relative to the vehicle-treated value taken as 1. Each value is the mean + S.D. of two replicate cultures.

As described above this pair of cell lines was chosen to study the association between ERα positivity and response to the anti-proliferative effects of retinoids. Indeed, ATRA (1 μM) inhibited the growth of MCF-7 cells, but not that of MDA-MB-231 cells (Fig. 17A). Differential sensitivity extended to the transcriptional effects of ATRA, as the retinoid-dependent reporter construct, RARE-tk-luc, showed much higher response to ligand

activation in MCF-7 cells (Fig. 17B). Ligand-dependent trans-activation of RARE-tk-luc was reduced by E_2 in MCF-7 cells.

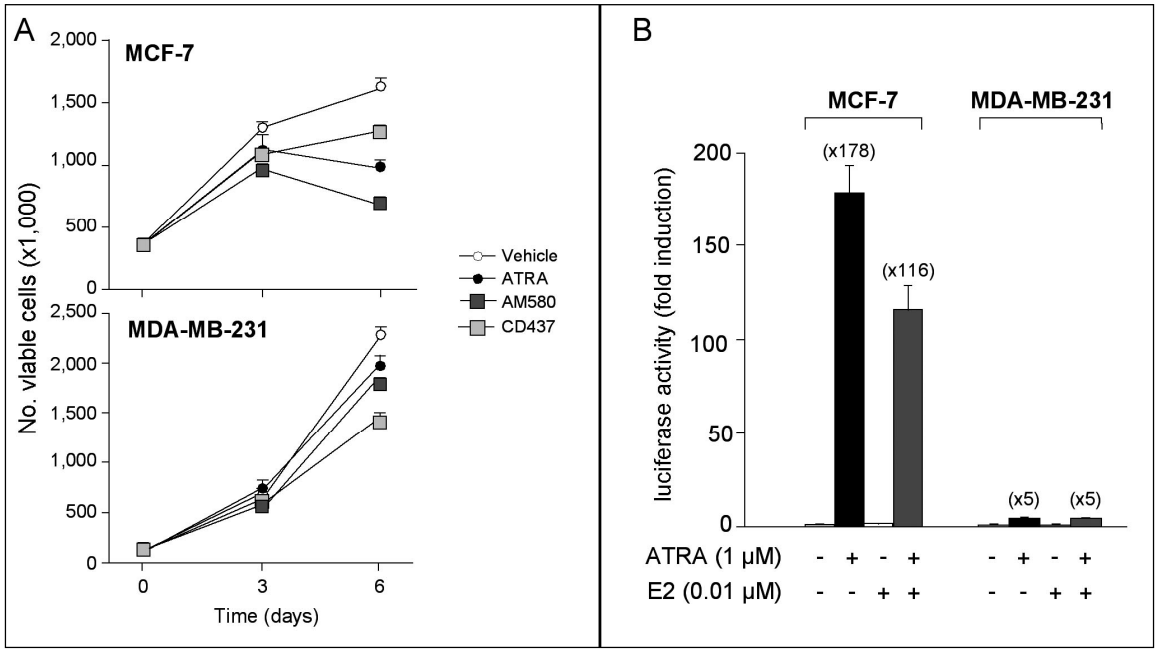

Figure 17: (A) The growth curves of MCF-7 and MDA-MB-231 cells incubated in the presence of vehicle (DMSO), ATRA (1 µM), AM580 (0.1 µM) or CD437 (0.1 µM) for the indicated amounts of time are shown. Each value is the mean + S.D. of three replicate cultures. **(B)** MCF-7 and MDA-MB-231 cells were transfected with the retinoid-dependent and firefly-luciferase-based reporter construct, RARE-tk-Luc, and the renilla luciferase control plasmid. After further 24 hours of treatment with the indicated concentrations of ATRA and/or E_2, cell extracts were used for the measurement of firefly and renilla luciferase activities. Results are expressed in arbitrary units of firefly luciferase following normalization for the transfection efficiency (renilla luciferase activity) and are the mean + S.D. of two replicate transfections. The number in parenthesis indicates the fold-induction value. The results shown are representative of three independent experiments.

In basal conditions (Fig. 18A), both MCF-7 and MDA-MB-231 cells express significant amounts of RARα, RARγ, RXRα and RXRβ mRNAs. The levels of these transcripts largely reflect the amounts of the corresponding protein products, as demonstrated for RARα, RARγ and RXRα (Fig. 18B). The major quantitative difference in the complement of nuclear retinoid receptors is observed for RARα, which is significantly more abundant in MCF-7 than in MDA-MB-231 cells (Rishi *et al*, 1995). This is consistent with the observation that ERα+ breast carcinomas are generally associated with higher levels of the RARα transcript, as illustrated by the meta-analysis (data not shown, visible in http://www.oncomine.org).

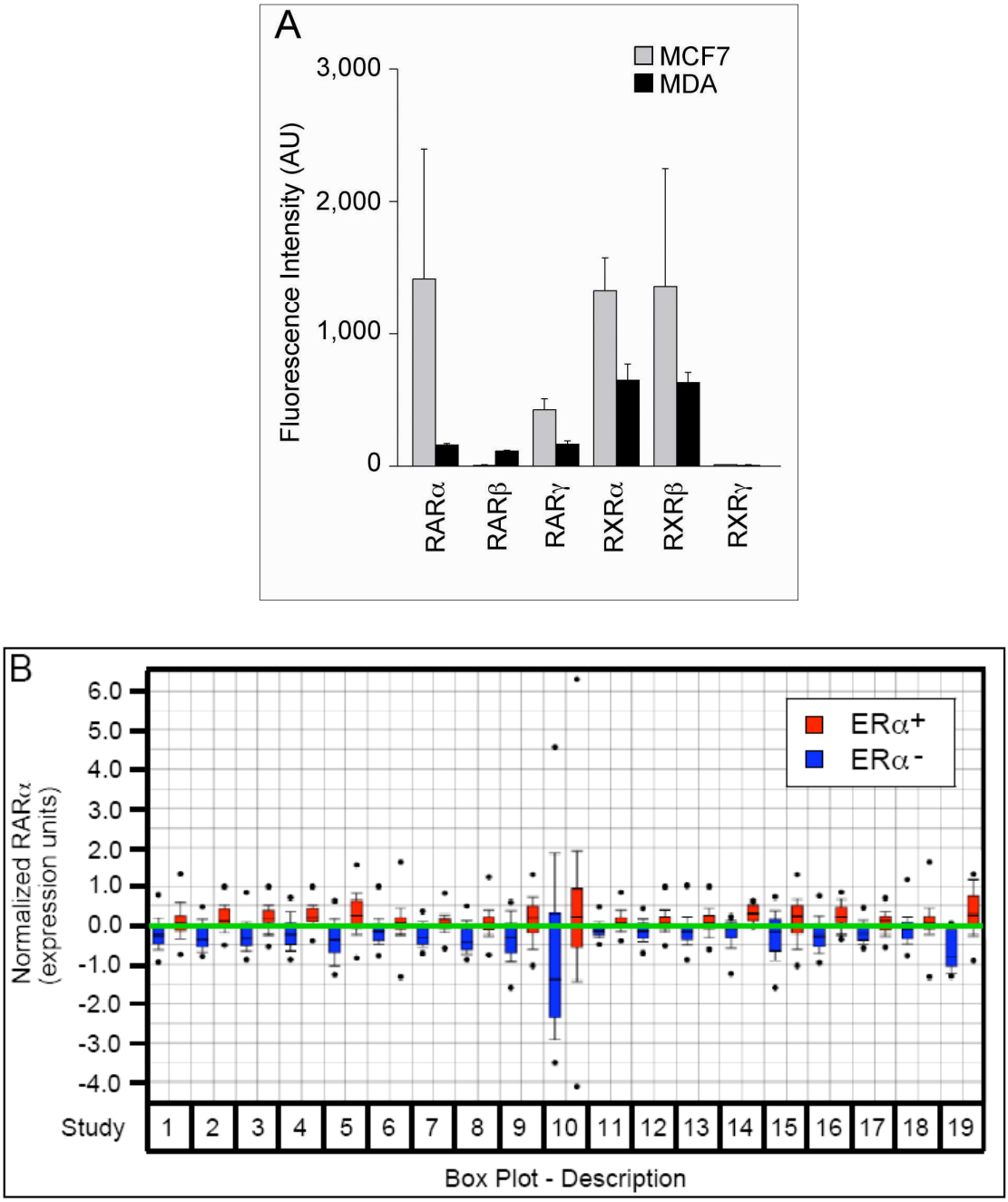

Figure 18: **(A)** The panel shows the levels of the transcripts encoding RAR and RXR nuclear retinoic acid receptors in MCF-7 and MDA-MB-231 cells grown in basal conditions. The results were obtained from the whole-genome gene expression microarray dataset and represent the mean + S.D. of 6 separate slides. Data are expressed in absolute fluorescent units and the values determined in MCF-7 and MDA-MB-231 are comparable directly. **(B)** The relative amounts of RARα mRNA present in ERα+ and ERα- breast carcinomas are shown. The results were obtained by an automatic metanalysis of the available data present in the Oncomine WEBsite (http://www.oncomine.org) using the "gene search" algorithm. In 19 of the studies analyzed, the levels of the RARα transcript are significantly higher in ERα+ cancers. The remaining 6 studies present in the database did not show significant differences in the levels of the mRNA in ERα+ and ERα- breast cancers and are not shown in the plot.

ATRA is a pan-RAR agonist with no selectivity for RARα or RARγ (Parrella *et al*, 2006; Rochette-Egly and Germain, 2009), the two RAR proteins expressed in MCF-7 and MDA-MB-231 cells. To demonstrate the relative functional importance of the two receptors in our cellular model, we compared the activity of ATRA and selective agonists of RARα (AM580) (Gianni *et al*, 1996) or RARγ (CD437) (Garattini *et al*, 2004; Mologni *et al*, 1999). In MCF-7 cells, AM580 (0.1 µM) inhibited the growth more efficiently than ATRA (1 µM) (Fig. 19) and was also a more powerful activator of the retinoid-dependent reporter construct, RARE-tk-Luc (Fig. 19). At receptor-selective concentrations (0.01 or 0.1 µM), CD437 exerted only modest anti-proliferative and transcriptional effects. At 1µM, the increase in RARE-tk-Luc activity in cells treated with CD437 is due to loss of RARγ selectivity (Garattini *et al*, 2004; Mologni *et al*, 1999). In MDA-MB-231 cells, AM580 and CD437 were devoid of significant growth inhibitory activity and caused an almost two order of magnitude lower activation of RARE-tk-Luc relative to what observed in MCF-7 cells. Altogether, these results indicate that RARα is a major determinant of the anti-proliferative and transcriptional effects of ATRA in MCF-7 cells.

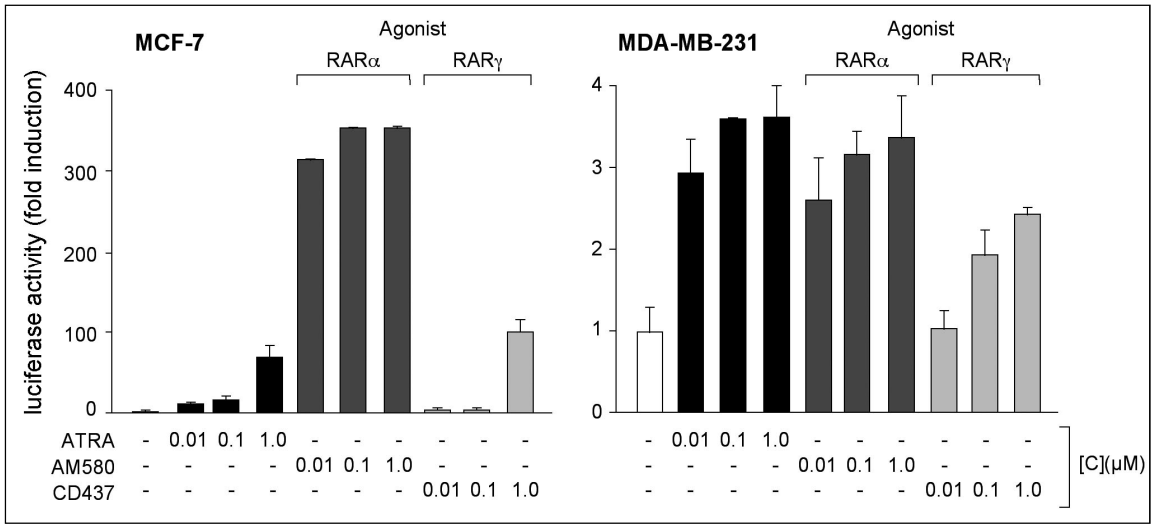

Figure 19: Cells were transfected with the retinoid-dependent reporter construct, *RARE-tk-Luc*, and treated for 24 hours with the indicated concentrations of ATRA, AM580 or CD437 (mean + SD, 2 replicate transfections). All the results are representative of at least 2 independent experiments.

1.2 Inducible over-expression and down-regulation of ERα in MDA-MB-231 and MCF7

It is known that the MCF7 respond to ATRA with growth inhibition, differentiation and apoptosis, in contrast, MDA-MB-231 are completely refractory to retinoid challenge. Indeed, we have tested for growth, viability and differentiation, the normal cell lines MCF7 and MDA-MB-231 using in the cell culture a medium containing charcoal-stripped foetal bovine serum (DCC-FBS) in the presence of vehicle alone, E_2, ATRA and with the combination E_2 with ATRA (Fig.20). Since both ATRA and E_2 are present in normal foetal bovine serum, the use of DCC-FBS allows us to score the specific effect due to the two steroid hormones on cell proliferation. As expected E_2 induces cell proliferation while ATRA triggers growth arrest in the ERα positive MCF7 cells, on the contrary, ERα negative MDA-MB-231 was completely refractory to the effects mediated by E_2 and ATRA. Moreover the inhibition of cell growth mediated by ATRA is stronger in the presence of E_2, indicating that ATRA interferes specifically with ERα triggered proliferation.

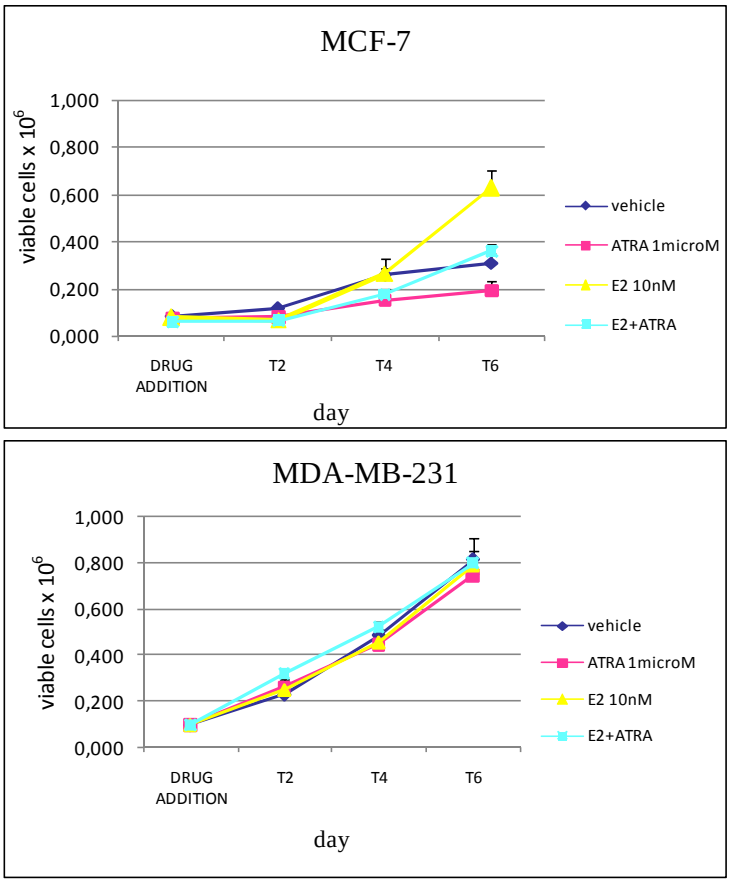

Figure 20: The growth curves of MCF-7 and MDA-MB-231 cells incubated in the presence of vehicle (DMSO), ATRA (1 μM), E_2 (10nM) and the combination E_2 with ATRA for 2-4-6 days.

To define the molecular mechanisms and determinants responsible for the ERα-dependent regulation of retinoid sensitivity/resistance in breast carcinoma we have established two complementary models of conditional ERα over-expression and silencing using paradigms of ERα-positive (MCF7) and ERα-negative (MDA-MB-231) breast cancer cells, as described in materials and methods.

1.3 Over-expression of ERα is not sufficient to restore ATRA sensitivity in MDA-MB231

MDA-MB-231/TR6 ERα stably over-expressing ERα by tetracycline induction were generated by sequential transfection of pcDNA6/TR and pcDNA4/TO ERα plasmids carrying the tetracycline repressor and ERα coding sequences respectively (see materials and methods). Clones (MDA-MB-231/TR6 ERα) selected for both blasticidin and zeocin (as guarantee for TetO and ERα cds insertions) were isolated and tested for the ability of inducibly express ERα. We as well produce LacZ inducible clones as a negative control (MDA-MB-231/TR6 LacZ). For each construct three clones were selected and analyzed. The figure 21 is representative of ERα over-expression induced by tetracycline in ERα transfectans.

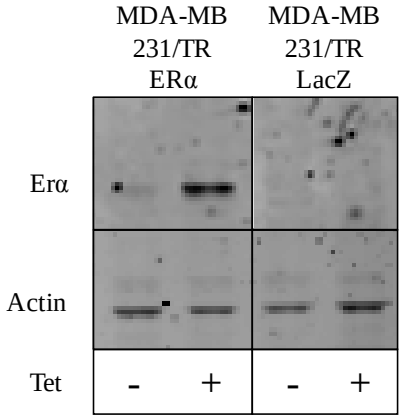

Figure 21: Western blot showing the ERα expression in MDA-MB-231/TR ERα and MDA-MB-231/TR LacZ inducibly clones, after tetracycline treatment.

To evaluate if the overexpressed ERα is biologically functional as a transcription factor, we analyzed the ability of our clones to modulate an ERα dependent promoter. Therefore we performed luciferase assay after transfection MDA-MB-231/TR6 ERα with the luciferase reporter plasmid ERE-tk-Luc (Ciana *et al.*, 2001) and subsequent tetracycline

addition (Fig. 22). As expected, tetracycline induces a strong trans-activation of ERE promoter. The phenomenon is magnified in the presence of the ligand E_2. As a matter of fact E_2 is required for the transcriptional activity of ERα and the spurious reporter activity measurable in absence of E_2 is probably due to the residual E_2 present in the medium combined to a residual leaky expression of ERα by the ERα inserted coding sequence.

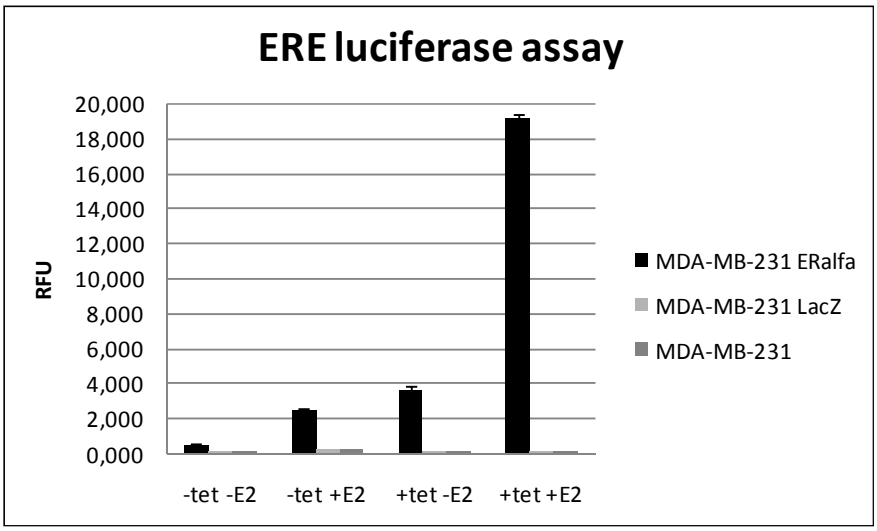

Figure 22: Luciferase assay after transfection of MDA-MB-231/TR6 ERα, MDA-MB-231/TR6 LacZ and MDA-MB-231/ with the luciferase reporter plasmid ERE-tk-Luc and in presence and absence of tetracycline and E_2 (10nM).

Since ERα is the major trigger of proliferation in ERα + breast cancer cell lines, we study the effect of ER over-expression on cell growth. Surprisingly tetracycline induction do not increase MDA-MB-231/TR6 ERα cell proliferation suggesting that the steroid nuclear receptor activated pathway is not sufficient by its own to interfere with the proliferative driving force of MDA-MB-231 genetic background.

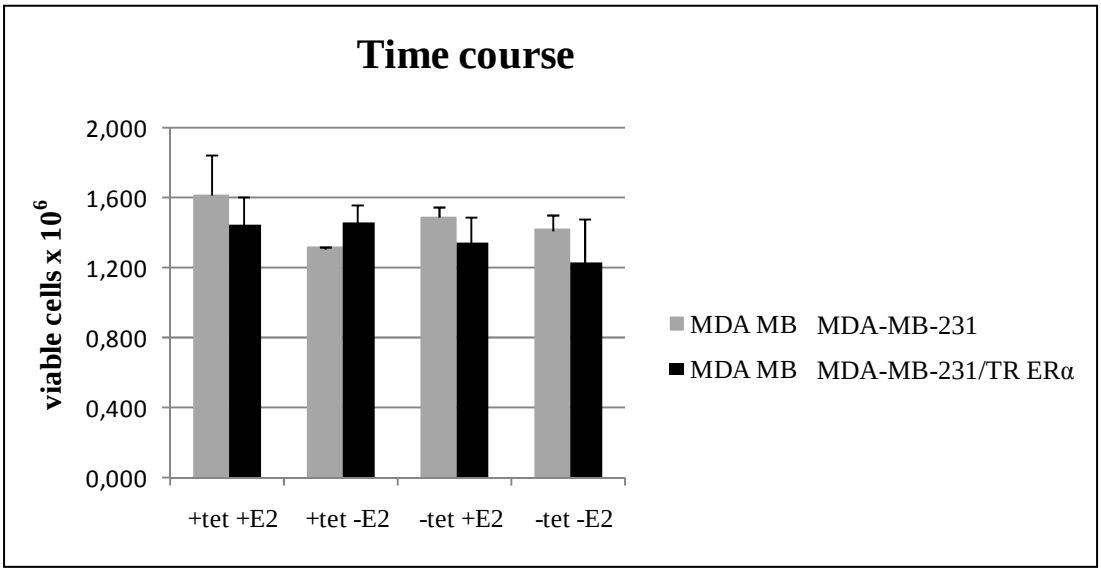

Figure 23: The growth curves of MDA-MB-231 and MDA-MB-231/TR6 ERα cells incubated in the presence or not of tetracycline and E_2 (10nM).

Altogether the data reported indicate above that the stable cell lines generated, correctly expressed a functional ERα but its over-expression do not affect cell proliferation. To evaluate if any change in retinoic acid sensitivity could still occur, we have characterized our clones for the growth and viability in presence or absence of tetracycline with ATRA and E_2. As shown in figure 24 no significant increase in ATRA sensitivity was acquired after tetracycline induction.

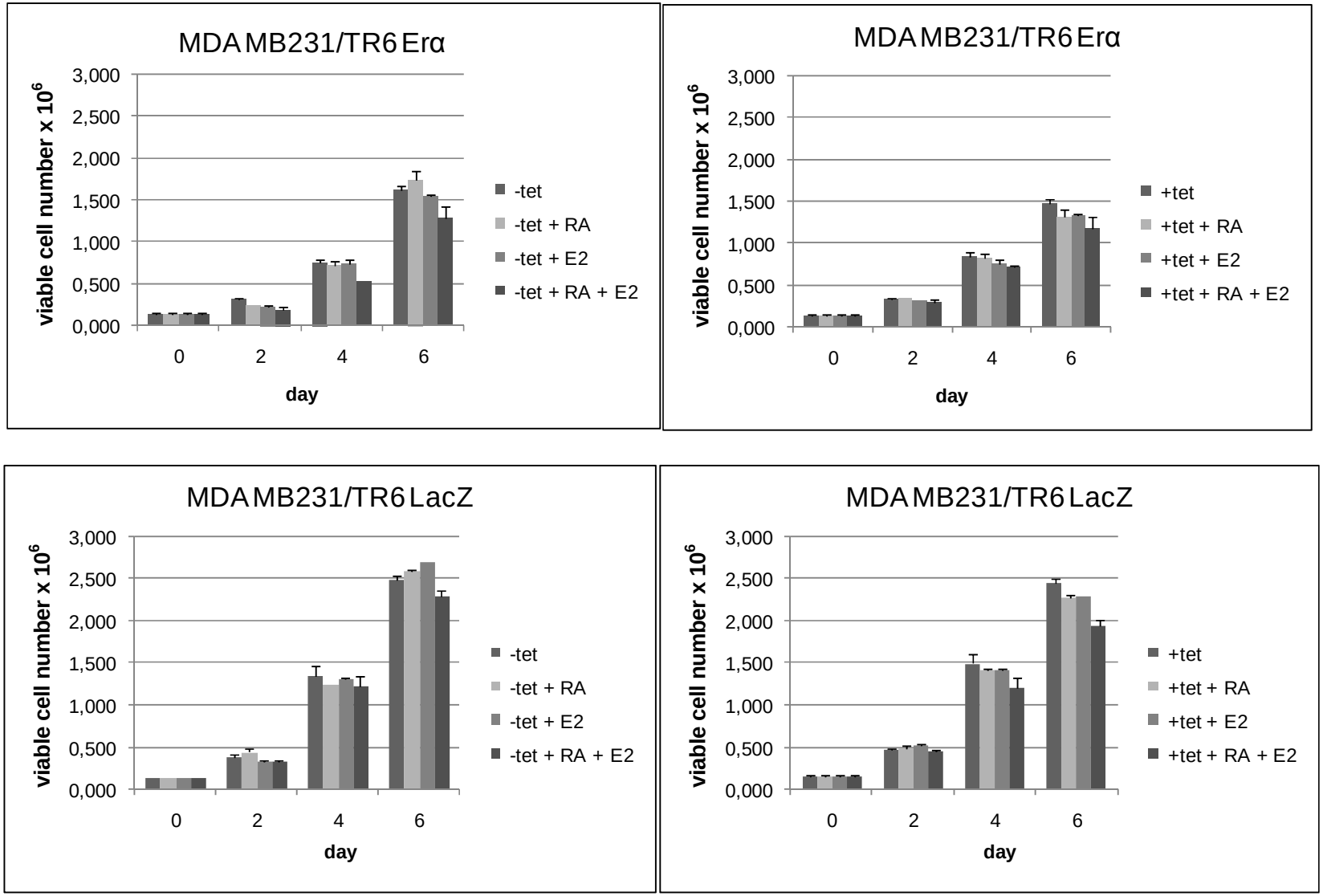

Figure 24: The growth curves of MDA-MB-231/TR6 ERα and MDA-MB-231/TR6 LacZ cells incubated in the presence of vehicle (DMSO), ATRA (1 μM), E_2 (10nM) and the combination E_2 with ATRA for 2-4-6 days.

We also performed luciferase assay after transfection of MDA-MB-231/TR6 ERα with the luciferase reporter plasmid RARE-luc (Gianni *et al*, 2009) and subsequent tetracycline and drugs addition. Again, no modulation of RARA dependent transcriptional activity could be observed after ERα induction (Fig. 25) in the MDA-MB-231/TR6 ERα clone or in the control clone MDA-MB-231/TR6 LacZ (data not shown).

Therefore, we can conclude that over-expression of ERα is not sufficient to modulate cell proliferation and to restore ATRA sensitivity in MDA-MB-231.

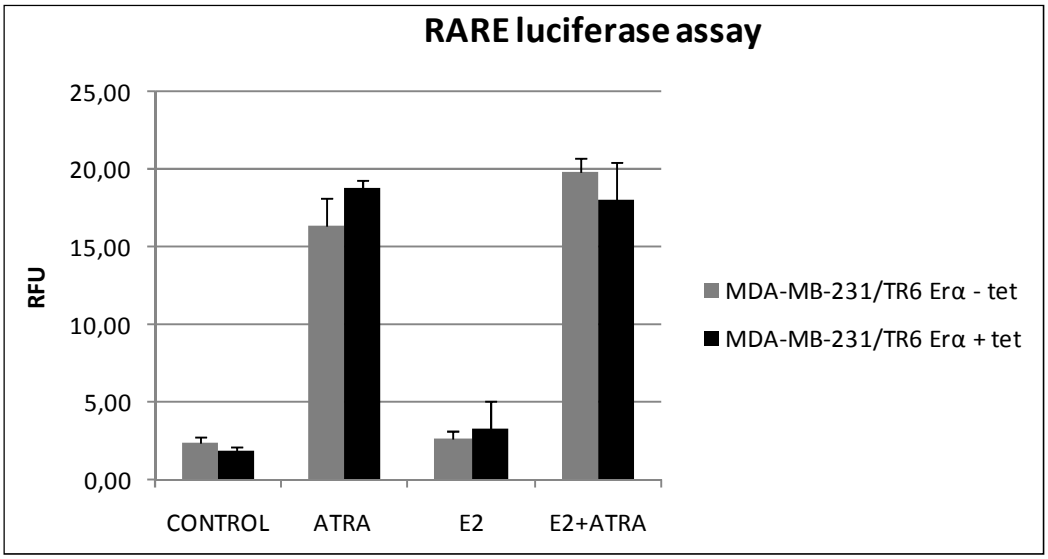

Figure 25: Cells were transfected with the retinoid-dependent reporter construct, *RARE-tk-Luc*, and treated for 24 hours in the presence of vehicle (DMSO), ATRA (1 μM), E_2 (10nM) and the combination E_2 with ATRA in presence and absence of tetracycline.

1.4 Down-regulation of ERα in MCF7 attenuates cell proliferation and demonstrates its role as a crucial determinant of ATRA sensitivity.

MCF7 have been engineered for the conditional expression of a short-hairpin RNA silencing ERα. To obtain the above mentioned inducible clone, we first produced a stable transfectant of the tetracycline repressor by transfection of pcDNA6/TR into MCF7. After isolation of the appropriate clones we transfected them with the vector pSingle-tTS-shRNA carrying the shRNA targeting ERα (see material and methods). As a control, clones conditionally expressing a non silencing shRNA sequence were also produced (control negative NSshRNA) (see material and methods section, Lin *et al*, 2006). After appropriate selection, the isolated clones were tested for the ability of inducible down-regulate ERα. For each construct, three clones were selected and were tested for their capacity to down-

regulate endogenous ERα expression in a tetracycline dependent manner by Western blot. As expected MCF7 clones stable expressing the shRNA against ERα (MCF7 TR6/shERα) exhibit a tetracycline-dependent down-regulation of the protein, while no modulation occurs in control clones (MCF7 TR6/shNS) (Fig. 26).

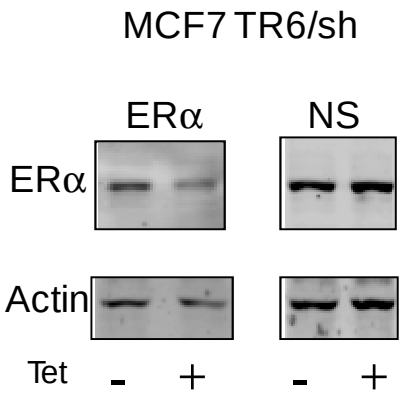

Figure 26: Western blot showing a lowest expression level of ERα in MCF7 TR6/shERα under tetracycline (tet) treatment. ERα expression is unchanged in ERα non silencing clones (NS).

To evaluate if the silencing we obtained in our clones correlates with a lower ERE-dependent transcriptional activity, we performed luciferase assays by transfection of the luciferase reporter plasmid ERE-tk-Luc after tetracycline addition. As expected, tetracycline induces a reduction of the ERE associated promoter activity (Fig. 27). As discussed in the case of MDA-MB-231/TR ERα clones, the down-regulation observed also in the case of absence of E_2 could be ascribed both to a leakage of the tet-system in the presence of residue E_2 or to an E_2 independent transcriptional activity of Erα.

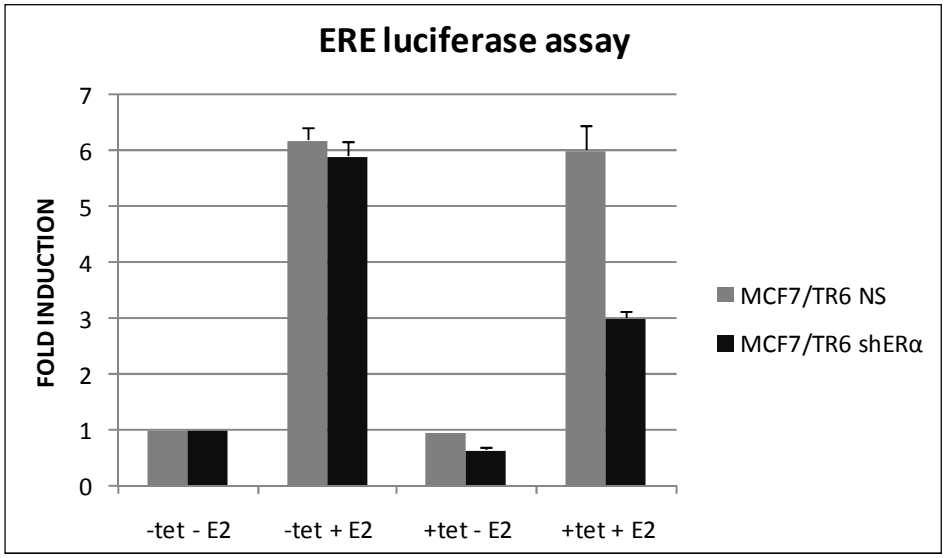

Figure 27: Luciferase assay after transfection of MCF7/TR6 NS and MCF7/TR6 sh ERα with the luciferase reporter plasmid ERE-tk-Luc and in presence or absence of tetracycline with or without E_2 (10nM).

We have also characterized our clones for their growth and viability state in the presence or absence of tetracycline with or without E_2 (Fig. 28). As shown in figure 28 down-regulation of ERα induces a wide and significant decrease of cell growth. Trypan blu analyses reveal that no cell death occurs indicating that the effect of ERα knock-down is anti-proliferative and not cytotoxic (data not shown). Growth inhibition is not complete and correlates quantitatively (about 50%) with the partial reduction of the ERα protein observed in clones. Indeed we were not able to obtain a clone with complete inhibition of the ERα protein possibly because of its crucial role in mediating cell viability in the studied cell line.

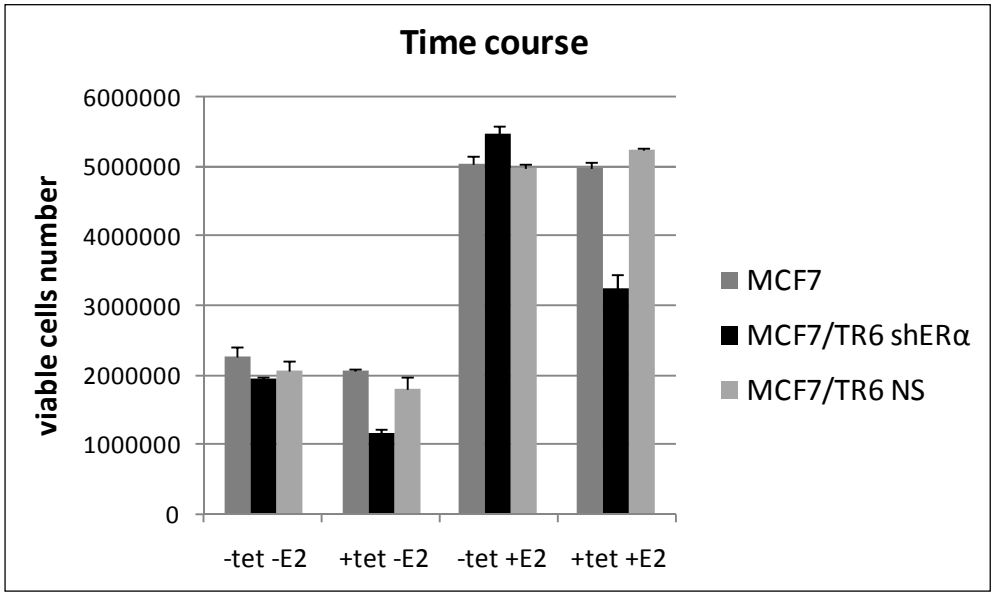

Figure 28: The growth curves of MCF7, MCF7/TR6 sh ERα and MCF7/TR6 NS cells incubated in the presence or not of tetracycline and E_2 (10nM).

Altogether the data above reported indicate that the stable cell lines generated, correctly down-regulate ERα protein inducing a specific decrease in ERE-dependent transcription and significantly impairing cell proliferation.

To evaluate if the modulation of ERα signaling could affect retinoid sensitivity in MCF7, we have characterized our clones for their growth and viability in the presence or absence of tetracycline followed by addition of ATRA and/or E_2. As shown in figure 29 no significant decrease in ATRA sensitivity was acquired after tetracycline induction.

ATRA induced growth inhibition is proportional to the proliferation status of the cells. Indeed, the ratio, in viable cells number, between E_2 *plus* ATRA and E_2 alone treated samples do not significantly differ after tetracycline induction.

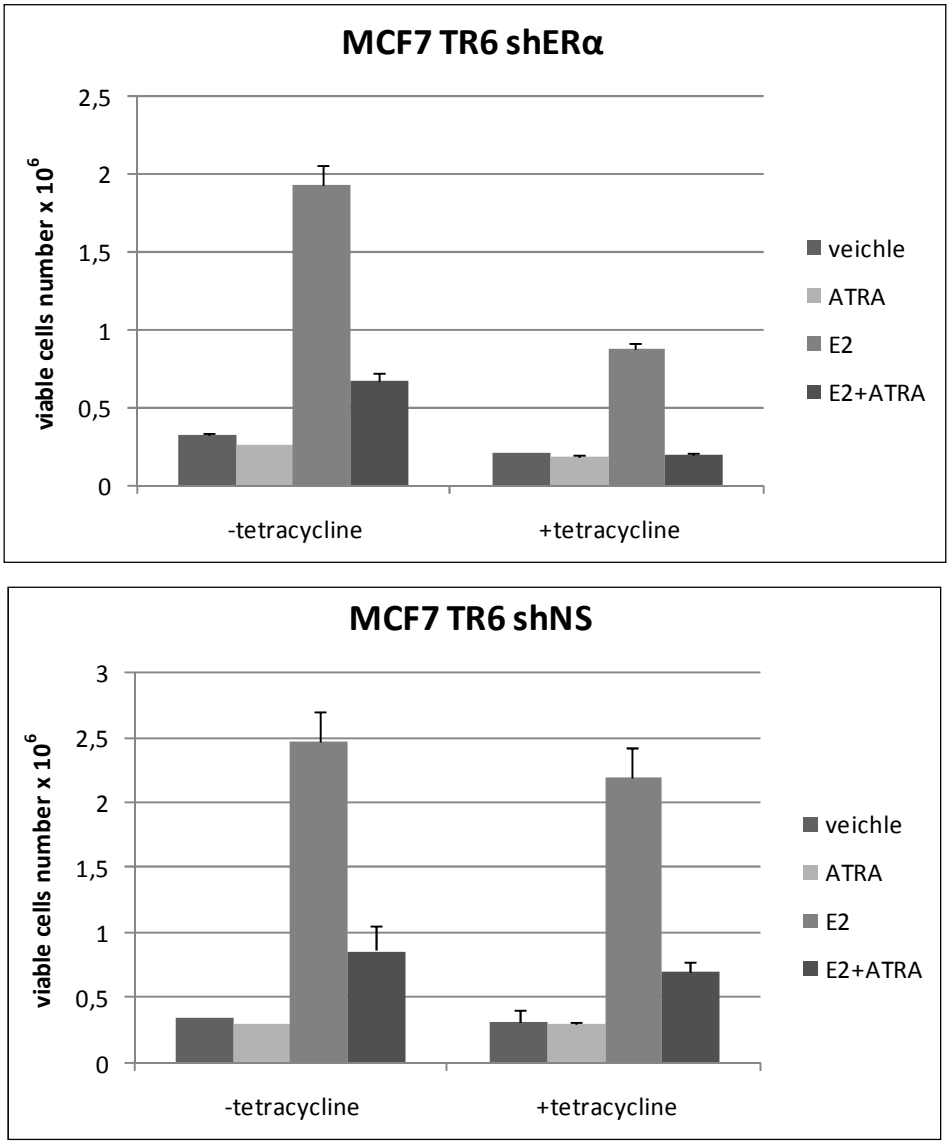

Figure 29: The growth curves of and MCF7/TR6 sh ERα and MCF7/TR6 NS cells incubated in the presence of vehicle (DMSO), ATRA (1 μM), E_2 (10nM) and the combination E_2 with ATRA after 6 days.

We also performed luciferase assays after transfection of MCF7 TR6/sh ERα with the luciferase reporter plasmid RARE-tk-luc and subsequent tetracycline and E_2/ATRA addition (Fig. 30).

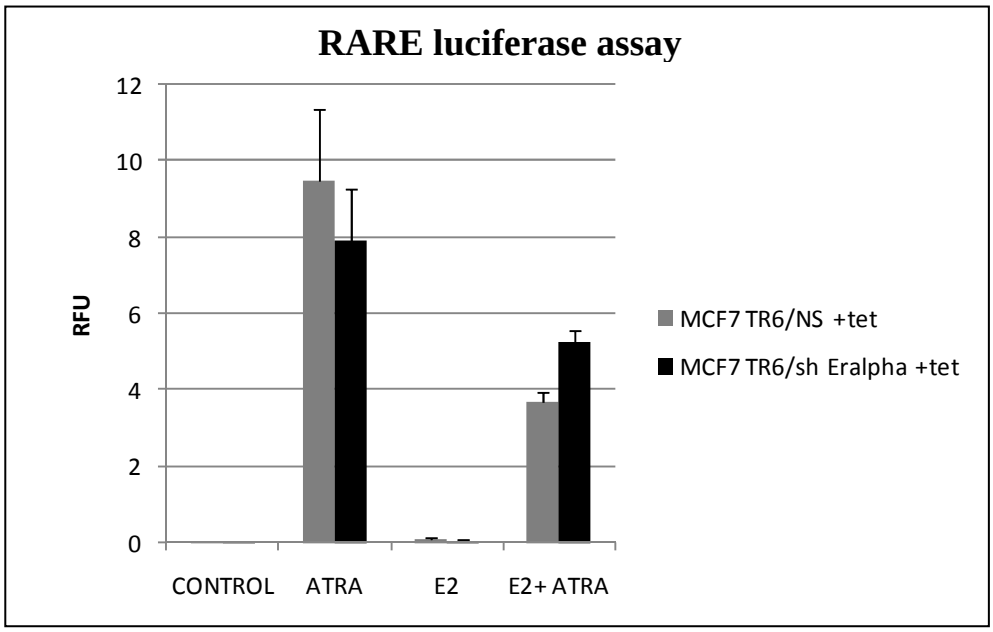

Figure 30: Cells were transfected with the retinoid-dependent reporter construct, *RARE-tk-Luc*, and treated for 24 hours in the presence of vehicle (DMSO), ATRA (1 μM), E_2 (10nM) and the combination E_2 with ATRA in presence and absence of tetracycline.

As already show in figure 17B, E_2 addition is able to reduce the RARE dependent transcriptional activity. This effect is reverted by ERα knock-down (compare the RFU ratio between ATRA and E_2 *plus* ATRA treated samples in fig. 30) as expected since ERα mediates E_2 transcriptional signaling in MCF7.

The data described above suggest that ATRA exerts its antiproliferative activity in MCF7 by specifically antagonizing E_2 triggered cell proliferation by ERα.

It is interesting to note, that White and colleagues have described a dose dependent inhibitory effect of retinoic acid on ERE-luc dependent promoter (White *et al*, 2005). The evidences here reported indicate a two-way cross-talk between E_2 and ATRA possibly mediated by ERα and RARα . They appear to counteract each other, ATRA inhibiting E_2 mediated proliferation and E_2 blocking ATRA induced cell growth arrest. These is supported also by a recent work, based on ChiP experiments, from Hua and colleagues claiming the existence of a genomic antagonism between retinoic acid and estrogen signaling in breast cancer (Hua *et al*, 2009).

To learn more about the mechanisms that underlie the cross-talk between ERα and RARα we have performed gene expression microarray since a huge insights in the molecular pathways involved in breast cancer have been disclosed by this approach.

1.5 Wide genome gene expression microarray reveals the activation of different gene pathways in MDAMB231 and MCF7 after treatment with retinoic acid.

The aim of our microarray study has been to compare the transcriptional action of retinoic acid in the two cell lines we have chosen as paradigms of ERα positive and negative breast cancers: MDA-MB-231 and MCF7. To take into account both early and late transcriptional events triggered by retinoic acid, MCF-7 and MDA-MB-231 cells were treated for 6 and 48 hours with ATRA, 1 μM. Total RNA was extracted and processed as described in materials and methods for the hybridization of the Whole Human Genome Oligo Microarray G4112F chip (Agilent). Each microarray chip that contains sequences representing over 41K human genes and transcripts, developed using data sourced from RefSeq, Goldenpath, Ensembl, Unigene, Human Genome Build 33, and others, represents a compiled view of the up to date human genome. In the subsequent analysis of the data obtained we looked for a subgroup of genes whose expression levels between replicate samples were similar, and whose expression levels between different conditions (untreated/treated or 6h/48h) were significantly different. By this approach we were able to identify reliable genes that were significantly regulated in response to ATRA treatment and we identified 473 such genes in MCF7 samples and 288 genes in MDA-MB-231 cells.

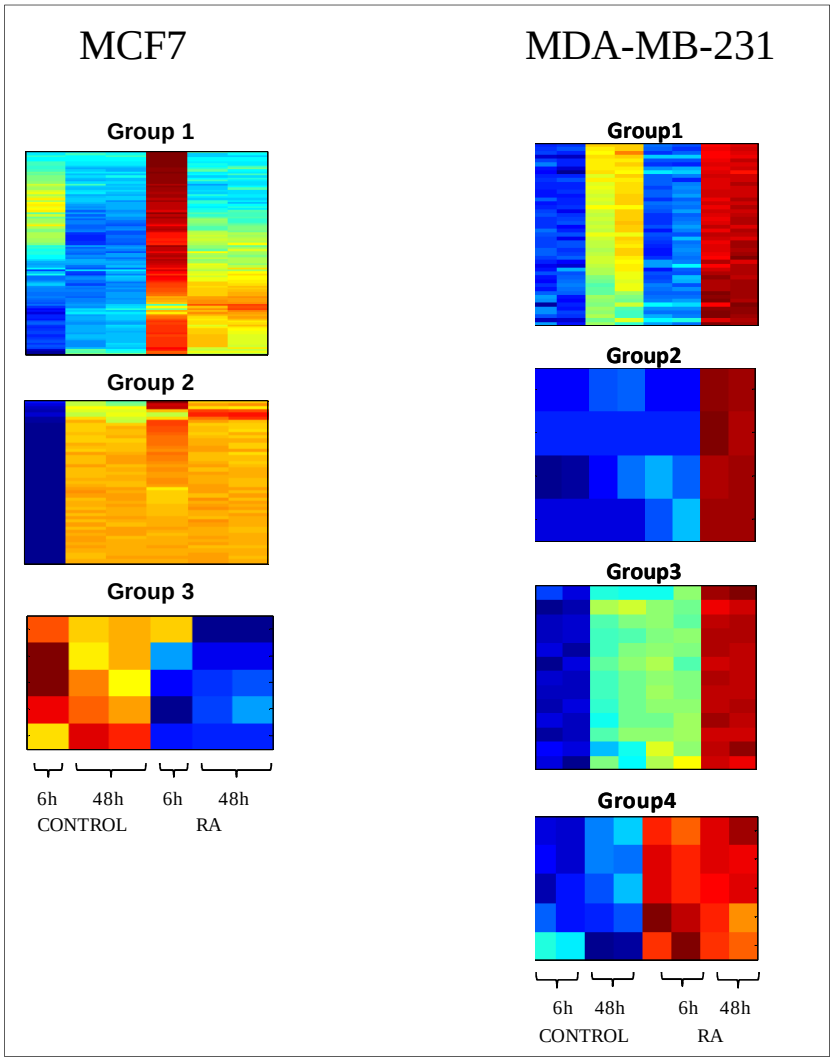

Figure 31: Results of neighborhood SPIN algorithm to represent the expression matrix of the sorted genes across MCF7 and MDA-MB-231 samples. 3 subgroups of genes, in MCF7 cells, and 4 subgroups of genes in MDA-MB-231, which are similar between replicate samples, and behave in a significantly different fashion between untreated cells and cells treated with ATRA.

The 473 genes identified in MCF7 has been divided in 3 subgroups of genes which are similar between replicate samples, and behave in a significantly different fashion between untreated cells and cells treated with ATRA.

Group1: Contains 78 genes that are up regulated in response to ATRA after 6 hours, and some of them remain up also after 48 hours relative to the control. Among these genes there are 12 known genes that are affected by ATRA (see Table 3).

Group2: Contains 50 genes that are up regulated in the untreated cells only after 48 hours, while in MCF7 cells treated with ATRA they are up regulated already after 6 hours. See Table 2 for known genes connected to ATRA.

Group3: Contains 5 genes which are down-regulated in response to ATRA.

MCF7	
GROUP 1	ACOX2, ADCY5, AHRR, AK023572, AK023757, AK024243, AK124576, ALS2CL, APOA1, ATP6V0A4, AW993939, AXIN2, A_24_P153363, A_24_P174353, A_32_P75141, BATF, BC042520, BDKRB1, BE004814, BTG2, C2orf54, C3orf32, CAPN13, CD14, CD52, CEACAM6, CNTD2, CSTA, CYP24A1, CYP26B1, DLX2, ELF3, ENST00000360329, EPAS1, EVA1, GDNF, GDPD5, GPR109B, H19, HOXA4, HOXA5, HOXB2, ID1, IGFBP6, LOC120376, LOC123688, LOC154822, LXN, LYZL2, MAG, N4BP3, NRXN3, NTN4, PADI3, PARP9, PHLDA1, PTGS1, RAP1GAP, RET, RFTN1, RGS16, S100P, SLC16A5, SLC4A8, SMPD3, SPOCK1, ST3GAL4, STRA6, THC2550463, TMC5, TMPRSS2, TMPRSS4, TNFAIP2, TNFSF10, TPPP3, USP35, WFDC5, ZBTB46
GROUP 2	ACTR2, ATP1A2, AW993939, A_24_P306754, A_24_P323778, A_24_P418106, A_32_P86264, BC041926, BCL2, BZW1, C14orf100, C18orf10, CDKN3, CIRBP, CKS1B, CKS2, CMTM6, CNOT7, CTNNB1, CYP51A1, DNAJA1, DTNA, FAM113B, GPR109B, HEXB, IARS2, ISOC1, KIAA0143, LOC120376, LSS, MYB, NDUFC1, PBX1, PDHB, PTTG2, RAN, RFC3, RPL22, SERBP1, SRP54, SRP9, SUMO1, TBC1D9B, TFRC, THC2691419, TMEM29, TUBB6, UBE2T, UTP11L, VDP
GROUP 3	BMP7, C21orf29, CLCA2, RDH16, SLC26A2

Table 3: Genes significantly different between untreated and ATRA treated samples in MCF7 cells, the red genes are connected to ATRA pathway.

With respect to MDA-MB-231, we divided the 288 identified genes in 4 subgroups of genes which are similar between replicate samples, and behave differently in untreated cells relative to cells treated with ATRA (Fig.31).

Group1: Contains 21 genes that behave in the same trend in the untreated and treated cells, but in response to ATRA, were significantly more up-regulated than without ATRA.

Group2: Contains 4 genes that are up regulated in response to ATRA after 48 hours.

Group3: Contains 5 genes which are up-regulated in response to ATRA after 6 and especially 48 hours.

Group 4: Contains 5 genes which are significantly up-regulated in response to ATRA after 6 and 48 hours relative to untreated cells.

MDA-MB-231	
GROUP 1	ABCG1, APOE, C21orf84, CFI, CHI3L2, CLDN7, DAPK2, ICAM1, ITGB3, KIAA1199, LAMA3, LTBP3, PTGS2, SERPINB3, SERPINB4, SIRPG, SLC2A5, STRA6, THC2650074, THC2699924,TNFSF10
GROUP 2	CFI, CYP26A1, RARRES1, UBD
GROUP 3	AK026140, ANGPTL4, FYB, GBP4, IL1B
GROUP 4	AK024680, A_32_P75141, BC045163, RARβ, SH2D1B

Table 4: Genes significantly different between untreated and ATRA treated samples in MDA-MB-231, the red genes are connected to ATRA pathway**.**

In conclusion, by limiting our analysis to reliable genes that are expressed in the same way in replicate samples, and significantly different in untreated and retinoic acid treated cells, we were able to identify 133 genes that are significantly regulated by ATRA in MCF7 cells, among them 16 known genes affected by ATRA. In the MDA-MB-231 cells we identified only 35 genes that are significantly regulated. This lower response is consistent with the evidence that MDA-MD-231 cells are not sensitive to ATRA treatment. However, the transcriptional response is not completely absent and includes the two metabolizing enzymes and six other genes which are likely to be related to ATRA (either because they are part of the nuclear receptor complex or because they are known to functionally interact with). It is worthwhile noticing that more than 10% of the identified genes are known to be related to ATRA supporting our approach in the identification of ATRA responsive genes.

The above described approach has allowed us to identified specific retinoid acid regulated genes in two breast cancer cell lines prototypes of ERα presence or absence as a proliferative driving force. Further exploration of these lists by such pathway enrichment analysis is needed to identify the specific molecular mechanism responsible for ATRA sensitivity in the two different cell lines and hopefully in their tumor counterpart.

1.6 Involvement of microRNAs in breast cancer

In the past decade microRNA have been demonstrated to be important regulators of transcription. Their role in mediating ATRA sensitivity in breast cancer in dependence of estrogen receptor status has been also the object of our studies in the attempt to understand the molecular relationship between retinoids and ERα.

The aim of microRNA array study is to compare the transcriptional action of ATRA in the two cell lines, in order to understand the spectrum of miRNAs over-expression or down-regulation by ATRA. Considering that the ER-negative cells (MDA-MB-231) are not phenotypically affected by ATRA, while the ER-positive (MCF7), differentiate and stop proliferation in response to ATRA.

1.6.1 MiR-21 is the sole miRNA induced by ATRA in ERα+ and retinoid sensitive MCF-7 cells

We determined the profiles of miRNAs in MCF-7 and MDA-MB-231 cells cultured with or without ATRA (Fig. 32). While ATRA had no effect on the miRNA profile of MDA-MB-231 cells, in the MCF-7 counterparts, the retinoid caused significant increases of a single miRNA, miR-21 (Fig. 32A and 17B). The result was confirmed by RT-PCR (Fig. 32C). Induction of miR-21 was detectable after 2 hours and plateaued at 24 hours (Fig. 32D). Consistent with elevated miR-21 expression in ERα+ compared to ERα- breast cancers (Mattie *et al.*, 2006), in basal conditions, the amounts of the miRNA were higher in MCF-7 than in MDA-MB-231 (Fig. 32B).

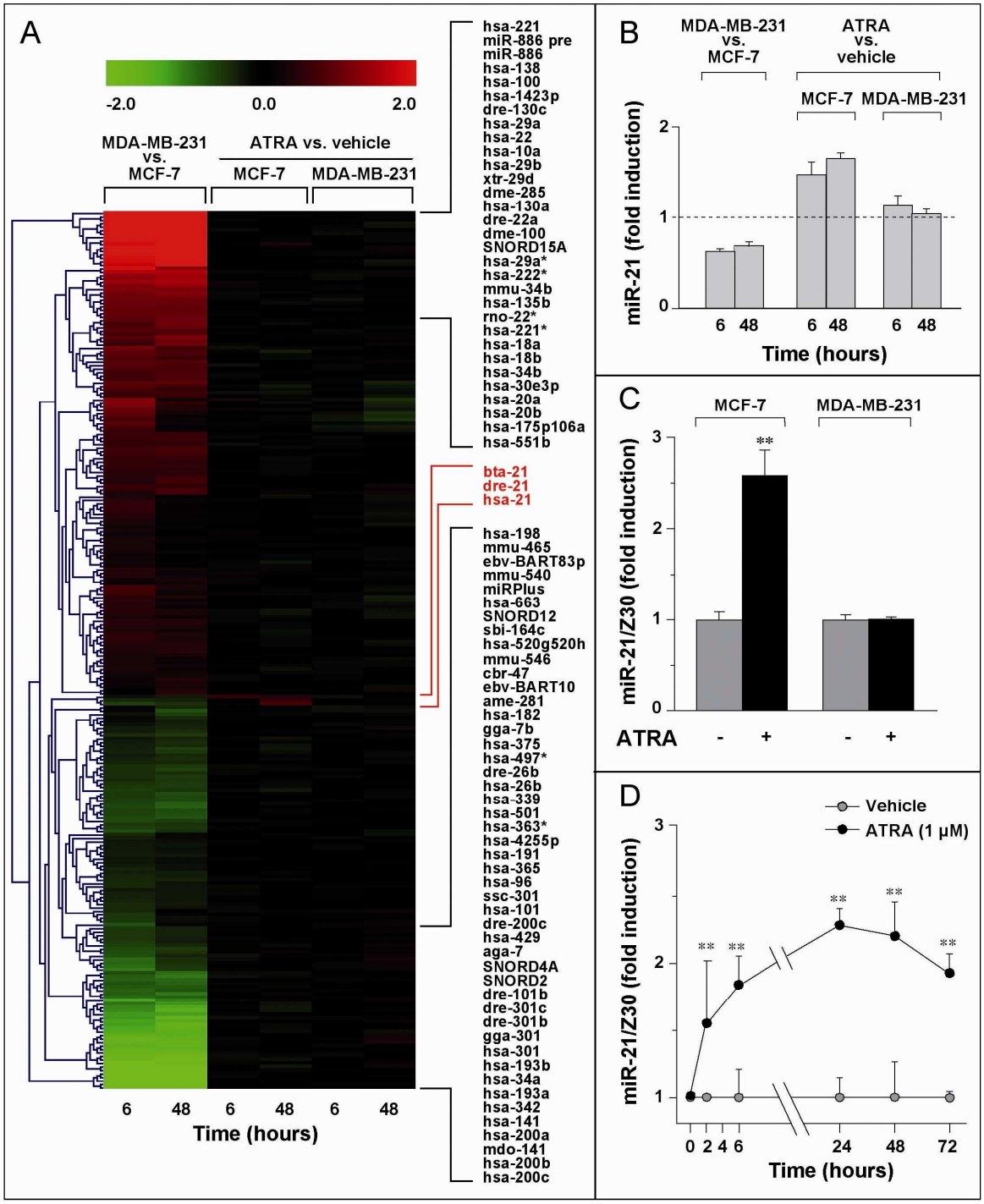

Figure 32: (A) Heat-map of microRNAs showing significant differences (adjusted p<0.05) in at least one of the comparisons indicated. Relevant microRNAs are listed on the right. **(B)** Relative levels of miR-21 (mean+S.D. of two independent microarray experiments performed in triplicate). (C) RTPCR validation of microarray results (mean+SD; 3 independent cultures). Cells were treated with ATRA (1 µM) for 6 hours. Data are representative of 3 independent experiments. ** Significantly higher than the corresponding control value (p<0.01, Student's t-test). (D) Time course of miR-21 induction. Results were obtained as in **(C)** and are representative of 2 independent experiments. ** Significantly higher than the corresponding control value (p<0.01, Student's t-test).

1.6.2 Ligand-dependent activation of RARα induces transcription of the MIR21 gene via functional retinoic acid responsive elements

To determine the molecular mechanism of miR-21 induction in MCF-7 cells, we measured the levels of the primary transcript (pri-miR-21) and the 77-nucleotide hairpin pre-miRNA (Fig. 33A). Both pri-miR-21 and pre-miR-21 were induced by ATRA, suggesting increased transcription of the MIR21 gene. In MCF-7 cells, induction of miR-21 was observed with the RARα ligand, AM580, but not with the RARγ agonist, CD437 (Fig. 33B). In line with the relative anti-proliferative and transcriptional potency of the two compounds (see Fig. 19), the inducing effect of AM580 was superior to that of ATRA. This indicates that transcriptional activation of the MIR21 gene in the ERα + cell line is mediated by RARα.

Two predicted retinoid-regulated elements (RARE-1 and RARE-2) are present in the 5'-flanking region of MIR21 (as described in materials and methods). To establish whether RARα is part of a retinoid - dependent transcriptional complex binding to the MIR21 promoter, we performed ChIP experiments (Fig. 33C). After treatment of MCF-7 cells with ATRA, RARα specific signals were determined with amplimers encompassing both the RARE-1 and RARE-2 containing regions of the MIR21 promoter, while no binding to a transcribed region of MIR21 was detected. The available evidence demonstrates that RARα is recruited to the promoter region of MIR21 after ligand activation. Hence, MIR21 seems to have all the characteristics of a direct retinoid-target gene. To define the functional activity of the predicted RARE-1 and RARE-2 sequences, the MIR21 5'-flanking region was inserted upstream of a luciferase reporter gene. In MCF-7 cells, the reporter was induced by ATRA and AM580 only when the promoter was in the sense orientation (Fig. 33D). Trans-activation of the MIR21 promoter construct was specific, as ATRA did not affect the activity of a control reporter (p-GL3). In MDA-MB-231 cells, the basal level of MIR21 promoter activity was left unaltered by ATRA (data not shown). Point mutations were introduced in critical residues of the RAR-binding consensus repeats (A/GGG/TTCA) of RARE-1 (*Mut1*: AGGACA, nt -4,218) and RARE-2 (*Mut2*: AGTGCA, nt -3,630) (Fig. 13). Only *Mut1* abrogated transcriptional activation of the *MIR21* promoter by ATRA, although a minor reduction was also observed with *Mut2* (Fig. 33E). The role of RARE-1 in ligand-dependent trans-activation of MIR21 transcription was confirmed by deletion of both RAR-binding consensus repeats (*ΔRARE1*). All this indicates that both RARE-1 and RARE-2 are functionally active and control the transcriptional activity of *MIR21* in a retinoid-dependent manner. Abrogation of the retinoid response with *Mut1* or

ΔRARE1 is consistent with a model predicting cooperative interaction between RARE-1 and RARE-2.

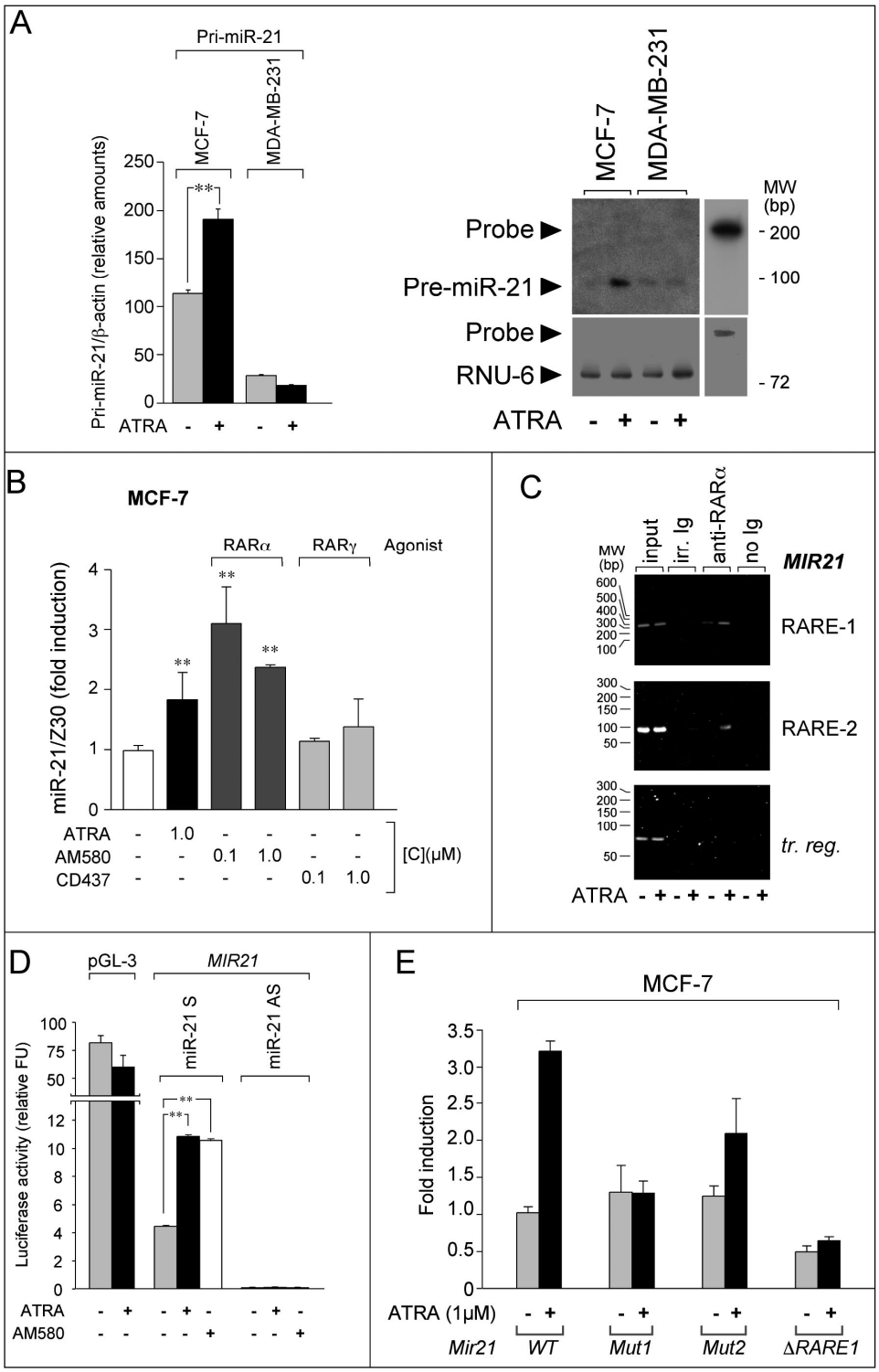

Figure 33 (A): Cells were treated with ATRA (1 μM) or vehicle (DMSO) for 48 hours. Left: RT-PCR analysis of pri-miR-21, using a primer pair corresponding to sequences placed outside the miR-21 containing stem-loop region of the primary transcript. Two sets of primers were selected from the published sequence (AY699265) and used for the amplification of pri-miR-21. The two transcripts were determined by a SYBR green based assay. Since the results obtained with the two pairs of primers were super imposable, only the

results obtained with the first pair are shown. Each value is the mean + S.D. of three separate cultures. Right: Northern blot analysis of pre-miR-21 (radio-labeled probe encompassing the stem-loop region). MW = molecular weight. The ethidium bromide staining of the gel is also shown. Results are representative of 2 independent experiments. **(B)** RT-PCR determination of miR-21 in *MCF-7* cells treated with the indicated concentrations of ATRA, AM580, or CD437, for 48 hours (mean+SD, 3 independent cultures). ** Significantly higher than control (Student's t-test, $p<0.01$). **(C)** ChIP assays of MCF-7 cells treated with vehicle or ATRA for 2 hours. For the amplification of the transcribed region (tr.reg.) of *MIR21*, we used the same pair of amplimers described in (A). **(D)** *MCF-7* cells were transfected with firefly luciferase reporter constructs driven by the 5'-flanking-region of human *MIR21* in the sense or antisense orientation. A plasmid containing a CMV-based enhancer/promoter was used as a negative control for the effect of ATRA (pGL-3). Cells were treated with ATRA (1 μM) for 24 hours. Results are expressed in arbitrary units following normalization with renilla luciferase (mean+SD, 2 replicate transfections). **(E)** Functional analysis of the putative RARE-1 and RARE-2 sequences. Constructs containing the firefly luciferase reporter gene driven by the wild type MIR21 promoter (WT) or the same promoter with point mutations in RARE-1 and RARE-2 (*Mut1* and *Mut2*, see text) or deletion of RARE-1 sequences (ΔRARE1) were transfected in MCF-7 cells, before treatment with vehicle or ATRA (1 μM) for 24 hours.

1.6.3 RARα expression and sensitivity to retinoids is not sufficient for the induction of miR-21

To investigate the determinants of cell-specific miR-21 induction, we selected three other breast carcinoma cell lines with different sensitivity to ATRA (Fig. 34) and distinct patterns of ERα/RAR/RXR expression (Fig. 18B). ERα-positive T47D cells were sensitive to the anti-proliferative action of ATRA and responded to the retinoid with miR-21 induction (Fig. 20A). In contrast, expression of the miRNA was unaltered in the two ERα-negative cell lines, SKBR3 and MDA-MB-453 cells, showing sensitivity and resistance to ATRA, respectively. Our results suggest a correlation between ERα-positivity and miR-21 up-regulation by ATRA. Furthermore, the data obtained with SKBR3 cells support the concept that sensitivity to retinoids and high levels of RARα in the context of ERα negativity are not sufficient to determine miR-21 induction by ATRA.

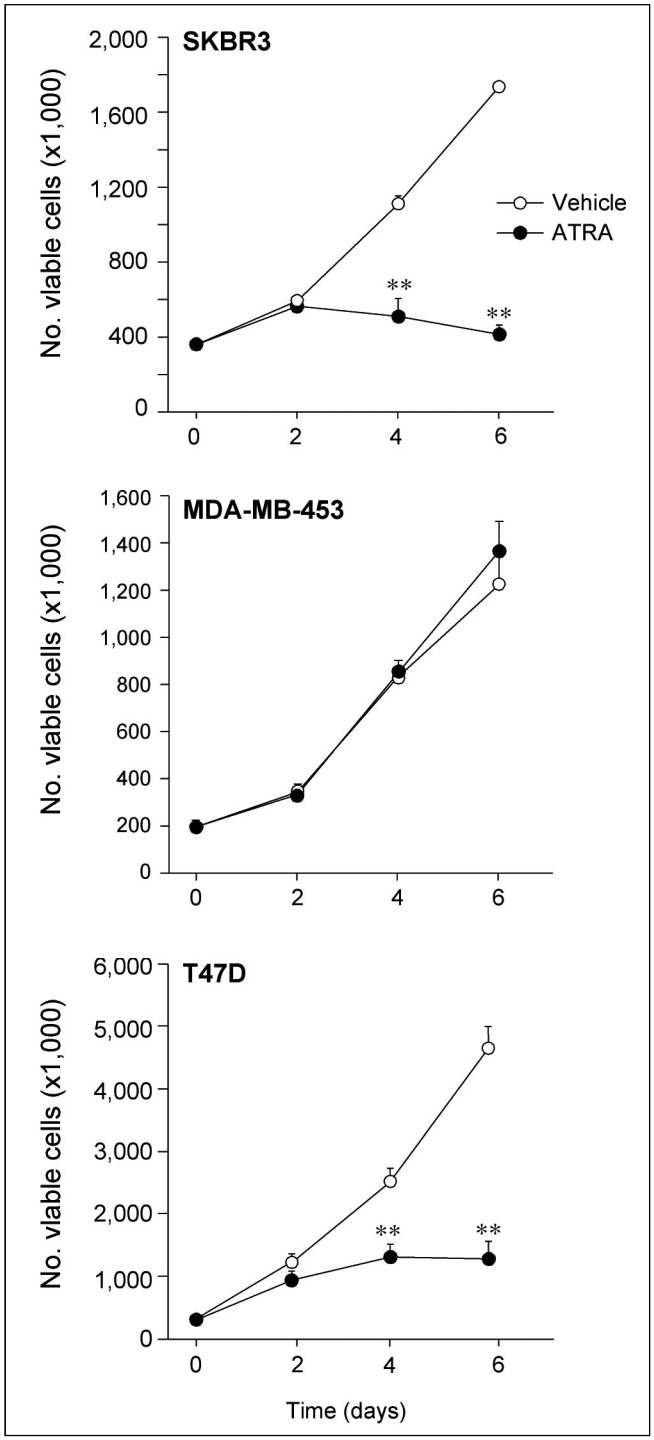

Figure 34: ERα - SKBR3 and MDA-MB-453 as well as ERα+ T47D cells were treated with ATRA (1 μM) for the indicated amount of time. The growth curves of the three cell lines are shown after staining with trypan blue for the determination of cellular viability. Each value is the mean + S.D. of three replicate cultures. ** Significantly lower than the corresponding vehicle-treated value (Student's t test, $p<0.01$).

Selective induction of miR-21 by ATRA in ERα+ cells may be controlled by multiple factors, including antagonistic (Hua *et al.*, 2009) (see also Fig. 2B) or cooperative (Ross-Innes *et al.*, 2010) interactions between the estrogen and the retinoid receptor pathways. A

direct correlation between miR-21 and ERα expression in breast cancer has been observed (Elgort *et al.*, 1996; Mattie *et al.*, 2006). On the other hand, E_2 has also been reported to exert the opposite effect, down-regulating miR-21 in ERα+ cells (Wickramasinghe *et al.*, 2009). For all these reasons, we evaluated whether induction of miR-21 by ATRA was influenced by E_2. MCF-7, T47D and MDA-MB-231 cells were estrogen starved for 5 days and subsequently exposed to ATRA, E_2 or E_2+ATRA for 48 hours (Fig. 20B). MiR-21 levels in MDA-MB-231 were unaffected by E_2 and/or the retinoid, whereas the estrogen inhibited miR-21 expression in MCF-7 (Wickramasinghe *et al.*, 2009) and T47D cells. In these conditions, increased expression of miR-21 by ATRA was evident both in the presence and absence of E_2. All this supports the idea that ligand-activated ERα does not exert a significant effect on miR-21 induction by ATRA and suggest that as yet undefined permissive mechanisms underlay retinoid-dependent up-regulation of miR-21 in ERα + cells.

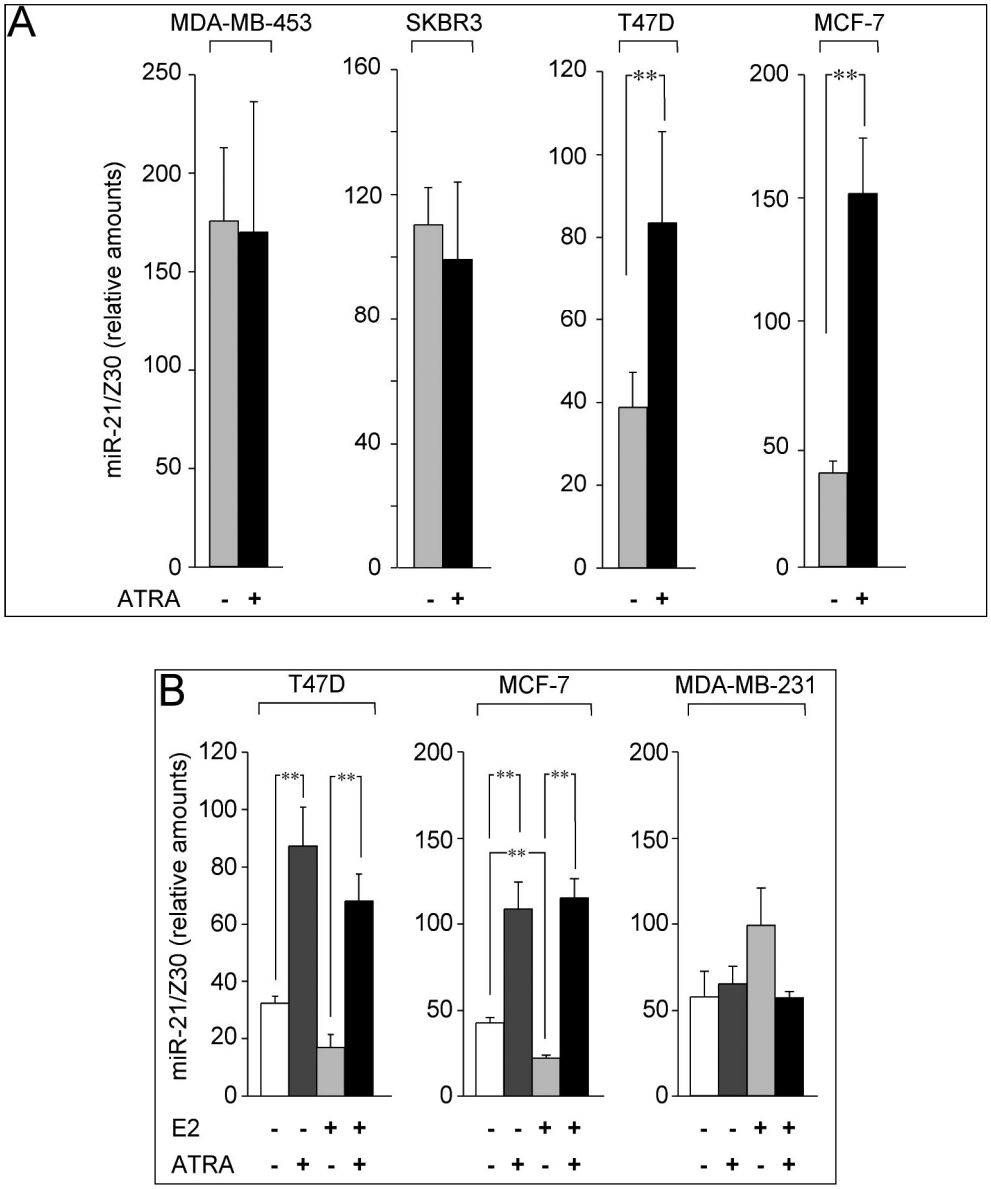

Figure 35: (**A**) The indicated cell lines were treated with ATRA (1 μM) for 48 hours. The microRNA fraction was extracted and subjected to the determination of mature miR-21 by quantitative RT-PCR. Data are normalized for the content of the Z30 control microRNA and are the mean+S.D. of the RNA extracted from three independent cultures. ** Significantly higher than the corresponding control (Student's t-test, $p<0.01$). The data are representative of at least two independent experiments. **(B)** The indicated cell lines were depleted of E_2 by culturing in F12 medium supplemented with charcoal stripped serum for 5 days. Cells (125,000/ ml) were replete and treated with vehicle, E_2 (0.01 μM), ATRA (1 μM) or the combination of the two agents for 48 hours. Mature miR-21 were determined as above. The data are the mean+S.D. of three independent cultures. The results shown are representative of two independent experiments. ** Significantly different (Student's t-test, $p<0.01$). Total RNA was extracted and subjected to quantitative RT-PCR for the determination of pri-miR-21. The data are the mean+S.D. of three independent cultures. The results shown are representative of two independent experiments. ** Significantly higher (Student's t-test, $p<0.01$).

1.6.4 MiR-21 modulates growth, senescence and motility in MCF-7 cells

Induction of miR-21 by an anti-proliferative agent like ATRA in ERα+ breast carcinoma cells was unexpected, as the miRNA is endowed with oncogenic properties and is over expressed in many tumors (Krichevsky and Gabriely, 2009). Thus, we studied the role of miR-21 induction in some of the cellular responses underlying the therapeutic activity of ATRA. To this purpose, we over-expressed or silenced miR-21 by transient transfection experiments of appropriate and validated oligonucleotides (*pre-miR-21* and *anti-miR-21*). *Pre-miR-21* stimulated *MCF-7* cell proliferation at both the time points considered (Fig. 36A). In complementary studies, *anti-miR-21* did not alter the proliferative action of E_2 in the same cell line (Fig. 36B). ATRA exerted only minimal effects on the growth of control oligonucleotide (*anti-NC*) treated and E_2 stimulated cells at these early time points. In contrast, significant growth inhibition by ATRA was observed upon miR-21 silencing, indicating sensitization of *MCF-7* cells to the anti-proliferative activity of the retinoid. These results suggest that miR21 induction is part of negative feed-back loop counteracting the anti-proliferative activity of ATRA. Senescence is a modality by which cells lose the ability to divide and is the final destiny of ATRA-treated *MCF-7* cells (Chen *et al.*, 2006). The effect of miR-21 silencing on ATRA-induced senescence was evaluated (Fig. 36C). Control-transfected cells became positive for the senescence marker after 4 days of treatment with ATRA. The percentage of senescent cells was significantly augmented in ATRA-treated cultures transfected with *anti-miR-21*. Thus, miR-21 induction counteracts not only the anti-proliferative but also the cell-aging program activated by the retinoid.

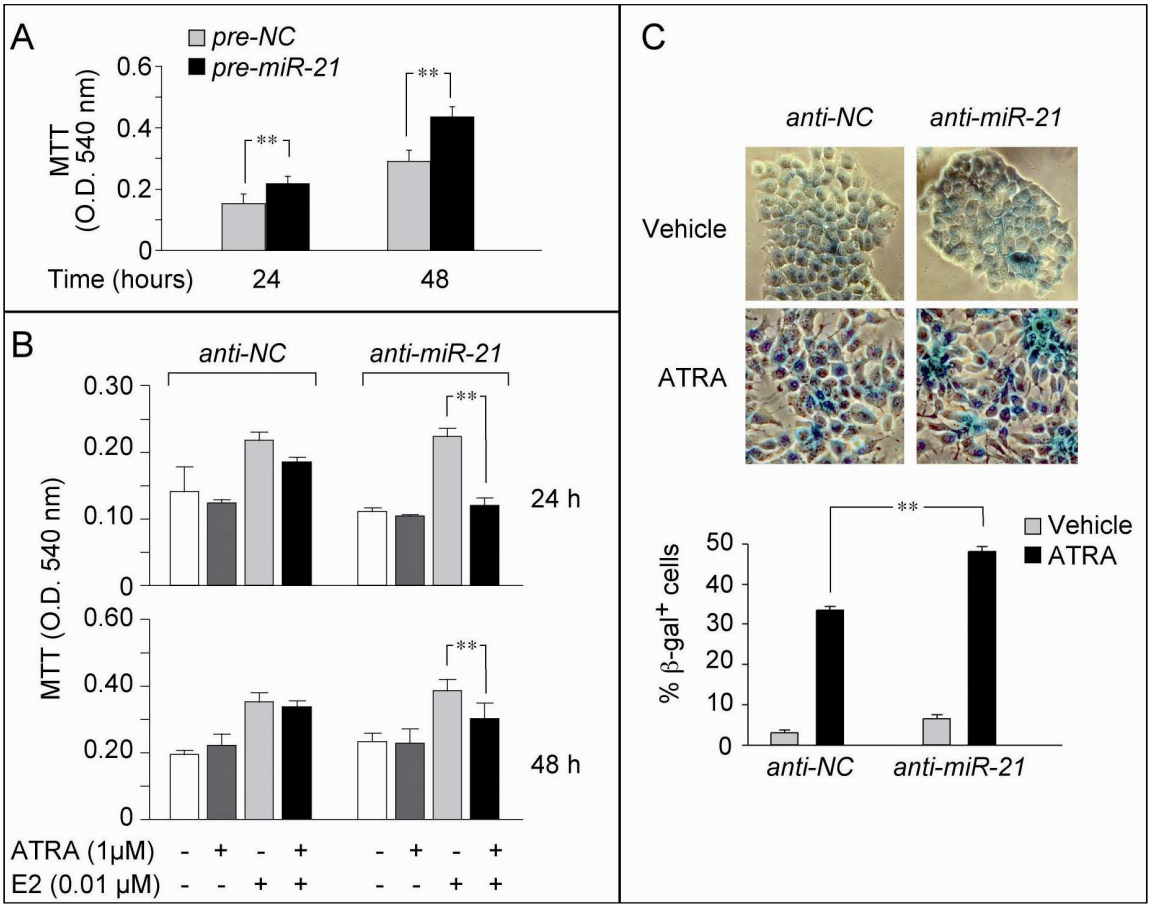

Figure 36: **(A)** MCF-7 cells were transfected with *pre-miR-21* or the negative control *pre-NC*. Cell growth was evaluated 24 or 48 hours later by the MTT assay (mean+SD, 3 independent cultures). **(B)** E_2- depleted MCF-7 cells were transfected with *anti-miR-21* or the negative control, *anti-NC*, 24 hours before treatment with ATRA and/or E_2 for 24 or 48 hours and proliferation evaluated as in **(A)**. **(C)** MCF-7 cells were transfected with *anti-miR-21* or *anti-NC*. After transfection cells were treated with vehicle or ATRA (1 μM) for a further 4 days in the presence of E_2 (0.01 μM) and stained for the senescence marker β-galactosidase. The pictures show representative light micrographs and the bar-graph illustrates the percentage of β-galactosidase-positive cells. More than 500 cells/field, 4 fields/experimental point, were counted (mean+SD, 3 independent cultures). (A), (B), (C) ** $p<0.01$ (Student's t-test). Results are representative of 3 independent experiments.

The metastatic process is a sequence of events that includes increase in cancer cell motility. As retinoids are endowed with anti-metastatic properties (Nwankwo, 2002), we evaluated the action of ATRA on random cell motility, using time-lapse microscopy. In our assay conditions, ATRA (1μM) inhibited the ability of both MCF-7 and T47D cell lines to move in a non directional manner, while exerting no appreciable effect on MDA-MB-231 cells (data not shown). AM580 (0.1 μM) suppressed random motility of MCF-7 cells, again indicating involvement of RARα (Fig. 37A). The significance of miR-21 for inhibition of cellular motility by ATRA was examined by knocking down the miRNA in MCF-7 and T47D cells. Retinoid-dependent inhibition of MCF-7 and T47D motility was

reproducibly reversed by transfection of *anti-miR21* (Fig. 37B). This implies that miR-21 induction mediates at least part of the reduction in cell motility afforded by ATRA in ERα+ breast cancer cells.

The results obtained in retinoid treated MCF-7 and T47D cells were unexpected, as a permissive role of miR-21 in cancer cell dissemination has been reported (Li *et al.*, 2009). Reversion of motility inhibition may be the consequence of a retinoid-dependent re-orientation of some cellular responses to miR-21. Indeed, in the absence of ATRA, transfection of *anti-miR 21* in both MCF-7 and T47D cells resulted in a reproducible decrease, rather than increase, in cell motility (Fig. 22B). This observation was supported by mirror studies involving transfection of MDA-MB-231 cells with *pre-miR-21*, which resulted in augmentation of random motility in an ATRA independent manner (Fig. 37C). We propose that exposure of ERα+ sensitive cells to retinoids are associated with a switch of this miR-21 biological activity.

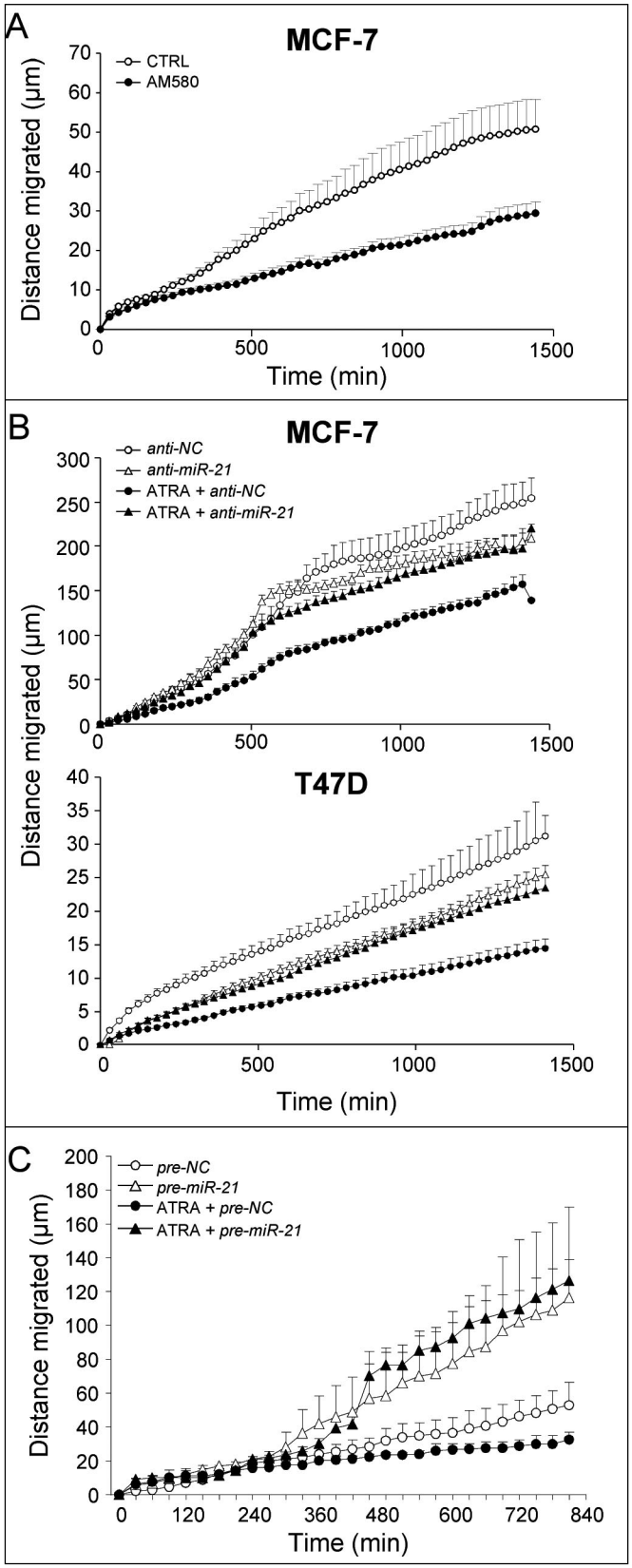

Figure 37

Figure 37: (**A**) *MCF-7* cells were treated with vehicle or AM580 (0.1 µM) for 48 hours. Single cell random motility was monitored by time-lapse microscopy for the indicated amount of time. Each value is the mean+SEM of at least 16 cells/experimental point. (**B**) E_2-depleted *MCF-7* or *T47D* cells (5 days) were transfected with *anti-miR-21* or *anti-NC* along with pEGFP-N1 (containing the enhanced green fluorescent protein gene, GFP) before treatment with ATRA (1 µM) or vehicle in the presence of E_2 (0.01 µM). The displacement of individually tracked GFP-positive cells (mean+SEM, at least 10 GFP-positive cells/experimental point) was measured by time-lapse microscopy, starting 48 hours after transfection. Results are representative of 3 independent experiments. All the points of the curves corresponding to the *anti-NC* and ATRA+*anti-NC* treatments are significantly different (Student's t-test, $p<0.01$). (**C**) *MDA-MB-231* cells were transfected with *pre-miR-21* or *pre-NC* along with pEGFP-N1 before treatment with ATRA (1 µM) or vehicle. Random motility was measured as in (**B**). Results are representative of 3 independent experiments. All the points of the curves corresponding to the *pre-miR-21* and *pre-NC* treatments are significantly different regardless of the presence/absence of ATRA (Student's t-test, $p<0.01$).

1.6.5 MiR-21 regulates the established target, maspin, in MCF-7 cells

To identify functionally relevant genes regulated by ATRA-dependent miR-21 induction, we focused our attention on the differential expression of established miR-21 targets (Krichevsky and Gabriely, 2009) in MCF-7 and MDA-MB-231 cells exposed to ATRA. The only gene whose expression was clearly reduced by ATRA selectively in MCF-7 cells was maspin. Subsequent RT-PCR experiments showed ATRA dependent down-regulation of maspin not only in MCF-7 but also in T47D cells and confirmed lack of modulation by the retinoid in MDA-MB-231 cells (Fig. 38A). In MCF-7 cells, down-regulation of maspin mRNA was evident at 6 and maximal at 48 hours (Fig. 38B). Consistent with the relative ability to induce miR-21, AM580 was more effective than ATRA in inhibiting the expression of maspin mRNA (Fig. 38C), which was not reduced by CD437. To gain direct evidence for miR-21 involvement in retinoid-dependent control of maspin expression, MCF-7 cells were transfected with anti-miR-21 and pre-miR-21 (Fig. 38D). Anti-miR-21 increased the basal levels and prevented down-regulation of maspin mRNA by ATRA. In contrast, pre-miR-21 enhanced this effect.

Altogether these results indicate that induction of miR-21 by ATRA controls the levels of maspin transcript in ERα+ cells.

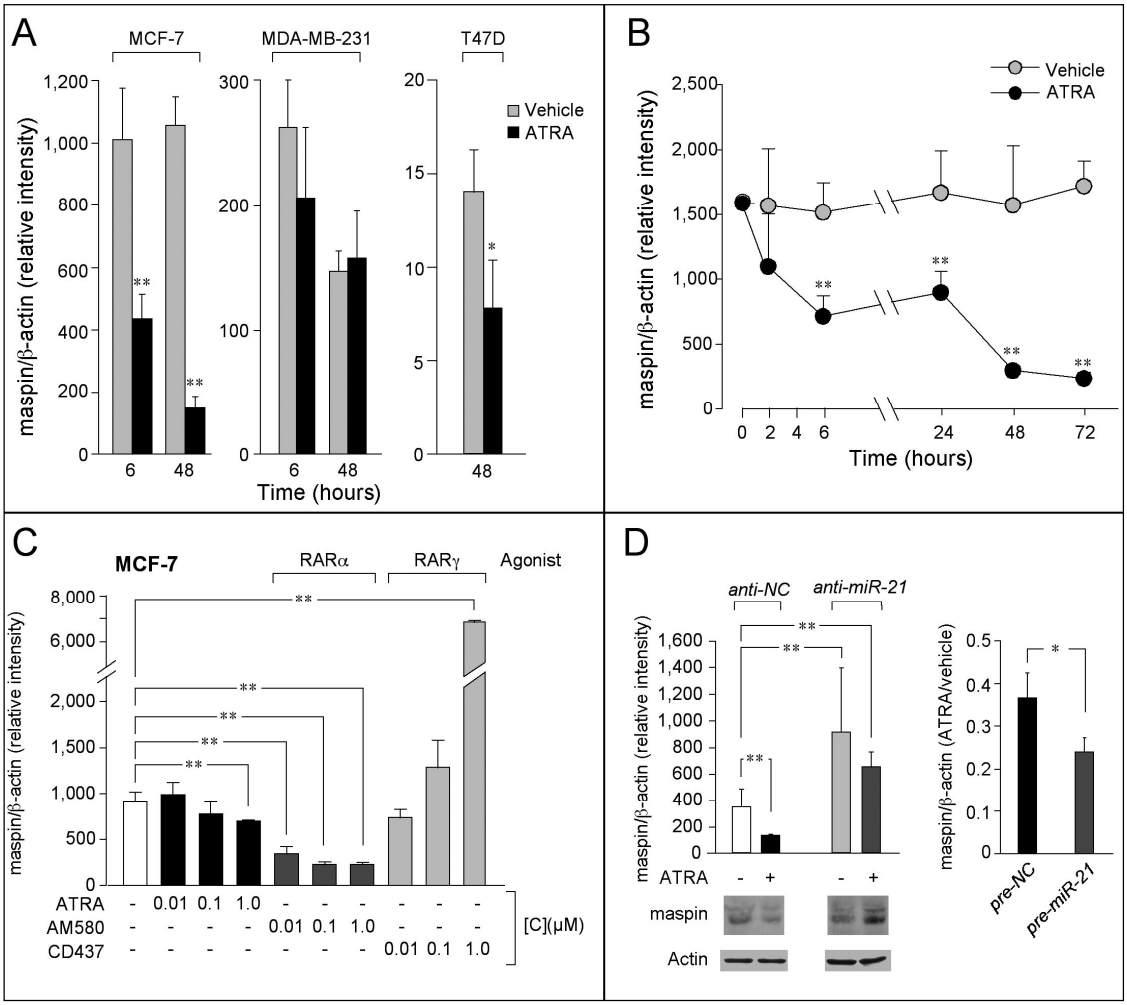

Figure 38: The levels of maspin transcripts were determined by RT-PCR using a Taqman assay (SERPINB5 (Maspin), Hs00985283_m1). Results are the mean+SD, 3 independent cultures). **(A)** MCF-7, MDA-MB-231 or T47D cells were treated with 1 μM ATRA. **(B)** MCF-7 cells treated with 1 μM ATRA (time course). **(C)** MCF-7 cells were treated with the indicated compounds. **(D)** Left: MCF-7 cells were transfected with anti-miR-21 or the negative control, anti-NC, before treatment with ATRA for 72 hours. Right: MCF-7 cells transfected with pre-miR-21or the negative control, pre-NC and cultured for 72 hours. Results are expressed as the ATRA/vehicle ratios. All the data are representative of at least 2 independent experiments. * $p<0.05$, ** $p<0.01$ (Student's t-test).

1.6.6 IL1B, ICAM1 and PLAT are direct miR-21 targets

We exploited our cellular model to identify novel transcripts negatively controlled by ATRA via miR-21, using gene-expression microarrays in combination with a bio-informatics approach. Given the large pool of miR-21 target genes predicted by the commonly available algorithms, we proceeded by progressively restricting the number of potential miR-21 targets. Initially, ATRA- induced changes in the transcriptomes of MCF7 and MDA-MB-231 cells were determined. As miR-21 was induced by ATRA only in MCF-7 cells, we focused on genes whose retinoid-dependent regulation was significantly

different ($p<0.01$) in the two cellular contexts. Out of the 481 genes identified, 276 were either down-regulated in MCF-7, but not in MDA-MB-231, or up-regulated by the retinoid in MDA-MB-231, but not in MCF-7 cells. This is the regulation pattern expected for direct targets of miR-21.

Subsequently, we focused on the 78 genes (20%) that were predicted to be potential miR-21 targets by at least one of the algorithms, Miranda, TargetScan or PITA. Genes involved in inflammation and immunity, including IL1B, are enriched in this last group. To examine the role of miR-21 in the regulation of these genes, MCF-7 cells were transfected with anti-miR-21 or anti-NC and treated with ATRA for 72 hours before analysis of global gene expression with microarrays. The two transcriptomes were compared and the data obtained for the 67 predicted miR-21 target genes were extracted. We expressed the results as the Log2 ratio of the anti-miR-21/anti-NC signal, anticipating that this value would be positive for direct miR-21 targets. Using a threshold value of 0.2, the prediction was fulfilled for 15 (22%) of the genes.

We selected 7 candidate miR-21 targets of biological interest. Differential regulation of the corresponding mRNAs by ATRA in MCF-7 and MDA-MB-231 cells was validated by RT-PCR, and the expression of IL1β was investigated also at the protein level (data not shown). CCR1, PLAT, PTX3 and TNFAIP3 were representative of potential miR-21 targets down-regulated by ATRA in MCF-7 cells. IL1B, ICAM1 and ITGB3 represented genes, whose ATRA-dependent up-regulation in MDA-MB-231 was not observed in MCF-7 cells possibly as the result of a suppressive effect exerted by miR-21 induction. Except for CCR1, all the selected transcripts were higher in MCF-7 than in MDA-MB-231 cells, consistent with the basal levels of miR-21 in the two cell lines (data not shown). To define whether any of these 7 genes is indeed a direct miR-21 target, we cloned the 3'UTR of the selected transcripts downstream of a luciferase reporter and evaluated the effect of miR-21 in 293T cells, which contain low levels of the miRNA (Zhu *et al.*, 2008) (Fig. 39B). MiR-21 inhibited the expression of the ICAM1, ITGB3, PLAT and IL1B constructs. The inhibitory effect was similar (PLAT) to or higher (IL1B, ICAM and ITGB3) than that observed with the 3'UTR of maspin. We confirmed the biological significance of miR-21 induction for the control of IL1B, ICAM1 and PLAT mRNAs in MCF-7 cells challenged with anti-miR-21 and ATRA, by RT-PCR (Fig. 39C). Silencing of miR-21 resulted in the appearance of detectable amounts of IL-1B after treatment with ATRA (48 and 72 hours). Similarly, in the presence of the retinoid, anti-miR-21 caused up-regulation of ICAM1 (72 hours) and relieved PLAT down-regulation (72 hours). The relevance of miR-21 induction

for the control of IL1B and PLAT is supported by mirror results obtained in MDA-MB-231 cells exposed to pre-miR-21 and ATRA (Fig. 39D). In these conditions, forced expression of miR-21 reduced ATRA-dependent induction of PLAT mRNA, as well as IL1B mRNA and the respective protein. ICAM1 was not down-regulated by pre-miR-21 in MDA-MB-231 cells, suggesting that direct regulation by miR-21 of the transcript is superseded by other regulatory circuits activated by ATRA in these cells.

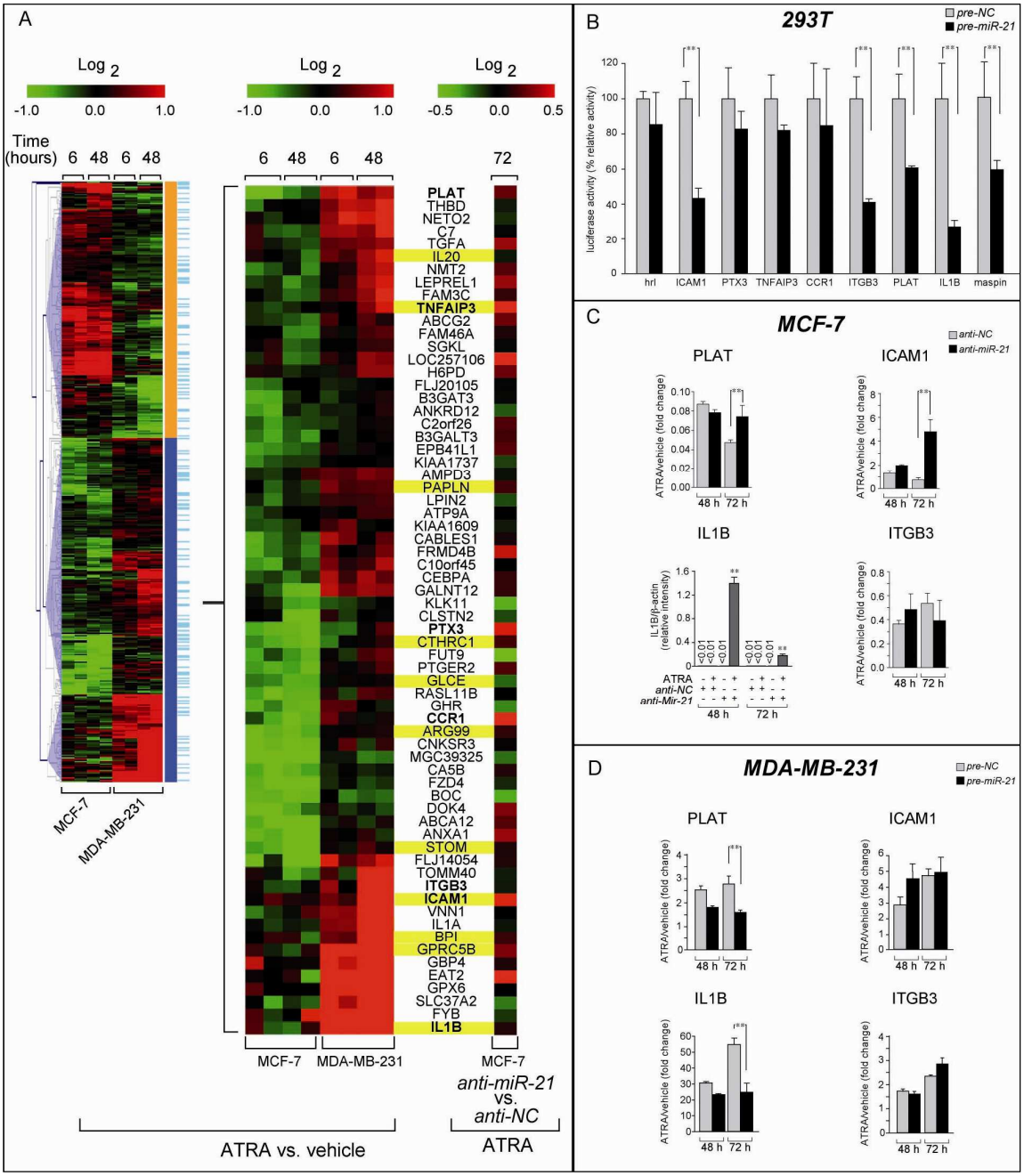

Figure 39: **(A)** Gene expression profiling of MCF-7 and MDA-MB-231 cells treated with vehicle or 1 μM ATRA (Log2 ratio of ATRA *vs.* vehicle). Left: heat-map of genes whose retinoid-dependent regulation is significantly different in the two cell lines ($p<0.01$) and classification in patterns consistent with miR-21 regulation (blue cluster) or not (orange cluster). Genes predicted to be target of miR-21 by Miranda, PITA or TargetScan are indicated in light blue. Middle: Partial blow-out of the blue cluster containing the 68 predicted and annotated targets of miR-21. Right: Heat-map of the same transcripts derived from MCF-7 cells treated with 1 μM ATRA (72 hours) after transfection with *anti-miR-21* or *anti-NC* (Log2 ratio of ATRA+*anti-miR-21* vs. ATRA+*anti- NC*). Genes belonging to inflammatory/immune responses/leukocyte migration pathways are marked in yellow. **(B)** 293T cells were co-transfected with *pre-NC* or *pre-miR-21* and plasmids containing the renilla luciferase reporter upstream of the indicated cDNAs' 3'-UTR. Forty eight hours later, luciferase activity was determined (Gregory PA, *et al.*, 2008). The results were normalized for the transfection efficiency using firefly luciferase. Each value is the mean + SD of three replicates and is representative of 3 independent experiments. Effect of anti-miR-21 in MCF-7 **(C)** and pre-miR-21 in MDA-MB-231 **(D)** cells on the ATRA dependent regulation of IL1B, PLAT, ICAM1 and ITGB3 mRNAs. Cells

were transfected with the indicated oligonucleotides and extracted RNA subjected to RT-PCR. Results were normalized for the expression of the β-actin mRNA, are the mean+SD of triplicate cell cultures and are representative of 3 independent experiments. ** Significantly different ($p<0.01$).

2. Characterization of retinoid sensitivity/resistance in Her2/Neu positive breast carcinoma cells

Breast carcinoma is traditionally classified according to the presence/absence of estrogen receptor α (ERα) and Her2/Neu, a membrane protein coded for by the ERBB2 gene. This classification has both prognostic and therapeutic value. Since breast carcinoma is a paradigmatic example of heterogeneity in the responses to retinoids, the second aim of the research program of my PhD was to characterize retinoid sensitivity/resistance in breast carcinoma and to define its molecular mechanism across the Her2 pathway.

We identified the Her2/neu+ subset as a group of tumors of particular interest for the development of new retinoid-based therapeutic strategies on the ground of a number of considerations and some data obtained.

The vast majority of Her2/neu+ carcinomas is the result of an amplification event involving the ERBB2 gene, interestingly, ERBB2 maps to chromosome 17 and is located at a short distance (0.65 Mb) from the RARA locus which encodes RARα. Different observations made in different breast cancer cell lines with RARα-specific agonists and antagonists suggest that RARα is a primary determinant of breast cancer cells' sensitivity to retinoids. This makes it plausible that a certain proportion of breast carcinoma with amplification of ERBB2 may carry an amplification of RARA.

2.1 Sensitivity of breast cancer cell lines to all-trans retinoic acid

To define the functional significance of RARα and/or other nuclear receptors, we evaluated the sensitivity of different breast carcinoma cell lines to the anti-proliferative effects of ATRA, which is a pan-RAR agonist, binding and activating all RAR isoforms with similar affinity. Logarithmically growing cells were challenged with increasing amounts of the retinoid and we determined half maximal effective concentrations (EC_{50}) (Fig. 40). The three cell lines, SKBR3, AU565 and UACC-812, characterized by co-amplification of ERBB2 and RARA showed the highest sensitivity to ATRA, with similar EC_{50} values falling in the low nanomolar range. These values more than one order of magnitude lower than the EC_{50} calculated for the two control cell lines MCF-7 and T47D, which represent popular models of the very well known association between ERα-positivity and sensitivity to retinoids in breast carcinoma. In contrast, MDA-MB453 and

HCC-1569 cells, which are characterized by an ERBB2-containing amplicon that does not extend to the RARA locus in the context of ERα-negativity, show the a higher refractoriness to the anti-proliferative effect of ATRA as the ERα- and ERBB2-negative MDA-MB-231 cell line. Interestingly, expression of ERα in the context of the ERBB2 gene amplification, as observed in the case of MDA-MB-361 cell line restores sensitivity to the growth inhibitory effect of the retinoid. As expected the ERα negative, ERBB2 not amplified MDA-MB-231 cell line is highly refractory to challenge with retinoids. Taken together, our results demonstrate that RARα expression above a certain threshold level, be it the result of RARA gene amplification, ERα induction or both mechanisms, confers sensitivity to ATRA.

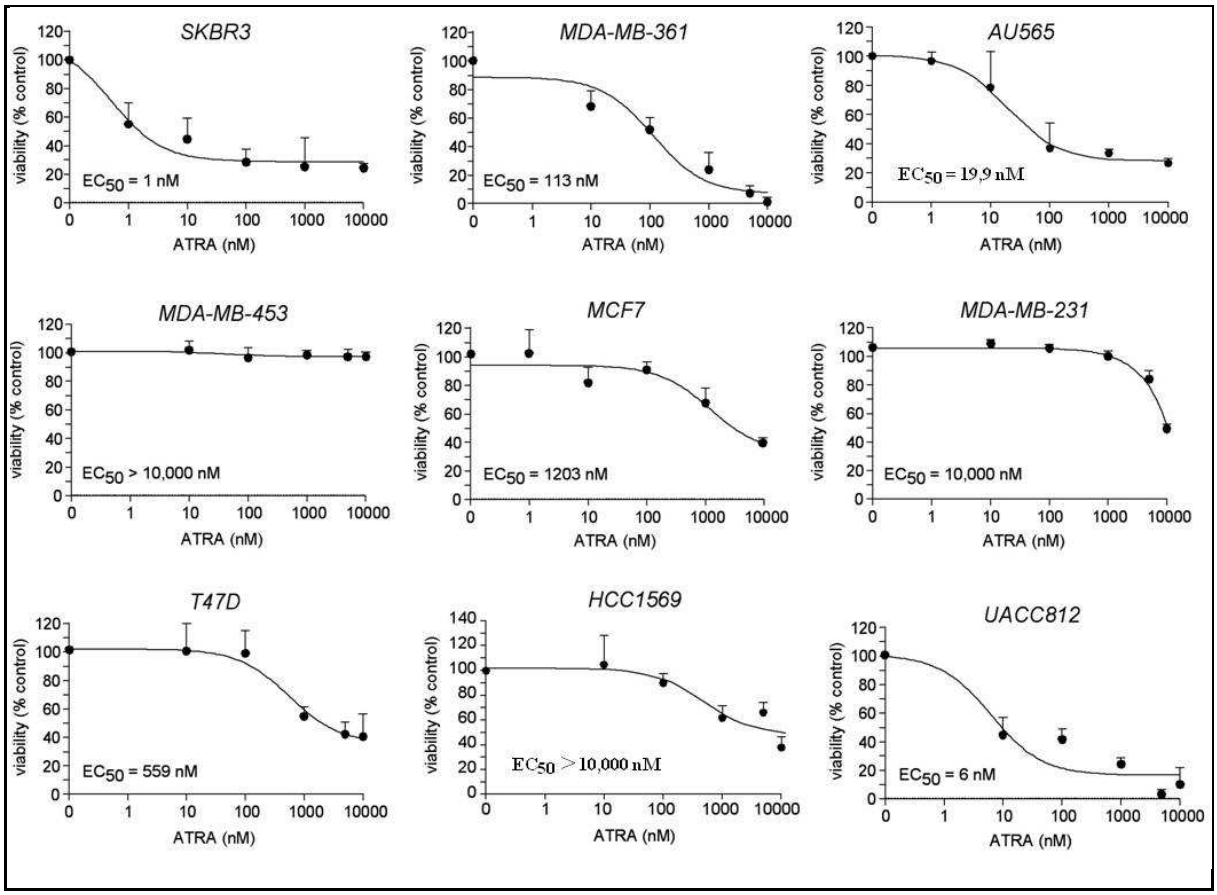

Figure 40: The indicated cells were challenged with increasing concentrations of ATRA and the anti-proliferative effect of the retinoid determined. The growth and viability of cells was measured with a coulter counter after staining with trypan-blue. The EC_{50} is the concentration of a drug that gives half-maximal response.

2.2 Role of retinoic acid receptors in mediating sensitivity to retinoids

As shown in fig 26, RARα is not or poorly expressed in cell lines refractory to ATRA (MDA-MB453, MDA-MB231, HCC1569), while cells that respond to the growth inhibitory effect of the retinoid express the nuclear receptor. As RARα is not the only nuclear retinoid receptor potentially active in breast carcinoma, expression of RARγ, RARβ and RXRα in our panel of cell lines was also evaluated by Western blot analysis (Fig. 41). Consistent with results obtained in other cell lines and breast carcinoma samples, the amounts of RARγ synthesized in the various cell lines are similar and no correlation is observed with RARα or Her2/neu expression. A similar situation is evident in the case of RXRα, which is likely to represent the major silent partner of the RXR/RAR complexes present in these as well as other breast carcinoma cell lines. Our cell lines do not show detectable amounts of RARβ in basal conditions, although treatment with ATRA causes induction of the receptor particularly in the majority of cells expressing high levels of RARα, regardless of the Her2/neu status.

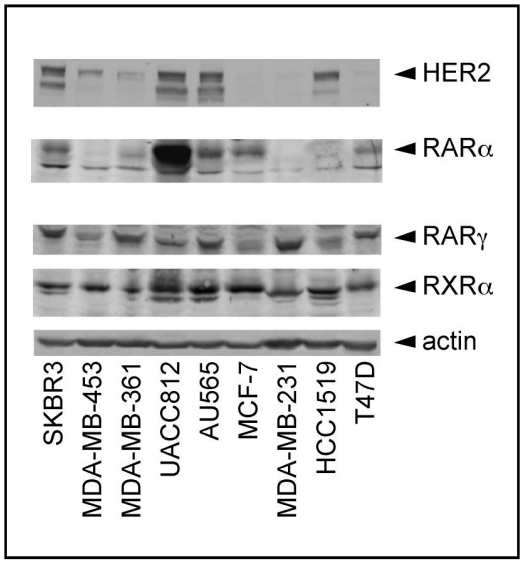

Figure 41: A panel of cell lines screened by Western blot analysis showing the levels of RARα, RARγ, RXRα and HER2 proteins in the cell lines considered.

We further evaluated the effect of ATRA treatment on RARγ or RARβ and Her2 on two cell lines representative of sensitivity/refractoriness to retinoids in the context of the ERBB2 amplification. We can see in figure 42 that ATRA down-regulates the expression of HER2 in SKBR3 cells and this may represent the mechanism underlying the anti-proliferative effect of the retinoid in cells with co-amplification of RARA and ERBB2 and

the ATRA-dependent down-regulation of RARα and RARγ is expected as it is the result of degradation coupled to activation of the two receptors. In contrast and as expected ATRA has no effect on MDA-MB-453 cells, which are characterized by an ERBB2-containing amplicon that does not extend to the RARA locus in the context of ERα-negativity even if they express RARγ. Therefore sensitivity to retinoids is not influenced by the presence of RARγ.

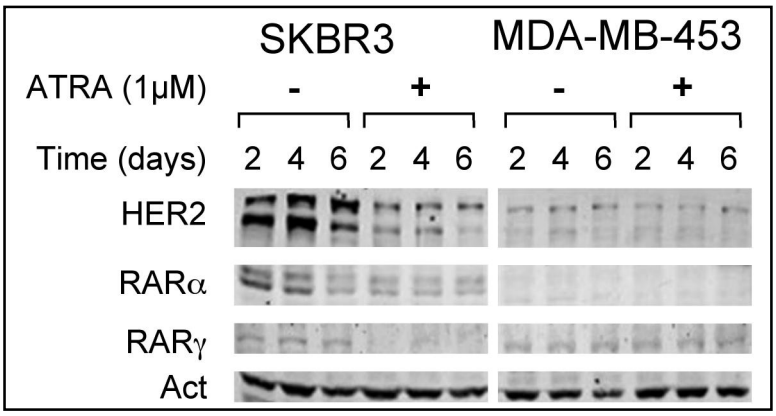

Figure 42: SKBR3 and MDA-MB-453 cells were challenged with vehicle (DMSO) or ATRA (1 μM) for 2, 4 and 6 days. A Western Blot analysis illustrating the levels of RARα, RARγ and HER2 proteins is shown.

Complementary evidence supporting a pivotal role for RARα in the observed effects was provided by the results obtained after treatment of SKBR3 cells with the three synthetic retinoids, AM580, CD437 and ATRA (Fig. 43). The RARα selective agonist, AM580, is the most powerful anti-proliferative agent in SKBR3 cells showing co-amplification of RARA and ERBB2. The responsiveness of SKBR3 cells to the anti-proliferative effects of AM580 is two orders of magnitude higher than that of HER2- and ER+ cells, T47D cells. This is the same type of differential sensitivity observed in the case of ATRA. Regardless the three synthetic retinoids were completely ineffective in the context of the MDA-MB-453 (characterized by an ERBB2-containing amplicon that does not extend to the RARA locus), lending support to the specificity of the results obtained.

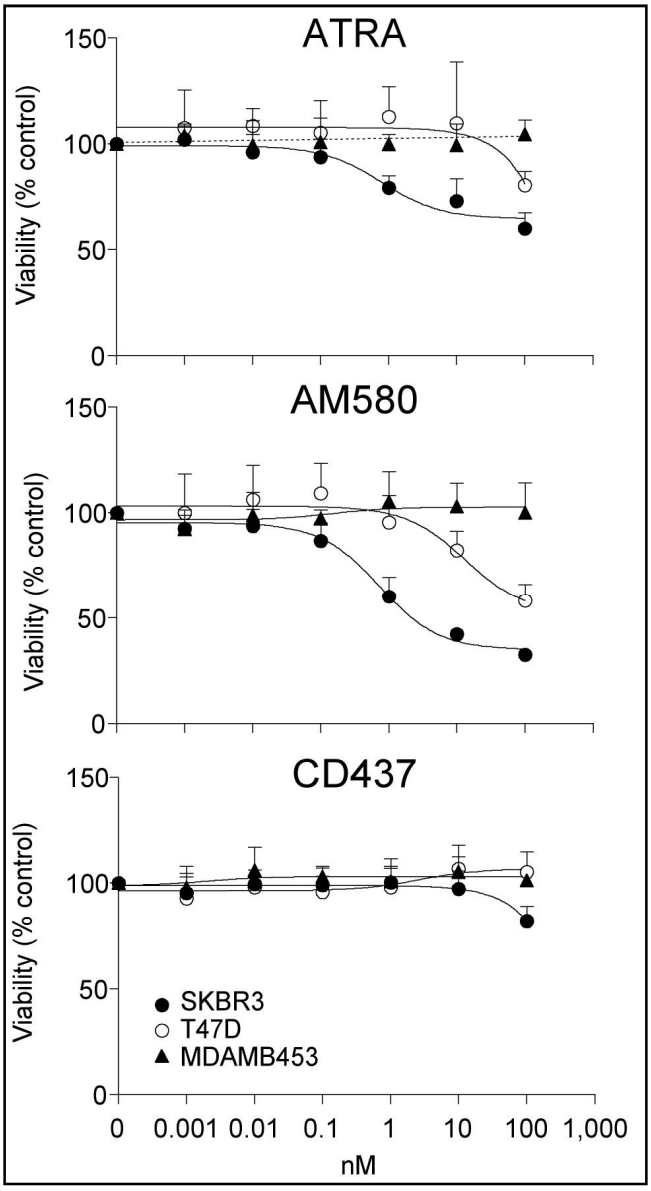

Figure 43: SKBR3, T47D and MDA-MB453 cells were challenged with increasing concentrations of the RARα selective agonist, AM580, the RARγ selective agonist, CD437 and the pan-RAR agonist ATRA as an internal reference. The growth of cells was measured using the Sulforhodamine assay.

Taken together, our results demonstrate that RARα expression above a certain threshold level, be it the result of RARA gene amplification, ERα induction or both mechanisms, confers sensitivity to ATRA. In addition, they indicate that sensitivity to retinoids is not influenced by the presence of RARγ. Finally, the data presented are in line with the idea that RARα expression is particularly effective in inducing retinoid sensitivity, when the breast carcinoma cell is endowed with amplification of ERBB2 and consequent expression of the Her2/neu protein. This suggests a specific effect of retinoids on the proliferative pathway(s) activated by Her2/neu in ERBB2-positive breast carcinoma cells.

2.3 Cell lines recapitulating ERBB2 and RARA co-amplification

To perform functional studies, cellular models recapitulating RARA gene-amplification in the context of Her2-neu-positivity were sought for, evaluating a panel of breast carcinoma cell lines with an RT-PCR approach (Fig. 44A). SKBR3, AU565 and UACC-812 cells were characterized by co-amplification of the ERBB2 and RARA genes. Similar relative ratios of co-amplification (4.4, 4.0 and 4.7, respectively) were observed in the three cell lines. MDA-MB361, MDA-MB453 and HCC-1569 cells were endowed with amplification of the sole ERBB2 gene. As expected, MCF-7 showed no amplification of ERBB2 and RARA genes. Co-amplification of RARA translated into increased basal levels of the corresponding protein product in SKBR3, AU565 and UACC-812 relative to MDA-MB-361 and MDA-MB-453 cells (Fig. 44B). The amounts of RARα synthesized in the Her2/neu-positive cell lines showing co-amplification of RARA were comparable to those present in MCF-7 and T47D cells (as described above). These two cell lines synthesize significant amounts of RARα as a consequence of the inducing effect exerted by ERα expression. With respect to this, it is interesting to notice that UACC812 and MDA-MB-361 are ERα-positive and are the cell types endowed with the largest amounts of RARα within the two distinct groups of cells characterized by simple and combined amplification of the ERBB2/RARA gene pair, respectively.

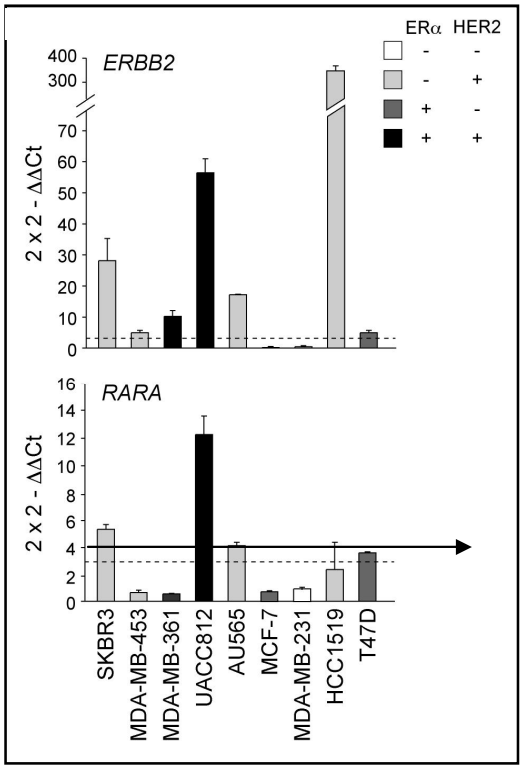

Figure 44: The specific RT-PCR assay described was used to define the copy number of RARA and ERBB2. The panel of cell lines screened includes cell lines already characterized for the HER2 and ER status, as indicated and Western blot analysis showing the levels of RARα, RARγ, RXRα and HER2 proteins in the cell lines considered (Fig. 41).

2.4 RARA and ERBB2 co-amplification in HER2+ breast carcinoma patients

So an important objective of the project is to establish whether a sub-population of patients suffering from Her2-positive breast carcinoma has the ERBB2-RARA amplification and if is responsive to treatments based on ATRA. We obtained the samples from the divisions of medical oncology at the hospital of Reggio Emilia. We have focused our attention on the sub-population of patients characterized by co-amplification of ERBB2 and RARA, with no distinction for the ERα status.

DNA was extracted from paraffin-embedded histological samples obtained from HER2+ breast carcinoma samples (from year 2006 on) of 76 cases classified as Her2/Neu+ by immune-cyto-chemistry and studied to amplification of the HER2 and RARA loci by RT-PCR (Fig. 45A). The patients showed amplification of HER2, indicating that a significant fraction of Her2/Neu^{+} breast carcinomas is characterized by this genetic abnormality. In 24% cases, we demonstrated simultaneous amplification of the *RARA* gene.

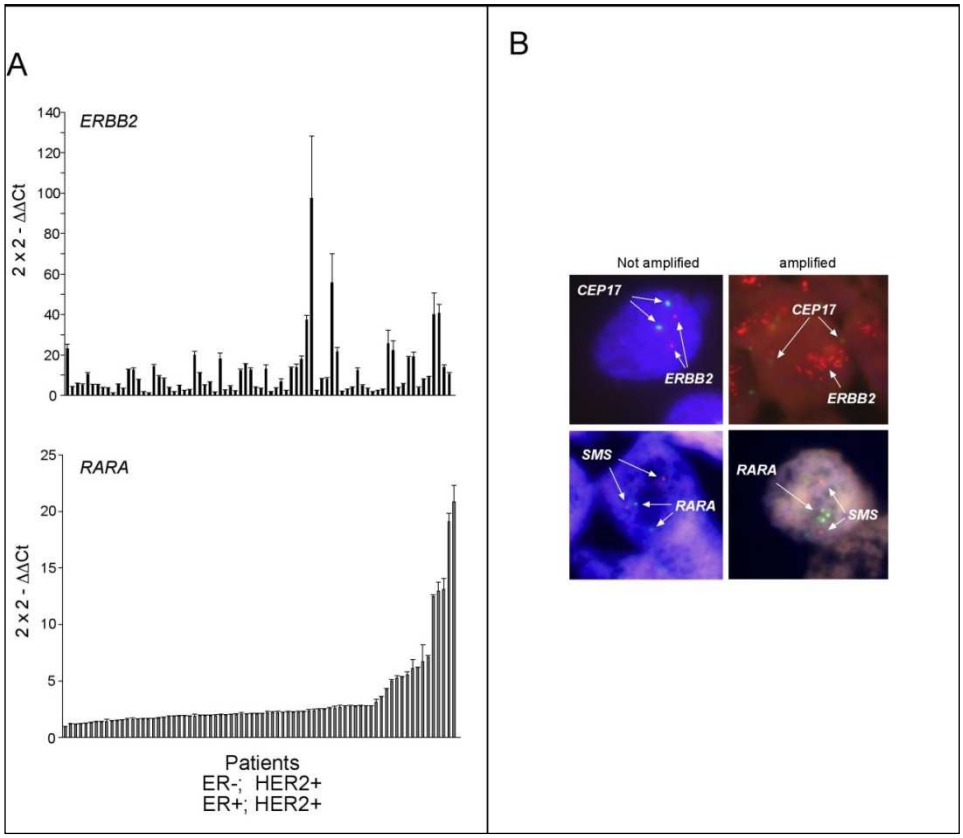

Figure 45: **(A)** A specific RT-PCR assay for the simultaneous determination of the *RARA* and *ERBB2* genes was developed. The method was implemented to determine the copy number of the two genes, using DNA extracted from paraffin-embedded histological samples obtained from HER2+ breast carcinoma samples (from year 2006 on). The cut-off used to define amplification of *RARA* and *ERBB2* is indicated by a dotted line. Each sample represent a single patient. **(B)** Most of the results obtained were confirmed by dual-color fluorescence in situ hybridization (FISH) analysis performed on interphase nuclei of the same histological sections. A representative case with amplification of both RARA and ERBB2 is shown.

2.5 Inhibition of Her2/neu by lapatinib enhances the anti-proliferative activity of retinoids in breast carcinoma cells with amplification of the ERBB2 and RARA genes

It is known that the anti-tumor activity of anticancer drugs can be enhanced by combining them with modulating agents to reduce their toxicity and/or boost their anti-tumor activity. This is particularly important considering that many human tumors have resistance mechanisms responsible for the failure of chemotherapeutic treatment so the use of resistance-counteracting compounds is potentially useful.

Given the peculiar sensitivity of breast carcinoma cells with co-amplification of ERBB2 and RARA to ATRA we evaluated whether targeting the retinoid and Her2/neu pathways simultaneously has the potential to represent a viable therapeutic strategy. To this purpose

the anti-proliferative effect of combinations between ATRA and lapatinib, a tyrosine kinase inhibitor used for the second-line treatment of Her2/neu-positive breast carcinoma (Medina *et al.*, 2008), was assessed in our panel of cell lines (Fig. 46A). Lapatinib has been described to inhibit the growth of Her2/neu-positive cell lines including the ones described in our study in a dose-dependent fashion (Konency *et al.*, 2006) (SKBR3, AU565, UACC-812, MDA-MB-453, MDA-MB-361 and HCC-1569). In contrast it does not exert any appreciable effect on the Her2/neu-negative cell lines MDA-MB-231, MCF-7 and T47D (Konency et al., 2006). To test the effect of the combination of ATRA and Lapatinib on an ERBB2/RARA amplified genetic background we used the compounds on the ERBB2/RARA amplified cell lines SKBR3 UACC-812. MDA-MB-453 was used as a negative control (Fig. 46). In agreement with retinoid sensitivity when Her2/neu-positive cell lines were challenged for 4 days with combinations of the lapatinib and ATRA cooperative interaction was observed only in the case of the SKBR3 and UACC-812 cells but not in the case of the negative control breast cancer cell line MDA-MB-453. Since MDA-MB-361 cell line has shown a peculiar sensitivity even in the absence of RARA amplification we studied the effect of the combination also in this cell line. As expected the combination results in cooperativity also in this case (Fig. 46).

To asses if the cooperative nature of the growth inhibitory effect is due to an additive or a synergistic interaction between the two compounds studied further experiments were carried out using the sulforhodamine -based proliferation assay (Skehan *et al*., 1990) on SKBR3 chosen as paradigm of ERBB2/RARA co-amplification. The anti proliferative effect of combined ATRA and Lapatinib treatments were evaluated in experiments challenging several combinations with drug concentrations spanning the whole efficacy range in SKBR3 cells (Fig. 46B). We used the isobologram analysis (Ubezio, 1985) for qualitative assessment of synergy or antagonism. As shown in fig. 46B the normalized concentrations of the combinations causing 50 or 70% growth inhibition could be evaluated by their position in relation to the line of additivity connecting all pairs of drug concentrations expected to produce 50 or 70% growth inhibition. Co-treatments were highly synergistic in SKBR3 (Fig. 46B).

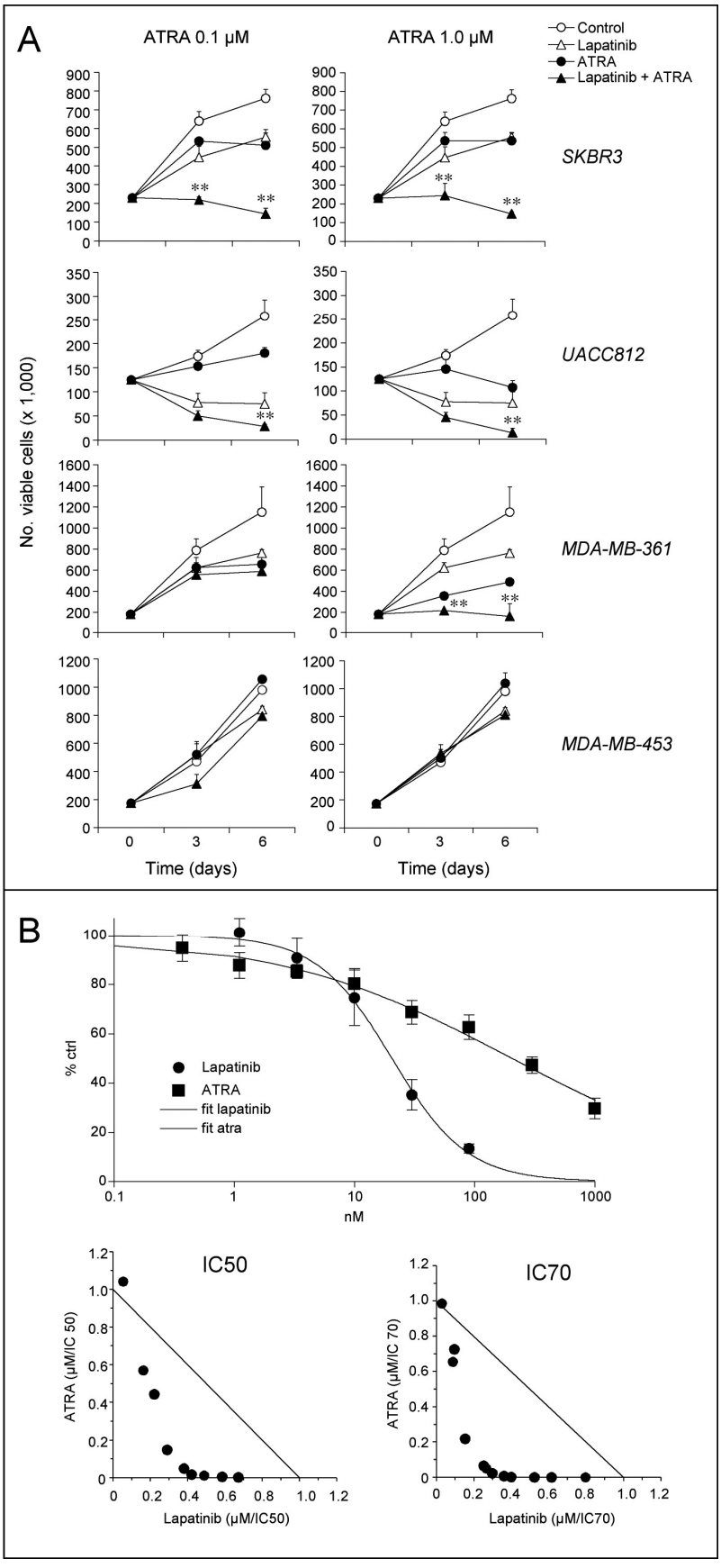

Figure 46 (A): The HER2+ cell lines, SKBR3, UACC-812, MDA-MB-361 and MDA-MB-453 cells were challenged with vehicle (DMSO), ATRA (0.1 µM), lapatinib (0.1 µM) for the indicated amount of time. The growth and viability of cells was measured with a coulter counter after staining with trypan-blue. Each value is the mean ± SD of three culture dishes.

(B): SKBR3 were treated with increasing concentrations of ATRA (0,33 to 1000nM) and Lapatinib (1 to 90 nM) for 96 hours and sulforhodamine assay was performed to asses cell proliferation. Isobolograms of ATRA and Lapatinib combinations in SKBR3 at the IC50 and IC70 levels. SKBR3 were treated with increasing concentrations of ATRA, Lapatinib or the combination for 96 hours and sulforhodamine assay was performed to asses cell proliferation. Abscissa: Lapatinib concentration, as a fraction of ATRA IC50 or IC70; ordinate: ATRA concentration, as a fraction of Lapatinib IC50 or IC70. The additivity line (dashed) separates the antagonistic (upper) from the synergistic (lower) region. The cells were exposed to a range of concentrations of ATRA plus Lapatinib. Combined concentrations producing 50 and 70% growth inhibition were calculated by fitting dose response curves of ATRA at each tested Lapatinib concentration and *viceversa.*

Figure 46

2.6 Combination of ATRA and Lapatinib cooperate to induce citotoxicity and apoptosis in SKBR3.

To establish the nature of the anti-proliferative effect of the combination ATRA/Lapatinib we evaluated the effects of the compounds on cell viability and apoptosis induction in a dose dependent manner. Treatment of SKBR3 cells for three days with two different concentrations (0.1 and 1μM) of ATRA or lapatinib determines a reduction of cell viability that is magnified by the combination of the two compounds (Fig. 47A). The loss of viability was associated with a proportional increase in cytotoxicity as determined by trypan blue analysis (Fig. 47D). The analysis of the appearance of apoptotic bodies in SKBR3 cells transfected with GFP and treated as indicated in fig. 47A revealed that the compounds combination, but not ATRA or lapatinib alone, is able to induce programmed cell death. Indeed the co-administration of ATRA and Lapatinib was able to trigger caspase-3 processing and PARP cleavage, a well known marker of apoptosis (Fig. 47B). To confirm that the synergistic cytotoxic effect observed is mediated by ATRA signaling thought RARα, we performed the trypan blue assay in the same condition above described but using specific retinoic acid receptor agonist. Fig 32A shows that the RARα selective agonist, AM580, is even more powerful that ATRA in inducing cytotoxicity in SKBR3 while the RARγ selective agonist, CD437 has no effect. In the same experimental conditions (data not shown) MDA-MB-453 did not show cytotoxicity or apoptosis after treatment with ATRA and in addition, the low cytotoxic and apoptotic effect afforded by lapatinib was not altered by addition of the retinoid.

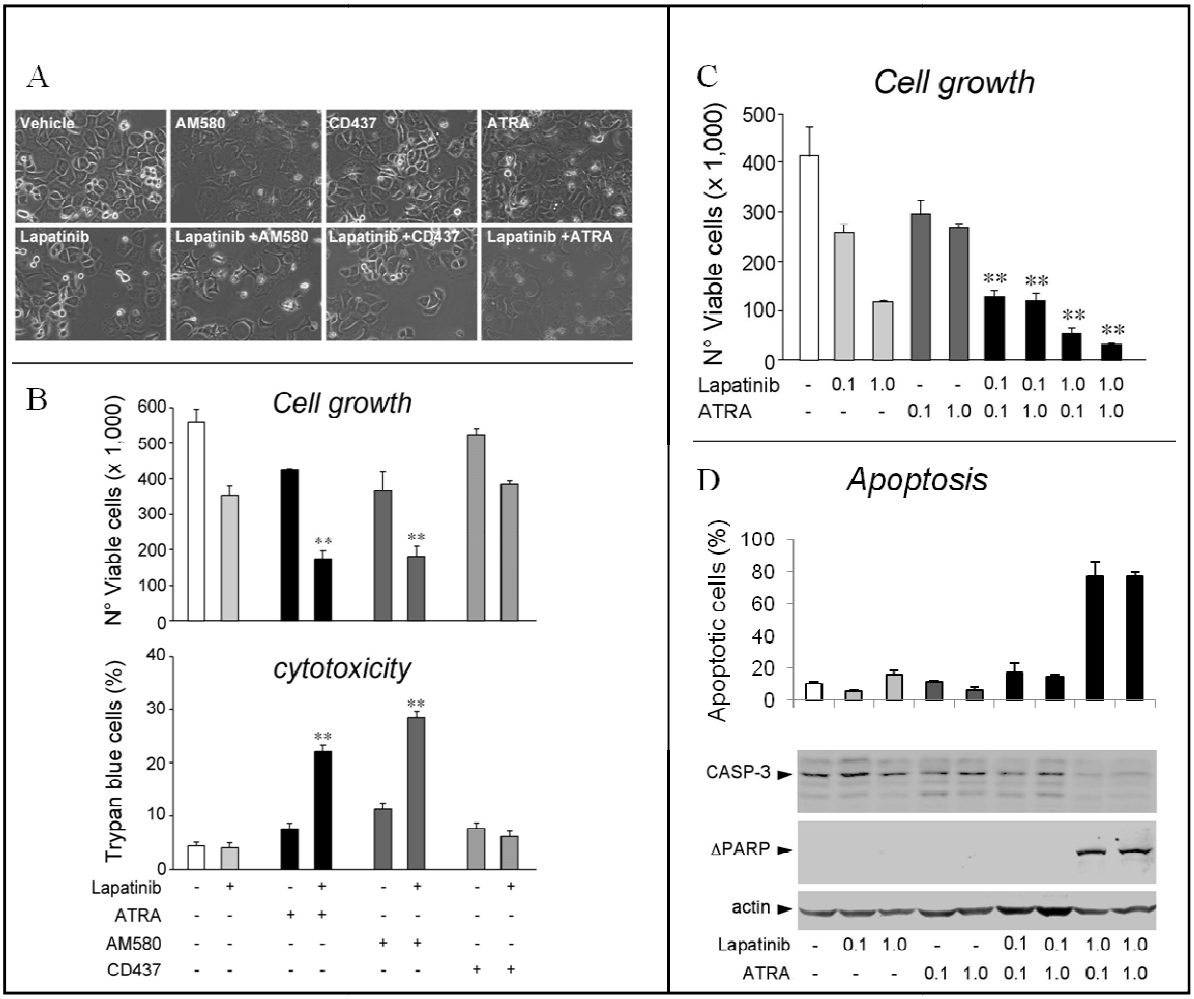

Figure 47: SKBR3 were challenged with the RARα selective agonist, AM580 (0.1 µM), the RARγ selective agonist, CD437 (0.1 µM) and the pan-RAR agonist ATRA (0.1 µM) alone or in combination with lapatinib (0.1 µM). The morphology **(A)** growth and viability **(B)** of cells was evaluated after trypan blue staining. Each result is the mean+SD of three replicate cultures. ** Significantly lower than ATRA ($p<0.01$). **(C)** SKBR3 cells were challenged with vehicle (DMSO), ATRA (0.1 or 1.0 µM), lapatinib (0.1 or 1.0 µM) and the combination of the two compounds for 4 days. The growth and viability of cells was measured with a coulter counter after staining with trypan-blue. Each value is the mean ± SD of three culture dishes. **(D)** The number of apoptotic cells was determined at the microscope, after trypan blue staining. Caspase-3 levels and degradation of PARP, two well known markers of apoptosis, were measured by Western Blot analysis.

2.7 Whole genome microarray analysis of the effect of the combination of ATRA and Lapatinib in SKBR3 reveal specific molecular pathways modulated by the compound combination.

To decipher the molecular pathways involved in the synergistic effect produced by co-administration of ATRA and Lapatinib in SKBR3, we took advantage of the whole genome microarray technique (see materials and methods). This approach was expected to reveal all the genes whose expression is influenced by the single compounds or by the combination of them. We have analyzed gene expression in a time dependent manner

looking for genes changed at 12 and 48 hours after administration of a fix concentration of ATRA (100nM) and Lapatinib (100nM) alone or in combination. We hypothesized therefore to obtain information on both early events (12h), when no detectable biologic effect occurs, and late events (48h), when the anti proliferative activity of the combination is evident. For the analysis of the obtained data, a Two-way ANOVA test was performed, for each time separately. This has lead to the identification of 2846 genes significantly affected at any time by any treatment. We subsequently performed a time course analysis using the BETR method, to select those with a temporal regulation of ATRA *vs* C, Lapatinib *vs* C, Lapatinib *plus* ATRA *vs* ATRA or Lapatinib *plus* ATRA *vs* Lapatinib, and finally Lapatinib *plus* ATRA *vs* C. We have thus identified 4 groups of genes, with relevant regulation patterns (Table 5). The fourth group is rather different from the others, as it is selected also with a maximal threshold (see also the different scale). The criteria for the identification of the four groups was based on the idea of isolating genes modified by the combination *versus* each single agent (group 1 and 2) or (group 4) by the combination only. To get insights into the molecular pathways mainly involved in each of the four groups, the EASE pathway enrichment analysis was performed, followed by Euclidean Hierarchical clustering. The principal gene pathways found modulated in each of the four groups are depicted in figures 48, 49, 50 and 51.

Group	**Selection, (BETR, comparisons)**	**Genes, (number)**
1	(LAP, vs, C, AND, LRA, vs, LAP), NOT, RA, vs, C	247
2	(RA, vs, C, AND, LRA, vs, RA), NOT, LAP, vs, C	190
Q	(LAP, vs, C, AND, LRA, vs, LAP), AND, RA, vs, C	167
R	(RA, vs, C, AND, LRA, vs, RA), AND, LAP, vs, C	163
3	Q, or, R	193
4	LRA, vs, C, not, (LAP, vs, C, or, RA, vs, C*)	182
	*with, no, significance, in, BETR, and, max, threshold, of, 0.5	

Table 5

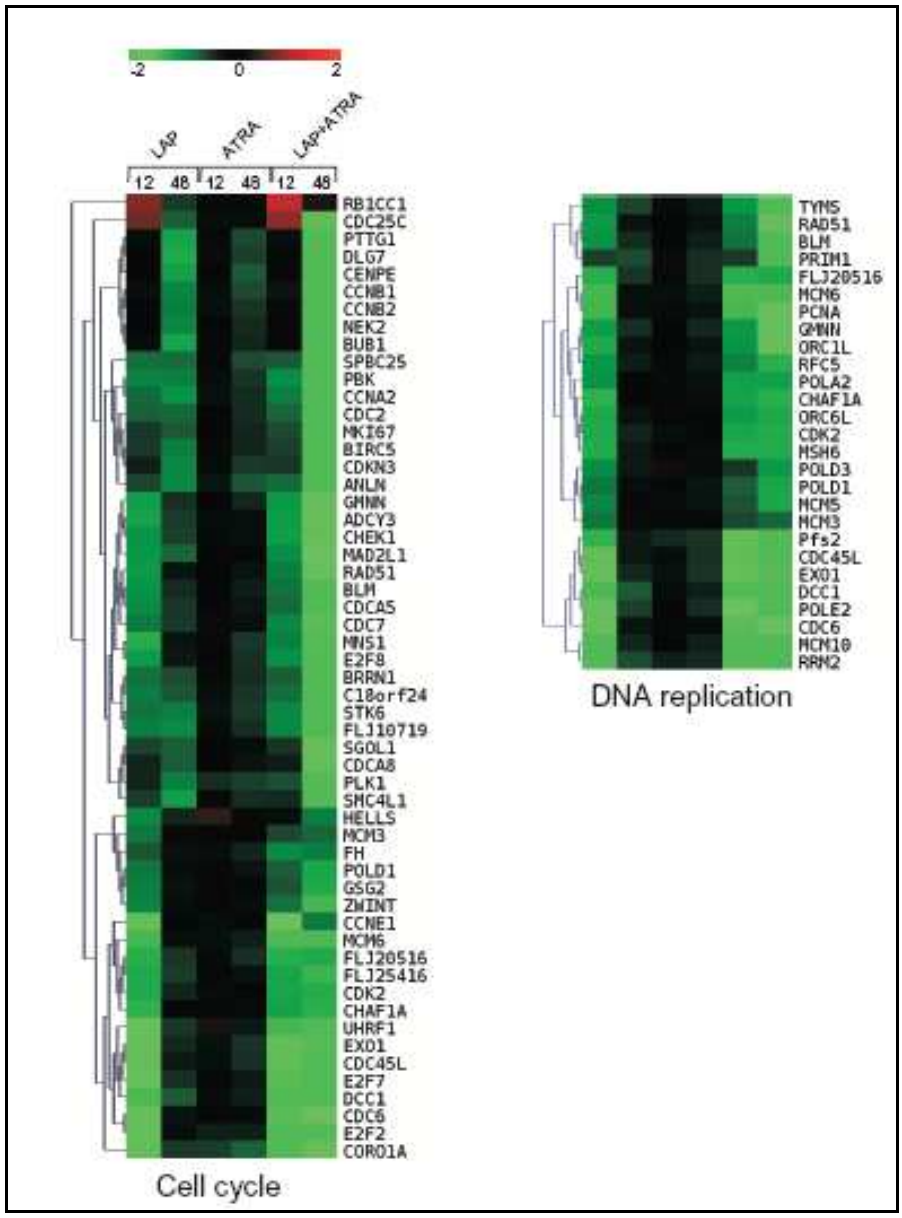

Figure 48 Cluster 1 with gene pathways found modulated.

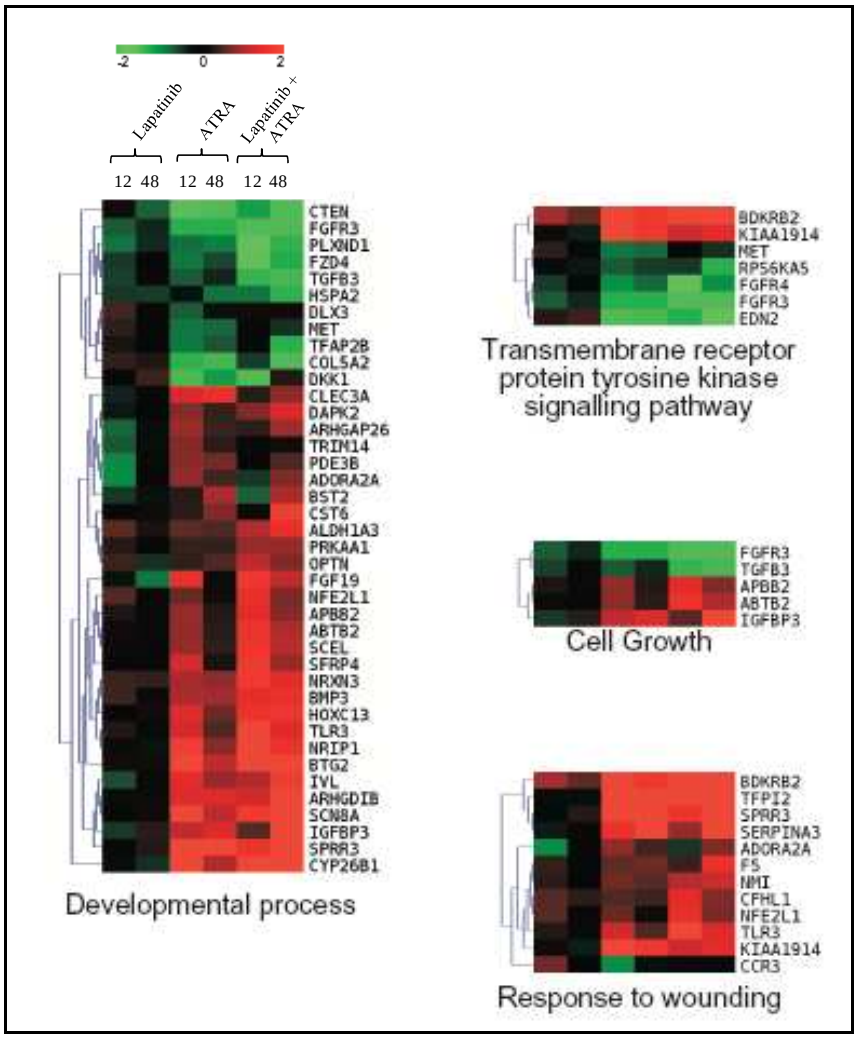

Figure 49 Cluster 2 with gene pathways found modulated.

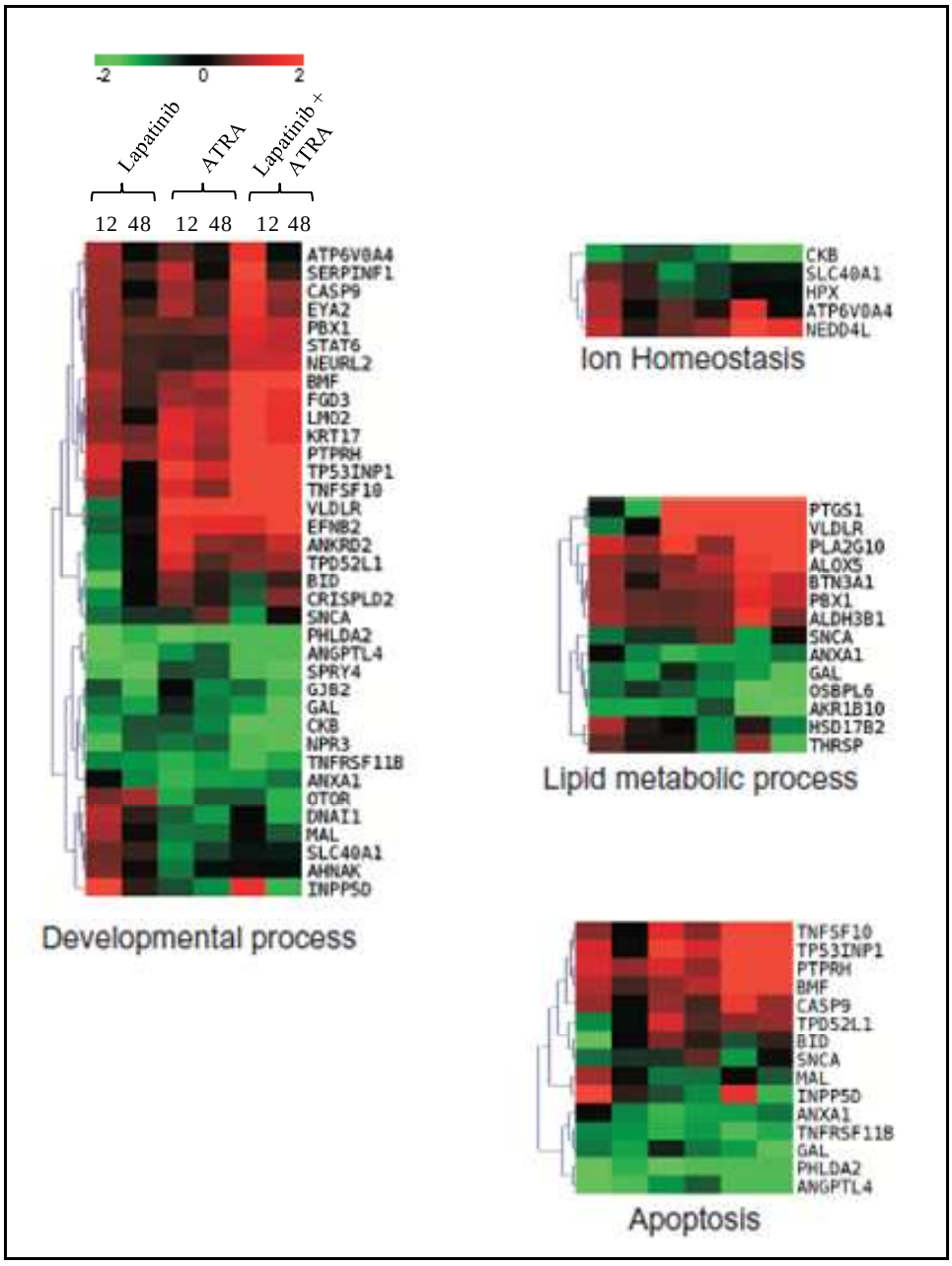

Figure 50 Cluster 3 with gene pathways found modulated.

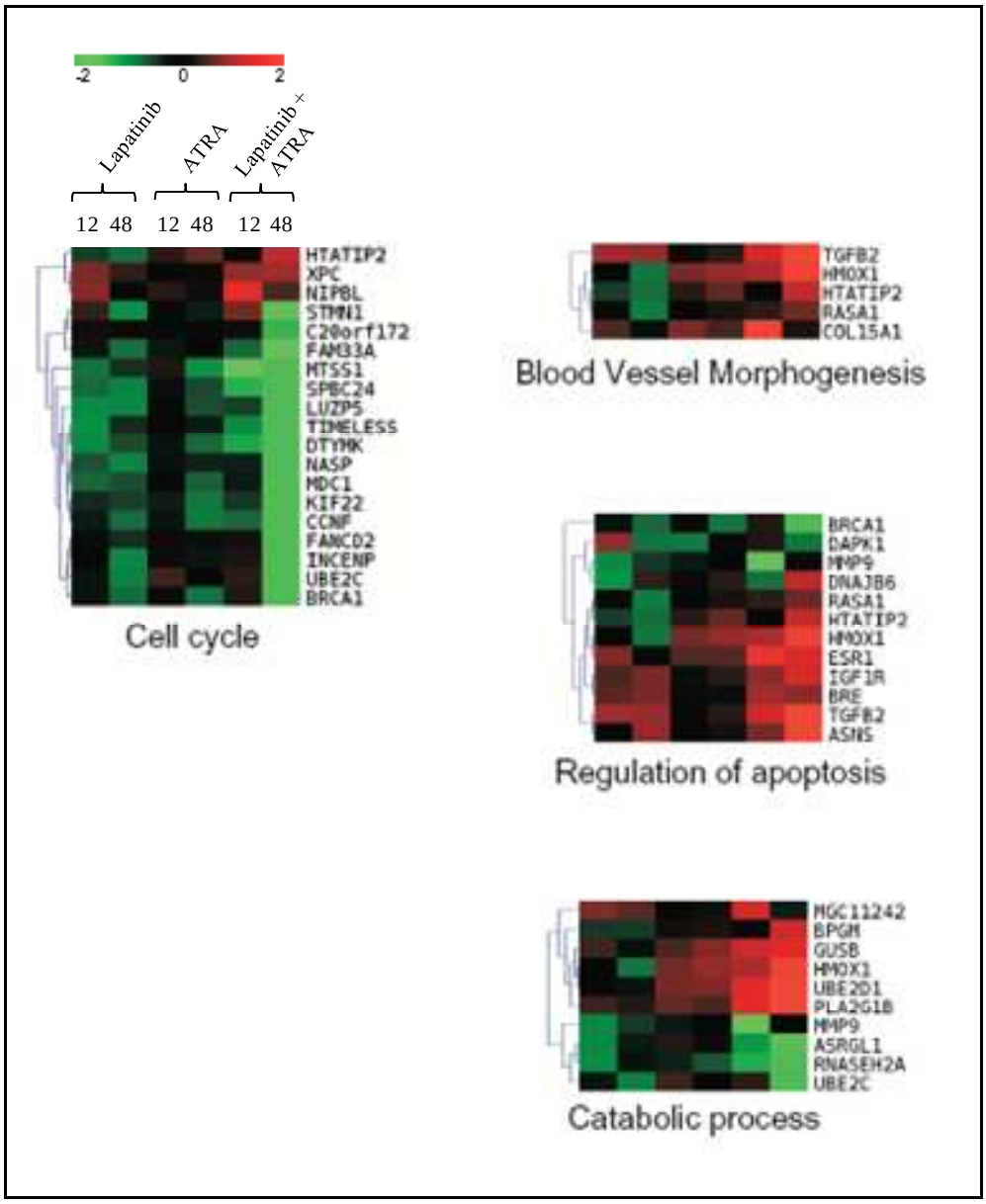

Figure 51 Cluster 4 with gene pathways found modulated.

2.8 Co-administration of ATRA and Lapatinib severely affects cell cycle progression while specifically inducing cell death.

From the microarray analysis it appears clear that one of the main pathways affected by the combination of ATRA *plus* Lapatinib *versus* each agent alone is the cell-cycle pathway. Indeed many crucial regulator of the cell cycle are differently modulated by the combination in respect to ATRA or Lapatinib alone. To better understand the relationship between cell cycle progression and the effect of our compounds, FACS analysis was performed on SKBR3 cells treated with ATRA, Lapatinib or the combination (Fig.52). After FACS analysis any effect of ATRA was detected for 24/48h. After that time no major alterations of cell cycle percentages were observed but the growth rate was reduced respect to controls associated to a minor number of cells in S and G2M phases. All together the data suggest a generalized cell cycle delay with a G1 delay somewhat stronger than in the other phases. An increase of the percentage of G1 cells was observed after 24h lapatinib treatment which however was not associate to a strong growth inhibition. This indicates that most of the cell divided in the first 24h but then were blocked in G1 phase. The G1 block was persistent and confirmed by the slow growth up to the end of the experiment. The G1 block is not complete however as a small fraction of cells was detected in S and G2/M phases at all times. The percentage of S, G2/M cells increased in time indicating that a subset of resistant cells is proliferating or/and G1 blocked cells are dying. While a subG1 population was observed in the combined treatment that was absent in both lapatinib and ATRA single treatments. The subG1population was already observed at 24h increased at 48h reaching a maximum at 72h. The G1 block induced by lapatinib is almost complete and persistent in the combination because no cells overcoming the block were observed suggesting a contribution of the long lasting cytostatic effect of ATRA. Altogether the data achieved by FACS analysis indicate that while both ATRA and Lapatinib are capable on their own to impair cell cycle progression, the combination is even more effective and only their combination is able to induce cell death supporting the idea that ATRA plus Lapatinib engages a synergistic pathway leading to cell death.

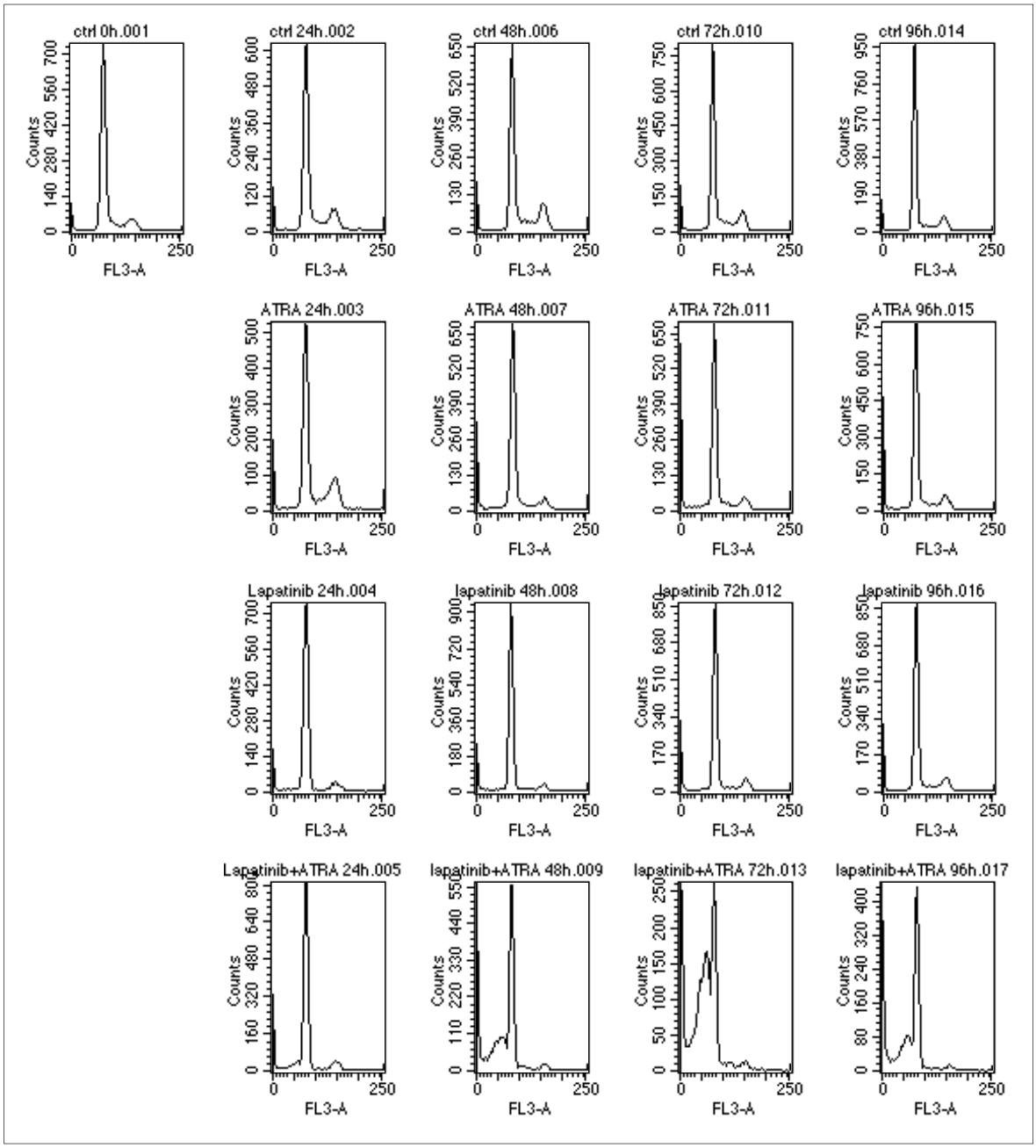

Figure 52: FACS analysis performed on SKBR3 cells treated with ATRA, Lapatinib or the combination for 24 and 48h.

DISCUSSION

The aim of my PhD project was to investigate the role exerted by all-*trans* retinoic acid (ATRA) and derivatives in breast carcinoma cells in order to better understand the nature of retinoid sensitivity/resistance in breast carcinoma and to define, by different approaches, its molecular mechanism focusing on the ERα and Her2 pathways.

Retinoid acid signaling and Estrogen Receptor status

ATRA and derivatives are interesting agents not only for the treatment of certain types of leukemia but also for the treatment and prevention of solid cancer (Altucci and Gronemeyer, 2001; Altucci *et al*., 2007; Garattini *et al*., 2007). Indeed, there is a wealth of data demonstrating that retinoids inhibit the progression of cancer from the premalignant to the malignant stage due to their ability to modulate cell growth, differentiation and apoptosis.

Retinoids are of therapeutic interest because of their efficacy in preventing carcinogen-induced rat mammary cancer (Anzano *et al*., 1994; Gottardis *et al*., 1996) and their antiproliferative effect towards breast cancer cells *in vitro* (Fontana, 1987; van der Burg, 1993; van der Leede *et al*., 1995; del Rincon, 2003). There is also clinical evidence that retinoids may be beneficial in breast cancer chemoprevention (Veronesi *et al*., 2006; Lawrence *et al*., 2001).

In vitro studies indicate that retinoids inhibit the growth of ERα-positive but not of *ERα*-negative breast cancer cell lines (Fontana, 1987; van der Burg, 1993; del Rincon, 2003). The molecular mechanisms responsible for the modulation of the cellular responses to retinoids by *ERα* are largely unknown. Clarification of these mechanisms will result in a better understanding of retinoids anti-tumor activity and is also likely to lead to the identification of specific molecular targets for the clinical management of breast carcinoma.

To study this aspect of retinoid biology we used the two cell lines MCF-7 and MDA-MB-231 that recapitulate sensitivity and refractoriness of estrogen receptor- positive (ERα+) and estrogen-receptor-negative (ERα-) breast carcinomas to the anti-proliferative activity of ATRA.

This part of project is expected to provide fundamental information on the molecular determinants responsible for the sensitivity of *ERα*-positive and the resistance of *ERα*-

negative breast carcinoma cells to the anti-tumor and/or chemopreventive action of *ATRA* and derivatives. A further fall-out of this research has been the identification of new molecular determinants (gene products and *miRNAs*) of functional significance for the anti-tumor activity of retinoids.

To define the molecular mechanisms and determinants responsible for the ERα-dependent regulation of retinoid sensitivity/resistance in breast carcinoma we have established two complementary models of conditional ERα over-expression and silencing using MDA-MB-231 and MCF7 breast cancer cells. Since it is known that the *MCF-7* respond to the challenge with retinoids by growth arrest, differentiation along the lactogenic pathway and eventually apoptosis. In contrast, retinoids exert no effect on MDA-MB231 (Fontana, 1987; van der Burg, 1993; del Rincon, 2003; Donato and Noy, 2005). We expected that the introduction of a conditional shRNA targeted against ERα in MCF7 should allow us to induce resistance to ATRA, in a reversible fashion by switching on and off the expression of the receptor, using tetracycline. Conversely, we expected to induce a reversible form of retinoid sensitivity in the MDA-MB231 cells engineered for the conditional expression of ERα using the same strategy.

To evaluate if the over-expressed ERα is biologically functional as a transcription factor, we analyzed the ability of our clones to modulate an ERα dependent promoter and the effect of ER over-expression on cell growth. To evaluate if any change in retinoic acid sensitivity could occur, we have characterized our clones for the growth and viability after ATRA and E_2 administration. Altogether the data obtained indicates that the stable cell lines generated correctly expressed a functional ERα but its over-expression do not affect cell proliferation suggesting that the steroid nuclear receptor activated pathway is not sufficient by its own to interfere with the proliferative driving force of MDA-MB231 genetic background. Moreover, in our clones after ERα induction, we cannot find any significant modulation of RARA dependent transcriptional activity.

Therefore, we can conclude that in MDA-MB231 the over-expression of ERα is not sufficient to modulate cell proliferation neither to restore ATRA sensitivity.

To better define the molecular mechanisms and determinants responsible for the ERα-dependent regulation of retinoid sensitivity/resistance in breast carcinoma we have replicated the same analyses described above for MDA-MB-231/TR6 ERα, for the MCF7 TR6/shERα. As expected, the stable cell line generated, after Erα silencing induction, exhibit a down-regulation of the protein and a reduction of ERE associated promoter

activity. Cell growth was significantly reduced by ERα silencing. Importantly, the effect exerted by ATRA was proportional to the magnitude of estrogen mediated proliferation both in silenced and in the non-silenced clones, indicating that ATRA affect specifically ERα driven cell growth. This suggests that ATRA exerts its antiproliferative activity in MCF7 by specifically antagonizing E_2 triggered cell proliferation via ERα.

The evidence reported indicates a two-way cross-talk between E_2 and ATRA possibly mediated by ERα and RARα. They appear to counteract each other, ATRA inhibiting E_2 mediated proliferation and E_2 blocking ATRA induced cell growth arrest. This is supported also by a recent work, based on ChiP experiments, claiming the existence of a genomic antagonism between retinoic acid and estrogen signaling in breast cancer (Hua *et al*, 2009).

To gain more insight into the mechanisms that underlie the cross-talk between ERα and RARα we have performed gene expression microarray to better understand the molecular pathways involved in breast cancer. We were able to identify, by this approach, 133 genes that are significantly modulated by ATRA in MCF7 cells, among them, 16 genes were already known to be affected by ATRA. In the MDA-MB-231 cells we identified only 35 genes that are significantly regulated. This lower response is consistent with the evidence that MDA-MD-231 cells are not sensitive to ATRA treatment. So we can conclude that the microarray approach has allowed us to identified specific retinoic acid regulated genes in two breast cancer cell lines.

Further exploration of these lists with pathway enrichment analysis is needed to identify the specific molecular mechanism responsible for ATRA sensitivity in the two different cell lines and hopefully in their tumor counterpart.

Retinoic acid modulation of microRNA in breast cancer

Breast cancer is a complex and phenotypically diverse disease, involving a variety of changes in gene expression and structure. Recent advances in molecular profiling technology have made great progress in the molecular taxonomy of breast cancer, which has shed new light on the etiology of the disease and also heralded great potential for the development of novel biomarkers and therapeutic targets. The recent discovery that miRNA expression is frequently deregulated in cancer has uncovered an entirely new repertoire of molecular factors upstream of gene expression, which warrants extensive investigation to further elucidate their precise role in malignancy (Verghese *et al.,* 2008).

MiRNA biogenesis in the human cell is a multi-step complex process and elucidating its mechanisms of action is still in its infancy. Nonetheless, some work in this area has

demonstrated that miRNAs may regulate cellular gene expression at the transcriptional or posttranscriptional level by suppressing translation of protein coding genes, or cleaving target mRNAs to induce their degradation, through imperfect pairing with target mRNAs of protein coding genes (Jackson and Standart, 2007).

To gain more insight into the mechanisms that underlie the initiation and progression of ERα positive breast cancers, many groups have studied the regulation of ERα gene expression. Regulation of the expression of ESR1 is complex and posttranscriptional regulation of ERα mRNA includes targeting by miRNAs. It is reported that relatively long 3'UTR of the human ERα mRNA is enriched in putative miRNA target sites, moreover the miRNAs that were increased in ERα negative *versus* ERα positive breast cancer might have a role in down-regulation of ERα expression (Adams *et al*., 2007).

We demonstrated that treatment of retinoid-sensitive ERα+ breast carcinoma cells with ATRA resulted in the induction of miR-21. Up-regulation of miR-21 was the consequence of increased transcription of the corresponding gene via selective activation of RARα. This was due to a direct effect of the ligand-activated receptor on two functional RAREs, mapping to the 5'- flanking region of *MIR21*. Induction of miR-21 by an anti-proliferative agent like ATRA in ERα+ breast carcinoma cells was unexpected, as the miRNA is endowed with oncogenic properties and is over-expressed in many tumors (Krichevsky and Gabriely, 2009). For this reason, we deemed it important to establish whether miR-21 induction was involved in some of the cellular responses underlying the therapeutic activity of ATRA. In the MCF-7 context, miR-21 protected cells from the antiproliferative effect of ATRA by inhibiting retinoid-induced senescence. This is consistent with the reported role of miR-21 on the growth and progression of breast carcinoma, suggesting that induction by ATRA is part of an uncharacterized negative feedback loop similar to the one activated by E2 in ERα+ cells (Bhat-Nakshatri *et al.*, 2009; Castellano *et al.*, 2009). Our data suggest that caution should be exercised in drawing conclusions as to the beneficial or detrimental significance of miR-21 induction for the overall therapeutic activity of retinoids. As miR-21 is predicted to control the activity of hundreds of direct targets, identification of the genes responsible for the growth-activating and anti-motility properties of the miRNA in ERα+ breast carcinoma cells treated with retinoids is challenging. Among the few validated miR-21 targets, maspin was the only one modulated by ATRA via induction of the miRNA in MCF-7 cells. Maspin may be directly involved in miR-21-dependent suppression of retinoid–dependent growth inhibition. In fact, maspin is endowed with onco-suppressor properties and inhibits the growth of breast carcinoma

(Joensuu *et al.*, 2009; Latha *et al.*, 2005). Given the reported role in suppressing the metastatic potential of cancer cells (Joensuu *et al.*, 2009; Latha *et al.*, 2005), it is unlikely that maspin plays any role in ATRA-dependent inhibition of cell motility. Selective modulation of miR-21 by ATRA was exploited to identify novel direct targets of the miRNA. ICAM1 is an adhesion molecule and its down-regulation by miR-21 may be relevant for the ATRA-dependent morphological alterations associated with changes in the adhesion properties of MCF-*7* cells (see Fig. 36C). PLAT, codes for a known fibrinolytic factor, whose induction in cells lacking miR-21 up-regulation may be part of a stereotyped cellular response to ATRA, which is endowed with anti-thrombotic activity (Marchetti *et al.*, 2003). Inhibition of PLAT expression by miR-21, as observed in MCF-7 cells, may be therapeutically desirable, as stimulation of a thrombotic response in cancer cells is associated with tumor growth and progression (Rickles, 2009). PLAT is also known to activate the motility of neural crest cells (Erickson and Isseroff, 1989), which is consistent with an involvement of the protein in the miR-21 dependent anti-motility effects observed in ATRA-treated ERα+ cells. Finally, IL-1β is an inflammatory protein and our microarray data indicate that ATRA up-regulates inflammation related genes in ERα- MDA-MB-231 cells, but not in ERα+ MCF-7 cells. Based on the presence of several potential miR-21 targets in this group of genes, including IL1B, we propose that miR-21 inhibits, either directly or indirectly, certain aspects of the inflammatory responses otherwise activated by retinoids in breast cancer cells. As inflammation is known to be involved in tumor growth *in vivo* (Mantovani *et al.*, 2008), the proposed anti-inflammatory action of miR-21 may play a positive role in the overall therapeutic responses to retinoids. In conclusion, this is the first demonstration that MIR21 is a direct retinoid target gene.

The results relating to the role of miR-21 in the anti-proliferative, pro-senescence and anti-motility effects, as well as the identification of IL1B, ICAM1 and PLAT as novel miR-21 target genes have far-reaching implications for the therapeutic use of retinoids in breast carcinoma and are the object of studies in progress.

Retinoid acid signaling and Her2 status

Co-amplification of RARA and ERBB2 in breast cancer: therapeutic implications

Tailoring of therapeutic approaches according to the molecular characteristics of the disease is one of the priorities and goals of modern medicine. This is particularly true in the realm of oncology, as tumors with similar histology of specific types of cancer are likely to be an important determinant of prognosis and response to therapy. Reclassification of common and rare types of tumors according to the gene expression profile to develop targeted and more rational therapeutic approaches is the objective of numerous studies. Often these studies aim at the identification of sub-population of patients characterized by a presence of a novel and druggable molecular targets or molecular targets of established and available anti-neoplastic drugs. Selection of patients that may obtain the greatest benefit from any given therapeutic regimen calls for the development of robust diagnostic tests capable of stratifying the oncologic population according to the presence/absence and level of expression of single or multiple molecular targets.

The accumulated knowledge of the mechanistic, molecular and pharmacological actions of retinoids, together with the possibility of creating novel types of retinoid-related molecules with defined activities, is the basis for the development of efficient anticancer therapies, as is obvious from the success story of treating APL with ATRA (Degos and Wang, 2001).

The identification of HER2/neu and the resultant development of Trastuzumab has been hailed as the most significant development in breast cancer therapeutics over the past twenty years (Glynn *et al.*, 2010).

Her2 is an attractive therapeutic target in breast cancers because of the tight correlation between over-expression and poor prognosis, and because normal cells express relatively low levels of HER2. Approximately 30% of breast cancers have an amplification of the HER2/neu gene or over-expression of its protein product (Zhou and Hung, 2003). Over-expression of this receptor in breast cancer is associated with increased disease recurrence and worse prognosis. The poor prognosis may be due to global genomic instability as cells with high frequencies of chromosomal alterations have been associated with increased cellular proliferation and aggressive behavior (Ellsworth *et al.*, 2008). The success demonstrated with Trastuzumab has stimulated the research community to go in search of further suitable therapeutic targets, including those genes located on chromosome 17q12-21.

The rationale behind the selection of Her2 as a potential target of retinoic acid combined therapy is based on the presence of RARA on chromosome 17 at 0,650 Mb from ERBB2. Since in most case, her2/Neu positivity is the result of an amplification event involving a variable portion of chromosome 17 where the ERBB2 gene is located, we expected to find in the amplicon RARA locus. This should result of an increase of Her2/Neu and RARα proteins.

For this reasons we evaluated the sensitivity of different breast carcinoma cell lines to the anti-proliferative effects of ATRA characterizing a panel of breast cell lines showing the amplification of the ERBB2 and/or not RARA.

The three cell lines, SKBR3, AU565 and UACC-812, characterized by co-amplification of ERBB2 and RARA showed the highest sensitivity to the anti-proliferative effects of retinoids, indicating a sensitivity which is higher than that observed in MCF-7 cells, showing no amplification of ERBB2 and RARA genes and considered the gold standard for sensitivity to retinoids. In general, the data obtained indicate that breast carcinoma cell lines with high levels of RARα, as a consequence of RARA co-amplification, are exquisitely sensitive to the anti-proliferative and differentiating activity of ATRA and derivatives.

Therefore, an important objective was to establish whether a sub-population of patients suffering from Her2-positive breast carcinoma with the ERBB2-RARA amplification is responsive to treatments based on ATRA. We have focused our attention on the sub-population of patients characterized by amplification of HER2, and in 24% cases we demonstrated simultaneous amplification of the RARA gene. On the other side, given the peculiar sensitivity of breast carcinoma cells with co-amplification of ERBB2 and RARA to ATRA we evaluated whether targeting the retinoid and Her2/neu pathways simultaneously has the potential to represent a viable therapeutic strategy.

HER2-positive breast carcinoma patients are the focus of our proposal because it is known that the patients with Her2/Neu-positive breast carcinoma used to have a poor prognosis until the introduction of HER2/Neu- targeted therapy (Gonzalez-Angulo *et al*., 2006). Currently, front-line treatment of this group of patients is based on trastuzumab, despite its efficacy; trastuzumab treatment is associated with resistance in a large portion of these patients (Galleshaw, 2003 and Board *et al*., 2006). Resistant patients benefit from second-line treatment with lapatinib, in spite of significant progress the management of this subgroup of breast cancer patients is still far from satisfactory (Frampton, 2009).

To this purpose we evaluated the anti-proliferative effect of combinations between ATRA and lapatinib. Lapatinib has been described to inhibit the growth of Her2/neu-positive cell lines and not exert any appreciable effect on the Her2/neu-negative cell lines (Konency *et al.*, 2006). In agreement with retinoid sensitivity when Her2/neu-positive cell lines were challenged with combinations of the lapatinib and ATRA a cooperative interaction was observed only in the case of cells responsive to ATRA such as the cell lines harbouring co-amplification of ERBB2 and RARA (e.g. SKBR3 and UACC-812).

To establish the nature of the anti-proliferative effect of the combination ATRA/Lapatinib we have chosen SKBR3 as our model of study to evaluate the effects of the two compounds on cell viability and apoptosis induction in a dose dependent manner. We can affirm that the growth inhibitory effect of two compounds is synergistic and the two types of compounds synergize in terms of anti-proliferative and apoptotic activity too. Treatment of SKBR3 cells with ATRA or lapatinib in fact, determines a reduction of cell viability that is magnificated by the combination of the two compounds and the loss of viability is associated with a proportional increase in cytotoxicity.

To decipher the molecular pathways involved in the synergistic effect produced by co-administration of ATRA and Lapatinib in SKBR3, we have used the whole genome microarray technique, with the aim to reveal all the genes whose expression is influenced by the single compounds or by the combination of them. The gene-expression microarray data demonstrate that treatment of cells with *ERBB2* and *RARA* co-amplification results in massive effects at the transcriptional level and we have identified four clusters with modulated gene pathways involving many genes of cell cycle, cell growth and apoptosis.

The data, confirmed by FACS analysis, indicate that while both ATRA and Lapatinib are capable by their own to impair cell cycle progression, the combination is even more effective and only their combination is able to induce cell death supporting the idea that ATRA *plus* Lapatinib engages a synergistic pathway specifically leading to cell death.

In conclusion we think that our findings provide a strong rationale to propose a combinatorial therapeutic approach based on retinoids and Her2 inhibitors for the management of a subset of her2/Neu-positive breast carcinoma patients characterized by co-amplification of the ERBB2 and RARA genes. Moreover, we are confident that the results of our research project will have an impact for the development of new diagnostic methodologies for breast cancer classification.

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PUBBLICATIONS

Inhibition of the peptidyl-prolyl-isomerase Pin1 enhances the responses of acute myeloid leukemia cells to retinoic acid via stabilization of RARalpha and PML-RARalpha.

Gianni' M, Boldetti A, **Guarnaccia V**, Rambaldi A, Parrella E, Raska I Jr, Rochette-Egly C, Del Sal G, Rustighi A, Terao M, Garattini E.

Cancer Res. 2009 Feb 1;69(3):1016-26. Epub 2009 Jan 20.

Induction of miR-21 by retinoic acid in estrogen-receptor-positive breast carcinoma cells: biological correlates and molecular targets.

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