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Antiproliferative effects of retinoic acid in breast cancer

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Antiproliferative effects of retinoic acid in breast cancer

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Résumé

Les rétinoïdes sont utilisés dans le traitement d'une variété de tumeurs malignes et lésions précancéreuses. Leurs effets dans des lignées cellulaires dérivées de tumeurs solides tel que le cancer du sein ont été étudiés extensivement. Cependant, les bénéfices dans le cancer du sein restent à date peu clairs. Ceci est probablement du à l'hétérogénéité des tumeurs mammaires et la réponse très variable aux effets antiprolifératifs de l'acide rétinoïque. Dans les lignées cellulaires cancéreuses mammaires, la réponse l'AR est fortement corrélée au niveau d'expression du récepteur aux estrogènes alpha (ERα), qui régule l'expression du gène qui encode le récepteur à l'acide rétinoïque alpha, RARA. Malgré cela, certaines lignées cellulaires ER-négatives, comme la lignée HER2-positive SK-BR-3, ont été décrites comme étant sensibles à l'AR.

Dans le Chapter 2: de cette thèse, nous avons étudié les mécanismes de la signalisation ER-dépendante et ER-indépendante dans les cellules cancéreuses mammaires. Nous avons utilisé des lignées ER-négatives et ER-positives pour démontrer qu'une partie de la réponse à l'AR est indépendante de la signalisation par ER. Nous avons identifié plusieurs gènes cibles primaires de l'AR qui ont des effets similaires à l'AR quand ils sont surexprimés dans des cellules mammaires cancéreuses. Cette étude apporte une meilleure compréhension des mécanismes complexes qui mènent à l'arrêt de croissance induit par l'AR dans les cellules cancéreuses mammaires.

Dans le Chapitre 3, nous avons regardé plus en détails la signalisation ER-indépendante par l'AR dans des cellules ayant une amplification des gènes HER2 et RARA et nous avons identifié une synergie entre l'AR et le Herceptin dans ces cellules. Nous proposons que les gènes FOXO jouent une rôle dans cette synergie. Les cellules SK-BR-3, ayant une coamplification HER2/RARA, pourraient représenter une classe de tumeurs qui pourraient bénéficier d'un traitement avec des rétinoïdes, en augmentent la réponse au Herceptin et potentiellement en réduisant la résistance au Herceptin.

En conclusion, les données présentées dans cette thèse aident à mieux comprendre les mécanismes menant à l'arrêt de croissance induit par l'AR dans les cellules cancéreuses

mammaires et fournissent une application potentielle pour l'utilisation de l'AR dans le traitement du cancer du sein.

Mots-clés : Cancer du sein, acide rétinoïque, HER2, ERα, Herceptin

Abstract

Retinoids are being used in the treatment of several malignancies and precancerous lesions. Their effects on cell lines derived from solid tumors, such as breast cancer, have also been described extensively. Their benefit in breast cancer, however, remains unclear. This might be because of the high levels of heterogeneity of breast tumors and the very variable response to the antiproliferative effects of retinoic acid. In mammary tumor cell lines, the response to retinoic acid is highly correlated with the expression of the estrogen receptor alpha (ERα), which regulates the expression of the retinoic acid receptor alpha gene RARA. However, some ER-negative cell lines, such as the HER2 positive SK-BR-3 cell line, have been reported to be RA-sensitive.

In Chapter 2: of this thesis we have investigated the mechanisms of ER-dependent and ER-independent RA signaling in breast cancer cells. Using ER-positive and ER-negative cell lines, we show that part of the response to RA is independent of ER signaling. Several direct retinoic acid targets were identified that could mimic antiproliferative effects of retinoic acid when overexpressed in breast cancer cells. This study has provided better insight in the complex mechanisms that lead to RA-induced growth arrest in breast cancer cells.

In Chapter 3: we looked further into the ER-independent RA signaling in HER2/RARA-amplified cells and identified a synergy between RA and Herceptin in these cells. We propose a role for FOXOs in mediating this synergy. HER2/RARA coamplified breast tumors might represent a subclass of tumors that could benefit from retinoid treatment, both increase antitumor effects of Herceptin, as well as in potentially reducing Herceptin resistance.

In conclusion, data presented in this thesis give better insight in the mechanisms of RA induced growth arrest in breast cancer cells and provide a potential application of retinoids in a subset of breast tumors.

Keywords : Breast cancer, retinoic acid, HER2, ERα, Herceptin

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List of abbreviations

ADH	Alcohol dehydrogenase
AF-1/2	Activation function 1/2
ALDH	Aldehyde dehydrogenase
ATP	Adenine triphosphate
CBP	CREB binding protein
CI	Combination index
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERa	Estrogen receptor alpha
FACS	Fluorescence activated cell sorting
FISH	Fluorescence in situ hybridization
GH	Growth hormone
GR	Glucocorticoïd receptor
GSEA	Gene set enrichment analysis
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HER2	Human EGF receptor 2
HMEC	Human mammary epithelial cell
HMT	Histone methyl transferase
IPA	Ingenuity Pathway Analysis
MAPK	Mitogen activated protein kinase
MMTV	Mouse mammary tumor virus

mRNA	messenger ribonucleic acid
NCOR	Nuclear corepressor
PCA	Principal component analysis
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
RA	Retinoic acid
RALDH	Retinal dehydrogenase
RAMBA	Retinoic acid metabolism blocking agent
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
ROR	RA receptor-related orphan receptor
RXR	Retinoid X receptor
SDR	Short-chain dehydrogenase/reductase
SMRT	Silencing mediator of RAR and TR
SRC	Steroid coreceptor
TDLU	Terminal ductal lobulo-alveolar unit
TEB	Terminal end bud
TR	Thyroid hormone receptor
VDR	Vitamin D receptor

À mes deux amours.

Because it's love that keeps us going.

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First part:

Chapter 1: Introduction

Chapter 1: Introduction

1.1 Metabolism and physiology of retinoids

Dietary vitamin A has been recognized for almost a century (1, 2) as essential for the life of all chordates. Since its discovery, a substantial amount of research has shown this vitamin to be essential for embryonic development, reproduction, vision, immune function and tissue homeostasis. Different types of active metabolites are responsible for controlling these functions. 11-*cis*-retinal play an important role in the visual cycle as a mediator of phototransduction. All-*trans*-retinoic acid (RA) and 9-*cis*-retinoic acid (9cRA) can regulate the expression of a wide range of target genes through the activation of retinoic acid receptors (RARs), and as such control multiple networks that are important for embryonic development and adult tissue maintenance (3).

1.1.1 Normal retinoid metabolism

1.1.1.1 Retinoids

Vitamin A (all-*trans*-retinol; atRol) and all its metabolites are collectively called retinoids. Natural retinoids share a chemical structure composed of a β-ionine ring, a polyene side chain and a polar end group ((4); Figure 1A). No animal species can make *de novo* vitamin A. They can however convert dietary carotenoids into active retinoids. Another source of retinoids from the diet are retinyl esters and retinol that can be found in some animal tissues such as the liver. RA, the most active retinoid metabolite, is formed in target cells from all-*trans*-retinol by a two step oxidation process: all-*trans*-retinol is converted into all-*trans*-retinal (at-Ral), which is subsequently converted into RA.

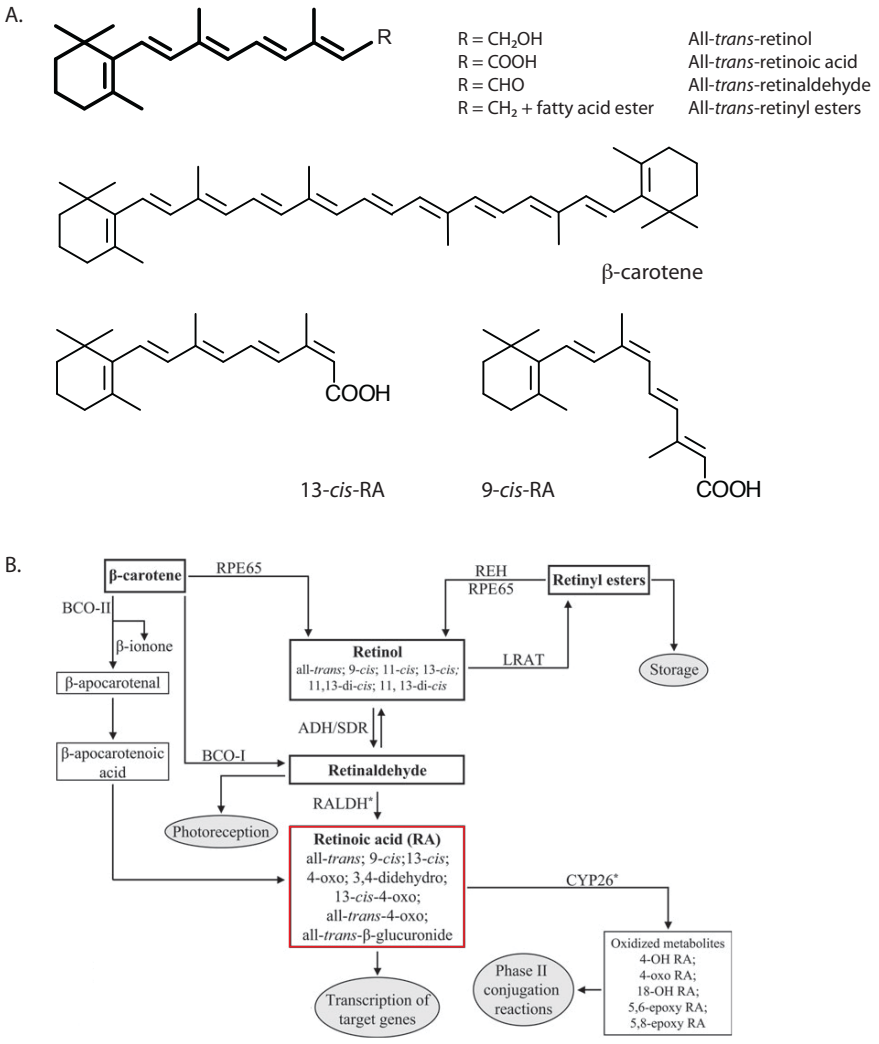

Figure 1 Structure and metabolism of retinoids

(A) Chemical structures of some naturally occuring retinoids. Adapted from (5). (B) Schematic overview of retinoid metabolism. Endogenous retinoids are boxed and gray circles highlight major physiologial responses to the different retinoids. The enzymes responsible for the various steps of retinoid metabolism are indicated. Certain enzymes can catalyze the synthesis or degradation of RA *in vitro*, indicated by asterisks. *ADH* Alcohol dehydrogenase (ADH1,3,4), *BCO-I* β,β-carotene-15,15'-monooxygenase, *BCO-II* β,β-carotene-9',10'-dioxygenase, *CYP26* cytochrome P450 family 26, *LRAT* lecithin:retinol acetyltransferase, *RA* retinoic acid, *RALDH* retinaldehyde dehydrogenases (RALDH1, 2, 3, 4), *REH* retinol ester hydrolase, *RPE65* retinal pigment epithelium-specific protein 65 kDa, *SDR* short-chain dehydrogenase/reductase. Adapted from (6).

1.1.1.2 Metabolic generation of RA

Active retinoid metabolites are generally believed to be synthesized in target tissues, where it acts in an autocrine and/or paracrine manner. In higher vertebrates, the most important source of this synthesis is all-*trans*-retinol that is taken up from the plasma (7). A

schematic overview of retinoid metabolism is shown in Figure 1B, and the process will be discussed in detail below.

Retinoid uptake and transporting proteins

Dietary provitamin A carotenoids such as β-carotene enter enterocytes in the small intestine by passive diffusion, to subsequently be cleaved into two molecules of retinal. This retinal will then be reduced to retinol by retinal reductases. Dietary retinyl esters will be converted into retinol in the intestinal lumen before uptake by the enterocytes (7). In the enterocytes, the retinol will bind to cellular retinol binding protein type II (CRBP-II). Most of the retinol in the enterocytes will then be re-esterified, a process that is facilitated by binding of CRBP-II. The retinyl esters are then incorporated in large lipoprotein complexes called chylomicrons and transported to the liver. Here, they will either be stored in stellate cells (mainly as retinyl esters) or be hydrolyzed and bound to retinol binding protein (RBP1). The retinol-RBP1 will then be released in the plasma and absorbed by target cells expressing the RBP1 receptor STRA6 (6). STRA6 is a multitransmembrane protein and widely expressed in mouse embryos, whereas the expression in the adult is much more restricted. STRA6 has been shown to be upregulated by retinoids in mouse mammary epithelial cells (8).

Retinol processing by retinol dehydrogenases

The first step in the production of all-*trans*-retinoic acid in target tissues is the oxidation of retinol into retinal (retinaldehyde). This process is mediated by retinol dehydrogenases, which are members of the families of cytosolic medium-chain alcohol dehydrogenases (ADH) or membrane-bound short-chain dehydrogenase/reductases (SDR) (9).

The ADHs ADH1, ADH3 and ADH4 are all capable of oxidizing at-Rol into at-Ral *in vitro*. ADH4 is the most efficient of the four. Contrary to vertebrates that have multiple ADHs, invertebrates only have one, most often ADH3. The latter is ubiquitously expressed, whereas ADH1 and ADH4 show a more tissue-specific expression pattern (6, 7). Studies with knock-out mice suggest that *Adh3* is the ubiquitous ADH under normal physiological conditions, whereas *Adh1* and *Adh4* seem to be necessary in cases of vitamin A excess or

deficiency, respectively (10-12). ADH1 and ADH4 can compensate for lack of ADH3 in the case of sufficiently high retinol levels, but are incapable of doing so in the case of limited retinol supply (13). The ADHs do not seem to be able to oxidize at-Rol when it is bound to RBP1 (7).

The other family of enzymes capable of oxidizing at-Rol are the SDRs. Contrary to the ADHs that are cytosolic, these enzymes are microsomal. Several members of this family that have been shown to function as retinol dehydrogenases include RDH1, RDH5, RDH10, RDH11, CRAD1, CRAD2, CRAD3 and retSDR1 (10). Genetic studies have been performed only with RDH5 and RDH10. Whereas RDH5 is important for vision, it does not seem to play a vital role in *in vivo* retinoic acid synthesis (10). RDH10 on the other hand is important for embryonic RA synthesis and its loss results in embryonic lethality (14). As opposed to ADHs, SDRs use RBP1-bound retinol as a substrate. RBP1 functions as a chaperone directing retinol to the metabolizing enzymes (15).

Retinal oxidation by retinal dehydrogenases

The retinal that is being produced by oxidation of retinol is then further processed into RA by a second, irreversible oxidation process. This oxidation of retinal is mediated by retinal dehydrogenases (RALDHs) (16). Vertebrates generally express four RALDHs, RALDH1 or ALDH1A1, RALDH2 or ALDH1A2 and RALDH3 or ALDH1A3 of the ALDH1A class, and RALDH4 of the ALDH8 class. Unlike the expression of ADH3 which is ubiquitous, the expression of the RALDHs shows a high level of tissue specificity. This implicates that although retinal can be produced in a ubiquitous manner in the organism, the production of RA is a localized process (17). Notably, the RALDHs are expressed specifically in epithelia whose the differentiation is regulated by RA (10). Besides the RALDHs, several cytochrome P450 enzymes (CYPs), CYP1A1, 1A2, 2C3 and 2J3, have also been shown to be capable to oxidize retinal *in vitro* (5).

RALDH1 expression is observed in the dorsal retina of embryos, as well as in several adult epithelial tissues, including testes, brain, lungs, kidneys and mammary epithelium (18-21). RALDH1 knock-out mice are morphologically normal, viable and fertile. Also the effects on the dorsal retina are fairly minor. *Raldh1-/-* mice do present with a very low

capacity to produce RA in the embryonic dorsal retina and the adult liver (22). When overexpressed in *Xenopus* embryos, RALDH1 leads to premature RA synthesis, showing that this enzyme can be a functional retinol dehydrogenase *in vivo* (10). It has been suggested that the main role of RALDH1 is in the catabolism of excess retinol (6). Several lines of evidence also suggest RALDH1 to be a marker of stem cells and progenitors in different tissue types, including muscle (23), brain (24), intestine and adipose tissue (25), prostate (24), the hematopoietic system (26, 27), as well as the mammary gland (19).

RALDH2 expression occurs in multiple tissues in both the embryo and the adult. *Raldh2-/-* mice show shortening of the anteroposterior axis and lack limb bud formation due to the absence of RA (28, 29). These embryos die at midgestation because of defects in heart development. These defects can be overcome to a large extent by the maternal administration of RA, leading to the conclusion that the main role of RALDH2 is to provide RA for embryonic development (28).

RALDH3 was isolated from human salivary glands (30) and was subsequently shown to be expressed in various adult tissues such as intestines, liver, prostate, pancreas and lungs (31). Unlike RALDH1 and RALDH2 that show activity with 9-*cis*-, 13-*cis*- and all-*trans*-retinal, this family member seems to be active solely with the all-*trans* isoform (32). In the developing embryo, RALDH3 expression is found in the retina, lens and olfactory pit, as well as in ureteric buds and surface ectoderm in the developing forebrain (10). RALDH3-null mice have severe cranio-facial defects and die shortly after birth because of respiratory distress. Defects during embryonic development of RALDH3-null mice can be partially overcome, like in the case of RALDH2, by administration of RA to the mother (33). This shows that RALDH3 also has important roles in RA synthesis during embryonic development. In spite of its broad expression in the adult, its role remains unclear. RALDH3 is also expressed in normal mammary epithelial, but the breast cancer cell line MCF-7, which lacks the capacity of RA synthesis, does not express this enzyme (34, 35).

RALDH4 is the least characterized of the RALDHs. It is expressed in mouse liver and kidney and preferentially functions with 9-*cis*-retinal, with which it is about two times as active as with the all-*trans* isoform (36). Therefore it has been suggested that this enzyme

plays a role in the synthesis of 9cRA, but due to the lack of studies performed on this subject, the *in vivo* role of RALDH4 remains largely unclear.

Retinoic acid transport by CRABPs

Cellular retinoic acid binding proteins type I and II (CRABP-I and CRABP-II) will bind the newly synthesized RA and transport it to either the nucleus where it will activate target gene transcription, or to nearby target cells. CRABP-I shows higher affinity for RA than CRABP-II and both have higher affinity for RA than for 9cRA (37).

CRABP-II was suggested to act as a facilitator of RA uptake and metabolism and to have a role as a cofactor in RA signaling (38). Its expression is cytosolic in the absence of ligand, but when RA is present it quickly translocates to the nucleus. Here, the RA-CRABP-II complexes interact directly with retinoic acid receptors, transferring the ligand to the receptor. CRABP-I on the other hand seems to play a role in regulating the metabolic inactivation of RA (39). Overexpression of CRABP-I in F9 cells by transfection led to higher levels of RA inactivation and loss of RA sensitivity (40, 41).

RA catabolism

To control the levels of RA in cells and tissues, a tight balance exists between the synthesis and catabolism of RA. The catabolism of RA is mediated mainly by the cytochrome P450 enzymes of the CYP26 family, although several other CYPs have also been implicated in RA modifictaion *in vitro* (6). The first CYP26 to be identified was CYP26A1, which was first cloned from zebrafish and the human variant was cloned not long after by the same group (42, 43). Subsequently, CYP26B1 and CYP26C1 have also been identified (44, 45), and like CYP26A1 these enzymes metabolize RA into more polar metabolites such as 4-*oxo*-RA, 8-*hydroxy*-RA and 15-*hydroxy*-RA (42, 44, 45). Different expression patterns of the three CYP26s suggest individual roles for each enzyme in RA catabolism (46).

Although RA metabolites were initially thought to be inactive, Pijnappel and colleagues have shown that 4-*oxo*-RA respecifies the head-to-tail axis in the *Xenopus* embryo and that it appears to bind and activate specific RARs, notably RARβ (47). In

addition, overexpression of CYP26 in embryonal carcinoma cells can induce neuronal differentiation (48).

1.1.2 Retinoic acid in normal physiology

1.1.2.1 Embryonic development

Retinoic acid is essential for embryonic development. Abnormalities in vitamin A deficient embryos were first described in 1933 (49) and many other malformations, affecting heart, bones, eyes, limbs, brain and nervous system, have been described since (50). Both excess and insufficient levels of RA during embryogenesis have teratogenic effects. Although studies with excess or insufficient vitamin A give good insight on its importance during embryonic development, such studies are not the best option for studying its physiological roles, since the thightly regulated homeostasis of retinoids makes it extremely difficult to achieve total depletion. A large body of loss-of function studies of RA synthesizing enzymes and receptors now provide extended information on the roles of RA throughout embryonic development. These roles are numerous and depend on the regulation of specific sets of target genes (16). Some well-studied examples will be described below.

Neural development

Treatment of mouse embryonic stem cells or embryonal carcinoma cells with high concentrations of RA can induce neural differentiation of these cells (51). In normal mouse development however, RA is not produced until well after induction of neuroectoderm and it has been demonstrated that neural induction does not require RA (52, 53). The role of RA in neural differentiation is to act on the neuroectoderm to induce its further differentiation. Data from knockout studies with *Raldh1/2/3* show that RA signaling in early neural development is only required in posterior structures such as the hindbrain, the spinal cord and eye (54, 55).

Hindbrain patterning

An important feature in the developing hindbrain is the differential expression along the anteroposterio axis of a cluster of genes called the Hox genes. The regulation of several of these genes by RA has been well documented and appears to be one of the major roles of RA in hindbrain patterning (56-58). For example, RA has been shown to directly regulate the expression of the Hox gene *Hoxb1* by both induction and repression, thereby tightly controlling the spatial expression of this gene (59, 60). Boundaries of RA signaling in the developing brain are created by the expression in the mid-and forebrain of the RA degrading enzymes *Cyp26a1* and *Cyp26c1*. Knockout models of these enzymes in developing mouse or zebrafish embryos lead to posteriorization of the expression pattern of *Hoxb1* and other RA target genes and subsequent posteriorization of the developing brain structures at the expense of the anterior structures (61, 62).

Anteroposterior patterning of the heart

RA signaling is also necessary for the anteroposterior patterning of the heart. Expression of RALDH2 just posterior of the developing heart leads to the production of RA that will localize to the posterior heart mesoderm (63). The role of RA in the development of the heart is repressive rather than inductive. These effects are mediated through the negative regulation of the growth factor *Fgf8* (64), normally expressed in the posterio-medial region of the heart tube. Mouse embryos that lack expression of RALDH2 show increased posterior expression of *Fgf8* and its downstream target *Isl1* (65, 66). Lack of RA signaling in the posterior part of the heart tube leads to a severely reduced inflow tract domain and abnormal cavity formation in the outflow tract domain (67).

Limb development

For a long time it was thought that RA plays an instructive role in limb development. Recently however, people have challenged this model and proposed a more permissive role. Originally, the model was that an anteroposterior gradient of RA is responsible for the induction of genes important for limb bud development and outgrowth, such as *Tbx5* and *Hand2*, which in turn regulates the induction of the essential gene *Shh* (68). However, the distribution of RA along the anteroposterior axis is equal, and instead a gradient is observed

along the proximodistal axis, where higher activity is observed in the proximal region (69). Other studies suggested that RA produced by Raldh2 in the flank will induce the proximal limb markers Meis1 and Meis2 and that these genes are repressed by the distally expressed Fgf8 (70). It has been shown that FGF signaling at the distal end is required for proximodistal patterning and distal expression of Cyp26b1 is necessary to avoid RA induced teratogenesis in the developing limb. However, the requirement of RA signaling in the proximal region for the formation of the proximodistal axis has not been clearly shown (68).

Knockout studies with Raldh2-/- mice showed lack of forelimb development, suggesting a role for RA signaling in the induction of limb bud development, prior to limb patterning (28). Treatment with maternal dietary RA supplements do induce limb development, resulting in close to normal hindlimbs and undersized forelimbs, with abnormal expression patterns of Shh and Fgf in the forelimb.

In recent studies using Raldh2 and Raldh3 knockouts, Zhao and colleagues provide proof for a model in which the repression of Fgf8 by RA in the proximal region is required for forelimb development, similar to the role of RA in heart development (64). They show direct binding of RARs to the Fgf8 promoter *in vivo* and show that the effects of RA deficiency on limb development can be overcome by the use of an FGF receptor antagonist. Thus, it appears that the effects of RA on limb development are by creating a permissive environment through the repression of Fgf8 expression.

1.1.2.2 Adult differentiation and tissue homeostasis

Aside from the multiple effects on embryonic development, RA also plays a vital role in adult tissue differentiation and homeostasis.

Skin differentiation

One of the physiological roles of RA signaling in the adult is the maintenance of skin epithelium integrity. Human skin is formed of two major compartments, the dermis and the epidermis, that are separated by the basement membrane. The epidermis, the outer protective layer of the skin, is made up primarily of keratinocytes (71). Treatment of the

skin with RA inhibits terminal differentiation of keratinocytes (72), stimulates the proliferation of keratinocytes and increases the thickness of the epidermis (73, 74). A recent study has identified several RA regulated genes implicated in this process (75). During their differentiation, keratinocytes will produce a specific type of fibrous proteins called keratins. Several studies have shown that animals on a vitamin A deficient diet will present with keratinization of the skin and muquous tissues. This can generally be treated with RA (76).

Immune function

The roles of retinoids in the immune system have long been recognized. Vitamin A deficiency leads to impaired functioning of various components of the innate as well as the adaptive immune system (77). Reduced vitamin A levels have been correlated with reduced natural killer (NK) cell number and function in rats and human (78, 79). T helper lymphocyte stimulated antibody production by B cells also requires vitamin A and it was shown that defects in this process in vitamin A deficient mice can be overcome by supplying them with T helper cells from vitamin A proficient mice (80). Antigen presenting cells express STRA6 and RALDH2 and produce RA (81). Upregulation of CD1d by retinoic acid leads to activation of invariant NK T cells (82). RA regulation of the matrix metalloprotease 9 (MMP-9) is important for migration of dendritic cells (DC) to inflammatory sites. Immature DC cells that were cultured in the presence of pharmacological doses of RA were found to be more migratory *in vitro* as well as when injected in tumors (83).

1.2 Retinoic acid signaling pathways

1.2.1 The concept of nuclear receptors

In the 1950s, it was thought that the small lipophilic hormones such as steroids, retinoids, thyroid hormones and vitamin D_3, all potent regulators of cell proliferation and differentiation, functioned through a series of oxidation steps which would liberate energy for such roles. These ideas changed radically because of pioneer work by E.V. Jensen. He used radioactively labeled hormones to show the existence of proteins that bound the hormones and subsequently translocated from the cytoplasm to the nucleus. The idea of intracellular hormone receptors was born, suggesting a link between transcriptional control and physiology (84, 85).

1.2.1.1 Cloning of the first nuclear receptors

To better understand the mechanisms of steroid hormones, the cloning of the receptors was absolutely essential. The first nuclear receptors to be cloned in the mid-1980s were the estrogen receptor (ER) and the glucocorticoid receptor (GR) (86-89). Based on the homology of these receptors to the *v-erbA* oncogene, this led to the subsequent discovery of the *c-erbA* locus as the thyroid hormone receptor (TR) (90, 91). Although the ligands are all chemically different, these receptors are structurally similar. The idea of a nuclear receptor superfamily (92) was reinforced in 1987, when two independent research groups cloned a nuclear receptor for retinoic acid (93, 94).

1.2.1.2 The nuclear receptor superfamily

Since the discovery of these first nuclear receptors, many more have been identified by homology in both vertebrates and invertebrates. The nuclear receptor superfamily is now comprised of 48 closely related family members in humans, 21 in *Drosophila* and no less than 270 in *C. elegans* (95-97).

The general structure of nuclear receptors

Nuclear receptors are ligand-dependent transcription factors that all share the same modular structure composed of six regions of homology (Figure 2). The highly variable N-terminal A/B region harbors the AF-1 (Activation Function 1) domain, a hormone-independent transactivation domain (98, 99).

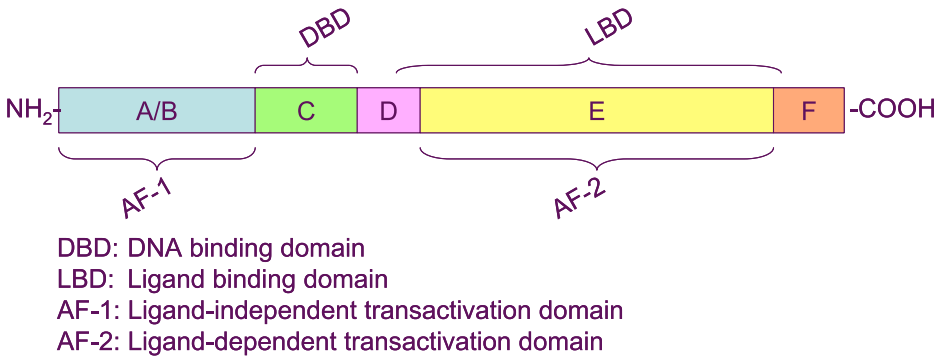

Figure 2 General structure of nuclear receptors

Nuclear hormone receptors are composed of a highly conserved DNA binding domain (C-region), a variable amino terminus (A/B-region), harboring the ligand-independent transactivation domain 1 (AF-1) and a conserved carboxy-terminus (D, E and F-region), harboring the ligand binding domain (LBD) and the ligand-dependent transactivation domain 2 (AF-2).

The DNA binding domain (DBD) is the most conserved region of the receptor structure, found in the C region. The DBD is essential for the recognition of DNA sequences called hormone response elements by the receptor. It contains two C_2-C_2 type zinc fingers, of which four highly conserved cysteine residues coordinate the binding of one Zn^{2+} ion. Additional residues in the N- and C-terminal of the DBD are necessary, however, for optimal DNA binding (100). The only human nuclear receptors that do not possess a DBD are SHP and DAX (101, 102).

The evolutionary conserved C-terminal domainis responsible for multiple functions, such as ligand binding and transactivation (103).The transactivation function of the AF-2 domain depends on the binding of the ligand to the ligand binding domain (LBD). The LDB is moderately conserved throughout the family, though highly within isotypes of a receptor, such as RARα, RARβ and RARγ. However, its structural organization is preserved within the NR superfamiliy. The LBD secondary structure is composed of 12

α-helices that together form the ligand binding pocket, the dimerization surface and the AF-2, and is much more conserved than the sequence (104, 105). In addition to the AF-2 transactivation domain in the C-terminus, a silencing function has been described that occurs in members of the thyroid hormone/retinoid receptor class of nuclear receptors (103).

Some nuclear receptors also contain a C-terminal F-region. This region is highly variable and its structure and function are not known.

Different classes of nuclear receptors and their mode of action

Nuclear receptors can be classified into four distinct classes based on their DNA binding and dimerization (85, 103).

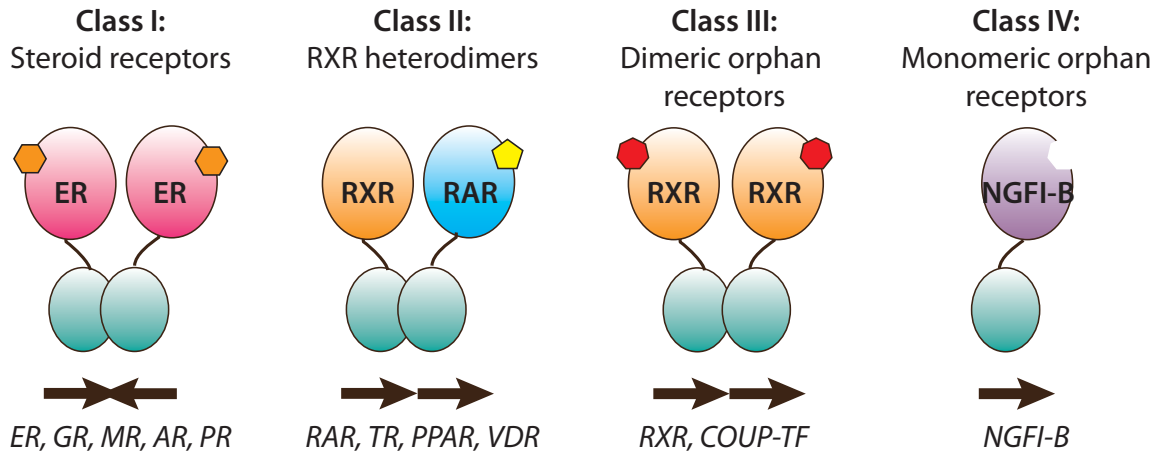

Figure 3 Classes of nuclear receptors

Four classes of nuclear receptors can be identified according to ligand binding, DNA binding, and dimerizationproperties: steroid receptors, RXR heterodimers, homodimeric orphan receptors, and monomeric orphan receptors. Representative receptors are presented for each group. Figure modified from Mangelsdorf *et al*. (85).

The first class is composed of steroid receptors such as GR, ER, AR and PR. These receptors associate with heat shock proteins and are localized in the cytoplasm in the absence of ligand. The binding of ligand induces a conformational change allowing for dissociation from the heat shock proteins and formation of homodimers. Class I receptors bind response elements with half-sites organized as inverted repeats (palindromes).

The second class contains receptors such as TR, PPAR and RAR, that instead of forming homodimers, function as heterodimers with RXR. These heterodimers are present on the DNA even in the absence of ligand. The conformational change that is induced by ligand binding results in transcriptional activation. Class II receptors preferentially bind to response elements of the direct repeat type, although everted and inverted repeats can also be bound by several heterodimers.

The last two classes are composed of orphan nuclear receptors, for which no ligand has been discovered. These receptors bind as dimers to direct repeats (class III) or as monomers to extended core sites (class IV).

1.2.2 RAR and RXR: ligand-dependent transcription factors

1.2.2.1 Retinoic acid receptors

Even before the discovery of the retinoic acid receptors, it was proposed by P. Chambon that RA could be the ligand of a nuclear receptor (92). Three retinoic acid receptor isoforms have now been identified. The first one was identified in 1987 and named RAR, later RARα (93, 94). Subsequently, two additional RARs, RARβ and RARγ have been identified (106, 107). Several isoforms exist of each isotype, produced by the use of different promoters, for example five isoforms are known to exist for RARβ (108, 109). The isoforms differ only in their N-terminus (105). Several of the RAR isoforms are themselves RA inducible, as summarized in Table I. The suggestion that RARα, RARβ and RARγ each have their own specific function followed from the observation that there is a much higher level of interspecies conservation among members of the RAR family than there is conservation of the three receptors within the same species (110).

Table I RAR isoforms and their RA regulation.

Receptor isoform	RA regulation	Species	References
RARα2	Induction	Mouse	(111)
RARβ2	Induction	Mouse, human	(112-114)
RARγ2	Induction	Human	(115)

Individual knockouts for the different RARs do not shown very drastic phenotypes. Knockout mice show some features of vitamin A deficiency, including growth deficiency and male sterility (116). Observations with the single knockout mice suggest high levels of functional redundancy, at least during development. The phenotype observed in double knockouts is much more serious and leads to reduced viability. Various malformations related to vitamin A deficiency cause these mice to die *in utero* or shortly after birth (6). The high level of resemblance between defects observed in vitamin A deprived mice or mice with defects in RA synthesis and the RAR knockout confirms the importance of the receptors in RA signaling.

1.2.2.2 Retinoid X receptors

The discovery of the retinoid X receptor meant a great leap forward in the understanding of the selectivity of DNA binding by non-steroid receptors, most of which form heterodimers with RXRs, as well as the discovery of a receptor for another retinoid, 9-*cis*-RA (117). Three isotypes exist, RXRα, RXRβ and RXRγ, the primary sequence of which differs substantially from the RARs (118). Like for the RARs, multiple isoforms have been identified.

Rxra-/- mice die around embryonic day 14.5 due to hypoplastic development of the ventricular chamber of the heart. Mice also have ocular malformations, and both these phenotypes are consistent with vitamin A deficient models, supporting the idea that RXRα plays a role in RA signaling *in vivo* (119). Malformations observed in RXRβ and RXRγ knockouts are fairly minor; the former results in 50% embryonic lethality with male sterility in surviving offspring (120), the latter have no obvious phenotype apart from thyroid hormone resistance (121). Knocking out both RXRβ and RXRγ together did not result in any obvious morphogenetic defects, even when one allele of RXRα was deleted at the same time. This shows that one copy of RXRα is sufficient to perform most RXR functions (121).

1.2.3 Regulation of gene expression by RAR/RXR heterodimers

As mentioned before, RARs are class II nuclear receptors that function as heterodimers with RXR. It has been shown in *in vitro* binding studies that both all-*trans*-RA and 9-*cis*-RA are high affinity ligands for RARs, but only 9-*cis*-RA is able to bind to RXRs (122). However, the *in vivo* existence and the physiological roles of 9-*cis*-RA are still highly disputed (123). Some other naturally occurring retinoids are also capable of binding and transactivating RARs, such as all-*trans*-4-oxo-RA and all-*trans*-4-oxo-retinol, but the most important ligand for the RAR-RXR heterodimer seems to be all-*trans*-RA (RA) (6).

1.2.3.1 Retinoic acid response elements

RAR-RXR heterodimers bind to the DNA on so-called RAREs (retinoid acid response element). Typically, these elements are composed of a direct repetition (DR) of the core motif PuG(G/T)TCA or closely related motifs (3). The alternative motif PuG(G/T)(G/T)(G/C)A is also used and represents a 10% consensus. Most frequently, the DRs are separated by 5 nucleotides (DR5), although DR1 and DR2 (1 or 2 nucleotide spacer, respectively) elements are also found frequently. Alternatively, DRs with varying spacers as well as inverted repeat (IR) elements have been described as functional RAREs (124). Various groups have been able to show that binding of the heterodimer to DR2 and DR5 elements occurs in the orientation 5'-RXR-RAR-3' (125-127), whereas DR1 elements are bound in the opposite orientation (128). Some examples of well characterized RAREs are listed in Table II.

1.2.3.2 Target gene repression in the absence of ligand

As previously discussed, unlike steroid hormone homodimers, the RAR-RXR heterodimers can bind DNA in the absence of ligand, if the conformation of the chromatin does not impede recognition of the RARE (105). This transcriptional repression is due to the association of the unliganded form of RAR with corepressors. These corepressors, such as NCoR (nuclear receptor corepressor) or SMRT (silencing mediator for RAR and TR), allow for the recruitment of specialized complexes with histone deacetylase (HDAC) activity. These HDAC complexes play well-known roles in transcriptional repression, by

deacetylating the N-terminal tails of histones. This leads to condensation of the chromatin at the promoter of target genes, rendering the transcriptional start site (TSS) inaccessible for the general transcription factors (105, 135).

Other corepressors recruited to RARs in a ligand-dependent mannder include RIP140/NRIP1 (receptor interacting protein of 140 kDa), PRAME (preferentially expressed antigen in melanoma) and TIF1α/Trim24 (transcription intermediary factor 1-α). It has been suggested that these corepressors could limit or end the RA signal (105). This is supported also by the fact that at least one of these corepressors, NRIP1, is by itself RA-inducible (136, 137), and may thus be implicated in a negative feedback mechanism.

Table II Examples of some known RAREs

Gene	Species	RARE type, sequence	Distance from TSS	Refs.
RARβ2	Mouse	DR5 : 5'-**GGTTCA**ccgaa**AGTTCA**-3'	-53 bp	(112)
CYP26A1	Mouse	DR5 : 5'-**AGTTCA**cccaa**AGTTCA**-3'	-223 bp	(129)
	Mouse	DR5 : 5'-**AGTTCA**caggc**AGTTCA**-3'	-2,005 bp	(130)
FOXA1	Mouse	DR5 : 5'-**AGGTCA**ggggg**AGGGGA**-3'	-1,300 bp	(131)
BTG2	Human	DR2 : 5'-**GGaTCA**cg**AGGTCA**ag**AGATCA**-3'	-3,357bp	(132)
CASP9	Human	DR2 : 5'-**AGGTCA**gg**AGTTCA**-3'	+9,461bp	(133)
CRBPI	Mouse	DR2 : 5'-**AGGTCA**aa**AGGTCA**-3'	-1,010bp	(134)

1.2.3.3 Activation of transcription by RA

It is now widely accepted that upon binding of RA to RAR a conformational change is induced that leads the corepressors that are bound to RAR-RXR heterodimers to be exchanged for coactivators. Upon ligand binding, helix 12 in the LBD gets reoriented and forms a charge clamp with helix 3, allowing for binding of the LXXLL motif of coactivators (105). Interaction with coactivators of the p160 subfamily of steroid receptors (SRC), SRC-1 (NCo-A1) (138), SRC-2 (TIF-2, GRIP-1) (139, 140) and SRC-3 (pCIP, ACTR, AIB1, TRAM1, RAC3) (141, 142), that act as adaptor proteins, leads to the

recruitment of other activator complexes, that have different enzymatic activities (105). Different classes of coactivators are (i) histone acetyl transferases, notably CBP/p300 (CREB binding protein) and p/CAF (p300/CBP associated factor); (ii) histone methyltransferases like CARM1 (coactivator associated arginine methyltransferase 1) and PRMT1 (protein arginine methyltransferase 1); (iii) ubiquitinases/deubiquitinases and (iv) ATP-dependent nucleosome remodeling complexes. The LXXLL motif is a recurrent structural attribute of most AF2-dependent nuclear receptor coactivators (143). All these complexes will alter the chromatin structure around the promoters of target genes, in a process referred to as derepression (135). Finally, it has been shown that activated RARs can directly interact with a subunit of the mediator complex, DRIP205/TRIM220, which contains two LXXLL motifs (144). This interaction leads to the recruitment of the mediator complex, the RNA polymerase II and the general transcription factors (105), allowing eventually for the initiation of transcription.

1.2.3.4 Modulation of RA signaling by other signaling pathways

Various major signaling pathways have been shown to influence the levels of RA signaling in cells. The most important ones will be discussed here.

MAPK signaling

RA rapidly induces the activation of the p38MAPK/MSK1 signaling pathway. Several phosphorylation events have been identified in the RARs, which appear to be regulated in a highly coordinated RA-induced phosphorylation cascade starting with RAR phosphorylation by MSK1 (145). Upon stimulation with RA, RARα rapidly gets phosphorylated on two serines, S369 in the LBD and S77 in the N-terminus (145). S77 is a target for the cdk7 subunit of the general transcription factor TFIIH (146). This phosphorylation event depends on the docking of Cyclin H at a site in the LBD (147) and is important for transcriptional activation (146, 148). S369 corresponds to a consensus site for multiple kinases, such as PKA and MSK1 and might integrate signals from various signaling pathways (145, 147). Phosphorylation of this site by MSK1 leads to increased interaction with Cyclin H and subsequently cdk7. The phosphorylation of S77 is thus a

downstream consequence of the phosphorylation of S369. The phosphorylation of S77 is conserved in RARγ, but it is not clear whether RARγ can be phosphorylated in the LBD by MSK1. RARγ can also be phosphorylated in the N-terminus by p38MAPK, but this event is not conserved in RARα. Finally, RA also activates p40/42MAPK, but it has not been shown whether this affects RAR phosphorylation (105).

MSK1 also contributes to activation of RA target genes by phosphorylating histone H3 in the promoter regions of these genes (145, 149).

Protein kinase C

Protein kinase C (PKC) is a protein family of serine/threonine kinases that have roles in the regulation of fundamental cellular processes such as proliferation, differentiation and apoptosis, as well as tumorigenesis (150, 151). Clues for a role of PKCs in RA signaling come from the fact that in several tumor cell lines, treatment with RA largely increases PKC levels (152, 153). Also, overexpression of PKCα in B16 mouse melanoma cells and F9 teratocarcinoma cells leads to phenotypical changes induced by RA (152, 154). RA induced differentiation of these cell lines also leads to strong induction of PKCα at both the mRNA and the protein level (152, 155, 156). PKCs, particularly PKCα, also appear to be important for the anti-proliferative effects of RA in several breast cancer cell lines (157-159). PKC inhibitors were shown to reduce the binding activity of a RARα/RXRα dimer to DNA and reduce transcriptional activity from an RA inducible promoter. PKCα overexpression was able to compensate for this effect and PKCα was found to phosphorylate RARα *in vitro* (160). Later, the same group identified the Ser157 site in the DNA binding domain as a PKC phosphorylation site and showed that this site can be phosphorylated by both PKCα and PKCγ. Phosphorylation of this site reduced transcriptional activity of RARα as well as its capacity to heterodimerize with RXRα (161). Another group however showed that this phosphorylation could not be observed *in vivo*, and instead showed that activation of PKC leads to an increase of RARα half-life and AF-2-dependent transcriptional activity (162). All in all, a cross-talk between PKC and RA pathways clearly exists, but more research will be needed to understand the full extent of it.

Phosphorylation of cofactors

In addition to the RARs, several coactivators and –repressors have also been shown to be modulated by MAPK and other kinases. SRC-3 and TBLR1, a corepressor, are phosphorylated respectively by p38MAPK and PKCδ in response to RA (163, 164). p160 family members, p300/CBP and SMRT and NRIP1 corepressors have been shown to be phosphorylated by MAPKs and other kinases, but whether these effects are RA-dependent remains to be investigated (105).

1.2.4 Non-canonical RA receptors

Apart from the well recognized interaction of RA with RAR-RXR heterodimers, various studies have come out in the last couple of years strongly suggesting that RA can also function through other nuclear receptors. This idea is also supported by observations such as the fact that RA is important for skin maintenance, but that this is not RAR-mediated.

1.2.4.1 RA signaling through PPARβ/δ

It has recently been shown by the group of N. Noy that RA can function as a high affinity ligand for the orphan nuclear receptor PPARβ/δ, when compared to PPARα and PPARγ (165). The same group next published a set of results that suggest RA could be directed to PPARβ/δ by FABP5, in a similar way as the shuttling of RA to RARs by CRABP-II. They used knockdown methods to alter FABP5/CRABP-II ratios and concluded that RA activation of PPARβ/δ leads to activation of pro-proliferative and anti-apoptotic effects, in contrast to the well-known anti-proliferative and apoptosis promoting effects of RAR activation by RA (166). Another group not long after studied the effects of PPARβ/δ by RA compared to known high- and low-affinity PPAR ligands and concluded RA is not likely to be a ligand for PPARβ/δ (167). The latter two studies did not used the same cellular models and reporter constructs, which might in part account for the discrepancies between them. Another point to be kept in mind is that in none of the above mentioned studies the affinities of RA towards FABP5 and PPARβ/δ were compared to those towards CRABP-II and RAR. It is possible that RA functions as a ligand for

PPARβ/δ in some context, but more studies are necessary to better understand the physiological relevance of these observations.

1.2.4.2 RA as a ligand for RORβ

A family of nuclear receptors that are closely related to the RARs is that of the RA receptor-related orphan receptors (RORs). RORs play important roles in cellular differentiation and development, but physiological ligands have not been identified (168). RA and several synthetic retinoids have been shown to be able to act as functional RORβ ligands, binding to the RORβ-LBD in *E. coli* binding assays. RA also partially inhibited transcriptional regulation by RORβ in neuronal HT22 cells, but not in NIH3T3, 293 or P19 cells. This suggests that the antagonistic effects of RA on RORβ are cell-type specific (169). More studies will be needed to understand the *in vivo* relevance of these observations.

1.2.4.3 RA activation of COUP-TFII

Finally, it has been shown that the chicken ovalbumin upstream promoter-transcription factor COUP-TF-II can be activated by micromolar concentrations of RA (170). COUP-TFI and II are amongst the most conserved nuclear receptors and play roles in organogenesis, angiogenesis, cell fate determination and neuronal development as well as metabolic homeostasis and circadian rhythm (6). Both RA and 9cRA can serve as low affinity ligands for COUP-TFII in cell culture models (170). However, concentrations needed for receptor activation make it highly doubtful that there is a physiological role to this activation. Nonetheless, this is interesting to keep in mind in cases where cells are treated with pharmacological doses of RA.

1.2.5 Transcriptional regulation through interaction with other transcription factors

An alternative mechanism of gene expression regulation by nuclear receptors is through interaction with and repression of the activity of other transcription factors (171).

This transrepression activity has been observed for several NR ligands, such as glucocorticoids and retinoids.

1.2.5.1 Transrepression of AP-1 activity

AP-1 is a transcription factor complex composed of members of the Jun (c-jun, junB, junD) and Fos (c-fos, FosB, Fra1, Fra2) families of proto-oncogenes (171). Its activity is associated with cellular proliferation and tumorigenesis (172). The transactivation of AP-1 is necessary for *in vivo* tumor promotion (173). AP-1 activity is highly responsive to extracellular stimuli because of regulation by a network of various protein kinases, such as JNK and SAPK for c-jun and FRK for c-fos (174-176).

Retinoids can alter the activity of AP-1 in several ways, that do not all seem to require RAR transcriptional activity. Several so-called dissociated retinoids exist that are not able to transactivate through RARs, but that do induce AP-1 transrepression (177-180). Also several RAR mutants have been described that have altered transactvating properties, but behave normally when it comes to transrepression (181). The contribution of the different RAR and RXR isoforms to AP-1 transrepression are not fully understood and seem to be very cell-type specific (182-184). Several reports suggest that the RAR DBD is required for AP-1 transrepression (185-187).

Direct binding of RAR to c-jun was shown to interfere with the binding of c-jun homodimers to AP-1 binding sites *in vitro* (186). RAR also interferes with the formation of c-jun/c-fos heterodimers in some cell types (188). A cooperation of COUP-TF with RAR to inhibit AP-1 activity has been shown in 1) its ability to increase RARβ2 promoter activity (189) and 2) inhibit c-jun DNA binding trough direct interaction (190). RARs were also reported to inhibit the expression of c-jun and c-fos in pituary cells and keratinocytes (183, 191). Finally, RARs might inhibit AP-1 activity by competition for available coactivators. Although this could be a plausible explanation of the transrepression effects, reports are contradictory. Also, this model would not explain functioning of dissociated retinoids (171).

1.2.5.2 Transcriptional regulation through modulation of Sp1

Sp1 and Sp3 are transcription factors that are members of the Specificity Protein/Krüpel-like Factor (SP/KLF) family. They are expressed in all mammalian cells and regulate genes involved in differentiation and cell cycle progression (192). Several nuclear receptors have been shown to be able to interact with Sp1, such as ER and COUP-TF (193, 194).

Suzuki and colleagues first showed the involvement of Sp1 in RA-mediated gene regulation for the induction of urokinase plasminogen activator (uPA). RAR/RXR directly interact with Sp1, increasing Sp1 binding to the GC box in the promoter of uPA and uPA transcription (195). Similarly, Sp1 binding to the promoter of the tPA (tissue-type plasminogen activator) gene is necessary for RA-mediated induction of this gene through a DR5 enhancer element. This is specific for Sp1 and similar results could not be observed for Sp3 (196). The enzyme 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) is responsible for the rapid conversion of estrogen to estrone. In human endometrial and placental cells HSD17B2 mRNA and enzymatic activity are induced by RA. This induction depends on availability of RARs and RXRs, as well as functional Sp1 and Sp3 binding to the promoter. RA was found to induce an *in vivo* interaction between RAR and Sp1/Sp3 (197).

Other genes that are regulated by RA through Sp1 include CREB ((198), reelin (199), secretin (200), lamin A and C (201, 202), BMP2 (203), folate receptor beta (204), CD18 (205) and IL-1β (206).

1.2.6 Non-genomic actions of retinoic acid

Aside from the classical signaling through RAR-RXR heterodimers and activation of transcription, it has been shown that RA can also function through activation of non-genomic pathways. These occur both in the presence and in the absence of RARs.

1.2.6.1 Non-genomic signaling of RARs

RARs have been implicated in mediating non-genomic actions of RA. RARα was shown to be required for RA-dependent homeostatic synaptic plasticity in neurons, in a

manner that is independent of transcription (207). RARα functions as an inhibitor of translation by binding to specific mRNAs, such as the mRNA for the glutamate receptor 1 (GluR1). RA binding to RARα diminishes the association with the mRNA and allows for translation to take place (207, 208). RARβ was shown to augment the frequency of transmitter release in developing neuromuscular synapses in *Xenopus* cell cultures (209). This was shown to be mediated PLCγ and PI3K signaling pathways and SRC activation that lead to changes in intracellular Ca^{2+} levels (210).

1.2.6.2 Interaction with the PI3K/Akt pathway

Several reports have described both positive and negative interactions between RA and PI3K/Akt signaling in numerous cell types. ATRA activates PI3K in HL-60 cells (monitored by PIP3 recovery) and this is required for RA induced granulocytic differentiation (211). In NIH3T3 cells, rapid induction of PI3K activity by RA, but not EGF, is required for RA-stimulated expression of tissue transglutaminase (TGase), as well as for its GTP binding activity (212). This RA-induced TGase expression was inhibited by EGF treatment through activation of Ras-ERK signaling (213). RA can induce neural differentiation in SH-SY5Y cells. This differentiation involves downregulation of ID1, ID2 and ID3, which requires RA-induced activation of the PI3K/Akt signaling pathway (214). The rapid activation of PI3K signaling in SH-SY5Y cells requires RARs, interacting in a complex with the p85 subunit of PI3K (215). Other interactions of RARs with PI3K subunits have been described. Farias *et al*. showed that CRBP-I inhibits the PI3K/Akt pathway in an RAR-dependent manner by decreasing p85-p110 heterodimerization in transformed MTSV1-7 breast epithelial cells. RA treatment was found to mimic these effects of ectopic CRBP-I (216). Retinoic acid also stimulates the sodium/iodide symporter (NIS) in MCF-7 breast cancer cells and this is mediated by the insulin growth factor-I/PI3K and p38MAPK signaling pathways (217). This RA induction of NIS in MCF-7 cells is was found to be mediated by rapid activation of the PI3K pathway and appears to involve direct interaction of p85 with RAR and RXR (218). RA-induced PI3K/Akt activation was also found to be implicated in induction of Nanog expression in the early stage of differentiation of F9 cells (219). Finally, using a DN Akt as well as PI3K inhibitors, it was shown that

downregulation of PI3K/Akt signaling is required for RA-induced RARγ2 phosphorylation by p38MAPK in RARγ2 transfected COS-1 cells (220).

1.2.6.3 Impact on PKCα signaling

Crosstalk between retinoids and PKCs, both positive and negative, has also been described by various groups. The effects of PKC on RARα and RA signaling have been discussed above. RA can also influence the activity of PKCs, and based on structure analysis and binding assays it was proposed that this is due to direct binding of RA to PKC (221). More recently it has been shown that RA binds directly to one of the regulatory units of PKCα, thus competing with binding of acidic phospholipids and acting as an inhibitor of PKCα signaling (222). PKCδ on the other hand was shown to be activated in an RA dependent fashion (223). RA also exerts classic genomic effects on some of the PKCs, as a functional RARE has been identified in the promoter of the murine PKCα gene (224).

1.2.7 Retinoic acid in human disease

The role of RA in mediating processes such as differentiation and proliferation have made it a molecule of outstanding interest for the treatment of various diseases. Already in 1925, the potential of retinoids as anti-cancer agents was recognized by Wolbach and colleagues (225). Various natural and synthetic ligands are being tested for the treatment of human cancers, as well as skin diseases such as psoriasis and acne (reviewed in (226, 227)). Retinoids have been found to be particularly useful in the treatment of acute promyelocytic leukemia, but also other cancers have been described to benefit from retinoid treatment, either in treatment or prevention settings.

1.2.7.1 Acute promyelocytic leukemia

Probably the best known application for RA in the treatment of cancer is in acute promyelocytic leukemia or APL (228). APL is due to chromosomal translocations of the RARα gene. This leads to gene fusions, most often including the N-terminal part of the PML gene (229, 230). The resulting PML-RARα fusion protein interferes with normal RA dependent transcriptional regulation (231). However, the protein remains RA-responsive

and treatment of APL patients with RA as a single agent can induce complete remission in most patients because of the reactivation of normal signaling pathways (227, 228, 232). However, remissions are not sustained. When consolidation chemotherapy is given after treatment with RA to newly diagnosed patients, this improves the remission duration (233). Clinical trials were done to compare treatment with RA and conventional chemotherapy, and investigate benefits of concurrent or sequential combination therapies. Early studies with RA compared its efficacy against classical chemotherapeutic agents such as ara-C, as well as the benefits of maintenance treatment with RA during one year after induction treatments. In this study, the best outcome was observed in patients who received RA for both induction and maintenance treatment, with up to 75% of patients apparently cured of their disease (234). More recent studies showed the concurrent use of RA and chemotherapy to be more efficient than subsequential use (235-237). Long-term follow-up of clinical trials with RA in APL confirmed the benefits of combination therapy with RA and chemotherapy (238, 239). Currently, treatment with RA and chemotherapy is the standard for newly diagnosed cases of APL.

Besides PML-RARα, other fusion proteins that are involved in PML include PLZF-RARα, NuMA-RARα, NPM-RARα and Stat5-RARα (240). The response of these fusion proteins to RA is variable. On the whole, treatment with RA has made APL the most curable type of acute myeloid leukemia in adults (241).

1.2.7.2 Retinoids in chemoprevention and treatment of solid tumors

In addition to the beneficial effects of RA in the treatment of APL, retinoids have been shown to be effective therapeutic and preventive agents in several other types of cancers and precancerous lesions. Early studies in rodents have shown that retinoids are effective in reducing the tumorigenesis of several types of epithelium, such as skin, respiratory, mammary, buccal and stomach epithelia (76). In humans, retinoids have been shown to be effective in reversing premalignant epithelial lesions, inducing myeliod cell differentiation and preventing liver, lung, breast and ovarian cancer (242-245). Furthermore, clinical trials are ongoing for the use of *all-trans* RA for the treatment of lung cancer, cervical cancer, kidney cancer, neuroblastoma, glioblastoma, lymphoma, leukemia and melanoma,

combined or not with other drugs (based on information available on www.clinicaltrails.gov using the search criteria « all trans retinoic acid cancer », on September 19, 2011).

Visibly, retinoids are a potent class of molecules with various potential applications in the field of cancer treatment and chemoprevention. Nevertheless, the frequent occurrence of unwanted effects and problems of resistance make further research into the molecular mechanisms of the antitumor effects of retinoids essential before standard implementation in treatment regimens.

1.3 The mammary gland : normal physiology and malignancy

1.3.1 Normal breast physiology and development

The principal function of the mammary gland is to secrete milk during lactation. It has a unique developmental program, with the majority of the development taking place after birth, in puberty and during pregnancy (246).

1.3.1.1 Mammary gland morphology

At birth, a rudimentary mammary gland is present in both females and males. This rudimentary breast is composed of 15-25 mammary ducts that come together in approximately 10 major ducts near the epidermis (247). Further development of the female breast starts in puberty, whereas the male breast will not undergo significant development after birth and will remain rudimentary (248).

The adult mammary gland is composed of three distinct types of structures: skin, subcutaneous tissue and breast tissue (248). The latter can be subdivided into the parenchyma, mammary epithelium organized in an extensively branched ductal-lobular system and the mesenchyma or stroma, a heterogeneous layer of connective tissue in which the parenchyma is embedded (249, 250). The parenchyma is composed of 15-20 segments, the lobules. These lobules drain in a network of milk collecting ducts. Around 10 major collecting ducts converge and open at the nipple ((248); Figure 4A). Stromal and epithelial cells in the mammary gland communicate though the extracellular matrix (ECM) and this is crucial for normal patterning and function of the gland. Disruption of this communication can lead to both induction and promotion of breast cancer (251).

Stroma

The biggest part of the human mammary gland is made up of stroma (Figure 4A). The stroma and the subcutaneous tissue of the mammary gland are composed primarily of adipose tissue and connective tissue, in which is contained an extensive network of blood vessels, nerves and lymphatics (248). The stroma is important not only as supportive tissue for the epithelium, but also plays an important role in the development and morphology of

the mammary gland (252, 253). Stromal cells were shown to secrete factors that can stimulate the growth of epithelial cells in a cell culture system (254). Injection of non-mammary stem cells in the mammary stroma was shown to induce their expression of mammary epithelial markers such as β-casein and ERα and induce stromal invasion and formation of ducts and lobules (255-257). This reinforces the idea that the stroma plays an important role in the development of the mammary epithelium.

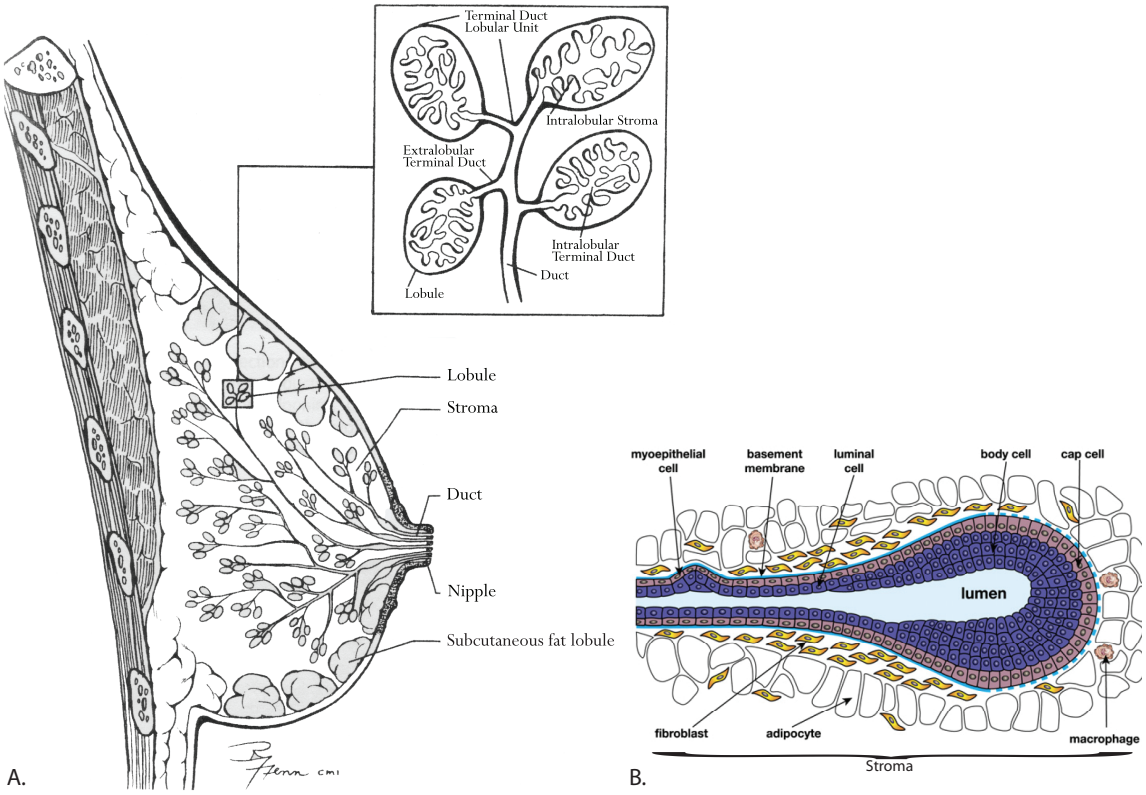

Figure 4 Schematic overview of the adult mammary gland (A) and a TEB (B)
Figure adapted from (258, 259)

Mammary epithelium : the parenchyma

The mammary epithelium or parenchyma is organized in a branched network of ducts ending in structures called terminal ductal lobulo-alveolar units (TDLUs or terminal end buds (TEBs) in mouse) (259). The TDLUs are clustered together in the before mentioned lobules (Figure 4A; inset). The normal mammary epithelium consists of a bilayer of inner luminal cells that are implicated in milk production and an outer basal layer composed

mainly of myoepithelial cells that eject milk into the ducts (260). The epithelial cells are separated from the stroma by the basement membrane ((259); Figure 4B).

The basal layer of mammary epithelium comprises all cells that are not in direct contact with the lumen. As mentioned above, this includes primarily myoepithelial cells, however this layer also contains mammary stem cells and progenitors (259). The myoepithelial cells, because of their contractile properties, are important in the excretion of milk into the ducts. These cells are characterized by the specific expression of markers such as cytokeratin 5 and 14, β2 and α2 integrins and smooth muscle actin (SMA) (261, 262).

The inner layer of luminal cells is composed of highly differentiated, polarized cells. Their most important role is the production and secretion of milk. Typical markers for this cell type are cytokeratin 8/18, FOXA1 and GATA-3 (262-264). Also, around 10-15% of luminal epithelial cells express the nuclear hormone receptors ERα and PR (progesterone receptor) (265). The existence of at least two distinct types of luminal cells has been described : ductal and lobular cells (263).

1.3.1.2 Post-natal mammary gland development

At puberty the rudimentary mammary gland that was formed during fetal development will continue its development. Hormones from the ovaries and the pituitary gland, as well as local growth factors and cytokines will initiate branching morphogenesis of the simple pre-puberty glands (266). This expansion is driven by the highly proliferative TEBs/TDLUs. These structures penetrate into the fat pad as the ducts are elongating and the mature epithelial ductal tree will be formed (267). Two types of cells are present in the TEBs : cap cells and body cells (Figure 4B). The cap cells, located in the outer layer of the TEB and in contact with the stroma, are progenitors for the myoepithelial cells, whereas the body cells are progenitors for cells of the luminal lineage (268).

Ductal branching morphogenesis is directed by the TEB and depends on the local availability of growth factors. The first cue for ductal morphogenesis comes from estrogens produced in the ovaries. Ovariectomized mice, as well as ERα deficient mice fail to develop a ductal network (269, 270). Expression of ERα is required in the epithelial cells

and not in the stroma, as can be seen from transplantation experiments using ERα$^{-/-}$ epithelial cells or fat pads (271). This estrogen-driven development of the mammary gland also requires growth hormones produced by the pituitary gland. The hormones GH (growth hormone) and Prl (prolactin) are both required and function through activation of the Jak-Stat signaling pathway (267). Stat5a knock-out mice have defects in lateral and secondary branching. IGF1 produced in the mammary gland acts as the local effector of GH (272). It is produced in both stromal and epithelial cells during postnatal mammary gland development and its absence leads to a strong reduction of the outgrowth potential (273, 274). Knock-out studies have shown that EGFR in the stroma is also essential for normal outgrowth of the mammary tree at puberty (275). HER2 signaling appears to be required for initiation stages of ductal morphogenesis (276).

The formation of a lumen is essential in the development of a functional ductal system in epithelial organogenesis of for example the mammary gland (268). This can occur in different ways, such as deformation of an epithelial sheet in for example lung development, and cavitation in breast development (277, 278). Epithelial cells in the breast are organized in so-called acini, spherical structures with a lumen that is formed by apoptotic clearance of the inner layers of newly formed branches of epithelial cells (277, 279). Apoptosis has been detected in the body cells of TEBs (Figure 4B; (280)). The maintenance of the lumen in expanding ducts appears to depend on caspase-dependent apoptosis, mediated by the pro-apoptotic factor BIM (281, 282).

The final step of mammary differentiation takes place during pregnancy, when alveolar differentiation and lactation are induced under the influence of rising levels of prolactin, estrogen and progesterone.

1.3.1.3 Mammary stem cells

The existence of mammary stem cells was suggested after transplantation experiments showed that explants taken from different regions of the mammary gland were able to reconstitute a fully functional mammary gland in the cleared fat pad of mice (283). Using MMTV-infected donor tissue Kordon and Smith were able to show the clonality of these mammary outgrowths, suggesting that a single stem cell was able to repopulate the entire mammary epithelium (284). Using flow cytometry, several different epithelial subpopulations have been identified based on the expression of several cell surface markers (Figure 5). A strong enrichment of mouse mammary cells (regenerating a fully functional mammary tree) was found in the $CD49f^{hi}CD29^{hi}CD24^{+}Sca^{-}$ subpopulation (285-287). Mammary stem cells are rare and even this highly enriched population comprises less than 5% of these cells. Mammary stem cells differ from luminal epithelial cells in that they have lower levels of CD24 (286). Human mammary cells have also been isolated by several groups (19, 288, 289). Notably, human mammary stem cells appear to be marked by higher ALDH1 activity, indicated by the observation that ALDEFLUOR-positive cells isolated from mammary epithelium posess stem cell like properties (19). Mammary stem cells appear to exist only in the basal layers and could not be detected in the luminal compartment (290).

The specification of various subtypes of progenitor cells in the differentiation hierarchy of the mammary epithelium from mammary stem cells is controlled by a network of transcription factors (Figure 5). GATA-3 is essential for the differentiation of the ductal and alveolar luminal lineages and is not expressed in the myoepithelial compartment (263, 291). Alveolar differentiation during pregnancy requires the additional activity of the ETS family member Elf5 (292). STAT5A was shown to be essential for the establishment of the luminal alveolar compartment. Knock-outs of this gene in the mammary gland resulted in a block in alveolar differentiation during pregnancy and these mice failed to lactate (293, 294). The CCAAT/enhancer binding protein beta (C/EBPβ) plays an essential role in proper lobuloalveolar differentiation (295). Finally ΔN-p63 is important for the development of basal cell lineages (296).

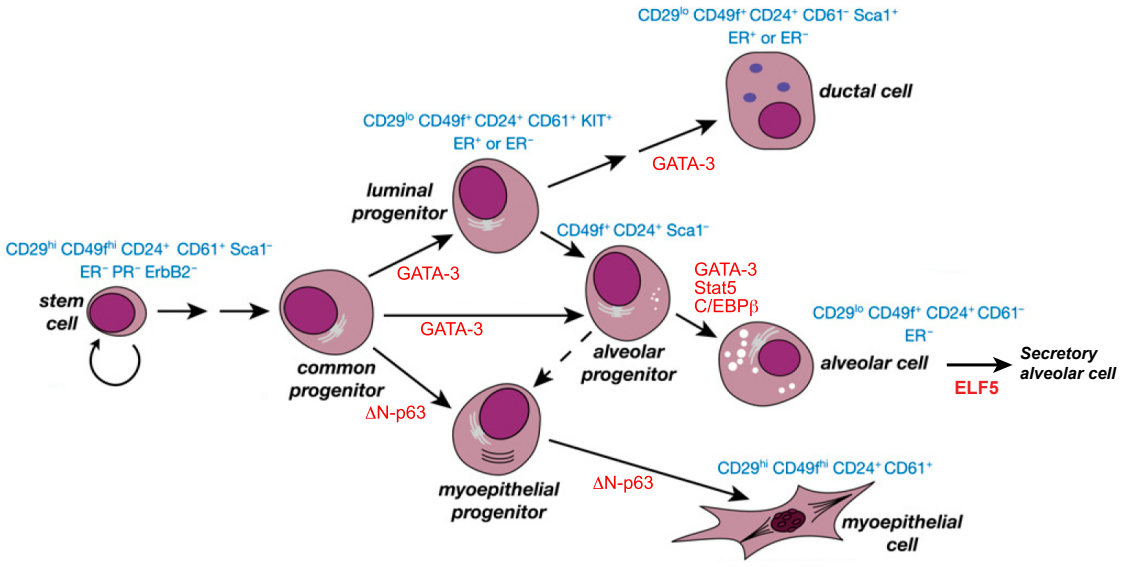

Figure 5 Model of mammary differentiation hierarchy

Markers for different populations of progenitors and terminally differentiated cells are indicated in blue (markers for mouse mammary cells are given). In red, transcription factors implicated in different steps of differentiation are indicated. Figure adapted from (259) and (296).

1.3.2 Breast cancer: Subtypes and their prognosis

Breast cancer is the most frequent cancer in women in developed countries. In Canada, one woman in nine will develop a breast cancer during the course of her life and one in twenty-eight will die of it. Breast cancer is a very heterogeneous disease, different types of breast tumors exist with different characteristics. To better target therapies, various groups are working on the better understanding and classification of these different tumors (297-300). Gene expression patterns have been used to classify breast tumors according to five distinct classes ((297, 298); Table III). It is also possible to classify cell lines into related classes using gene expression profiles.

Table III Classification of breast tumors.

Based on data from refs. (297, 298, 301, 302). Cell lines that were used in the studies presented in this thesis (Chapters 2 and 3) are indicated next to the most relevant class. Note that SK-BR-3 cells are often classified as luminal B cells, probably because of the presence of low levels of ERα mRNA. However, since no ERα protein can be detected in these cells, they are considered 'HER2-overexpressing' for the work presented here.

Class	Markers	Survival rate	Cell lines
Luminal A	ER+/PR+	61,2%	MCF-7
Luminal B	ER+/PR-/HER2+/-	48,4%	BT-474; SK-BR-3
Basal	ER-, PR-, HER2-, CK5/6+ and/or HER1+	60,9%	MDA-MB-231
Normal-like / Triple negative	ER-, PR-, HER2-	80,4%	
HER2-overexpressing	ER-, PR-, HER2+	51,8%	SK-BR-3

1.3.2.1 Origins of different breast cancer subtypes

Several lines of evidence seem to show that different types of breast cancer are not derived from each other, but rather have different origins. For example, it has been shown that around 65% of low grade, ER-positive tumors have lost the long arm of chromosome 16. This was observed in only 16% of the high grade, ER-negative tumors (303). Tumor grades classify tumors according to histological and clinical properties, with low grade tumors being less aggressive, slow growing and well differentiated and high grade tumors being more aggressive and generally undifferentiated. According to these observations, for a tumor to progress from low grade to high grade, it would have to regain genetic material, a highly unlikely event (260). Currently the model that seems most appealing is one where different tumor types arise from cancer stem cells (CSCs) or progenitors that are present in the constantly developing mammary gland. Figure 6 illustrates this model in a schematic overview of normal vs. tumor differentiation and molecular pathology.

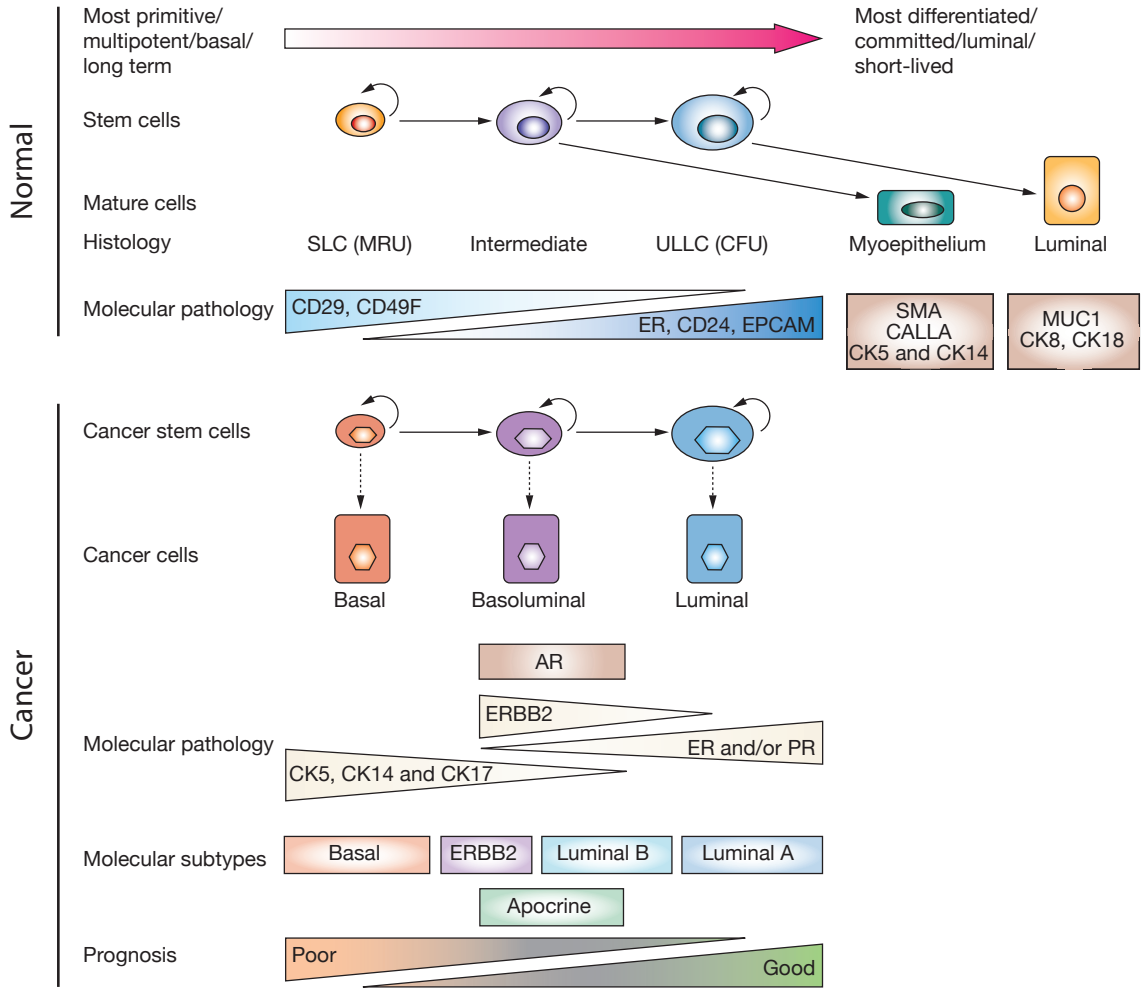

Figure 6 Origins of breast cancer subtypes based on stem cell hierarchy

A model of normal vs. cancer stem cell hierarchy and how this hierarchy could lead to the occurrence of different subtypes of breast cancer. AR, androgen receptor; CFU, colony forming unit; EPCAM, epithelial cell adhesion molecule; MRU, mammary repopulating unit; SLC, small light cell; ULLC, undifferentiated large light cell. Figure adapted from (260).

1.3.2.2 Hormone dependent, ER-positive breast cancer

About 70% of all breast tumors express the nuclear hormone receptor ERα, estrogen receptor alpha. These tumors are called hormone-dependent, since the growth of such tumors is dependent on the presence of estrogens in the tumor microenvironment. Because ERα drives the proliferation of these ER-positive tumors, it is an ideal target for treatment of these tumors (304).

Antiestrogen therapies

Due to their estrogen dependency, ER-positive breast tumors can be efficiently targeted by endocrine therapies, that is to say, therapies that either block estrogen production or ER function and by doing so inhibit estrogen-mediated cell proliferation (305). Antiestrogens, antagonists of estrogen receptors, can block their transcriptional effects. Two classes of antiestrogens exist, partial antiestrogens and total antiestrogens. The partial antiestrogens, also called selective estrogen receptor modulators (SERMs), have a tissue- and gene-dependent partial estrogenic activity (306). They function by inhibiting the AF-2 domain of ER (307). Whereas these compounds are ER antagonists in the breast, they are agonists in for example bone (308). The best known partial antiestrogen is tamoxifen, successfully tested for breast cancer treatment in the 1970s (309). Full antiestrogens block ER transcriptional activity in all target tissues. These compounds inhibit both AF-1 and AF-2 functions and also induce the degradation of ER (310, 311). The full antiestrogen fulvestrant is currently being used for treatment of postmenopausal women with recurrent ER-positive breast cancer (312).

Aromatase inhibitors

Another way of targeting ER-induced tumor cell proliferation is by inhibiting the production of estrogens. While circulating estrogen levels are very lwo in post-menopausal women, there is also local production from androgens by aromatase expressed in the stroma in the vicinity of the tumor (304). Aromatase inhibitors (AIs) inhibit this conversion and thus prevent estrogen signalling in the tumor. AIs treatment is associated with a slightly better prognosis and longer disease-free survival than tamoxifen in postmenopausal women (313).

1.3.2.3 HER2 overexpressing tumors

The oncogene HER2/neu encodes a 185 kDa transmembrane protein that is a member of the Epidermal Growth Factor Receptor family, a subgroup of receptor tyrosine kinases (RTKs), that play important roles in cell proliferation, metabolism, differentiation and survival and are often implicated in various kinds of cancers (314). The family is composed

of four members, namely EGFR (HER1 or Erb-B1), HER2/neu (Erb-B2), HER3 (Erb-B3 and HER4 (Erb-B4). HER2 is overexpressed in about 25-30% of breast tumors. In the majority of cases, this overexpression is due to an amplification of the corresponding gene located on the long arm of chromosome 17 (17q12). The overexpression of HER2 in breast tumors is associated with reduced levels of disease-free and overall survival in metastatic breast cancer ((315, 316); Table III).

The HER2 gene amplification

The HER2 amplicon (Figure 7) has been extensively studied in order to better understand its impact on breast cancer treatment (317). A minimal region of amplification (small amplicon) of 280-750 kb was identified and may contain >20 genes (318, 319). A larger amplicon (long amplicon) may contain up to 40 genes (320) and extend as far as the TOP2A gene on 17q21 (321). Several of the genes that can be co-amplified with HER2 have potential therapeutic applications (317).

The amplification and overexpression of the TOP2A gene was shown to correlate with the response of tumors to anthracycline-based chemotherapeutics (322-325). It has been proposed that the amplification or deletion of TOP2A could be used as a predictive marker for response of HER2-positive patients to this kind of chemotherapy. Either amplification or deletion of the TOP2A gene occurs in up to 50% of all HER2-amplified tumors (321, 326, 327).

Another gene that can be co-amplified is STARD3, and the amplification of this gene has been associated with shorter overall and disease-free survival in breast cancer patients (328). The GRB7 gene has been found in tumor biopsies to be co-amplified with HER2 (329). The knockdown of both STARD3 and GRB7 in HER2 overexpressing SK-BR-3 or BT-474 cells resulted in reduced cell proliferation, although not to the same extent as the knockdown of HER2 (330). The GRB7 gene is included in the prognostic gene-signature Oncotype DX (Genomic Health, Redwood City, CA, USA) for response to chemotherapy.

Finally, it is important to note that the gene encoding the RARα receptor is also located within the large amplicon of HER2 and was found to be co-amplified with HER2 in 2/6 tumor samples tested (321). However, overexpression of HER2 has been described to lead to RA resistance in mammary cells (331), thus the impact of RARA gene amplification is unclear.

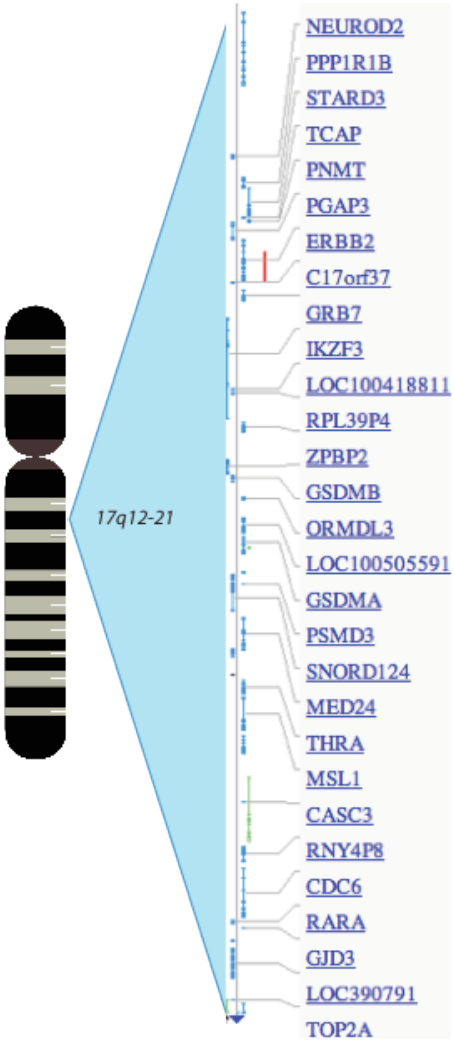

Figure 7 The HER2 locus on chromosome 17

Ideogram of chromosome 17, focusing on the region from 17q12 to 17q21. Know RefSeq genes within the region are shown on the right, using the Gene_Seq track from the NCBI MapViewer, build 37.2 (November 2010).

The 717 kb amplification region in between the HER2 and TOP2A genes contains about 35 genes, several of which, as noted above, are potentially interesting for cancer

treatment. However, due to the existance of breakpoints within the region, not all of these genes are similarly amplified. Also, the products of these genes are not necessarily all easily drugable targets. Given these facts, and before of our particular interest in the use of retinoids in breast cancer treatment, data presented in this thesis will focus on the amplification of RARA in combination with HER2.

Targeting HER2 for breast cancer treatment : Herceptin™

Because of the frequent strong and very uniform overexpression of HER2 in breast tumor cells and correlation with the prognosis of breast cancer, HER2 it has become an important therapeutic target (332). The first FDA-approved HER2 targeting therapy was Herceptin™ (trastuzumab; Genentech; South San Francisco, CA, USA). Herceptin™ is a humanized monoclonal antibody, that targets the extracellular domain of the HER2 protein. It is active as a single agent and as an adjuvant treatment in combination with chemotherapy (333). Several mechanisms of action of Herceptin™ on HER2 overexpressing tumors have been described in both in vitro and in vivo models. These mechanisms are summarized in Table IV.

Table IV Proposed mechanisms of Herceptin™ action.

Several mechanisms of the inhibitory actions of Herceptin™ on HER2-overexpressing tumors have been described in both *in vitro* and *in vivo* models. Table adapted from refs. (332) and (334).

Mechanism	Evidence	Reference
Internalization and degradation of HER2.	HER2 was shown to be downregulated in SK-BR-3 and MDA-MB-453 cells after Herceptin™ treatment.	(335)
G1 phase cell cycle arrest.	Herceptin™ treatment of SK-BR-3 and BT-474 cells resulted in increased levels of $p27^{Kip1}$ and $p27^{Kip1}$-Cdk2 complex formation, leading to reduced Cdk2 activity.	(336)
Disruption of PI3K/Akt signaling.	Herceptin™ decreased Akt phosphorylation and activity and increased the membrane localization of the PI3K/Akt inhibitor PTEN.	(337) (338)
Inhibition of angiogenesis.	Decreased levels of VEGF; decreased microvessel density in breast cancer xenografts.	(339)
Inhibition of DNA damage repair.	Inhibition of repair of DNA adducts after cisplatin treatment; block of unscheduled DNA synthesis after radiation.	(340-342)
Inhibition of HER2 extracellular domain cleavage.	Herceptin™ decreased proteolysis of the HER2 extracellular domain *in vitro*, as well as in a Herceptin-docetaxel phase II trial.	(343, 344)
Stimulation of natural killer cells and activation of ADCC.	Strong NK cell activation was observed in patients and ADCC levels correlated with response. ADCC activation was also observed in xenograft models of BT474 cells and multiple breast cancer cell lines.	(345) (346, 347)

Resistance to Herceptin™ treatment

As mentioned above, Herceptin™ can be used as a single agent as well as in combination therapy with chemotherapy. In a mono-therapy setting response rates are low, with a median duration of 9 months. Primary resistance to Herceptin™ mono-therapy ranges between 66% and 88% (348-350). Combined therapy with chemotoxins such as paclitaxel or docetaxel significantly increase response rates and overall survival compared to mono-therapy (343, 351, 352). However, even patients that initially respond to

Herceptin™ based regimens in combination therapy will develop resistance within the first year (333). Several molecular mechanisms of Herceptin™ resistance have been proposed and will be discussed below.

One way in which tumor cells can achieve resistance to Herceptin™ treatment is by increasing signaling from other receptors, either within the HER family or of other receptor families. Some examples include increased levels of HER family ligands such as heregulin and EGF, as well as increased signaling through other HERs, including HER2/HER3 and HER2/EGFR heterodimers. Particularly important in this respect is signaling through HER3/EGFR heterodimers and EGFR homodimers (353, 354). Although HER2 is the preferred dimerization partners of all other HER family members, dimers without HER2 can be formed, and such dimers will not be inhibited by Herceptin™ due to its specificity towards HER2 (334). Another pathway that has been described to be upregulated in Herceptin™ resistance is the insulin growth factor 1 receptor (IGF1R) signaling pathway. Several groups have shown that overexpression of IGF1R in otherwise Herceptin™ sensitive cells results in resistance to the drug (355-357). This resistance can be overcome by the overexpression of IGFBP3, an inhibitor of IGF1 mediated activation of IGF1R (356). Increased IGF1 signaling leads to increased levels of the $p27^{Kip1}$ ubiquitin ligase SKP2 and thus to lower levels of $p27^{Kip1}$, an event that depends on PI3K/Akt signaling. Inhibition of IGF1R signaling restores Herceptin™ sensitivity.

Alterations in the interaction between ligand and receptor can also lead to resistance. In this respect, the membrane associated glycoprotein MUC4, a member of the mucin family, has been proposed to play a role in Herceptin™ resistance (333). MUC4 inhibits the immune recognition of cancer cells, suppresses apoptosis and promotes tumor progression and metastasis. It can also interact with and activate HER2 (358). One study using a Herceptin™ resistant cell line showed an inverse correlation between MUC4 levels and Herceptin™ binding to HER2. A knockdown of MUC4 in these cells restored Herceptin™ sensitivity (359). Another way of disrupting ligand-receptor interaction would be through mutations in the extra-cellular domain of HER2. Such mutations have however not been described to date for HER2 (334).

Altered expression of and signaling by components of the HER2 downstream signaling pathway have also been implicated in Herceptin™ resistance. Increased levels of active Akt were found in Herceptin™ resistant BT-474 cells, compared to sensitive parental cells (360). Decreased levels of the PTEN phosphatase decreased the sensitivity of breast cancer cell lines to Herceptin™ and loss of PTEN in HER2 positive tumors correlated with poor response to Herceptin ™-based treatments (338). Loss of $p27^{Kip1}$ or its nuclear localization have also been correlated with Herceptin™ resistance in breast cancer cell lines (361).

Finally, the full-length HER2 protein can be cleaved by matrix metalloproteases into an extracellular domain, which can be secreted in the culture medium or in the serum *in vivo*, and a truncated transmembrane domain with increased kinase activity (362-364). Increased levels of circulating ECD have been correlated with poor prognosis in patients with advanced stage breast cancer and might be implicated in Herceptin™ resistance through competition for antibody binding (365). On the other hand however, circulating levels of pre-treatment serum HER2 ECD seem to also be positively correlated with response (366) and Herceptin™ inhibits the cleavage of HER2 (344).

1.3.3 Expression of ERα and HER2 and the response to RA

The response of breast cancer cell lines to the antiproliferative actions of retinoic acid is variable and appears to depend highly on the expression of RARα (367-369). Schneider *et al.* have shown that maximum retinoid responses can be obtained in ER-negative and ER-positive cell lines by activating solely RARα by means of specific ligands (370).

1.3.3.1 ER-positivity and response to RA

Various reports in the literature show a positive correlation between the expression of ERα and the response to RA (367, 371-373). Both ER-positive cell lines and tumors have been shown to have higher levels of RARα RNA and protein levels (367, 374, 375). Estrogen has been shown to upregulate the levels of both RNA (376) and protein (377) of RARα in ERα-positive cells. Furthermore, it has been demonstrated that the re-introduction of either ERα or RARα in ER-negative cell lines sensitizes them to the growth inhibitory effects of RA (378, 379). Thus, the correlation between ERα expression

and RA sensitivity is the result of the regulation of RARα by estrogens. Nonetheless, various research groups have reported RARα expression and sensitivity to retinoids in ER-negative cell lines, such as SkBr-3 and MDA-MB-435 (380, 381). Fitzgerald *et al*. showed retinoid sensitivity of two ER-negative cell lines, SkBr-3 and Hs578T. Response to 9cRA and a panel of synthetic retinoids correlated with high to moderate expression of RARα, respectively. No correlation was observed with the expression of RARβ or RARγ (369).

Inversely, RAR-agonists have been shown to increase response to antiestrogens in ER-positive cell lines (382, 383). RA and tamoxifen were shown to inhibit the growth of berast cancer cells in a synergistic manner. Further studies in MCF-7 cells showed that this is due to a selctive synergistic effect on the activation of apoptosis through downregulateion of Bcl-2 mRNA and protein levels (382).

1.3.3.2 HER2 overexpression and response to RA

Contrary to ERα expression, the overexpression of HER2 is negatively correlated with the response of breast cancer cells to retinoid treatment (331). In an MMTV/neu mouse model (mammary specific overexpression of Her2), RA has even been shown to promote tumor growth and negatively impact survival (166). Tari and colleagues have shown that the overexpression of HER2 or Heregulin (HER2/3 activating ligand) in otherwise RA-sensitive MCF-7 cells significantly reduces the anti-proliferative response of these cells after treatment with RA. On the other hand, inhibition of HER2 signaling by pretreating with Herceptin™ induced RA sensitivity in HER2-overexpressing cell lines BT-474 and MDA-MB-453 (331). Authors show that this induction of RA resistance depends on the activation of Grb-2 and Akt. Later it has been shown that HER2 signaling reduces RAR binding on RAREs and that this inhibition of binding also passed primarily through Akt (384). Akt has been shown to be able to phosphorylate RARα in non-small cell long cancer (NSCLC). This phosphorylation on Ser96, a residue located in the DNA binding domain of the receptor, inhibited RAR transactivation and contributed to RA resistance of NSCLC cells (385). It has also been shown that HER dependent tyrosine kinase pathways regulate the expression of RARα at the levels of the mRNA (380) and protein (331).

1.3.4 Retinoic acid signaling and breast cancer

1.3.4.1 Alterations of RA metabolism and signaling in breast cancer

Several modifications of normal retinoid metabolism and signaling have been described in breast cancer cells. Such changes have the potential of conferring a growth advantage to tumor cells.

With respect to metabolism, it appears that various breast cancer cell lines have lost the capacity of RA synthesis from retinol, compared to normal human mammary epithelial cells (HMECs) or immortalized cell lines (34). For MCF-7 cells it has been shown that their incapacity to synthesize RA from retinol can be overcome by the expression of RALDH3, which is normally not expressed in this cell line, but is expressed in HMECs (35). On the other hand, the RA catabolizing enzyme CYP26A1 was found to be highly expressed in 42% of human cancers and its expression increases tumorigenicity of mouse mammary cells (386). Taken together, these observations suggest that tumor cells gain a growth advantage form creating an environment with low levels of RA.

Another way for tumors to escape the inhibitory effects of RA is by reducing the expression levels of the RARs. RARα2 was shown to be epigenetically silenced in MCF-7 cells and this event was suggested to be important for the loss of RA signaling in mammary tumorigenesis (387). This idea was supported by the observation that in breast cancer cell lines expressing RARα, full retinoid responses could be obtained by activating only this receptor (370). Expression of RARβ2 has also been shown to be frequently lost in mammary tumorigenesis (388, 389). This was found to be at least in part due to the hypermethylation of the promoter (390, 391). Treatment of breast cancer cell lines with the demethylation agent 5-aza-2deoxycytidine leads to re-expression of RARβ2 and induction of cell cycle and growth arrest (392). However, the hypermethylation could not fully explain loss of RARβ2 expression (393). Loss of heterozygosity of the RARβ locus on chromosome 3p24 has also been described, but did not correlate with loss of RARβ2 expression (394). Yet another study showed that the PI3K/Akt signaling pathway induces

transcriptional repression of the RARβ2 gene by increased phosphorylation and enhanced recruitment of SMRT to the RARβ2 promoter (395).

A more recent study suggested that the loss of response to the anti-proliferative effects of RA in advanced stages of mammary tumorigenesis is not correlated with lower expression of RARα, RARβ or RARγ, but was instead associated with the overexpression of HER2 (396).

1.3.4.2 The effects of retinoic acid on breast cancer cell lines

As already mentioned before, retinoic acid can provoke anti-proliferative effects in various breast cancer cell lines. Various responses and implicated signaling pathways have been described and will be summarized below.

Reintroduction of RARβ in ER-positive MCF-7 cells resulted in the induction of growth inhibition followed by apoptosis after 4-6 days of RA treatment, whereas in ER-negative MDA-MB-231 cell growth inhibition without apoptosis was observed. In MDA-MB-231 cells, but not in MCF-7 cells, the growth inhibition was associated with downregulation of c-myc mRNA (397). In HMECs however, the same group showed that RA only induces a G1 arrest, which is associated with reduced levels of hyperphosphorylated retinoblastoma (Rb) protein (398). Another group recently suggested that the induction of apoptosis in MCF-7 cells by RA is mediated by the regulation of antioxidant enzymes (399). RA was also shown in MCF-7 cells to specifically decrease protein levels and IGF-I induced tyrosine phosphorylation of the IGF-IR downstream target IRS-1, impairing Akt but not ERK1/2 activity and resulting in growth inhibition (400). This downregulation was later also shown in the ER-positive cell lines T47D and Zr75.1, but could not be observed in the ER-negative cell lines MDA-MB-231 and MDA-MB-453. The downregulation of IRS-1 was shown to be a result of increased degradation due to PKC-dependent activation of the ubiquitin-proteasome pathway (401).

Nakagawa *et al*. showed that the RA induced growth arrest in ER-negative SkBr-3 cells corresponds to a G1 arrest and decreased levels of ERK phosphorylation. These effects could be overcome by overexpression of PKCα (158). Also using SkBr-3 cells, it

was shown that prolonged exposure (3-9 days) to a high dose of RA (2 μM) reduced acetylation at the hTERT promoter and decreased telomerase activity (402). The authors also observed a significant decrease in colony formation in soft agar when cells were treated with RA, as well as a strong induction of apoptosis in the first 6 days of treatment (402). RA and 9cRA, as well as the RARα specific ligand TTAB, also induce epithelial differentiation and cell-cell adhesion in SkBr-3 cells (403). This appears to be mediated through stabilization and translocation to the cell membrane of β-catenin protein.

9cRA and some receptor-selective retinoids were shown to induce a G1 cell cycle block in several normal and malignant mammary cell lines through modulation of Cyclin D1 and D3 (404). All tested retinoids in this study inhibited the phosphorylation of Rb. In normal 184 cells and T47D tumor cells 9cRA induced low levels of apoptosis, as measured by caspase 3 and Annexin V assays (404).

1.3.4.3 Direct RAR targets in breast cancer

A number of direct RAR target genes has been identified that have been suggested to play a role in the antiproliferative effects of RA in breast cancer cells.

HOXA5 was shown to be a direct target of RA and an RARE located in the 3' end of the gene was found to be bound by RARβ (405). The expression HOXA5 was found to be lost through promoter methylation in 16 out of 20 p53-negative tumor samples (406). The loss of HOXA5 in a $p53^{+/-}$ background was suggested to trigger mammary tumor development (407). It has been shown that this gene induces apoptosis in breast cancer cell lines, through p53 and caspase 2/8 dependent pathways (406, 408). Also, the knockdown of HOXA5 reduced RA-induced apoptosis and promoted cell survival after RA treatment (405).

SOX9 expression was shown to be rapidly induced by RAR pan-agonists and the RARα selective agonist Am580 in the ER-positive cell lines MCF-7 and T47-D (409). Upregulation of this member of the high mobility group of transcription factors in mouse and human melanomas inhibited their growth and restored their sensitivity to RA (410). Overexpression of SOX9 mimicked RA treatment and using a dominant negative form of

SOX9 it was shown that the induction of this gene is essential for RA-induced cell cycle arrest in T47-D cells (409). Furthermore, it was shown that SOX9 regulates the expression of the HES-1 gene (411), which was shown to be important for the response of MCF-7 cells to RA (412).

BTG2 is a p53 induced gene (413) and has been shown to inhibit G1/S transition of the cell cycle in an pRb dependent manner through the inhibition of cyclin D1 transcription (414). It is also a direct RA target gene in MCF-7 cells and its induction leads to a decrease in Cyclin D1 expression (132). The authors therefore conclude that BTG2 mediates at least part of the antiproliferative actions of RA in MCF-7 cells.

The PDCD4 gene was shown to be regulated by RAR agonists, antiestrogens and HER2 antagonists in breast cancer cells. Induction of this gene by such a broad range of antiproliferative compounds suggests a prominent role in breast cancer cell growth inhibition. When overexpressed in T47-D or MDA-MB-231 cells PDCD4 induced apoptosis (415).

1.3.4.4 Use of retinoids in the treatment of breast cancer

It may be clear from the effects observed in mammary tumor cell lines that retinoids are interesting candidates for cancer treatment (416-418). Several papers describe the use of natural and synthetic ligands in mouse models as well as clinical trials. Already some 30 years ago it was shown that the synthetic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR or fenretinide) is capable of reducing the incidence and growth of chemically induced mammary lesions in mice (419, 420). The RARα selective retinoid AM580 inhibits the formation of mammary tumors in the MMTV-neu and MMTV-Wnt1 models (421). The rexinoid (RAR and RXR ligand) bexarotene can prevent the occurrence of premalignant lesions in an MMTV-ErbB2 mouse model (422).

Various clinical trials show that the effects of retinoids on mammary tumors are generally beneficial, although there is a lack of strong objective response and severe side effects have been described (383, 423-425). The retinoid fenretinide appears to be of particular interest in the prevention of cancer (426). Because of its selective accumulation in breast tissue and favorable profile of secondary effects, it is the most studied retinoid in

trials of breast cancer chemoprevention (427). It has been shown to strongly reduce recurrence of breast cancer in premenopausal women after a median follow-up of 97 months (428). 15 year follow-up of the same study showed marginally significant reduction of occurrence of second breast cancer incidence overall (17%) and a strong reduction of 38% in premenopausal women (429). Bexarotene is also being tested in the clinic and appears to be beneficial in about 20% of patients with metastatic breast cancer (430).

1.4 Objectives

Although the potential benefits of administering retinoids to (breast) cancer patients has been recognized for several years now, in practice the benefits of retinoid treatments have been limited in breast cancer. In order to better employ the benefits of retinoid acid to the advantage of cancer treatment, it is of capital importance to better understand the signaling pathways leading to antiproliferative responses. Other problems lay in the difficulty of identifying classes of tumors that would benefit from such a treatment. Therefore, it is also important to find better identifiers for the possibly relatively small subclass of tumors that can really benefit from retinoid treatment.

Several groups have shown the importance of functional signaling by RARs in breast tumor cells is essential for antiproliferative responses. This points toward an important role for direct RA target genes in the regulation of these responses. A number of direct RA targets have been shown or suggested to mediate at least part of the RA response in some cell lines. We hypothesize that by performing large-scale gene regulation analyses we could identify more genes important for the cellular responses to RA in breast tumor cell lines. Particularly, we are interested in comparing the signaling in ER-positive and ER-negative RA-sensitive cells in order to identify common and specific mediators of response. Using such a study will allow us to identify specific gene regulatory programs that determine the anti-proliferative and pro-apoptotic responses to retinoic acid treatment.

Although most RA-sensitive cell lines are ER-positive and an importance for ER signaling in RA mediated growth responses has been suggested, some ER negative cells have also been shown to be growth inhibited by RA. The best know example is the cell line SK-BR-3, which in spite of being HER2 amplified and ER-negative, is particularly sensitive to RA. Since it has been suggested that the RARA gene is included in the HER2 amplicon in this cell line, it could represent a subclass of breast tumors that may benefit from treatment with retinoids. We propose that in such RA/HER2 amplified cells there could be an additional benefit in combining treatments directed against HER2 and RAR. This appears particularly interesting because of known interactions between the two

signaling pathways. We therefore investigated the possibility of a synergy between RA and Herceptin treatments in this type of tumor using SK-BR-3 cells as a model.

Second part:

Chapter 2: Molecular Basis of the Antiproliferative Actions of Retinoic Acid in Breast Cancer

Chapter 3: Synergistic Growth Inhibition by Retinoic Acid and Herceptin in HER2/RARA-Amplified Breast Cancer Cells

Chapter 2: Molecular Basis of the Antiproliferative Actions of Retinoic Acid in Breast Cancer

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Manuscript in preparation for submission to Molecular and Cellular Biology

As first author, I performed all cell proliferation essays, western blots and siRNA/FACS experiments, prepared cells for microarray analysis, performed RNA extractions and several Q-PCR studies. I realized all electroporation/FACS experiments in close collaboration with Martine Bail. Finally, I made all the figures and helped Dr. Mader writing the manuscript.

Molecular basis of the antiproliferative activity of RA in sensitive breast cancer cells

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List of non-standard abbreviations: ERα, estrogen receptor alpha; HER2, human epidermal growth factor receptor 2; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR retinoid X receptor.

SUMMARY

Retinoids are used clinically for the treatment of specific malignancies and precancerous conditions, but their benefit is unclear in breast cancer, possibly due in part to the variable sensitivity of breast tumor types to the antiproliferative effects of retinoic acid. In breast tumor cell lines, sensitivity generally correlates with expression of estrogen receptor alpha (ERα), which regulates expression of the RARA gene. However, the ER-negative SK-BR-3 cell line is also sensitive to the antiproliferative effects of RA and of RARα-selective ligands. We report here that patterns of gene regulation in two RA sensitive cell lines, ER-positive MCF-7 and ER-negative SK-BR-3 cells, are largely overlapping, indicating that part of the antiproliferative effects of RA is independent of estrogen signaling. Compatible with direct response element recognition by RARs, we found a strong enrichment of RA response elements (RAREs) in primary up-regulated RA target genes in both cell lines. Several other transcription factor binding sites were also over-represented, suggesting the involvement of tethering or non-genomic mechanisms in the regulation of primary RA targets. While a subset of common up-regulated RA target genes were regulated in an opposite manner by RA and estrogens in MCF-7 cells, most were sensitive to cycloheximide for down-regulation by estrogens, indicating that protein synthesis is required for this antagonistic effect. Several primary RA target genes, including the transcription factors SOX9 and FOXA1, were found to inhibit cell cycle progression when overexpressed in ER-negative SK-BR-3 cells. Overexpression of these two genes also lead to the transcriptional regulation of secondary RA target genes with antiproliferative activity. Finally, consistent with our observation that RA directly up-regulates expression

of proteins involved in the control of ER expression and signaling, such as FOXA1 and GATA3, RA primary target genes identified in ER-negative SK-BR-3 cells were found to discriminate between ER+ and ER- tumors, suggesting that RA signaling contributes to luminal breast cancer cell differentiation.

Keywords: retinoic acid, breast cancer, estrogen receptor, luminal differentiation

INTRODUCTION

Retinoids include natural forms of vitamin A such as retinol and its derivatives, retinal and RA. RA acts by binding retinoic acid receptors (RARs), which belong to the nuclear receptor superfamily (1-3). RA target genes are regulated by heterodimers between RARs and members of a second family of nuclear receptors, the RXRs (4, 5). Both RAR and RXR families are composed of three members: α, β and γ, each of which is expressed as several N-terminal variant isoforms (4). Two main isomers of RA, all-*trans*- and 9-*cis*-RA bind these receptors. All-*trans* RA (ATRA) is the natural ligand for RARs, whereas 9-*cis* RA binds both RARs and RXRs. RAR-RXR heterodimers bind response elements in the form of direct repeats of PuG(G/T)TCA motifs separated by 1, 2 or 5 base pairs (DR1, DR2 or DR5 elements) (6-9). RA binding induces recruitment of coactivator complexes required for transcriptional activation, including p160 coactivators (NCOA1/2/3), the CREB binding protein (CBP) and its homologue p300, which possess histone acetyltransferase (HAT) activity, and large complexes of proteins required for recruitment of the transcriptional machinery such as the DRIP/TRAP complex (10, 11).

The roles of the RARs and RXRs during mammalian development have been demonstrated by gene ablation studies (see (2) for a review). Vitamin A deficiency in adult murine models results in squamous metaplasia of numerous epithelia. Animal models have also demonstrated potent chemopreventive effects of retinoids on epithelial tissues exposed to carcinogens (12). The inverse relationship between incidence of various cancer types and serum vitamin A or β-carotene levels prompted investigation of the use of retinoids for

cancer treatment or prevention, in particular for acute promyelocytic leukemia and head and neck cancer (13-15). In breast cancer, in spite of promising results on reduction of second malignancies by RA (14, 16) and on a decrease in rates of contralateral tumors in premenopausal women with stage I breast cancer by the synthetic retinoid fenretinid (17, 18), retinoid-based therapies have failed to achieve a significant break-through. This may be due to a failure to predict accurately which tumors will exhibit sensitivity to retinoids.

In cultured breast cancer cell models, sensitivity to RA is cell line-specific. ER-positive cells are sensitive to the antiproliferative effects of ATRA, while the majority of ER-negative cells are not (19-21). This may be due to the induction of RARα expression by estrogens (20, 22, 23). In addition, recent large-scale chromatin immunoprecipitation experiments have identified a large overlap in the chromatin regions bound by ER and RARα, suggesting that an interplay between retinoic and estrogen signaling (24, 25). Surprisingly however, ERα-negative, ERBB2-positive SKBR-3 cells are very sensitive to the anti-proliferative effects of ATRA. This may be explained by the high expression levels of RARα in this cell line (21), and suggests that sensitivity to the anti-proliferative effects of RA is not dependent on ER expression.

In RA-sensitive cell lines, retinoids have antiproliferative and/or proapoptotic activities through mechanisms that remain incompletely understood (26-30). The antiproliferative effects of RA in MCF-7 breast cancer cells have been linked to decreased expression and phosphorylation of Rb, as well as decreased expression of cyclin D3 and CDK4 (31) and/or cyclin D1 (32). In SK-BR-3 cells, RA treatment was shown to also decrease Rb phosphorylation and expression of cyclins A and E (33). Induction of several pro-apoptotic

genes has been observed in MCF-7 cells, including CASP7 and 9 (32). Several RA target genes have been proposed to act as mediators of these effects. BTG2 is an RA- and p53-target gene that regulates phosphorylation of Rb and modulates expression of cyclin D1 and cyclin E in mouse fibroblasts (34, 35). SOX9 also plays roles in the control of the cell cycle in breast cancer cell lines sensitive to RA, increasing the percentage of cells in G0/G1 (36). In addition, induction of HOXA5 was shown to contribute to the pro-apoptotic effects of RA in MCF-7 cells (37). Nevertheless, the overall mechanisms of gene regulation and of the antiproliferative effect of RA in sensitive breast cancer cell lines remain incompletely understood.

In this article, we characterized the effects of RA on primary and secondary target gene expression in both ER-positive MCF-7 and ER-negative, HER2-positive SK-BR-3 cells. Our results indicate a large overlap in regulation of both primary and secondary target genes, suggesting ER-independent mechanisms of regulation in both cell lines. RARE motifs were found enriched specifically in primary up-regulated target genes in both cell lines. In addition, other types of transcription factor binding sites were over-represented in primary responsive genes, suggesting a contribution of tethering or non-genomic mechanisms to primary target gene regulation. We identify transcription factors FOXA1 and SOX9 as RA targets in both cell lines and demonstrate that their overexpression results in antiproliferative effects and modulation of expression of several secondary RA target genes in SK-BR-3 cells. Finally, RA regulation of luminal differentiation genes FOXA1 and GATA3 in SK-BR-3 cells suggests a role of RA in the initiation and/or maintenance of

luminal differentiation. Accordingly, several primary up-regulated RA target genes are strongly associated with the luminal phenotype and ER-positive status in breast tumors.

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MATERIALS AND METHODS

Cell culture and treatments

MCF-7 and SK-BR-3 cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada). MDA-MB-231 cells were maintained in DMEM supplemented with 5% FBS. Three days before experiments, cells were plated in DMEM supplemented with 10% or 5% charcoal-stripped FBS, respectively. For treatments, cells were seeded at a density of 1-1.5 million cells per 10 cm plate in medium containing charcoal-stripped FBS and the next day cells were treated with *all trans*-retinoid acid (RA, 100 nM, Sigma) or vehicle (0.1% DMSO) for indicated periods of time. Where mentioned cells were pretreated for one hour before RA treatment with cycloheximide (CHX, 10μg/ml, Sigma).

Growth assays

Cells were seeded in 6-well plates at a density of 30,000 cells per well and treated every 2-3 days with vehicle (0, 0.1% DMSO), retinoic acid (RA) or AM580 (J. Gleason, McGill University, Montreal, QC) at indicated concentrations in medium containing 5% charcoal-stripped FBS. After 9 days, protein concentrations were measured as described previously (38).

RNA purification and DNA microarrays

Three days prior to the experiments, cells were switched to medium containing charcoal stripped medium to eliminate estrogenic activity. One day before RA treatment, cells were plated at a density of 1-1.5 million cells per 10 cm plate in medium containing charcoal-stripped FBS. 8 hours after treatment with vehicle or RA (100 nM) cells were collected in 1 ml of TRIZOL (Invitrogen). Total RNA was extracted as recommended by the manufacturer and further purified with RNeasy MinElute Cleanup Kit (QIAgen, Mississauga, ON, Canada). cRNA synthesis from total RNA, labeling and hybridization to Affymetrix HG-U133 2.0 Plus gene chips (>54,000 probe sets to cover >47,000 transcripts and variants) were performed at the Genome Quebec and McGill University Innovation Center using standard protocols (http://www.genomequebec.mcgill.ca/). Experiment #1: SK-BR-3, MCF-7 and MDA-MB-231 were RA treated in the absence of cycloheximide (CHX) and in addition MCF-7 cells were analyzed in the presence of 10 μg/ml of CHX added one hour before RA treatment. Three biological replicates for each condition were used for microarray analysis. Experiment #2: SK-BR-3 cells were treated for 8 hours with 100 nM RA in the presence or absence of 10 μg/ml CHX added one hour before RA treatment. Four biological replicates were used. The 8 hour time point was chosen to allow for the identification in the same samples of both primary (early induced) and secondary targets (induced at later times).

Affymetrix microarrays were normalized with the affy bioconductor package using the Robust Multi-array Average (RMA) normalization method (39). Illumina microarrays were normalized with the lumi biobonductor package using the quantile method and the variance

stabilizing transform (40). Moderated *t*-statistics (41) from the limma bioconductor package were used to identify statistically significant change of gene expression between vehicle and RA treatments. Genes deemed significantly regulated were those with 1.4-fold change, average log2-expression levels greater than 5 across all samples (A-value) and a P-value smaller than 0.01. P-values were adjusted to control the false discovery rate with the Benjamini and Hochberg method (42).

For overexpression studies, SK-BR-3 cells were electroporated with plasmids of interest and RNA was extracted 48 hours after transfection as mentioned above. 24h RA treated samples were inculded as controls. Triplicate samples were analyzed for each condition. cRNA synthesis from total RNA, labeling and hybridization to Illumina WG-6 v3.0 BeadChips were performed at the Genome Quebec and McGill University Innovation Center.

Screening for transcription factor binding sites

Human Genomic sequences +/- 10 Kbp around the transcription start sites (TSS) were extracted for all annotated gene in the RefSeq track (43) from the UCSC Genome Browser Database (hg17, May 2004) (44). Custom RAR matrices and matrices from TRANSFAC 2010.2 (45) were used to screen these sequences for transcription factor binding sites using a base score cutoff of 65% and 5% increments as described previously (46). For each transcription factor, four cutoffs were chosen with frequencies in all gene promoters closest to those of RAREs DR5 (70%: 1.14 DR5/gene, 75%: 0.65 DR5/gene, 80%: 0.21 DR5/gene, 85%: 0.05 DR5/gene). *Z*-scores and *P*-values from a Fisher exact test were used to evaluate

the significance of the observed enrichment in promoters of different sets of regulated genes versus those of all annotated genes. The Z-scores and P-values were calculated with programs adapted from oPOSSUM perl application programming interface (API) using the cutoff (*Z*-scores >10, *P*-value <0.01) recommended by the authors (47).

Gene expression quantification

Cells were treated with RA (100 nM) for the indicated time periods and total RNA was extracted as described earlier. Aliquots of 2 μg were reverse transcribed using the RevertAid H first minus strand cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada) as recommended by the manufacturer. Reverse transcription products were diluted 10 times in pure water prior to real-time quantitative PCR. Gene expression levels were determined using primer and probe sets from the Universal Probe Library (https://www.roche-applied-science.com) as previously described (46).

Cell cycle analysis

For analysis of the effect of RA and Am580 on cell cycle distribution, cells were seeded at a density of 1-1.5 million cells per 10 cm dish and treated for 48 hours at indicated concentrations. Cells were then trypsinized and fixed and permeabilized for at least 16 hours in 70% EtOH. Cells were then stained for 20' at room temperature with a solution containing 50 mg/ml propidium iodine (Sigma-Aldrich) and analyzed on a Canto flow cytometer with FACS Diva software (BD Biosciences). At least 15,000 single cell events

were used for cell cycle analysis using ModFit LT 3.2 software (Verity Software House, Topsham ME).

For analysis of the effect of RA target gene overexpression, SK-BR-3 cells were electroporated (5 million cells, 240 V, 950 μF) with 6 μg of either the parental pCMV_XL5 vector or vectors expressing selected RA target genes (Open Biosystems and Origene), together with a ten-fold lower amount of an expression vector for membrane-targeted EGFP (pEGFP-spectrin, (48)). Cells were seeded in 10 cm plates in DMEM (Wisent) supplemented with 10% FBS (Sigma-Aldrich) and 1% Penicillin/Streptomycin (Sigma-Aldrich). After 72 h cells were trypsinized and fixed in 70% EtOH. Cells were stained with propidium iodine (Sigma-Aldrich) and analyzed on a Canto flow cytometer with FACS Diva software (BD Biosciences). At least 15,000 GFP-positive single cell events were used for each condition for cell cycle analysis with ModFit LT 3.2 software (Verity Software House, Topsham ME).

Western blotting

For detection of RAR and ERa proteins by western blot, cells were lysed for 30 minutes on ice in E1A lysis buffer (ELB; 150 mM NaCl, 50 mM Hepes pH 7.5, 5 mM EDTA, 0.1% Nonidet P-40, supplemented with protease inhibitors (Sigma-Aldrich)). Bradford method was used to quantify protein samples. 35 μg of protein were used for analysis on 8% SDS-PAGE gels and subsequent blotting onto PVDF membranes (Millipore). Antibodies for RARα (C-20), RARβ (C-19) and RARγ (C-19) rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology and used at a dilution of 1/2500. Anti-ERa rabbit

monoclonal antibody (clone 60C; Millipore, Billerica, MA, USA) was diluted 1/1000. Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA) and used at a dilution of 1/10,000. The blots were developed using enhanced chemiluminescence using home-made solutions.

RESULTS

Sensitivity to the antiproliferative effects of RA correlates with differential expression of RARα

The anti-proliferative effects of RA have been associated with ER-positive (luminal) breast cancer cells (19-21). However, the SK-BR-3 cell line is known to be sensitive to RA treatment in spite of lack of ER protein expression and overexpression of ERBB2 (21, 33). Direct comparison of the effects of RA on ER-positive MCF-7 cells and ER-negative SK-BR-3 cells indicates that RA was more potent in SK-BR-3 cells compared to MCF-7 cells, while ER-negative MDA-MB-231 cells were, as previously reported (49, 50), insensitive to the effects of RA (Figure 1A). The antiproliferative activity of RA in SK-BR-3 cells corresponded to an increase at 48h in the percentage of cells in G0/G1 (Figure 1B). RARα has been proposed to be the key regulator of RA-induced antiproliferative and apoptotic events in breast cancer cells (21). Accordingly, RARA mRNA and protein levels were found to be at least 2-fold higher in SK-BR-3 cells compared to ER-positive MCF-7 cells, while RARβ protein was expressed to similar levels and RARγ was expressed to lower levels. The decrease in RARα expression levels in the presence of RA likely results from the reported induction of proteasomal degradation of this receptor (51). Very low levels of RARβ and no detectable expression of RARα and RARγ proteins were observed in MDA-MB-231 cells (Figure 1C-D). Consistent with previous reports of antiproliferative activity of RARα-selective ligands (21, 52), the RARα-selective AM580 retinoid (53) inhibited proliferation of both SK-BR-3 cells and MCF-7 cells, but with greater potency in SK-BR-3

cells (Figure 1A). Finally, the antiproliferative effects of RA were reduced in SK-BR-3 cells and nearly abolished in MCF-7 cells by sh-RNA mediated partial down-regulation of RARα (Suppl. Figure 1). Together, these results suggest that high levels of RARα in SK-BR-3 cells contribute to their sensitivity to RA in spite of lack of ERα expression at the protein level (Figure 1D).

Sensitivity to the antiproliferative effects of RA correlates with transcriptional regulation

To investigate the mechanisms of transcriptional regulation by RA in sensitive breast cancer cells, we screened Affymetrix HG-U133 Plus 2.0 chips in triplicates for SK-BR-3, MCF-7 and MDA-MB-231 cells treated or not with RA for 8 h within the same experiment. Regulated genes were identified by assessing the ratio between the average of the replicates treated with RA versus the vehicle control. A cutoff of 1.4-fold differential expression was applied and adjusted p-values smaller than 0.01 were considered (please refer to Materials and Method section for details), identifying regulated genes in SK-BR-3 (196 regulated genes) and MCF-7 (341 regulated genes) cells (Table I; top ranked regulated genes in SK-BR-3 and MCF-7 cells can be found in Suppl. Table I and Suppl. Table II, respectively). To assess reproducibility of results, the experiment in SK-BR-3 was repeated using the same experimental conditions, except that quadruplicates were used instead of triplicates. While the number of genes found to be regulated in a statistically significant manner was much larger (878 instead of 196), 90% of the target genes identified in the first experiment were also observed in the second experiment (Suppl. Figure 2). Variability between the two

experiments affected mostly down-regulated genes, with 22.7% of the genes in the smaller dataset not reproduced in the larger one compared to 1.7% in the up-regulated genes. Substantial overlap in regulated genes was observed between MCF-7 and SK-BR-3 cells using either SK-BR-3 datasets, especially in up-regulated target genes. Overlap between the MCF-7 and the first SK-BR-3 dataset represented 16.5% of the MCF-7 dataset and 35.5% of the first SK-BR-3 dataset for up-regulated target genes (43 genes), but 7.4% of MCF-7 and 8% of the first SK-BR-3 dataset for down-regulated target genes (6 genes). Similarly, overlap between the MCF-7 and the second SK-BR-3 dataset represented 37.3% of the up-regulated genes in MCF-7 cells and 20.4% of the ones in the second SK-BR-3 dataset (97 up-regulated genes), compared to 24.7% of the down-regulated genes in MCF-7 and 5% of the ones in the second SK-BR-3 dataset (20 down-regulated genes) (Figure 2).

In contrast to what was observed in luminal cells, only 2 genes (NRIP1, DHRS3) were found regulated using adjusted p-values in RA-insensitive MDA-MB-231 cells (Table I). Both genes were also identified as up-regulated RA target genes in SK-BR-3 and MCF-7 cells. Additional genes (70 total) were regulated in MDA-MB-231 cells when using non-adjusted p-values, but differed largely from those identified in MCF-7 and SK-BR-3 cells (14 identical target genes over 70, 20%, Suppl. Figure 3).

Together, these results indicate that RA sensitivity in growth assays correlates with transcriptional response, and that RA signaling is largely conserved between ER-positive MCF-7 and ER-negative SK-BR-3 cells.

Overlap in primary target genes regulated by RA in MCF-7 and SK-BR-3 cells.

To investigate whether common gene expression in MCF-7 and SK-BR-3 cells results from a common set of primary target genes in both cell types, we compared results from microarrays performed in MCF-7 and SK-BR-3 cells with those obtained from the same cell lines after pre-treatment with the protein synthesis inhibitor cycloheximide (CHX) before treatment with RA (8h) (experiment 1 for MCF-7 and experiment 2 for SK-BR-3 cells). Proportions of primary target genes, defined as genes regulated in the presence of CHX, were highly similar in SK-BR-3 and MCF-7 cells (28.1% for SK-BR-3 vs. 27.5% for MCF-7 cells (Figure 3A-B). 41 genes were identified as direct targets in both cell lines (39 up-regulated, 2 down-regulated), and this number increased to 78 (69 up, 9 down) when genes that were primary in one cell line and secondary in the other were also taken into account (Figure 3C, Table II).

Genes common to MCF-7 and SK-BR-3 cells include genes participating in RA transport and metabolism and in RAR activation, such as the receptor for retinol/retinol binding protein STRA6, the metabolic enzyme retinal short chain dehydrogenase DHRS3/RetSDR1, involved in accumulation of retinyl esters and often deleted in human neuroblastoma cell lines (54), and CYP26A1 and CYP26B1, involved in the inactivation process of RA (55). RAR coactivator NCOA3 and corepressors NCOR2 and NRIP1 (56-58) were also common targets.

In addition, a common set of genes with proposed or known antiproliferative/apoptotic/tumor suppressor functions was identified in both cell lines, including the transcription factors BTG2 and SOX9 (see Figure 4 for induction kinetics in

MCF-7 and SK-BR-3 cells), which have demonstrated antiproliferative activity in ER-positive MCF-7 or T47-D breast cancer cells (34-36), the proposed tumor suppressor and ER corepressor AHRR (59, 60) and the proposed tumor suppressor TFPI2 (61). In addition, RA up-regulated dual specificity phosphatase 4 (DUSP4), a likely cause of the reported RA-induced decrease in ERK MAP kinase phosphorylation (33).

RA also induced expression of primary target genes with roles in apoptosis, including the tumor protein p53 inducible nuclear protein 1 (TP53INP1), both a target and cofactor of p53 that triggers G1 arrest as well as increasing p53-mediated apoptosis (62, 63) (see Figure 4 for induction kinetics of TP53INP1 in MCF-7 and SK-BR-3 cells). Ingenuity pathway analysis indicated that when considering all target genes (primary and secondary), apoptotic signaling was more represented in SK-BR-3 than in MCF-7 cells, with induction of APAF1, BID, CAPN5, CASP3, 8, 9 (64), MAP3K5, MAPK8, and TNFSF15 (65). RA regulated several genes in the TGFβ pathway, inducing SMAD3, a known mediator of the apoptotic effects of TGFβ in breast cancer cells (66-68). TIAF1 is a 12 kDA TGF-β1-induced gene whose overexpression induces growth inhibition and apoptosis of several cell types including MCF-7 cells, while its inhibition by antisense RNA enhances epithelial cell proliferation; TIAF1 upregulates p53 and p21 expression but represses ERK phosphorylation (69), thus acting as another potential mediator of the reported effects of RA on suppression of MAPK activation (33). RA exerted multiple effects on the TGFβ signaling pathway, inducing the TGFβ-regulated protein DEC1/STRA13, associated with the hypoxic response and with high tumor grades in breast cancer (70, 71), but repressing

TGFβ-family member BMP7, an inducer of apoptosis through telomere shortening in breast cancer cells (72).

Differential regulation in MCF-7 and SK-BR-3 cells results in part from differences in basal expression levels of target genes.

Several primary RA target genes were differentially regulated in SK-BR-3 and MCF-7 cells. To investigate whether this may be due to different kinetics of regulation, we monitored expression of differentially regulated target genes by RT-qPCR and confirmed MCF-7-specific regulation of HOXA5, CBFA2T3, and PRKCD, and SK-BR-3-specific regulation of GATA-3 and UBD (Figure 4). FOXO3A was regulated more strongly in SK-BR-3 cells than in MCF-7 cells, where a 2-fold regulation was detected with similar kinetics as in MCF-7 cells. Conversely, FOXA1 was up-regulated at early time points in MCF-7 cells, but at later time points in SkBR3 cells (Figure 4).

Lack of regulation in one of the two cell lines could be attributed in some cases to different levels of basal gene expression. For instance, basal levels of GATA3 expression are much higher in MCF-7 cells (26 fold for the average of the three probes in the Affymetrix arrays), where its expression is not affected by RA, than in SK-BR-3, where it is up-regulated up to 4-fold (Figure 4). Conversely, PRKCD is much more expressed in SK-BR-3 cells (28-fold) and is regulated only in MCF-7 cells. These results suggest that up-regulation by other transcription factors can saturate or mask regulation by RA in a cell-specific manner.

DR5 elements are enriched in up-regulated, but not down-regulated primary RA target genes in SK-BR-3 and MCF-7 cells.

RARs regulate gene expression via binding as heterodimers to response elements composed of Pu$G^{G}/_{T}$TCA motifs arranged as direct repeats with 1, 2, or 5 bp spacing. We examined whether DR5, DR2 or DR1 elements are found enriched in the flanking sequences of RA primary up- or down-regulated primary and secondary target genes compared to average representation in similar windows (2.5, 5 or 10 kb around the transcriptional start site; over-representation by more than 1.5 fold was considered if associated p-values from a Fisher's exact tests were lower than 0.01 and Z scores higher than 10). DR5 response elements were enriched in up-regulated primary target genes in both SK-BR-3 and MCF-7 cells. This enrichment was detected at up to 10 kb from the transcriptional start site (Figure 5B-C). Enrichment in DR2 elements was also detected in up-, but not down-regulated primary genes. DR1 were not detectably enriched in either up- or down-regulated target genes, but DR0, DR4 and DR6 elements were significantly over-represented in primary up-regulated targets in SK-BR-3 cells (Suppl. Table III). We also detected enrichment in EREs (IR3) as well as in IR2/4/5/6 elements. Everted repeats with variable spacings (ER0/2/3/4/5) were also enriched more than 1.5 fold. Down-regulated primary target genes were not found enriched in canonical RAREs, although half sites were over-represented with respect to overall genomic distribution.

Sites for other transcription factors were found enriched in the flanking regions of either up- or down-regulated primary target genes. Comparison with sites enriched in published large-scale ChIP experiments (24, 25) suggests that some of these factors may be involved

in recruitment of RAR via tethering. For instance, sites for TFs HNF4, LRH1, ARNT, MYC/MAX, MYOD, SREBP, TEF1, NFIA/C, AML (RUNX1/2), NERF (ELF2), ETS1/2, HFH3, FOXO1, CTCF, Sp1, CACC binding factor, ZNF263, AP1/MAF (NF2L2), and EBF were found enriched in primary up-regulated RA target genes in SK-BR-3 cells and/or MCF-7 cells (Suppl. Table III), as well as in ChIP-chip and/or ChIP-seq experiments in MCF-7 cells (data not shown). In primary down-regulated RA target genes in either cell line, binding sites for HNF3/FOX, Sp1, GATA, AP1, AP2, CEBP, Pax, Oct, AML/PEBP (RUNX1/2), HNF1(TCF1) were found over-represented as well as in ChIP experiments (Suppl. Table III and data not shown).

In addition, some TF binding sites enriched in primary RA target genes were not found enriched in RAR-binding chromatin regions. This suggests that the action of those transcription factors is mediated through non-genomic regulation by RA, not necessitating RAR binding to the DNA. These TF binding sites include sites for CP2, EGR/KROX, SMAD, ZFX, SOX9, NFKB, STAT1/4, E2F1, TAL1, POU3F2, STAT, GATA, SMAD, and MYB (Suppl. Table III).

Identification of primary target genes with opposite regulation by RA in sensitive MCF-7 and SK-BR-3 cells and by estradiol in MCF-7 cells

Several of the RA target genes that are primary target in SK-BR-3 and/or MCF-7 cells and commonly regulated in the absence of CHX were regulated in opposite directions by estrogens in MCF-7 cells, consistent with potential roles in cell proliferation (Table III). All genes for which opposite regulation was observed were induced by RA and repressed by

estradiol, mostly as secondary targets (23 genes with repression by E2 below the threshold of 1.4 fold in the presence of CHX). However, TP53INP1, SCNN1A, CYP26A1, GPR160, FGD3, KLHDC2, IDH1 and GABBR2 are exception, being primary targets of E2 in MCF-7 and RA in MCF-7 and/or SK-BR-3 cells (genes were considered primary targets of E2 when significantly regulated in the presence of CHX, even if regulation did not reach significance in its absence. SOX9 was excluded from this list because of opposite regulation by E2 in the presence and absence of CHX).

An example of a gene differentially regulated by RA and E2 is BTG2, a primary RA target and secondary E2 target in MCF-7 cells. BTG2 is a p53 target gene and is also up-regulated by activated PKCδ. Of note, PKCδ is also induced by RA in MCF-7 cells (PRKCD, Figure 4 and Suppl. Table II). BTG2 induces cell cycle arrest and/or apoptosis by p53- and Rb- dependent and independent mechanisms, including relocalization of Pin1 to the cytoplasm, reduction of CCND1 expression and inhibition of CDC2 kinase activity (73). BTG2 expression has also been shown to be inhibited by estrogen both in breast cancer cell lines *in vitro* and in the rat mammary gland during pregnancy and lactation, while it is induced during involution (74). In addition, BTG2 was shown to interact with estrogen receptor via LXXLL motifs (75) and may therefore also act as an RAR cofactor.

In comparison with RA target genes with opposite regulation in MCF-7 cells, a smaller fraction of RA primary target genes (11 genes) were regulated in the same direction by RA and E2, with most target being regulated by E2 in the presence of CHX. These included the nuclear receptor co-repressor NCOR2 and RA inactivating enzyme CYP26B1, suggesting cross-talk between estrogen and RA signaling through target gene regulation.

These results indicate that the interplay between RA and E2 signaling is complex, including effects in the same or opposite direction. However, the observation that regulation by RA is observed in SK-BR-3 cells as well as in MCF-7 cells strongly suggests that RA is not acting mainly as a co-factor of ER in regulation of these target genes, but as a primary regulator.

Several common direct RA target genes inhibit proliferation in ER-negative SK-BR-3 cells

Because the anti-proliferative effects of RA are the result, at least in part, of a cell cycle arrest in the G0/G1 phase, we investigated the effect of several primary target genes common to MCF-7 and SK-BR-3 cells with opposite regulation by E2 in MCF-7 cells on progression through the cell cycle (SOX9, ELF3, SMAD3, BTG2, PLA2G10, TMPRSS2, TP53INP1). In addition, we also investigated the effect of modulated expression of GATA3 and FOXO3A (specific to SK-BR-3 cells) and of FOXA1 (primary in MCF-7 cells and regulated at later time points in SK-BR-3 cells) due to their reported roles in the control of ER expression and/or luminal differentiation (76-79). The pro-apoptotic transcription factor HOXA5 was also included to verify its lack of anti-proliferative effects. To this end, we transiently transfected SK-BR-3 cells by electroporation and analyzed cell cycle distribution 72 hours later (Figure 6A). Suppl. Figure 4A shows the levels of overexpression of the various genes, which are always at least at the same level as the induction by RA. Transfection of Cyclin D1 (CCND1) decreased the proportion of cells in G0/G1 significantly. On the other hand, no significant effects were observed with most of

the genes tested, except for 3 genes that increased the proportion of cells in the G0/G1 phase of cell cycle, mimicking the effect of RA (Figure 1B). These three genes include the known RA target and inhibitor of proliferation SOX9 (36), the Ets domain transcription factor ELF3, which has mixed anti-tumoral and tumor promoting activities (80-82), and the pioneer transcription factor FOXA1 (76, 78, 79). On the other hand, no significant effect of transfection of GATA3 was observed (data not shown). Unexpectedly, BTG2, another previously identified RA target with demonstrated anti-proliferative effects in MCF-7 cells, also had no significant effect under our experimental conditions.

To verify whether the SOX9, ELF3 and FOXA1 transcription factors participate in the antiproliferative effects of RA, we transiently transfected siRNAs against SOX9, ELF3 and FOXA1 in SkBR3 cells treated or not with RA. SiRNA against SOX9 and FOXA1 reduced the effect of RA on the distribution of cells in the different phases of the cell cycle. Combining siRNAs against SOX9 and FOXA1 almost fully abolished the effect of RA. No significant effects were observed with siRNAs against ELF3, possibly due to insufficient depletion (Figure 6B). Efficiency of the knockdowns is shown in Suppl. Figure 4B.

In conclusion, our results indicate that, in addition to SOX9, FOXA1 is a target gene of RA that mediates part of the antiproliferative action of RA in SkBR3 cells.

Secondary networks contributing to RA signaling.

To identify the respective target genes of SOX9 and FOXA1 in SkBR3 cells, these transcription factors were transiently overexpressed in SK-BR-3 cells. Microarray analysis

was conducted on the Illumina platform after 24h expression, and RA treatment was also performed for 24 h.

SOX9 over-expression led to modulated expression of 180 genes. 52 of these putative SOX9 target genes were also targets of RA in SK-BR-3 cells at 8 and/or 24 h, 41 being regulated in the same direction and 11 in opposite directions (Suppl. Table IV). The RA target genes whose expression is modulated by SOX9 include repressed secondary target genes E2F2 and MCM6 and transforming growth factor-beta superfamily member TGFB3. TGFβ family members BMP7 (repressed primary RA target) and GDF15 (up-regulated primary target) were also regulated by SOX9 over-expression, suggesting its role in amplifying RA effects on these genes. NFKB cofactor NFKBIZ was also found to be induced by SOX9 expression and by RA in SK-BR-3 cells and in MCF-7 cells.

FOXA1 overexpression resulted in modulated expression of 548 (394 up-regulated and 154 down-regulated) genes. 116 genes were common with RA target genes identified 8 hr or 24h after RA treatment (Suppl. Table IV). Out of these, most (81) were regulated in the same direction by RA and FOXA1 (35 in opposite direction). 25 of the 116 FOXA1 and RA target genes were also regulated by SOX9. Although some of these genes may represent artefacts of transient gene expression, several (15) were not observed regulated by overexpression of GATA3 (Suppl. Table IV), which does not have antiproliferative properties in SK-BR3 cells (data not shown). Genes common to FOXA1 and SOX9, but not GATA3 included E2F2, MCM6 and TGFB3. These results suggest that FOXA1 and SOX9 relay the antiproliferative activity of RA in SK-BR3 cells through activation of partially overlapping transcriptional programs.

Primary RA target genes discriminate between luminal and basal tumors.

Primary target genes of RA include two known modulators of luminal cell differentiation and ERα gene expression, GATA3 (up-regulated in SK-BR-3 only) and FOXA1 (up-regulated in both cell lines with different kinetics). In addition, we observed a modest up-regulation of ESR1 at the mRNA level, but not at the protein level, in SK-BR-3 cells (Figure 4). These results suggest that RA signaling contributes to luminal differentiation. To investigate this hypothesis further, we used the primary up-regulated RA target genes identified in ER-negative SK-BR-3 cells for non-supervised partitioning of breast tumors based on expression levels of these genes. This indicated a statistically significant association with tumor type (association with luminal vs basal tumors) and with ER-positive status in several datasets of breast tumor gene expression profiles (Figure 7 and data not shown). In addition, there was also a less robust association with grade (Figure 7 and data not shown). Several of the genes most strongly with the luminal status included up-regulated ER target genes such as NRIP1, CA12, SERPINA3 (46) (see also Table III) (83). However other genes strongly associated with the luminal status and ER positivity were down-regulated E2 target genes (SMAD3, GDF15, SCNN1A, PLA2G10, CYP26A1) and/or functioned in the repression of ER signaling (NRIP1, SMAD3, COUP-TFB), indicating that association with the luminal phenotype is not due only to an overlap between RAR and ER primary up-regulated target genes. Finally, several of these genes have antiproliferative activity in cancer cell lines and/or are putative tumor suppressors, including calcium/calmodulin-dependent protein kinase II inhibitor 1 CAMK2N1 (84),

TFPI2 (61) and SEMA3B (85). In addition, SCNN1A is subject to silencing by methylation in breast (86) and neuroblastoma tumor cells (87). These results are consistent with a role of several RA primary target genes in luminal cell differentiation rather than ER signaling itself.

Contrary to the above-described RA primary target genes that are positively associated with ER status, a few RA up-regulated primary target genes were negatively associated with ER status and presented higher mRNA levels in basal tumors, including TRPV6, GABBR2, TMPRSS2, KLHL24 and SOX9. Further experiments will be required to determine whether this reflects partially conserved RA signaling or constitutively high expression of the corresponding mRNAs in basal tumors.

DISCUSSION

In the present study we have sought to better understand the mechanisms leading to the anti-proliferative effects of RA in breast cancer cell lines by comparing target gene regulation in ER-positive MCF-7 cells and ER-negative SK-BR-3 cells. SK-BR-3 cells are classified as luminal cells although they express much reduced levels of ERα mRNAs compared to MCF-7 cells, and undetectable protein levels (88). SK-BR-3 also carry an amplification of the ERBB2 gene, and ERBB2 signaling has been suggested to inhibit the antiproliferative activity of RA (89, 90). However, SK-BR-3 cells are extremely sensitive to RA or to RARα-selective ligand AM580 (Figure 1A-B). Our results indicate that expression levels of RARα are determinant for the antiproliferative response to RA, as shRNA-mediated suppression inhibited growth-suppression by RA (Suppl. Figure 1). The high levels of RARα in SK-BR-3 cells in spite of lack of estrogen-induced RARA transcription (20, 22, 23), may result from co-amplification of the RARA gene with ERBB2; indeed, RARA is located in the 17q12-q21 long amplicon characterized in about half of ERBB2-amplified tumors (91-93). This amplification may be responsible for increased RARα levels and the resulting sensitivity to RA. Indeed, sensitivity to the antiproliferative effects of RA was restored in ER-negative MDA-MB-231 cells by expression of RARα (49). We observed that resistance to RA in MDA-MB-231 resulted from absence of gene regulation, likely reflecting the very low levels of RA receptors (Table I; Figure 1D). Expression of RARβ, detectable at the protein level in these cells, is

apparently insufficient for robust signaling, but may be responsible for the induction of NRIP1 and DHRS3, which are also primary RA targets in MCF-7 and/or SK-BR-3 cells.

Recent genome-wide characterization of chromatin binding regions of RARs in MCF-7 cells have uncovered colocalization of RAR and ER in about 50% of these regions, leading to the suggestion that RA signaling interferes with that of E2 for transcriptional regulation. Both antagonistic action of RA and E2 on target genes and a role of RARα as a cofactor for ERα have been proposed (24, 25). The observation that RA is antiproliferative in an ER-negative cell line and that there is a large overlap in RA target genes in ER-positive and ER-negative cell lines indicates that regulation of common target genes is independent of estrogenic signaling pathway. Thus it is likely that RAR binds to regulatory regions in these genes and modulates their transcription through processes that do not involve ERα. Accordingly, we observed an enrichment of RAREs (DR5 and DR2 types) in the flanking regions of up-regulated primary RA target genes in MCF-7 and SK-BR-3 cells (Figure 5B-C). Of interest, several primary RA target genes identified in this study, including FOXA1, SOX9 and BTG2, contained overlapping RARα and ERα chromatin binding sites in MCF-7 cells (data not shown). Potential RAREs were identified in the FOXA1, SOX9 and BTG2 RAR ChIP regions (data not shown), consistent with primary binding by RARs. Further, down-regulation of BTG2 by E2 was cycloheximide-sensitive, like that of most RA target genes down-regulated by E2 (Table III), suggesting that a simple mechanism of competition for binding sites is unlikely to explain the antagonism between ERα- and RARα-mediated signaling. In addition, the enrichment of several TF binding sites in the flanking regions of primary up- or down-regulated target genes as well as in ChIP regions

suggests a role in the mediation of transcriptional regulation through mechanisms of tethering or cooperativity with RAREs. Whether some of these factors could recruit both ERα and RARα remains to be investigated. Finally, we note that our array conditions are optimized for the identification of estrogen-independent effects, as MCF-7 cells were maintained in low estrogen concentrations (charcoal-stripped medium in the presence of the weak estrogen phenol red) for these experiments. Therefore, it is possible that a larger fraction of genes regulated in MCF-7 cells may be specific to ER-positive cells when patterns of gene expression are examined in the presence of high concentrations of estrogens. However, our results demonstrate that RA has antiproliferative effects through transcriptional regulation of genes in an ER-independent manner.

While about half of RAR primary target genes are common to both cell lines, other genes are regulated differentially (Figure 2). This can result from differences in kinetics of regulation, as observed for FOXA1 (Figure 4). Also, elevated expression in a cell line may mask regulation by RA through action of a stronger constitutive activator, as is likely the case for GATA3. Conversely, it is possible that absence of regulation in a given cell line may result from incorporation into heterochromatin. Finally, differences between the two cell lines in expression patterns of RARs (MCF-7 express higher levels of RARγ; Figure 1D) and in other cell signaling pathways, such as increased PI3K and MAP kinase activity in ERBB2 cells (94), could contribute to differential gene regulation in the two cell lines.

Primary target genes of RA mediate its antiproliferative effects via both regulation of cell cycle progression and control of apoptosis. Several primary and secondary target genes in SK-BR-3 cells are known inducers or effectors of apoptosis, including APAF, BID,

CASP 3, 8, and 9. RA also modulated expression of components of apoptosis-regulating pathways, such as the TGFβ pathway. Induction of several pro-apoptotic genes (APAF, BID) was cycloheximide-sensitive, this indirect regulation being consistent with the late induction of apoptosis after RA stimulation.

Contrary to induction of apoptosis, cell cycle arrest by RA can be observed at early time points after RA treatment (48h; Figure 1B). We examined the effects of several primary target genes of RA on cell distribution in the different phases of the cell cycle and observed that FOXA1 and ELF3 in addition to SOX9 increased the proportion of cells in the G0/G1 phase. Lack of effect of other expression vectors may be due to insufficient protein expression or expression of inappropriate isoforms. In addition, lack of effect of FOXO3A could be due to inactivation via ERBB2 signaling-induced inactivating phosphorylation. On the other hand, as RA appears to downregulate ERBB2 downstream signaling in SK-BR-3 cells through upregulation of the negative PI3K regulator PIK3IP1 (95, 96) and through secondary downregulation of AKT1, these effects could synergize with transcriptional induction of FOXO3A.

The antiproliferative effects of FOXA1 and SOX9 are supported by the inhibition of RA-induced accumulation of cells in G0/G1 via siRNA-mediated suppression of expression. Notably, combined inhibition of FOXA1 and SOX9 led to a more complete inhibition of accumulation of cells in G0/G1 than either siRNA separately, suggesting that both proteins contribute to the antiproliferative effects of RA (Figure 6B). Overexpression of FOXA1 and SOX9 both led to regulation of RA target genes, with a larger number of genes regulated in the same manner as by RA, consistent with a role of these genes in

propagating the RA-induced reprogramming of gene expression. Regulation of genes that were not identified as RA targets may be due to artificially high expression levels achieved in the transient transfection experiments, but also to modulation of the action of these transcription factors by coregulated RA targets. Of interest, several secondary RA targets regulated at 24h but not at 8h (with or without CHX) were co-regulated by FOXA1 and SOX9, including cell cycle control genes E2F2 and MCM6, consistent with induction of partially overlapping antiproliferative gene expression programs by the two transcription factors.

FOXA1 has been characterized as a pioneer factor important for luminal cell differentiation, ERα gene expression and transcriptional regulation by ERα (78, 79, 97-100). Our results indicating regulation of FOXA1 by RA in MCF-7 and, with delayed kinetics, in SK-BR-3 cells, suggest that RA controls the luminal differentiation program. This is consistent with the reported observation that the RA synthetic enzyme ALDH1A3 (observed here to be an RA-induced target gene) is expressed in luminal-committed precursors, suggesting that RA is synthesized during luminal differentiation (101). Significantly, GATA3, another important modulator of luminal cell differentiation and ER synthesis (77, 102-104), was also found in our microarrays as a primary target gene of RA in SK-BR-3 cells, while its levels were constitutively high in ER-positive MCF-7 cells. In this respect, it is important to stress that ERBB2-amplified breast cancer cells such as SK-BR-3 cells are thought to represented cell population blocked at an intermediate level in the differentiation process leading to luminal cell differentiation, and that SK-BR-3 cells have been classified as luminal in spite of their lack of expression of ERα and low

expression of GATA3. We suggest that this is due to their high levels of RARα expression, likely resulting from co-amplification of the RARA gene with ERBB2. Consistent with this interpretation, non-supervised classification of tumors based on expression levels of primary RA-upregulated target genes resulted in statistically significant sorting of tumors according to luminal or ER+ status (Figure 7). The identity of the genes most strongly associated with a luminal status indicates that while some of these genes are also up-regulated by E2, others are associated with limiting E2 signaling or action as tumor suppressors. Finally, we note that while the RA target gene SOX9 has antiproliferative action in SK-BR-3 luminal breast cancer cells (this study) as well as in T47D cells (36), its mRNA levels are more strongly associated with basal cells than with luminal cells. Further studies will be necessary to determine whether SOX9 protein levels are also higher in these tumors, and whether SOX9 intra-cellular localization is different in luminal and basal cells, as localization in the cytoplasm was found to correlate with a worse prognosis (105, 106).

In conclusion, our results indicate that RA activates gene expression programs with antiproliferative components that contribute to initiation/maintenance of luminal differentiation at least in part independently from ERα expression. Future studies will be needed to determine whether RARα, which mediates the effects of RA on induction of FOXA1 and GATA3 transcription and cross-talks with ER genomic signaling through still imperfectly understood mechanisms, qualifies as a pioneer transcription factor in luminal breast epithelial cells.

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FIGURE LEGENDS

Figure 1: Importance of RARα for the anti-proliferative effects of RA in breast cancer cells.

(A) Cells were treated every 2 days with vehicle (DMSO, 0.1%), RA or AM580 at indicated concentrations. After 9 days protein concentrations were measured to assess effects on cell growth. (B) Cell cycle analysis of SK-BR-3 cells treated with vehicle or retinoids for 48h. (C) RARα mRNA levels in cells treated for 8 hours with vehicle or 100 nM RA. Primers used detect both RARα isoforms. (D) Western blot analysis comparing levels of RARα, β and γ as well as ERα levels in breast cancer cell lines. Where indicated cells were treated with 100 nM RA for 24 hours. All results shown are representative examples of at least two separately performed experiments.

Figure 2: Sensitivity to the antiproliferative effects of RA correlates with transcriptional regulation.

Microarray analysis was performed with three biological replicates for each condition (see Material and Methods section) from MCF-7 and SK-BR-3 cells after 8 h treatment with vehicle or RA (100 nM). Numbers of genes significantly regulated (fold change between vehicle and RA treatments ≥1.4, amplitude of array signals across all conditions ≥5 and P-value based on moderated *t*-statistics ≤0.01) are indicated for each category.

Figure 3: Cycloheximide-sensitive and resistant RA target genes in SK-BR-3 and MCF-7 cells.

Microarray analysis was performed as in Figure 2, except that cells were pre-treated for one hour before RA treatment with cycloheximide (CHX, 10 µg/ml). Regulated genes are listed in Table II.

Figure 4: Kinetics of RA target gene induction.

MCF-7 and SK-BR-3 cells were treated with 100 nM of RA in the absence or presence of CHX (10 µg/ml) for indicated time periods. Regulation of selected genes was analyzed by Q-PCR. Represented are mean values from one experiment using three technical replicates. Error bars represent standard deviations.

Figure 5: Mechanisms of gene regulation by retinoic acid in MCF-7 and SK-BR-3 cells.

(A) Position weight matrix used for identification of putative RAREs in the vicinity of target genes. The DR5 element is shown as example. (B-C) Enrichment of DR5 (B) and DR2 (C) RAREs in a 10 kb window around the TSS of upregulated genes (±5 kb). *, enrichment significant in two statistical tests (Z-score > 10 and p-value < 0.01), °, enrichment significant for one of the two statistical tests.

Figure 6: Selected RA target genes play a role in growth inhibition in SK-BR-3 cells.

(A) SK-BR-3 cells were transfected with indicated RA target genes and cell cycle distribution was analyzed by FACS 72h later. Bars represent the average of at least 3 independent experiments. Error bars represent standard error of the means (S.E.M.). (B) SK-BR-3 cells were transfected with siRNAs against the indicated RA target genes. 24 hours after transfection cells were treated with 100 nM RA or vehicle and cell cycle distribution was analyzed by FACS 72h later. Bars represent the average of 3 independent experiments. Error bars represent standard error of the means (S.E.M.)

Figure 7: Primary RA target gene expression correlates with ER expression and tumor subtype.

Hierarchical clustering (euclidian distance, ward linkage) of 230 stage I-III breast cancers (MAQC-II dataset) (107) based on the expression of primary RA-induced genes. Histo-pathological variables of each tumor sample are presented in the boxes below each sample (shaded boxes indicate positive status). Intrinsic subtypes in the PAM50 classifier are as follows: green, normal-like; dark blue, luminal A; light blue, luminal B; pink, HER2; red, basal. Significant associations of each cluster with clinical characteristics assessed by a one-way Fisher exact test are presented below the relevant cluster.

Table I: Gene regulation by RA in SK-BR-3, MCF-7 and MDA-MB-231 cells.

Microarray analysis was performed with three biological replicates for each condition from SK-BR-3, MCF-7 and MDA-MB-231 cells after 8 h treatment with vehicle or RA (100 nM) (four replicates for SK-BR-3 #2). SK-BR-3 and MCF-7 cells were treated with 10 μg/ml of CHX where indicated. Numbers of genes significantly regulated (fold change between vehicle and RA treatments ≥1.4, amplitude of array signals across all conditions ≥5 and P-value based on moderated *t*-statistics ≤0.01) are indicated for each category.

Table II: Primary RA target genes in both MCF-7 and SK-BR-3 cells.

List of RA targets that were regulated in both RA-sensitive cell lines and that were CHX-insensitive in at least one cell line.

Table III: Comparison of RA and estrogen target genes.

Common RA regulated genes (MCF-7 and SK-BR-3) from the present study were compared to genes regulated by E2 in MCF-7 cells (46).

SUPPLEMENTARY DATA

Suppl. Figure 1: Knockdown of RARA reduces the response of breast cancer cell lines to RA.

MCF-7 and SK-BR-3 cells were infected with pLKO1-shRARA or non targeting (shNT) vectors at MOI 2. After selection, 9 day growth assays were performed in the presence of indicated concentrations of RA. Western blots confirm significant but incomplete knockdown of RARα protein with the two RARA targeting hairpins. The experiment was performed twice with comparable results and one experiment is shown. Error bars represent standard deviations of three technical replicates.

Suppl. Figure 2: Reproducibility of microarray analysis in SK-BR-3 cells.

Suppl. Figure 3: Numbers of RA target genes in SK-BR-3, MCF-7 and MDA-MB-231 cells.

Non-adjusted p-values were used for analyses.

Suppl. Figure 4: Modulation of RA target gene levels.

(A) Q-PCR analysis of the overexpression of RA target genes by electroporation. One out of two experiments with similar results is shown. Error bars represent standard

deviations of three technical replicates. (B) Western blot analysis of siRNA knockdown of selected RA targets. Experiment was performed twice with comparable results. NT, not transfected.

Suppl. Table I: Top 100 ranked genes regulated in SK-BR-3 cells without and with CHX.

Genes were ranked based on a combination of three parameters, fold-change between RA and vehicle treatments, amplitude of array signals across all conditions and P-value based on moderated t-statistics. Genes in bold are those found significantly regulated both in the absence and presence of cycloheximide (CHX).

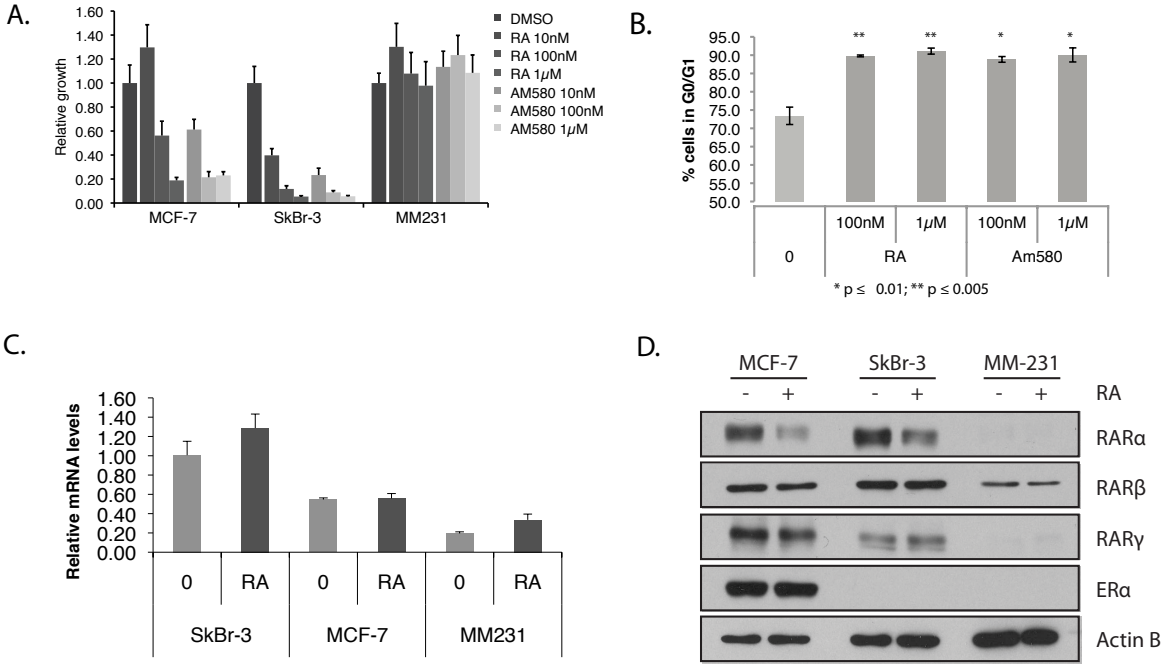
Suppl. Table II: Top 100 ranked genes regulated in MCF-7 cells without and with CHX.

Genes were ranked based on a combination of three parameters, fold-change between RA and vehicle treatments, amplitude of array signals across all conditions and P-value based on moderated t-statistics. Genes in bold are those found significantly regulated both in the absence and presence of cycloheximide (CHX).

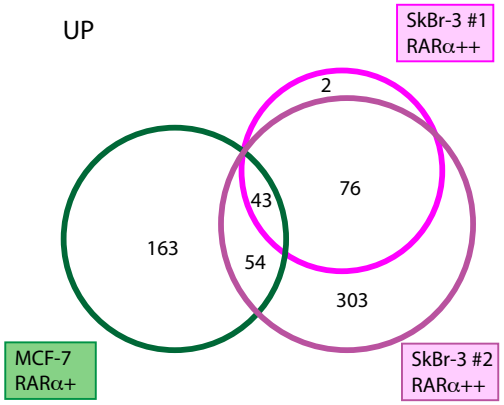
Suppl. Table III: Enriched transcription factor binding sites in ChIP-chip and ChIP-seq regions, as well as in promoters of RA regulated genes.

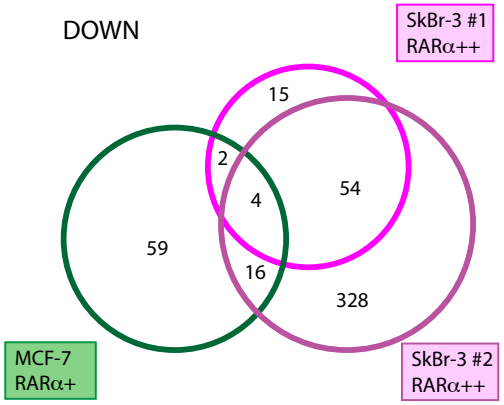
Suppl. Table IV: Gene regulation by overexpression of FOXA1, SOX9 or GATA-3 in SK-BR-3 cells and comparison with primary or secondary RA target genes.

ROZENDAAL ET AL, FIGURE 1

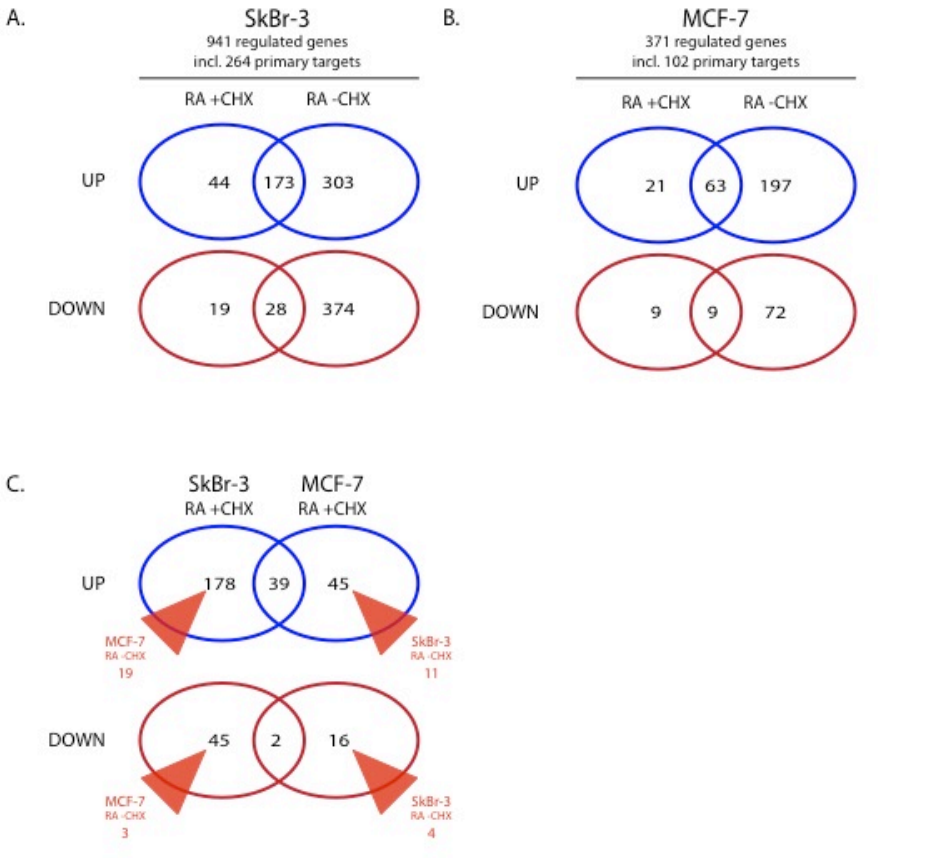


ROZENDAAL ET AL, FIGURE 2

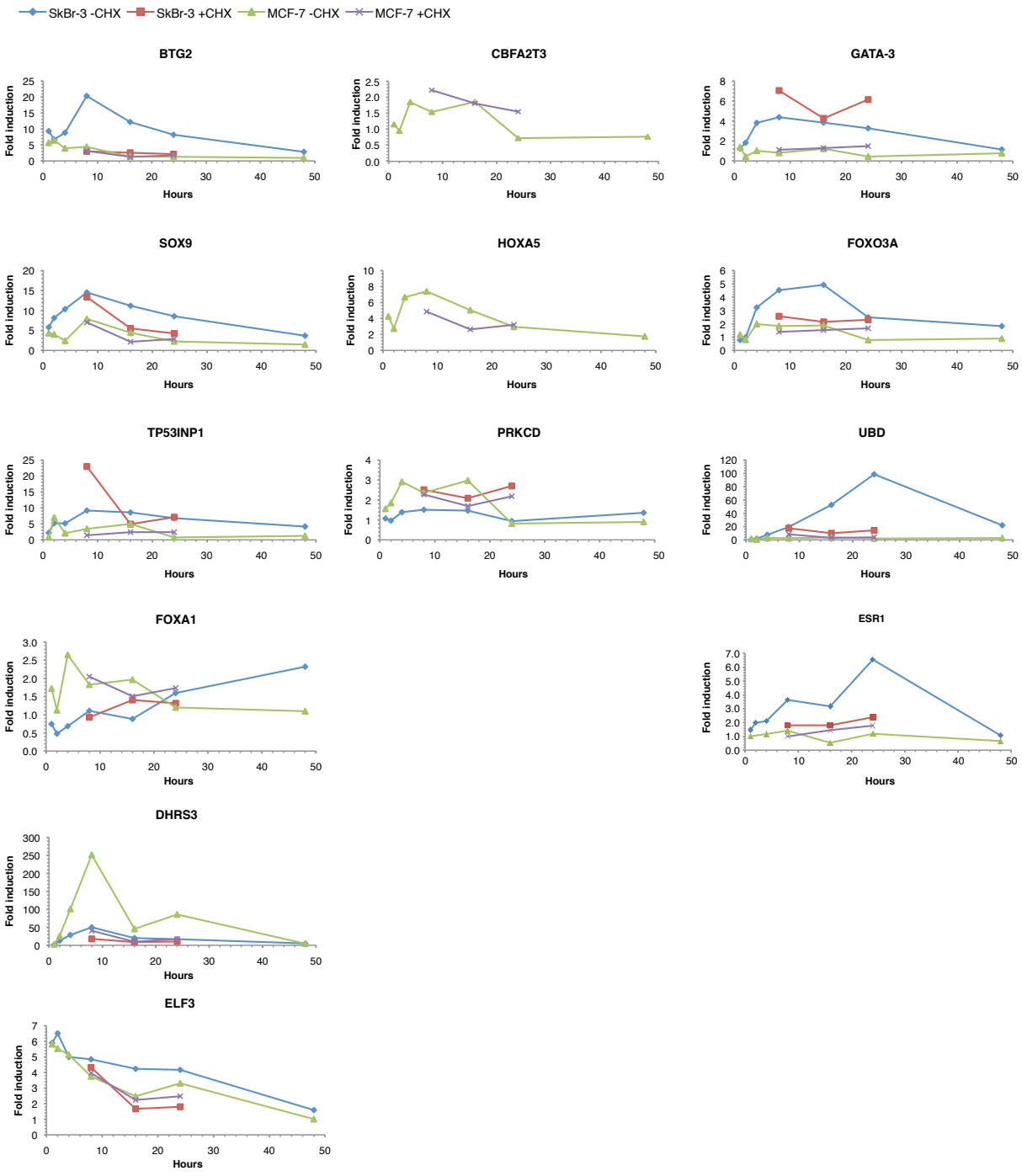


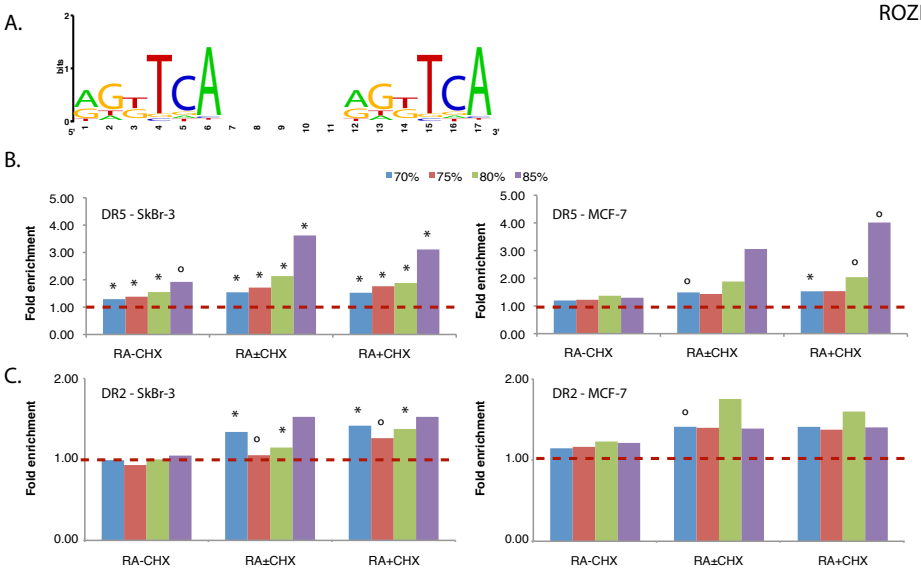


ROZENDAAL ET AL, FIGURE 3



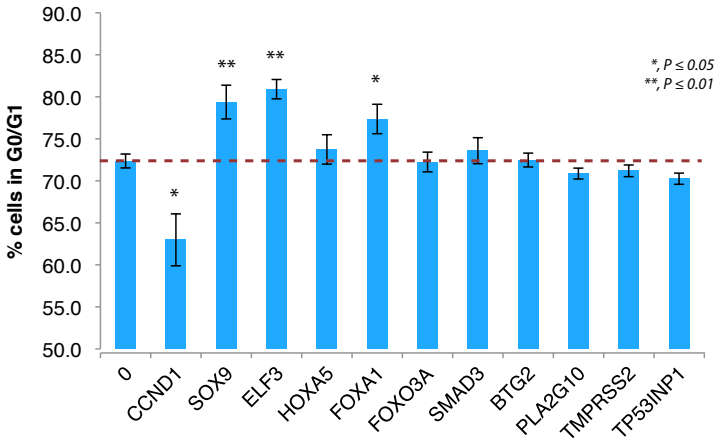
ROZENDAAL ET AL, FIGURE 4



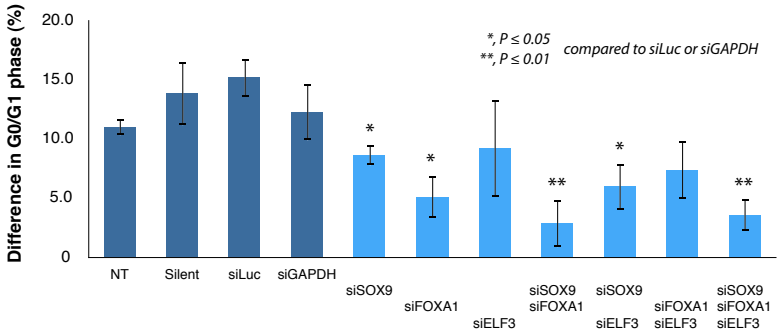

A.
B.
70%
75%
80%
85%
DR5 - SkBr-3
DR5 - MCF-7
Fold enrichment
5.00
4.00
3.00
2.00
1.00
0.00
RA-CHX
RA±CHX
RA+CHX
C.
DR2 - SkBr-3
DR2 - MCF-7

ROZENDAAL ET AL, FIGURE 6

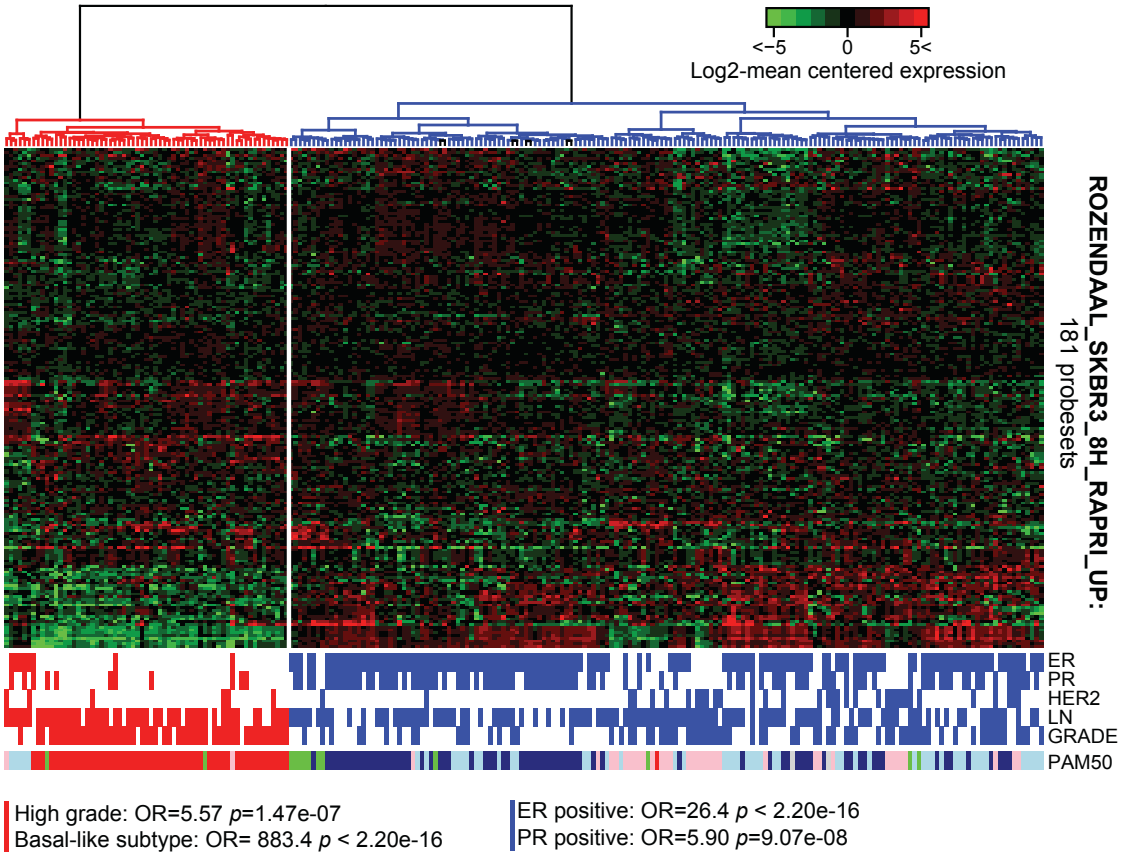
A.



B.



ROZENDAAL ET AL, FIGURE 7



ROZENDAAL ET AL, TABLE I

	SkBr-3 #1	**SkBr3 #2**		**MCF-7**		**MDA-MB-231**
	RA-CHX	RA-CHX	RA+CHX	RA-CHX	RA+CHX	RA-CHX
UP	121	476	217	260	84	2
DOWN	75	402	47	81	18	0

ROZENDAAL ET AL, TABLE II

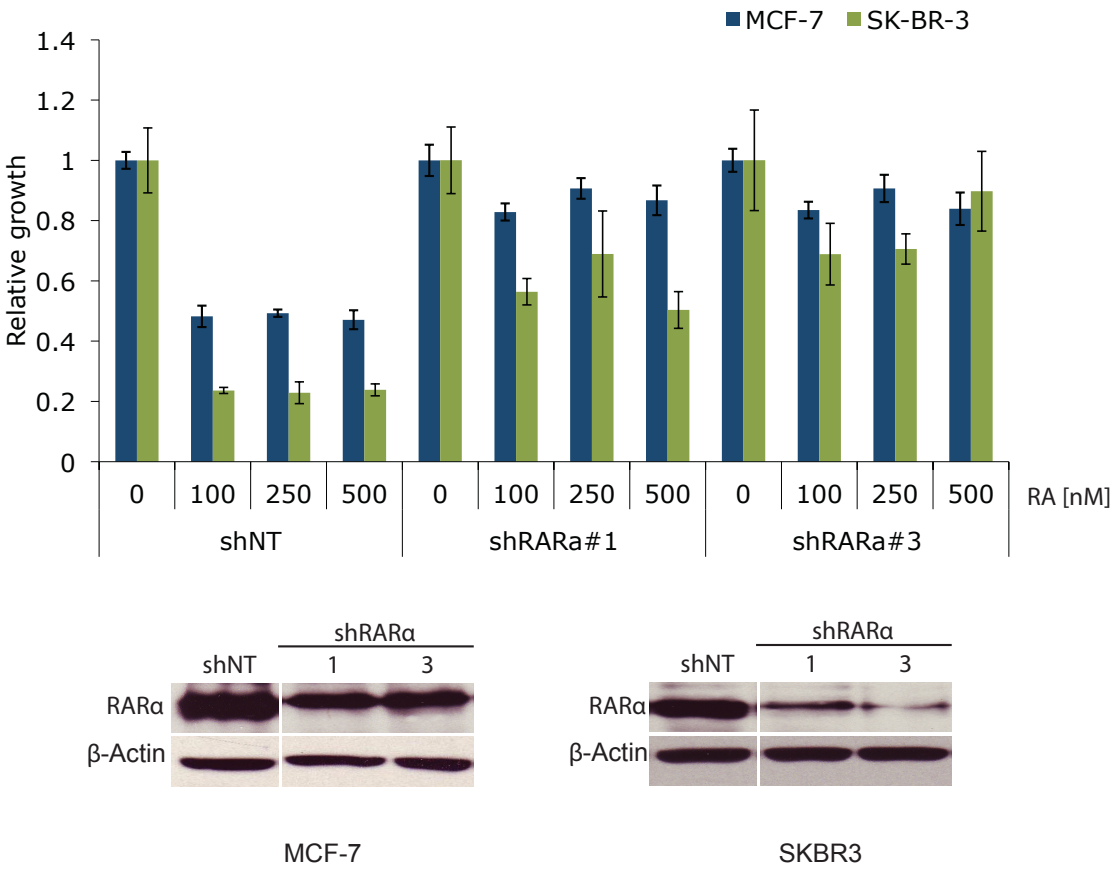
+CHX SK-BR-3; +CHX MCF-7	+CHX SK-BR-3; -CHX MCF-7	-CHX SK-BR-3; +CHX MCF-7
	Upregulated genes	
AHRR	AHRR	
AP1G1	AP1G1	AP1G1
	BAMBI	
	BCL3	
BDKRB2	BDKRB2	BDKRB2
BHLHB2	BHLHB2	BHLHB2
		BTG2
	C10orf58	
	C15orf39	
CA12	CA12	CA12
CNP	CNP	CNP
	CORO2A	
CYP26A1	CYP26A1	CYP26A1
	CYP26B1	
DHRS3	DHRS3	DHRS3
DUSP4		DUSP4
		DYRK2
ELF3	ELF3	ELF3
		EPAS1
EPB41L4A	EPB41L4A	EPB41L4A
FAM43A	FAM43A	FAM43A
FBXO34	FBXO34	FBXO34
		FGD3
	FOXO3	
	GABBR2	
Gcom1	Gcom1	Gcom1
	GDF15	
GPR160	GPR160	GPR160
GPRC5A	GPRC5A	GPRC5A
	HIVEP3	
		IDH1
JAG2		
		KLHDC2
	MID1IP1	
MYADM	MYADM	MYADM
		NANS
NCOR2	NCOR2	NCOR2
NRIP1	NRIP1	NRIP1
PARP9	PARP9	PARP9
PCTP		PCTP
	PBX1	
		PFKFB3
	PHLDA1	
		PHF8
PLA2G10	PLA2G10	PLA2G10
RAP1GAP		RAP1GAP
	RHOU	
RNF207	RNF207	
SCNN1A	SCNN1A	SCNN1A
SERPINA3		SERPINA3
SLC22A5	SLC22A5	SLC22A5
	SMAD3	
SOX9	SOX9	SOX9
STRA6	STRA6	STRA6
SYNJ2	SYNJ2	
TFPI2	TFPI2	TFPI2
TGFBI	TGFBI	TGFBI
	TIAF1	
TMPRSS2	TMPRSS2	TMPRSS2
	TMPRSS4	
TNFAIP2		
TP53INP1	TP53INP1	TP53INP1
TRAF4		TRAF4
		TSC22D3
UBD		UBD
VPS13D		VPS13D
	XYLT1	
		ZEB1
	ZNRF1	

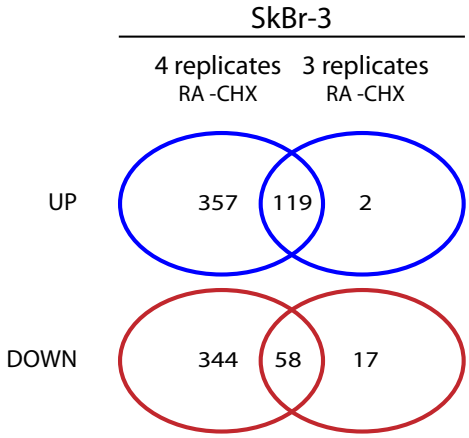
+CHX SK-BR-3; +CHX MCF-7	+CHX SK-BR-3; -CHX MCF-7	-CHX SK-BR-3; +CHX MCF-7
	Downregulated genes	
CLDN1		
		CPEB2
	DKK1	
		EDG3
		EFEMP1
		ELF5
	PCDH7	
PTGER4		
	SSBP2	

ROZENDAAL ET AL, TABLE III

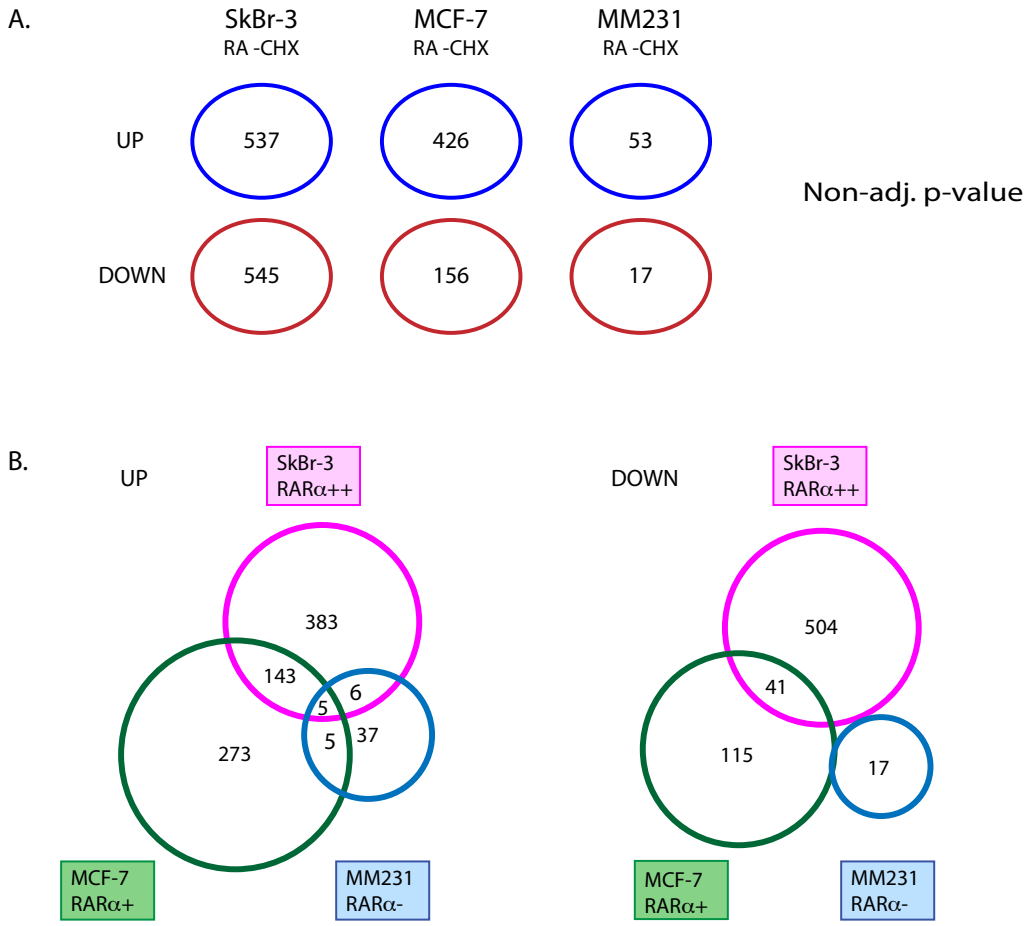
Probeset	mRNA	Symbol	RA regulation		Fold RA SkBr-3		Fold RA MCF-7		Fold E2 MCF-7	
			SkBr-3	MCF-7	-CHX	+CHX	-CHX	+CHX	-CHX	+CHX
Opposite regulation RA/E2 (UP/DOWN)										
229354_at	NM_020731	**AHRR**	primary	primary	1.05	1.46	2.12	1.77	-3.98	-1.28
201170_s_at	NM_003670	**BHLHB2**	primary	primary	2.12	1.48	1.49	1.52	-1.60	1.07
206424_at	NM_000783	**CYP26A1**	primary	primary	27.06	33.02	25.35	44.86	-1.54	-1.81
238032_at	NM_004753	**DHRS3**	primary	primary	4.03	2.55	3.11	2.60	-1.50	-1.08
202481_at	NM_004753	**DHRS3**	primary	primary	3.79	2.17	11.47	4.71	-2.37	-1.22
204014_at	NM_001394	**DUSP4**	primary	primary	1.53	2.41	1.45	1.73	-1.70	1.03
210827_s_at	NM_004433	**ELF3**	primary	primary	1.93	1.75	2.92	1.95	-1.86	-1.01
228256_s_at	NM_022140	**EPB41L4A**	primary	primary	1.62	1.67	1.97	1.96	-1.54	-1.02
228568_at	NM_001018100	**Gcom1**	primary	primary	1.62	1.61	1.86	2.18	-1.37	1.52
223423_at	NM_014373	**GPR160**	primary	primary	3.32	1.58	2.36	1.59	-1.51	-1.55
32137_at	NM_002226	**JAG2**	primary	primary	1.14	2.30	-1.08	1.65	-1.17	-1.59
209784_s_at	NM_145159	**JAG2**	primary	primary	1.09	2.28	-1.13	1.63	-1.22	-1.65
223220_s_at	NM_031458	**PARP9**	primary	primary	2.18	1.50	1.67	1.51	-1.70	-1.14
218676_s_at	NM_021213	**PCTP**	primary	primary	1.86	2.52	1.28	1.70	-1.44	-1.11
207222_at	NM_003561	**PLA2G10**	primary	primary	1.87	2.79	2.25	1.90	-1.73	-1.09
1555870_at	NM_207396	**RNF207**	primary	primary	1.31	1.81	2.03	1.89	-2.16	-1.31
203453_at	NM_001038	**SCNN1A**	primary	primary	1.92	1.42	3.79	2.92	-2.51	-1.46
202935_s_at	NM_000346	**SOX9**	primary	primary	6.08	3.65	9.50	2.56	-1.42	2.47
202936_s_at	NM_000346	**SOX9**	primary	primary	5.21	3.34	7.62	1.89	-1.54	2.05
201506_at	NM_000358	**TGFBI**	primary	primary	2.26	1.69	4.76	2.16	-2.24	-1.27
211689_s_at	NM_005656	**TMPRSS2**	primary	primary	5.18	2.41	3.66	2.33	-2.70	-1.35
225912_at	NM_033285	**TP53INP1**	primary	primary	4.10	4.08	1.82	2.06	-2.74	-1.40
204908_s_at	NM_005178	**BCL3**	primary	secondary	1.12	1.51	1.68	1.42	-1.30	1.53
209990_s_at	NM_005458	**GABBR2**	primary	secondary	3.35	1.64	2.08	1.66	-5.42	-1.57
221577_x_at	NM_004864	**GDF15**	primary	secondary	2.63	1.89	3.12	1.55	-1.72	-1.02
205397_x_at	NM_005902	**SMAD3**	primary	secondary	3.08	1.72	1.59	1.56	-1.60	-1.04
218284_at	NM_005902	**SMAD3**	primary	secondary	2.65	1.71	1.83	1.42	-1.90	-1.07
205398_s_at	NM_005902	**SMAD3**	primary	secondary	2.58	1.79	1.71	1.34	-1.56	-1.09
202039_at	NM_004740	**TIAF1**	primary	secondary	2.38	1.48	1.93	1.55	-1.47	1.01
201236_s_at	NM_006763	**BTG2**	secondary	primary	2.73	1.38	2.65	1.67	-2.43	1.02
200878_at	NM_001430	**EPAS1**	secondary	primary	1.48	1.19	3.59	1.77	-2.68	-1.12
227811_at	NM_001083536	**FGD3**	secondary	primary	1.56	1.36	1.29	1.53	-2.21	-1.40
1555037_a_at	NM_005896	**IDH1**	secondary	primary	1.94	1.35	2.57	2.04	-1.44	-2.04
201193_at	NM_005896	**IDH1**	secondary	primary	1.77	1.35	2.47	1.99	-1.45	-2.00
217906_at	NM_014315	**KLHDC2**	secondary	primary	2.13	1.36	2.40	2.27	-1.46	-1.38
202464_s_at	NM_004566	**PFKFB3**	secondary	primary	2.15	1.15	3.09	1.97	-1.60	1.33
207001_x_at	NM_001015881	**TSC22D3**	secondary	primary	1.51	1.21	1.16	2.17	-1.76	-1.32
Same regulation (UP)										
215867_x_at	NM_001128	**AP1G1**	primary	primary	2.63	1.79	2.40	2.22	1.80	2.92
214164_x_at	NM_206925	**CA12**	primary	primary	2.53	1.75	2.32	2.18	1.73	2.66
203963_at	NM_001218	**CA12**	primary	primary	2.46	1.88	2.89	2.72	2.00	3.43
207760_s_at	NM_006312	**NCOR2**	primary	primary	1.57	1.66	1.91	1.69	1.19	1.61
202376_at	NM_001085	**SERPINA3**	primary	primary	1.60	1.47	1.74	2.83	1.77	1.55
233388_at	NM_001218	**CA12**	primary	secondary	1.67	2.76	1.75	1.23	1.77	3.06
219825_at	NM_019885	**CYP26B1**	primary	secondary	2.37	2.63	4.79	1.76	3.85	7.46
225842_at	NM_007350	**PHLDA1**	primary	secondary	2.60	3.98	2.44	1.06	1.20	1.41
223168_at	NM_021205	**RHOU**	primary	secondary	2.11	1.69	2.08	1.20	1.17	1.54
Same regulation (DOWN)										
204897_at	NM_000958	**PTGER4**	primary	primary	-1.51	-1.98	-1.31	-2.11	-2.37	-1.40
203787_at	NM_012446	**SSBP2**	primary	secondary	-1.57	-1.40	-1.67	-1.22	-1.18	-1.46
228176_at	NM_005226	**EDG3**	secondary	primary	-1.42	-1.08	-2.12	-1.88	-2.66	-1.22
201842_s_at	NM_001039348	**EFEMP1**	secondary	primary	-1.41	-1.31	-1.96	-1.77	-1.34	-1.92

ROZENDAAL ET AL, SUPPL. FIGURE 1

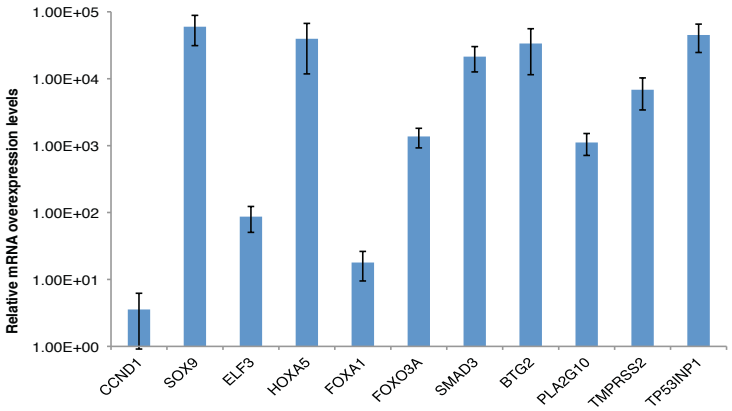



SkBr-3
4 replicates
RA -CHX
3 replicates
RA -CHX
UP
357
119
2
DOWN
344
58
17

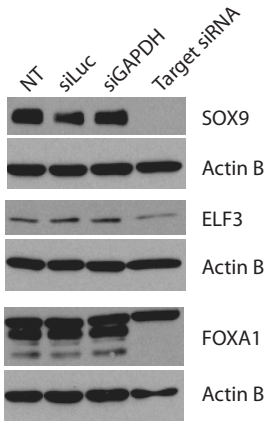
ROZENDAAL ET AL, SUPPL. FIGURE 3



A.


Relative mRNA overexpression levels
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CCND1
SOX9
ELF3
HOXA5
FOXA1
FOXO3A
SMAD3
BTG2
PLA2G10
TMPRSS2
TP53INP1

B.


NT
siLuc
siGAPDH
Target siRNA
SOX9
Actin B
ELF3
Actin B
FOXA1
Actin B

Rank	Gene	Fold	A	adj. p-value	Regulated RA+CHX
Genes -CHX					
1	**CYP26A1**	**27.06**	**6.65**	**1.54E-15**	**CYP26A1**
2	**PTGS1**	**19.89**	**7.07**	**1.15E-12**	**PTGS1**
3	**CDH5**	**6.95**	**8.56**	**7.44E-14**	**CDH5**
4	**SOX9**	**6.08**	**8.54**	**3.11E-13**	**SOX9**
5	**TMPRSS4**	**5.54**	**7.77**	**1.52E-11**	**TMPRSS4**
6	**GPRC5A**	**4.15**	**10.03**	**9.91E-12**	**GPRC5A**
7	**TMPRSS2**	**5.18**	**7.64**	**4.49E-11**	**TMPRSS2**
8	**BDKRB2**	**4.35**	**9.09**	**4.00E-10**	**BDKRB2**
9	**FAM83A**	**5.84**	**6.63**	**2.53E-12**	**FAM83A**
10	**NRIP1**	**3.62**	**10.48**	**4.77E-09**	**NRIP1**
11	PHLDA2	-3.98	9.16	5.42E-12	
12	**DHRS3**	**3.79**	**9.17**	**7.10E-11**	**DHRS3**
13	**GPR160**	**3.32**	**10.36**	**1.79E-08**	**GPR160**
14	**KRT40**	**5.22**	**6.42**	**6.76E-12**	**KRT40**
15	**EPDR1**	**5.09**	**6.25**	**3.05E-12**	**EPDR1**
16	**SLC26A2**	**3.46**	**9.02**	**5.42E-12**	**SLC26A2**
17	**CA12**	**3.05**	**10.11**	**1.43E-08**	**CA12**
18	STEAP4	3.95	7.70	4.02E-10	
19	**PHLDA1**	**4.72**	**6.44**	**2.96E-09**	**PHLDA1**
20	**PNKD**	**2.84**	**10.53**	**1.20E-10**	**PNKD**
21	**ID4**	**-3.41**	**8.44**	**2.15E-10**	**ID4**
22	**LASP1**	**2.58**	**10.98**	**7.03E-11**	**LASP1**
23	**CNP**	**2.57**	**10.92**	**4.47E-09**	**CNP**
24	**AP1G1**	**2.63**	**10.65**	**1.53E-10**	**AP1G1**
25	**SLC16A5**	**4.18**	**6.61**	**1.16E-09**	**SLC16A5**
26	**TFPI2**	**3.14**	**8.75**	**4.46E-09**	**TFPI2**
27	**KLHL24**	**3.14**	**8.74**	**6.59E-08**	**KLHL24**
28	**VLDLR**	**4.88**	**5.52**	**1.71E-11**	**VLDLR**
29	**FOXO3**	**2.82**	**9.51**	**1.71E-11**	**FOXO3**
30	**TP53INP1**	**4.10**	**6.51**	**2.40E-10**	**TP53INP1**
31	**TSPAN14**	**2.80**	**9.12**	**4.35E-11**	**TSPAN14**
32	**CTGF**	**3.02**	**8.38**	**4.41E-11**	**CTGF**
33	**NR2F2**	**3.30**	**7.62**	**1.52E-11**	**NR2F2**
34	KLHDC2	2.13	11.69	6.45E-10	
35	LOC26010	2.44	10.06	2.15E-10	
36	**SLC22A5**	**2.72**	**9.00**	**6.03E-11**	**SLC22A5**
37	S100A9	2.22	10.95	1.62E-08	
38	**TNFSF15**	**3.99**	**6.08**	**4.75E-10**	**TNFSF15**
39	**FGD6**	**3.52**	**6.88**	**1.78E-04**	**FGD6**
40	**PTGES**	**2.55**	**9.48**	**3.66E-08**	**PTGES**
41	**RAI14**	**3.05**	**7.91**	**1.02E-10**	**RAI14**
42	**MID1IP1**	**2.76**	**8.70**	**2.72E-10**	**MID1IP1**
43	**C10orf58**	**2.01**	**11.90**	**7.59E-10**	**C10orf58**
44	FAM46B	-2.55	9.37	3.91E-08	
45	NANS	2.05	11.49	4.07E-08	
46	**TIAF1**	**2.38**	**9.85**	**4.85E-07**	**TIAF1**
47	TPD52L1	2.34	10.02	6.53E-06	
48	**XYLT1**	**3.02**	**7.63**	**1.68E-07**	**XYLT1**
49	**C2orf54**	**2.77**	**8.27**	**4.48E-07**	**C2orf54**
50	**CHN2**	**3.25**	**7.06**	**1.20E-10**	**CHN2**
51	PGM2L1	2.92	7.84	1.01E-09	
52	**ELF3**	**1.93**	**11.80**	**2.15E-09**	**ELF3**
53	**TEAD2**	**3.78**	**6.00**	**2.05E-09**	**TEAD2**
54	**MAN1C1**	**3.38**	**6.68**	**8.07E-12**	**MAN1C1**
55	**PSMB9**	**3.18**	**7.07**	**2.25E-08**	**PSMB9**
56	VIPR1	-2.52	8.86	1.20E-10	
57	**RGS10**	**2.09**	**10.69**	**1.85E-09**	**RGS10**
58	**SMAD3**	**3.08**	**7.25**	**1.23E-08**	**SMAD3**
59	**GABBR2**	**3.35**	**6.66**	**1.17E-09**	**GABBR2**
60	**KLF4**	**-2.39**	**9.29**	**1.80E-08**	**KLF4**
61	**SYTL2**	**2.70**	**8.23**	**6.53E-10**	**SYTL2**
62	**AJAP1**	**4.07**	**5.45**	**9.49E-11**	**AJAP1**
63	**ZMIZ1**	**1.89**	**11.65**	**3.13E-05**	**ZMIZ1**
64	**KIAA1026**	**2.55**	**8.54**	**7.80E-09**	**KIAA1026**
65	**C10orf54**	**2.70**	**7.98**	**3.70E-09**	**C10orf54**
66	**DIO2**	**-2.90**	**7.41**	**8.45E-07**	**DIO2**
67	**TRIM31**	**3.78**	**5.62**	**5.28E-08**	**TRIM31**
68	**SCNN1A**	**1.92**	**11.03**	**1.99E-09**	**SCNN1A**
69	GALM	2.66	7.95	6.12E-08	

Rank	Gene	Fold	A	adj. p-value
Genes +CHX				
1	CYP26A1	33.02	6.65	5.66E-16
2	PTGS1	5.74	7.07	2.53E-09
3	NRIP1	3.54	9.85	5.63E-11
4	BDKRB2	3.61	9.09	7.86E-09
5	SOX9	3.65	8.54	5.63E-11
6	TNFSF15	4.42	6.08	5.44E-10
7	CDH5	3.13	8.56	1.83E-10
8	TP53INP1	4.08	6.51	7.30E-10
9	PHLDA1	3.98	6.56	2.52E-10
10	UBD	3.81	6.50	9.51E-10
11	GPRC5A	2.46	10.03	1.02E-08
12	GATA3	2.26	10.18	3.83E-09
13	NAV1	2.53	8.98	3.83E-09
14	DUSP4	2.41	9.16	3.11E-08
15	C10orf54	2.77	7.98	9.00E-09
16	MAG	3.71	5.86	7.29E-08
17	CA12	1.88	11.22	4.71E-06
18	EPDR1	3.37	6.25	3.36E-10
19	ZMIZ1	1.80	11.65	2.04E-04
20	ELF3	1.75	11.80	6.12E-08
21	KLHL24	2.34	8.74	7.32E-06
22	TFPI2	2.32	8.75	6.20E-07
23	C10orf58	1.70	11.90	9.16E-08
24	CNP	1.84	10.92	3.02E-06
25	ARL8A	2.17	9.18	2.47E-07
26	DHRS3	2.17	9.17	2.47E-07
27	C2orf54	2.38	8.27	9.69E-06
28	TRIP10	2.20	8.85	1.42E-07
29	CAMK2G	1.75	10.99	9.16E-08
30	PLA2G10	2.79	6.87	2.51E-06
31	AP1G1	1.79	10.65	3.25E-07
32	DDIT4	1.94	9.75	2.56E-07
33	PCTP	2.52	7.49	3.55E-09
34	KIAA1026	2.20	8.54	2.23E-07
35	NXT1	1.83	10.29	1.35E-06
36	TMPRSS2	2.41	7.64	3.85E-07
37	FBXO32	1.91	9.50	9.16E-08
38	LASP1	1.62	10.98	1.14E-06
39	OLAH	2.45	7.23	1.14E-06
40	ST3GAL1	2.23	7.95	9.69E-06
41	FAM83A	2.66	6.63	1.24E-08
42	NANOS1	2.07	8.53	8.49E-08
43	VLDLR	3.19	5.52	3.02E-09
44	NCOR2	1.66	10.50	4.41E-06
45	MT1X	1.53	11.15	4.67E-04
46	HOXC13	1.65	10.33	5.34E-06
47	MID1IP1	1.95	8.70	1.89E-07
48	PSCD3	1.67	10.16	8.74E-08
49	RGS10	1.58	10.69	2.14E-06
50	KLF4	-1.82	9.29	5.52E-06
51	TSPAN14	1.83	9.12	1.28E-07
52	SLC16A5	2.51	6.61	8.77E-07
53	JAG2	2.30	7.20	7.76E-08
54	MYADM	1.60	10.33	2.15E-06
55	RHOU	1.69	9.75	4.02E-06
56	BAMBI	1.52	10.82	1.39E-06
57	GPR160	1.58	10.36	3.39E-03
58	RAI14	2.07	7.91	8.74E-08
59	TMC5	2.64	6.16	1.70E-06
60	SLC22A5	1.81	9.00	1.56E-07
61	IER3	1.57	10.29	2.17E-05
62	SYTL2	1.96	8.23	2.81E-07
63	CITED4	1.70	9.53	1.80E-03
64	AUTS2	2.23	7.24	2.01E-07
65	SLC26A2	1.75	9.17	1.38E-05
66	GZF1	1.81	8.86	2.57E-07
67	MT1P2	1.54	10.40	3.60E-04
68	DUSP1	-1.50	10.63	1.30E-05
69	CASP9	2.07	7.67	1.42E-07

Rank	Gene	Fold	A	adj. p-value	Regulated RA+CHX
Genes -CHX					
70	TRIB3	-1.91	11.07	3.70E-09	
71	**IRF1**	**2.63**	**8.03**	**2.47E-08**	**IRF1**
72	**OLAH**	**2.96**	**7.12**	**5.17E-09**	**OLAH**
73	IDH1	1.94	10.79	4.10E-07	
74	RASA3	-2.50	8.32	1.19E-08	
75	**RHOU**	**2.11**	**9.75**	**1.75E-08**	**RHOU**
76	ACADM	1.91	10.72	3.99E-05	
77	MTUS1	2.64	7.74	1.56E-03	
78	MUC20	2.99	6.83	1.99E-09	
79	ATP8B1	2.12	9.62	6.37E-08	
80	BTG2	2.73	7.43	5.44E-09	
81	**M-RIP**	**1.86**	**10.89**	**2.16E-08**	**M-RIP**
82	CYP1A1	-1.98	10.22	6.20E-08	
83	CLIC3	-2.58	7.83	1.40E-07	
84	**ITGAL**	**3.92**	**5.05**	**1.31E-09**	**ITGAL**
85	**SERPINB1**	**3.13**	**6.33**	**6.67E-10**	**SERPINB1**
86	**BAMBI**	**1.83**	**10.82**	**4.46E-09**	**BAMBI**
87	**NAV1**	**2.06**	**9.55**	**1.13E-06**	**NAV1**
88	**DUSP1**	**-1.85**	**10.63**	**2.42E-08**	**DUSP1**
89	ENPP4	2.63	7.46	1.72E-07	
90	TRIM47	-2.03	9.60	4.01E-08	
91	**GATA3**	**1.90**	**10.18**	**2.65E-08**	**GATA3**
92	**EMP1**	**2.22**	**8.70**	**8.53E-10**	**EMP1**
93	RBPMS	2.03	9.46	1.26E-06	
94	CD24	1.63	11.85	4.50E-03	
95	SLC31A2	2.07	9.27	3.70E-09	
96	**CAMK2G**	**1.67**	**11.42**	**1.86E-07**	**CAMK2G**
97	ODC1	-1.81	10.54	6.06E-08	
98	ELF5	-2.80	6.81	6.67E-10	
99	**PCDH20**	**3.09**	**6.13**	**1.96E-10**	**PCDH20**
100	ARHGDIB	1.94	9.79	1.29E-06	

Rank	Gene	Fold	A	adj. p-value
Genes +CHX				
70	MTHFR	2.19	7.20	3.65E-06
71	SCNN1A	1.42	11.03	1.46E-05
72	VASN	1.50	10.48	1.78E-03
73	SLC40A1	-1.71	9.07	3.58E-06
74	KRT40	2.41	6.42	7.15E-08
75	MT1H	1.55	9.99	1.54E-03
76	DKK1	-2.38	6.46	7.84E-07
77	SLC45A3	1.63	9.45	3.23E-05
78	PNKD	1.46	10.53	1.19E-04
79	NR2C2	1.83	8.37	8.77E-07
80	ARHGEF10L	2.45	6.20	4.52E-08
81	SDC4	1.40	10.92	7.02E-03
82	C15orf39	1.85	8.20	2.01E-05
83	FAM113B	1.71	8.78	2.37E-06
84	ZNRF1	1.55	9.61	2.99E-04
85	TMEM64	-2.00	7.42	6.33E-04
86	FBXO34	1.68	8.83	1.82E-05
87	CAMK2N1	1.47	10.06	4.97E-06
88	DIO2	-2.00	7.41	3.60E-04
89	NR2F2	1.73	8.54	3.02E-06
90	SERPINF1	2.67	5.51	2.84E-07
91	ARHGAP8	1.49	9.86	7.49E-04
92	GALNAC4S-6ST	1.65	8.81	5.89E-07
93	TRAF4	1.75	8.24	2.60E-07
94	TIAF1	1.48	9.85	8.08E-03
95	CASP3	1.63	8.84	6.15E-07
96	ALDH3B1	1.57	9.16	4.56E-04
97	C20orf175	1.83	7.85	1.90E-05
98	BIN1	1.73	8.26	2.34E-05
99	MT1G	1.44	9.96	1.33E-03
100	SMAD3	1.71	8.32	4.16E-07

Rank	Gene	Fold	A	adj. p-value	Regulated +CHX	Rank	Gene	Fold	A	adj. p-value
Genes -CHX						*Genes +CHX*				
1	**CYP26A1**	**25.35**	**7.96**	**5.88E-13**	**CYP26A1**	1	CYP26A1	44.86	7.96	3.55E-14
2	**DHRS3**	**11.47**	**7.48**	**5.88E-13**	**DHRS3**	2	DHRS3	4.71	7.48	3.32E-09
3	**SOX9**	**9.50**	**8.45**	**5.88E-13**	**SOX9**	3	BDKRB2	4.09	7.04	5.43E-06
4	**HOXA5**	**8.19**	**7.31**	**5.36E-10**	**HOXA5**	4	SCNN1A	2.92	9.25	1.14E-07
5	**BDKRB2**	**6.41**	**7.04**	**3.90E-08**	**BDKRB2**	5	CA12	2.72	9.28	2.12E-06
6	**TGFBI**	**4.76**	**7.47**	**1.12E-10**	**TGFBI**	6	KLHDC2	2.27	10.22	7.39E-06
7	**SCNN1A**	**3.79**	**9.25**	**9.95E-10**	**SCNN1A**	7	NRIP1	2.03	10.90	8.36E-05
8	**EPAS1**	**3.59**	**9.47**	**9.13E-09**	**EPAS1**	8	TSC22D3	2.25	9.62	6.82E-06
9	AMIGO2	-2.73	10.12	3.90E-07		9	SOX9	2.56	8.45	8.55E-07
10	**PFKFB3**	**3.09**	**8.92**	**9.21E-09**	**PFKFB3**	10	HOXB3	3.42	6.29	3.30E-07
11	MAFB	-3.61	7.45	5.35E-08		11	AP1G1	2.22	9.27	6.82E-06
12	**CA12**	**2.89**	**9.28**	**2.49E-07**	**CA12**	12	IDH1	1.99	9.90	1.95E-05
13	LXN	2.34	11.25	1.14E-05		13	SERPINA3	2.83	6.88	1.75E-04
14	CYP26B1	4.79	5.49	4.82E-07		14	TFPI2	2.95	6.57	6.61E-07
15	PPM1E	3.56	7.21	2.13E-07		15	TP53INP1	2.06	9.09	1.01E-04
16	**ELF3**	**2.92**	**8.69**	**1.73E-07**	**ELF3**	16	GPRC5A	2.07	8.98	2.36E-05
17	**BTG2**	**2.65**	**9.24**	**9.48E-08**	**BTG2**	17	MBOAT1	2.84	6.50	4.14E-06
18	**KLHDC2**	**2.40**	**10.22**	**1.02E-06**	**KLHDC2**	18	CNP	2.45	7.49	5.43E-06
19	**IDH1**	**2.47**	**9.90**	**1.81E-07**	**IDH1**	19	PFKFB3	1.97	8.92	2.36E-05
20	GDF15	3.12	7.77	2.28E-07		20	ELF3	1.95	8.69	1.95E-04
21	**SKAP2**	**2.92**	**8.14**	**2.36E-08**	**SKAP2**	21	SOX2	-1.80	9.34	1.16E-03
22	CAPN13	4.58	5.19	1.16E-08		22	ZEB1	2.08	8.07	2.65E-04
23	**MYADM**	**2.49**	**9.49**	**5.06E-07**	**MYADM**	23	EPAS1	1.77	9.47	8.09E-04
24	SELL	4.01	5.63	3.58E-07		24	MYADM	1.75	9.49	6.45E-04
25	**TSC22D3**	**2.32**	**9.62**	**1.39E-06**	**TSC22D3**	25	MPPED2	2.85	5.71	4.86E-04
26	**AP1G1**	**2.40**	**9.27**	**6.50E-07**	**AP1G1**	26	AKAP1	1.73	9.37	1.16E-03
27	**GPR160**	**2.36**	**9.41**	**9.21E-07**	**GPR160**	27	TGFBI	2.16	7.47	6.82E-06
28	HK2	3.46	6.40	2.13E-08		28	CRISPLD2	2.14	7.54	1.72E-04
29	**HOXB3**	**3.48**	**6.29**	**5.08E-08**	**HOXB3**	29	TNFAIP2	2.72	5.89	2.90E-06
30	STK39	2.66	8.16	2.61E-06		30	SLC35C1	1.87	8.46	1.02E-04
31	**MBOAT1**	**3.22**	**6.50**	**2.35E-07**	**MBOAT1**	31	CBFA2T3	1.83	8.65	9.11E-05
32	LITAF	1.98	10.51	3.73E-05		32	FAM3C	2.11	7.43	7.95E-05
33	**GPRC5A**	**2.31**	**8.98**	**1.15E-06**	**GPRC5A**	33	NRCAM	-1.80	8.75	2.55E-03
34	LIMA1	2.31	8.88	6.42E-06		34	SLC22A5	2.01	7.77	5.02E-05
35	**ST8SIA4**	**-3.54**	**5.75**	**2.57E-07**	**ST8SIA4**	35	EFEMP1	-1.77	8.75	7.76E-04
36	**SLC22A5**	**2.62**	**7.77**	**2.35E-07**	**SLC22A5**	36	SKAP2	2.11	7.34	1.04E-03
37	LAMC1	2.59	7.80	2.47E-06		37	BTG2	1.67	9.24	8.18E-04
38	**AKAP1**	**2.14**	**9.37**	**9.17E-06**	**AKAP1**	38	RAP1GAP	2.24	6.81	1.12E-05
39	DKK1	-1.89	10.51	3.24E-05		39	FBXO34	1.76	8.57	7.89E-04
40	LOC400451	2.58	7.70	2.60E-05		40	GPR160	1.59	9.41	3.94E-03
41	PHLDA1	2.35	8.39	8.04E-07		41	ZNF503	2.10	7.08	1.88E-04
42	NQO1	1.69	11.69	2.58E-04		42	PLA2G10	1.90	7.80	1.01E-03
43	**TMPRSS2**	**3.66**	**5.32**	**5.08E-08**	**TMPRSS2**	43	FOXA1	1.65	8.92	1.18E-03
44	NFKBIZ	2.12	9.09	4.03E-05		44	NET1	1.49	9.88	9.58E-03
45	KITLG	-2.05	9.34	2.59E-05		45	MBP	2.38	6.03	2.01E-04
46	**NANS**	**2.19**	**8.70**	**1.64E-06**	**NANS**	46	SDC2	-2.09	6.84	2.87E-04
47	HIG2	1.99	9.54	4.01E-06		47	ADCY1	-1.80	7.87	3.42E-03
48	ZFP36L1	1.91	9.82	1.43E-04		48	DYRK2	1.84	7.68	1.50E-04
49	**FAM43A**	**2.33**	**7.96**	**3.67E-06**	**FAM43A**	49	NCOR2	1.69	8.32	7.19E-03
50	EPHA7	-3.05	6.06	6.10E-06		50	NANS	1.60	8.70	1.98E-03
51	KYNU	-1.67	11.03	5.34E-04		51	PCDH10	-1.88	7.31	4.02E-05
52	**MLPH**	**1.66**	**10.95**	**4.06E-04**	**MLPH**	52	PCTP	1.70	8.05	1.18E-03
53	SEMA3C	-1.78	10.19	5.60E-04		53	HOXA5	1.87	7.31	6.63E-03
54	**SOX2**	**-1.94**	**9.34**	**1.14E-04**	**SOX2**	54	APOBEC3B	1.74	7.69	2.87E-04
55	**PCDH10**	**-2.47**	**7.31**	**1.10E-07**	**PCDH10**	55	EDG3	-1.88	7.13	6.02E-04
56	TRIM16	1.97	9.14	9.17E-06		56	SULT1A1	1.82	7.37	4.94E-04
57	RNASE4	3.56	5.04	2.18E-07		57	AHRR	1.77	7.53	7.26E-04
58	**PLA2G10**	**2.25**	**7.80**	**2.48E-05**	**PLA2G10**	58	INSIG1	1.70	7.82	2.41E-03
59	GALNT7	1.83	9.58	7.43E-05		59	RNF207	1.89	6.93	4.18E-04
60	NTN4	2.48	7.06	9.00E-07		60	FAM43A	1.64	7.96	5.06E-03
61	**FBXO34**	**2.03**	**8.57**	**1.95E-05**	**FBXO34**	61	JAG2	1.65	7.84	1.18E-03
62	GPR30	-2.12	8.21	2.43E-06		62	FLJ10081	1.58	8.19	5.76E-03
63	SIX4	1.97	8.81	8.77E-05		63	ST8SIA4	-2.23	5.82	7.84E-03
64	LMCD1	2.04	8.44	1.92E-05		64	CPEB2	-1.59	8.17	9.38E-03
65	**EFEMP1**	**-1.96**	**8.75**	**4.15E-05**	**EFEMP1**	65	DUSP4	1.73	7.38	1.25E-03
66	CAV2	-1.83	9.33	1.43E-04		66	Gcom1	2.18	5.77	6.63E-05
67	**TFPI2**	**2.60**	**6.57**	**8.04E-07**	**TFPI2**	67	BHLHB2	1.52	8.35	6.30E-03
68	**ADCY1**	**-2.16**	**7.87**	**6.88E-05**	**ADCY1**	68	EPB41L4A	1.96	6.41	2.55E-03
69	DOCK8	2.34	7.21	2.44E-04		69	TMPRSS2	2.33	5.32	4.49E-05

Rank	Gene	Fold	A	adj. p-value	Regulated +CHX
Genes -CHX					
70	TRAK1	2.03	8.31	3.34E-05	
71	**CRISPLD2**	**2.23**	**7.54**	**3.07E-05**	**CRISPLD2**
72	TMTC1	-2.49	6.75	1.75E-04	
73	TPD52L1	1.53	10.91	1.64E-03	
74	**ZEB1**	**2.06**	**8.07**	**9.63E-05**	**ZEB1**
75	CTSH	2.01	8.27	1.54E-05	
76	BZW1	1.60	10.43	1.89E-03	
77	**NRIP1**	**1.53**	**10.90**	**5.79E-03**	**NRIP1**
78	ITPR1	2.41	6.88	4.01E-07	
79	**TP53INP1**	**1.82**	**9.09**	**3.06E-04**	**TP53INP1**
80	BCL6	1.73	9.53	8.96E-05	
81	TRIM14	2.30	7.17	4.09E-06	
82	**CPEB2**	**-2.02**	**8.17**	**3.91E-05**	**CPEB2**
83	ANXA9	2.13	7.73	9.32E-05	
84	DLX2	-1.85	8.88	5.78E-05	
85	EPB41L4B	1.85	8.88	2.85E-05	
86	ANXA1	-2.44	6.73	6.10E-06	
87	**SLITRK6**	**-2.62**	**6.24**	**1.08E-06**	**SLITRK6**
88	IRF2BP2	1.58	10.35	1.52E-03	
89	SMAD3	1.83	8.89	6.77E-05	
90	MYC	2.28	7.13	5.03E-06	
91	**SLC35C1**	**1.92**	**8.46**	**2.42E-05**	**SLC35C1**
92	WWP1	1.61	10.08	2.46E-03	
93	SAT1	1.84	8.78	4.34E-05	
94	MTHFD2	1.58	10.17	1.42E-03	
95	BAMBI	1.72	9.30	4.76E-04	
96	**AHRR**	**2.12**	**7.53**	**9.94E-06**	**AHRR**
97	RBPMS	1.97	8.07	4.42E-05	
98	**NCOR2**	**1.91**	**8.32**	**2.99E-04**	**NCOR2**
99	CAV1	-1.80	8.80	1.04E-04	
100	XBP1	1.61	9.82	1.59E-03	

Rank	Gene	Fold	A	adj. p-value
Genes +CHX				
70	PTGER4	-2.11	5.86	4.86E-04
71	SQSTM1	1.72	7.12	1.21E-03
72	SYNPO2	2.05	5.91	1.46E-04
73	MLPH	1.59	7.55	8.00E-03
74	C16orf14	1.53	7.76	3.84E-03
75	SLITRK6	-1.88	6.24	6.02E-04
76	HHEX	1.90	6.15	1.41E-03
77	PARP9	1.51	7.74	6.91E-03
78	RBMS1	-1.56	7.36	7.76E-03
79	LOC57228	1.61	7.18	8.06E-03
80	FRMD3	1.51	7.60	7.59E-03
81	PHF8	1.78	6.38	6.02E-04
82	FOXC1	2.03	5.48	4.21E-03
83	BCAS1	1.61	6.93	7.87E-03
84	KLHL28	1.47	7.55	8.53E-03
85	TNFRSF1A	1.59	6.96	2.41E-03
86	PREP	-1.72	6.41	5.43E-03
87	TMEM46	1.74	6.21	1.52E-03
88	CBLB	1.54	7.00	3.45E-03
89	FJX1	-1.57	6.81	7.87E-03
90	VPS13D	1.59	6.68	2.96E-03
91	ELF5	-1.58	6.68	5.43E-03
92	CLDN1	-1.98	5.25	1.27E-03
93	TGM2	1.78	5.85	3.11E-03
94	SYNJ2	1.54	6.77	7.02E-03
95	UBD	1.84	5.56	6.38E-04
96	PRKCD	1.68	5.94	3.38E-03
97	SERPINA5	1.87	5.33	7.49E-03
98	TRAF4	1.59	6.11	2.12E-03
99	STRA6	1.84	5.14	1.08E-03
100	FGD3	1.53	6.04	9.47E-03

TRANSFAC gene id	Gene	TFCat DBD Classification (Protein Group/Protein Family)	Description	Synonyms	Matrix	consen sus	TFBS enrichment	1.5 Fold RARa ChIP-chip MCF7	2 Fold RARa ChIP-chip MCF7	1.5 Fold RARa ChIP-seq MCF7
G000076	T	Beta-Hairpin-Ribbon Group/Transcription Factor Domain	T, brachyury homolog (mouse)	T; T, brachyury homolog (mouse); TFT	V_BRACH_01.M00108	WGMTTACACCTAGGTGTGAAATT				
G000076	T		T, brachyury homolog (mouse)	T; T, brachyury homolog (mouse); TFT	V_BRACH_01.M00108	WGMTTACACCTAGGTGTGAAATT				
G000432	TBX5	Beta-Hairpin-Ribbon Group/Transcription Factor N Domain	T-box 5	HOS; T-box 5; TBX5	V_TBX5_01.M01019	SSAGGTGTGASS				
G000432	TBX5		T-box 5	HOS; T-box 5; TBX5	V_TBX5_02.M01020	SSRGGTGTGC				
G000432	TBX5		T-box 5	HOS; T-box 5; TBX5	V_TBX5_Q5.M01044	CTCACACCTT				
G000432	TBX5		T-box 5	HOS; T-box 5; TBX5	V_TBX5_Q5.M01044	CTCACACCTT				
G000432	TBX5		T-box 5	HOS; T-box 5; TBX5	V_TBX5_Q5.M01044	CTCACACCTT				
G002602	ARID5B	Helix-Turn-Helix Group/ARID Domain Family	AT rich interactive domain 5B (MRF1-like)		V_E2A_Q6.M00973	CACCTGSNT				
G002602	ARID5B		AT rich interactive domain 5B (MRF1-like)		V_E2A_Q6.M00973	CACCTGSNT				
G002602	ARID5B		AT rich interactive domain 5B (MRF1-like)	ARID5B; AT rich interactive domain 5B; AT rich interactive domain 5B (MRF1-like); DESRT; FLJ21150; modulator recognition factor 2; Mrf2	V_E2A_Q6.M00973	CACCTGSNT				
G002602	ARID5B		AT rich interactive domain 5B (MRF1-like)		V_EBOX_Q6_01.M01034	SCACSTGRCN				
G002602	ARID5B		AT rich interactive domain 5B (MRF1-like)		V_EBOX_Q6_01.M01034	SCACSTGRCN				
G002602	ARID5B		AT rich interactive domain 5B (MRF1-like)		V_EBOX_Q6_01.M01034	SCACSTGRCN				
G002602	ARID5B		AT rich interactive domain 5B (MRF1-like)		V_MRF2_01.M00454	AMSCACAATACSTA				
G002603	ALX1	Helix-Turn-Helix Group/Homeodomain Family	ALX homeobox 1		V_CART1_01.M00416	SRSTAATTRSCATTASCN				
G002603	ALX1		ALX homeobox 1		V_CART1_01.M00416	SRSTAATTRSCATTASCN				
G002603	ALX1		ALX homeobox 1	ALX homeobox 1; Alx1; Cart1; cartilage paired-class homeoprotein 1	V_CART1_02.M01362	WGRSTTAATTARTRSHN				
G002603	ALX1		ALX homeobox 1		V_CART1_02.M01362	WGRSTTAATTARTRSHN				
G002603	ALX1		ALX homeobox 1		V_CART1_03.M01453	WSRSTT AATTAARRSHN				
G002603	ALX1		ALX homeobox 1		V_CART1_03.M01453	WSRSTT AATTAARRSHN				
G001915	BARX1	Helix-Turn-Helix Group/Homeodomain Family	BarH-like homeobox gene 1	BarH-like homeobox 1; Barx1	V_BARX1_01.M01340	WSRSTAATTRSTRSAWM				
G001915	BARX1		BarH1-like homeobox gene 1	BarH-like homeobox 1; Barx1	V_BARX1_01.M01340	WSRSTAATTRSTRSAWM				
G001921	BARX2		BarH1-like homeobox gene 2	BarH-like homeobox 2; BARX2	V_BARX2_01.M01431	TAAATAATTAATTRSTTN				
G001921	BARX2		BarH1-like homeobox gene 2	BarH-like homeobox 2; BARX2	V_BARX2_01.M01431	TAAATAATTAATTRSTTN				
G004019	CDX1	Helix-Turn-Helix Group/Homeodomain Family	caudal type homeo box transcription factor 1	caudal type homeo box transcription factor 1; CDX1	V_CDX1_01.M01373	SRRGGTMATAAAARRHN		V_CDX1_01.M01373		
G004019	CDX1		caudal type homeo box transcription factor 1	caudal type homeo box transcription factor 1; CDX1	V_CDX1_01.M01373	SRRGGTMATAAAARRHN		V_CDX1_01.M01373		
G004019	CDX1		caudal type homeo box transcription factor 1	caudal type homeo box transcription factor 1; CDX1	V_CDX_Q5.M00991	WATRRHHARAATTHATAAA				
G004019	CDX1		caudal type homeo box transcription factor 1	caudal type homeo box transcription factor 1; CDX1	V_CDX_Q5.M00991	WATRRHHARAATTHATAAA				
G002226	CDX2	Helix-Turn-Helix Group/Homeodomain Family	caudal type homeobox 2	caudal type homeobox 2; caudal type homeobox 2; Cdx-3; CDX2; CDX3	V_CDX_Q5.M00991	WATRRHHARAATTHATAAA				
G002226	CDX2		caudal type homeobox 2		V_CDX_Q5.M00991	WATRRHHARAATTHATAAA				
G002226	CDX2		caudal type homeobox 2		V_CDX2_01.M01449	SRRGGTMATAAAARRHN				
G002226	CDX2		caudal type homeobox 2		V_CDX2_01.M01449	SRRGGTMATAAAARRHN				
G002226	CDX2		caudal type homeobox 2		V_CDX2_Q5.M00729	AMAATTTTATRKCC				
G002226	CDX2		caudal type homeobox 2		V_CDX2_Q5.M00729	AMAATTTTATRKCC				
G002226	CDX2		caudal type homeobox 2		V_CDX2_Q5_01.M01659	SRHATAAARRH				
G002226	CDX2		caudal type homeobox 2		V_CDX2_Q5_01.M01659	SRHATAAARRH				
G004498	CRX	Helix-Turn-Helix Group/Homeodomain Family	cone-rod homeobox	Cone-rod homeo box-containing gene; cone-rod homeobox CORD2; CRD; CRX	V_CRX_02.M01436	YSRRGGGGATARCCH				
G004498	CRX		cone-rod homeobox		V_CRX_02.M01436	YSRRGGGGATARCCH				
G004498	CRX		cone-rod homeobox		V_CRX_Q4.M00623	YSRRTAATCYSRH				
G004498	CRX		cone-rod homeobox		V_CRX_Q4.M00623	YSRRTAATCYSRH				
G004498	CRX		cone-rod homeobox		V_CRX_Q4_01.M01712	YTAATC				
G004498	CRX		cone-rod homeobox		V_CRX_Q4_01.M01712	YTAATC				
G002028	DLX4	Helix-Turn-Helix Group/Homeodomain Family	distal-less homeobox 4	BP1; distal-less homeobox 4; DLX4; DLX7; DLX8; DLX9	V_DLX7_01.M01486	WCSRRHATAATTACMRHC				
G002028	DLX4		distal-less homeobox 4	BP1; distal-less homeobox 4; DLX4; DLX7; DLX8; DLX9	V_DLX7_01.M01486	WCSRRHATAATTACMRHC				
G002028	DLX4		distal-less homeobox 4	BP1; distal-less homeobox 4; DLX4; DLX7; DLX8; DLX9	V_DLX7_01.M01486	WCSRRHATAATTACMRHC				
G002012	DLX5		distal-less homeobox 5	distal-less homeo box 5; distal-less homeobox 5; DLX5	V_DLX5_01.M01388	WSRGTAATTRTTRRHN				
G002012	DLX5		distal-less homeobox 5	distal-less homeo box 5; distal-less homeobox 5; DLX5	V_DLX5_01.M01388	WSRGTAATTRTTRRHN				
G010054	DMBX1	Helix-Turn-Helix Group/Homeodomain Family	dienocephalonmesencephalon homeobox 1	dienocephalon/mesencephalon homeobox 1; Dmbx1; MBX; otx3; pax6	V_OTX3_01.M01403	WSRRHRMGGATTARRNTRH				
G010054	DMBX1		dienocephalonmesencephalon homeobox 1		V_OTX3_01.M01403	WSRRHRMGGATTARRNTRH				
G008021	EMX2	Helix-Turn-Helix Group/Homeodomain Family	spiracles homolog 2 (Drosophila)	spiracles homolog 2 (Drosophila); EMX2	V_EMX2_01.M01461	WRRRCTAATTAGTTRTRH				
G008021	EMX2		spiracles homolog 2 (Drosophila)	spiracles homolog 2 (Drosophila); EMX2	V_EMX2_01.M01461	WRRRCTAATTAGTTRTRH				
G002103	EN1	Helix-Turn-Helix Group/Homeodomain Family	engrailed homeobox 1	EN1; engrailed homeobox 1; engrailed homolog 1	V_EN1_01.M00396	GTAATTHN				
G002103	EN1		engrailed homeobox 1	EN1; engrailed homeobox 1; engrailed homolog 1	V_EN1_01.M00396	GTAATTHN				
G002103	EN1		engrailed homeobox 1	EN1; engrailed homeobox 1; engrailed homolog 1	V_EN1_02.M01365	WRHAAC TAATTARRTH				
G002103	EN1		engrailed homeobox 1	EN1; engrailed homeobox 1; engrailed homolog 1	V_EN1_02.M01365	WRHAAC TAATTARRTH				
G002104	EN2	Helix-Turn-Helix Group/Homeodomain Family	engrailed homeobox 2	En-2; EN2; engrailed homeobox 2; engrailed homolog 2	V_EN2_01.M01455	WGRRTTAATTANTRRHRN				
G002104	EN2		engrailed homeobox 2	En-2; EN2; engrailed homeobox 2; engrailed homolog 2	V_EN2_01.M01455	WGRRTTAATTANTRRHRN				
G004483	HOXA1	Helix-Turn-Helix Group/Homeodomain Family	homeo box A1	homeo boxA1; Hox1; HOX1F; HOXA1; MGC45232	V_HOXA1_01.M01487	SYRAGCTAATTARRTRTT				
G004483	HOXA1		homeo box A1	homeo boxA1; Hox1; HOX1F; HOXA1; MGC45232	V_HOXA1_01.M01487	SYRAGCTAATTARRTRTT				
G013852	HOXA10	Helix-Turn-Helix Group/Homeodomain Family	homeo box A10	homeo boxA10; HOX18; HOX1H; HOXA10; MGC12859; PL	V_HOXA10_01.M01464	SRRGTAATAAAAATRHM		V_HOXA10_01.M01464		
G013852	HOXA10		homeo box A10	homeo boxA10; HOX18; HOX1H; HOXA10; MGC12859; PL	V_HOXA10_01.M01464	SRRGTAATAAAAATRHM		V_HOXA10_01.M01464		
G013852	HOXA10		homeo box A10	homeo boxA10; HOX18; HOX1H; HOXA10; MGC12859; PL	V_HOXA10_01.M01464	SRRGTAATAAAAATRHM		V_HOXA10_01.M01464		
G013853	HOXA2	Helix-Turn-Helix Group/Homeodomain Family	homeo box A2	homeo boxA2; HOXA2	V_HOXA2_01.M01402	ARGGTAATTAHCRRAH				
G013853	HOXA2		homeo box A2	homeo boxA2; HOXA2	V_HOXA2_01.M01402	ARGGTAATTAHCRRAH				
G013854	HOXA3	Helix-Turn-Helix Group/Homeodomain Family	homeo box A3	homeo boxA3; HOX1E; HOXA3; MGC10155	V_HOXA3_01.M00395	CGTARRHHRH				
G013854	HOXA3		homeo box A3	homeo boxA3; HOX1E; HOXA3; MGC10155	V_HOXA3_02.M01337	GARRRHHTAATTARY				
G006215	HOXA7	Helix-Turn-Helix Group/Homeodomain Family	homeo box A7	Antp; homeo box A7; HOX11; HOX1A; HOXA7; Quox-1	V_HOXA7_01.M01108	YCAATCT				
G013857	HOXA9	Helix-Turn-Helix Group/Homeodomain Family	homeo box A9	ABD-B; homeo box A9; HOX17; HOX1G; HOXA9; MGC1934	V_HOXA9_01.M01351	RCGGYCATAAAAATAHM				
G013857	HOXA9		homeo box A9	ABD-B; homeo box A9; HOX17; HOX1G; HOXA9; MGC1934	V_HOXA9_01.M01351	RCGGYCATAAAAATAHM				
G013857	HOXA9		homeo box A9	ABD-B; homeo box A9; HOX17; HOX1G; HOXA9; MGC1934	V_HOXA9_01.M01351	RCGGYCATAAAAATAHM				
G013857	HOXA9		homeo box A9	ABD-B; homeo box A9; HOX17; HOX1G; HOXA9; MGC1934	V_MEIS1AHOXA9_01.M00420	TGACAGRTTTATGA				
G013857	HOXA9		homeo box A9	ABD-B; homeo box A9; HOX17; HOX1G; HOXA9; MGC1934	V_MEIS1BHOXA9_02.M00421	TGACAATTTTMATRR				
G009913	HOXB5	Helix-Turn-Helix Group/Homeodomain Family	homeo box B5	HHOC10; homeo box B5; HOX2; Hox21; HOX2A; HOXB5; HU	V_HOXB5_01.M01319	AAGGTAATTAACSRAAH		V_HOXB5_01.M01319		
G009913	HOXB5		homeo box B5	1	V_HOXB5_01.M01319	AAGGTAATTAACSRAAH		V_HOXB5_01.M01319		
G009916	HOXB9	Helix-Turn-Helix Group/Homeodomain Family	homeo box B9	homeo box B9; Hox-2E; HOX2; HOX2E; HOXB9	V_HOXB9_01.M01426	SRRGCHATAAAAATRHN				
G009916	HOXB9		homeo box B9	homeo box B9; Hox-2E; HOX2; HOX2E; HOXB9	V_HOXB9_01.M01426	SRRGCHATAAAAATRHN				
G006220	HOXC10	Helix-Turn-Helix Group/Homeodomain Family	homeo box C10	homeo box C10; HOX3I; HOXC10; MGC5259	V_HOXC10_01.M01361	SRRRRGTCGTAAAARRHN				
G006220	HOXC10		homeo box C10	homeo box C10; HOX3I; HOXC10; MGC5259	V_HOXC10_01.M01361	SRRRRGTCGTAAAARRHN				
G006221	HOXC11	Helix-Turn-Helix Group/Homeodomain Family	homeo box C11	homeo box C11; HOX3H; HOXC11; MGC4906	V_HOXC11_01.M01329	SAAAYGTCGTAAAARRHN				
G006221	HOXC11		homeo box C11	homeo box C11; HOX3H; HOXC11; MGC4906	V_HOXC11_01.M01329	SAAAYGTCGTAAAARRHN				
G006225	HOXC4	Helix-Turn-Helix Group/Homeodomain Family	homeo box C4	cp19; homeo box C4; HOX3; HOX3E; HOXC4	V_HOXC4_01.M01369	CSRRTTTAATTAARRATRH				
G006225	HOXC4		homeo box C4	cp19; homeo box C4; HOX3; HOX3E; HOXC4	V_HOXC4_01.M01369	CSRRTTTAATTAARRATRH				
G006234	HOXD13	Helix-Turn-Helix Group/Homeodomain Family	homeo box D13	homeo box D13; HOX4I; hoxd13; SPD	V_HOXD13_01.M01404	SRRTYTAATAAAARRHN				
G006234	HOXD13		homeo box D13	homeo box D13; HOX4I; hoxd13; SPD	V_HOXD13_01.M01404	SRRTYTAATAAAARRHN				
G006236	HOXD3	Helix-Turn-Helix Group/Homeodomain Family	homeo box D3	homeo box D3; Hox-4.1; HOX4; HOX4A; HOXD3; MGC10470	V_HOXD3_01.M01338	YTGRRRTAATTARRRHN		V_HOXD3_01.M01338		
G006236	HOXD3		homeo box D3	homeo box D3; Hox-4.1; HOX4; HOX4A; HOXD3; MGC10470	V_HOXD3_01.M01338	YTGRRRTAATTARRRHN		V_HOXD3_01.M01338		
G004093	LHX2	Helix-Turn-Helix Group/Homeodomain Family	LIM homeobox gene 2	hLhx2; LH2; LHX2; LIM homeobox 2	V_LH2_01.M01325	WSRACTAATTAGRRHRN				
G004093	LHX2		LIM homeobox gene 2	hLhx2; LH2; LHX2; LIM homeobox 2	V_LH2_01.M01325	WSRACTAATTAGRRHRN				
G014352	LHX3	Helix-Turn-Helix Group/Homeodomain Family	LIM homeobox 3	CPHD3; DKFZp762A2013; Lhx3; LIM homeobox 3; Lim3; M2-LHX3	V_LHX3_01.M00510	AATTAATTAA				
G014352	LHX3		LIM homeobox 3		V_LHX3_01.M00510	AATTAATTAA				
G014352	LHX3		LIM homeobox 3		V_LHX3_02.M01471	WAAATTAATTAAMRHRN				
G014352	LHX3		LIM homeobox 3		V_LHX3_02.M01471	WAAATTAATTAAMRHRN				
G014352	LHX3		LIM homeobox 3		V_LHX3_02.M01471	WAAATTAATTAAMRHRN				
G014352	LHX3		LIM homeobox 3		V_LHX3_02.M01471	WAAATTAATTAAMRHRN				
G037451	LHX4	Helix-Turn-Helix Group/Homeodomain Family	LIM homeobox 4	CPHD4; Gsh-4; Gsh4; Lhx4; LIM homeobox 4	V_LHX4_01.M01421	HARRCTAATTAGRTWTG				
G037451	LHX4		LIM homeobox 4	CPHD4; Gsh-4; Gsh4; Lhx4; LIM homeobox 4	V_LHX4_01.M01421	HARRCTAATTAGRTWTG				
G002211	LHX5	Helix-Turn-Helix Group/Homeodomain Family	LIM homeobox gene 5	Lhx5; LIM homeobox 5	V_LHX5_01.M01353	WRHATTAATTAAMRHRN				
G002211	LHX5		LIM homeobox gene 5	Lhx5; LIM homeobox 5	V_LHX5_01.M01353	WRHATTAATTAAMRHRN				
G002228	LMX1B	Helix-Turn-Helix	LIM homeobox protein 1B	LIM homeobox transcription factor 1 beta; LMX1B; LMX1B	V_LMX1B_01.M01363	SRRRRTTAATTAATRHN				

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
A2ML1	A2ML1	---	-1.42	---	---	---	---	---	---
ABCA3	ABCA3	---	2.59	---	---	---	---	---	---
ABCB1	ABCB1	---	1.73	---	---	---	---	---	---
ABHD11	ABHD11	---	-1.53	---	---	-1.45	---	---	---
ABHD6	ABHD6	---	1.53	---	---	---	---	---	---
ACSL1	ACSL1	---	1.9	---	---	---	---	---	---
ADORA2A	ADORA2A	---	-1.48	---	---	---	---	---	---
ADPRHL2	ADPRHL2	---	1.4	---	---	---	---	---	---
AFG3L2	AFG3L2	---	1.47	---	---	---	---	---	---
AFMID	AFMID	---	-1.6	---	---	---	---	---	---
AGR2	AGR2	---	1.42	---	---	-1.65	---	---	---
AK3	AK3	---	1.47	---	---	---	---	---	---
ALG6	ALG6	---	1.85	---	---	---	---	---	---
ALS2CR4	ALS2CR4	---	3.92	---	---	---	---	---	---
ANG	ANG	---	1.5	---	---	---	---	---	---
ANTXR2	ANTXR2	---	1.71	---	---	---	---	---	---
ANXA3	ANXA3	---	1.69	---	---	---	---	---	---
AP4B1	AP4B1	---	-1.43	---	---	---	---	---	---
ARHGAP24	ARHGAP24	---	1.77	---	---	---	---	---	---
ARHGEF2	ARHGEF2	---	1.48	---	1.43	1.48	---	---	---
ARID5B	ARID5B	---	1.47	1.69	---	-1.99	---	-1.62	---
ARPM1	ARPM1	---	3.45	---	---	---	---	---	---
ASPM	ASPM	---	-1.59	-1.49	---	---	---	---	---
ATAD2	ATAD2	---	-1.59	---	---	---	---	---	---
ATP6V1B1	ATP6V1B1	-1.67	-1.82	---	---	---	---	---	-2.03
ATP7B	ATP7B	---	1.64	---	---	---	---	---	---
B2M	B2M	---	1.51	---	---	---	---	---	---
B4GALT5	B4GALT5	1.43	1.49	---	---	---	---	---	---
BAMBI	BAMBI	---	1.87	---	1.52	1.83	---	1.72	1.64
BATF2	BATF2	---	1.66	1.56	---	---	---	---	---
BCKDHB	BCKDHB	---	1.42	---	---	---	---	---	---
BCL2L13	BCL2L13	---	1.45	---	---	---	---	---	---
BCL6	BCL6	---	1.83	---	---	---	---	1.73	---
BCLAF1	BCLAF1	---	-1.42	---	---	---	---	---	---
BFSP1	BFSP1	---	-1.52	---	---	-1.56	---	---	---
BLMH	BLMH	---	2.39	---	---	---	---	---	---
BLOC1S1	BLOC1S1	---	1.74	---	---	---	---	---	---
BTG1	BTG1	---	1.4	---	---	---	---	---	---
BTN2A1	BTN2A1	---	1.55	---	---	---	---	---	---
BTN3A1	BTN3A1	---	1.44	---	---	---	---	---	---
BTN3A2	BTN3A2	---	1.71	1.44	---	1.49	---	---	---
BTN3A3	BTN3A3	---	1.97	1.43	---	1.72	---	---	---
C11ORF51	C11orf51	---	2.05	---	---	---	---	---	---
C11ORF67	C11orf67	---	1.54	---	---	---	---	---	---
C12ORF23	C12orf23	---	1.54	---	---	---	---	---	---
C14ORF147	C14orf147	---	-1.44	---	---	---	---	---	---
C14ORF85	---	---	1.49	---	---	---	---	---	---
C17ORF37	C17orf37	---	1.51	---	---	---	---	---	---
C17ORF58	C17orf58	---	1.86	---	---	---	---	---	-1.81
C18ORF55	C18orf55	---	-1.44	-1.41	---	---	---	---	---
C19ORF43	C19orf43	---	1.59	---	---	---	---	---	---
C19ORF66	FLJ11286	---	1.56	---	---	---	---	---	---
C2ORF44	C2orf44	---	1.48	---	---	---	---	---	---
C3ORF57	C3orf57	---	1.44	---	---	---	---	---	-3.45
C4ORF34	C4orf34	---	1.82	---	---	---	---	---	---
C5ORF39	C5orf39	---	3.78	---	---	---	---	---	---
C6ORF115	---	---	1.95	---	---	---	---	---	---
C7ORF68	HIG2	---	1.45	---	---	---	---	1.99	---
C8ORF55	C8orf55	---	-1.46	---	---	---	---	---	---
C9ORF140	C9orf140	---	-1.61	-1.45	---	-1.42	---	---	-1.70
CA12	CA12	---	-1.57	---	1.88	3.05	2.88	3.80	1.51
CASP4	CASP4	---	1.43	---	---	---	---	---	---
CAST	CAST	---	1.66	---	---	---	---	1.58	---

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
CBARA1	CBARA1	---	2.01	---	---	---	---	---	---
CBLB	CBLB	---	1.48	1.72	---	---	1.54	1.64	---
CCDC136	CCDC136	---	1.71	---	---	---	---	---	---
CCDC24	CCDC24	---	1.52	---	---	---	---	---	---
CCL22	CCL22	2.08	1.76	2.07	---	---	---	---	---
CCL5	CCL5	2.24	1.71	2.08	---	---	---	---	---
CCNDBP1	CCNDBP1	---	1.45	---	---	1.58	---	---	---
CD24	---	---	-1.41	---	---	---	---	---	---
CD55	CD55	---	6.36	1.52	---	---	---	---	---
CD9	CD9	---	1.52	---	---	---	---	---	-1.43
CDC25B	CDC25B	---	-1.4	---	---	---	---	---	---
CDC45L	CDC45L	---	-1.48	-1.54	---	---	---	---	---
CDC6	CDC6	---	-1.44	---	---	---	---	---	---
CDKN2AIP	CDKN2AIP	---	-1.52	---	---	---	---	---	---
CEACAM1	CEACAM1	1.43	1.64	1.78	---	---	---	---	---
CENPF	CENPF	---	-1.74	---	---	---	---	---	---
CFB	CFB	1.83	1.71	2.08	---	---	---	---	---
CGA	CGA	---	1.72	---	---	---	---	---	---
CHAF1B	CHAF1B	---	3.25	---	---	1.65	---	---	1.63
CHMP5	CHMP5	---	1.46	---	---	---	---	---	---
CHP	CHP	---	1.71	---	---	---	---	---	---
CISH	CISH	---	-1.44	---	---	---	---	---	---
CITED4	CITED4	---	-1.48	---	1.70	---	---	---	---
CLDND1	CLDND1	---	1.48	---	---	---	---	---	---
CLIC3	CLIC3	---	-1.45	---	---	-2.58	---	---	-2.64
CMPK2	LOC129607	---	1.64	1.50	---	---	---	---	---
CNN3	CNN3	---	1.54	---	---	---	---	---	---
CNO	CNO	---	1.62	---	---	---	---	---	---
COBL	COBL	---	1.46	---	---	-1.47	---	---	-1.42
COL3A1	COL3A1	---	3.21	---	---	---	---	---	---
COL4A5	COL4A5	---	1.48	---	---	---	---	---	---
CORO1A	CORO1A	---	-1.57	-1.40	---	---	---	---	-1.98
CPA4	CPA4	---	-1.41	---	---	-1.42	---	---	-3.06
CPT1C	CPT1C	---	1.42	---	---	---	---	---	---
CRIP2	CRIP2	---	-1.7	---	---	-1.41	---	---	-1.44
CRLF3	CRLF3	---	1.46	---	---	---	---	---	---
CRY1	CRY1	---	1.48	---	---	---	---	---	---
CSNK1G1	CSNK1G1	---	2.04	---	---	---	---	---	---
CSNK1G2	CSNK1G2	---	9.38	---	---	---	---	---	---
CT45A4	CT45-4	---	1.46	---	---	---	---	---	---
CWF19L2	CWF19L2	---	1.7	---	---	---	---	---	---
CX3CL1	CX3CL1	1.85	2.16	1.57	---	---	---	---	---
CXADR	CXADR	---	-1.43	---	---	---	---	---	---
CXCL1	CXCL1	---	1.7	---	---	---	---	---	---
CXCL10	CXCL10	2.59	3.73	3.10	---	---	---	---	---
CXCL11	CXCL11	1.57	1.8	1.61	---	---	---	---	---
CXXC5	CXXC5	---	-1.62	---	---	---	---	---	---
CYP1B1	CYP1B1	---	1.45	---	---	---	---	---	---
CYP2J2	CYP2J2	---	1.55	---	---	---	---	---	---
DBNDD1	DBNDD1	---	1.71	---	---	---	---	---	---
DCAF6	IQWD1	---	1.46	---	---	---	---	---	---
DCPS	DCPS	---	-1.45	---	---	---	---	---	---
DCTPP1	XTP3TPA	---	-1.48	---	---	---	---	---	---
DDIT4	DDIT4	---	-1.41	---	1.94	---	---	---	---
DDIT4L	DDIT4L	---	1.64	---	---	2.67	---	---	2.34
DDR2	DDR2	---	1.85	---	---	---	---	---	---
DDX17	DDX17	---	1.44	---	---	---	---	---	---
DECR1	DECR1	---	1.61	---	---	---	---	---	---
DHRS2	DHRS2	---	1.46	---	---	---	---	1.79	---
DHX58	LGP2	---	1.53	---	---	---	---	---	1.40
DISP1	DISP1	---	1.66	---	---	---	---	---	---
DKFZP667M2411	---	---	1.5	---	---	---	---	---	---
DKFZP761P0423	---	---	-1.55	---	---	---	---	---	---

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
DKK1	DKK1	---	1.76	---	-2.38	-2.14	---	-1.89	---
DNHD2	---	---	1.77	---	---	---	---	---	---
E2F2	E2F2	---	-1.52	-1.44	---	---	---	---	-1.67
E2F5	E2F5	---	1.45	---	---	---	---	---	---
EDN1	EDN1	---	1.76	---	---	---	---	-1.52	1.58
EEF2K	EEF2K	---	-1.61	---	---	---	---	---	-1.58
EFNB2	EFNB2	---	1.48	---	1.59	2.12	---	---	2.29
EHD4	EHD4	---	1.7	---	---	1.87	---	---	1.64
ELF5	ELF5	---	-1.45	---	---	-2.80	-1.58	---	---
ENDOG	ENDOG	---	-1.43	---	---	---	---	---	---
ENOPH1	ENOPH1	---	-1.44	---	---	---	---	---	---
EPRS	EPRS	---	-1.49	---	---	---	---	---	---
ESPNL	ESPNL	---	1.97	---	---	---	---	---	---
EXOG	ENDOGL1	---	2.15	---	---	---	---	---	---
EXT1	EXT1	1.67	1.42	1.75	---	-1.60	---	---	---
EYA2	EYA2	---	-1.43	---	---	2.05	---	---	1.63
FAM115A	KIAA0738	---	1.45	---	---	---	---	---	---
FAM177A1	C14orf24	---	1.44	---	---	---	---	---	---
FAM188A	C10orf97	---	1.7	---	---	---	---	---	---
FAM46A	FAM46A	---	1.44	1.53	---	---	---	---	---
FAM46B	FAM46B	---	-1.79	---	---	-2.55	---	---	-1.80
FAM62B	FAM62B	---	-1.68	---	---	-1.66	---	---	---
FBLN1	FBLN1	---	-1.4	---	---	---	---	---	---
FBP1	FBP1	---	-1.95	---	---	---	---	---	-1.72
FBXO34	FBXO34	---	2.71	---	1.68	1.80	1.76	2.03	1.57
FBXO6	FBXO6	---	1.43	1.51	---	---	---	---	---
FEN1	FEN1	---	-1.44	---	---	---	---	---	---
FGB	FGB	---	7.33	---	---	---	---	---	---
FH	FH	---	1.41	---	---	---	---	---	---
FKBP3	FKBP3	---	1.58	---	---	---	---	---	---
FLJ13305	FLJ13305	---	1.45	---	---	---	---	---	---
FLJ37078	FLJ37078	---	1.49	---	---	---	---	---	---
FLJ46309	---	---	1.48	---	---	---	---	---	---
FOXA1	FOXA1	---	3.98	---	---	---	1.65	1.77	---
FOXC1	FOXC1	---	1.62	---	---	---	2.03	---	---
FREQ	FREQ	---	1.98	---	---	---	---	---	---
FSTL5	FSTL5	---	1.52	---	---	---	---	---	---
FTHL11	---	---	1.42	---	---	---	---	---	---
FTHL12	---	---	1.48	---	---	---	---	---	---
FTHL2	---	---	1.48	---	---	---	---	---	---
FTHL3	---	---	1.4	---	---	---	---	---	---
FTHL8	---	---	1.46	---	---	---	---	---	---
FUNDC1	FUNDC1	---	1.45	---	---	---	---	---	---
GADD45B	GADD45B	---	1.56	---	1.47	1.43	---	---	---
GALNT10	GALNT10	---	-1.48	---	---	---	---	---	---
GBP1	GBP1	1.59	1.57	1.53	---	1.53	---	---	1.72
GBP4	GBP4	1.79	1.9	1.84	---	---	---	---	---
GCA	GCA	---	1.5	---	---	---	---	---	---
GCHFR	GCHFR	-1.42	-1.96	-1.68	---	---	---	---	---
GDF15	GDF15	---	1.57	1.62	1.89	2.63	---	3.12	2.34
GFOD1	GFOD1	---	4.84	---	---	---	---	---	---
GK	GK	---	4.3	---	---	---	---	---	---
GKAP1	GKAP1	---	1.44	---	---	---	---	---	---
GLRX	GLRX	1.52	2.25	---	---	---	---	---	---
GLYATL2	GLYATL2	-1.51	-2.01	-1.43	---	---	---	---	1.85
GMPR	GMPR	1.64	1.62	1.66	---	---	---	---	---
GNA15	GNA15	---	2.41	---	---	---	---	---	---
GNL3L	GNL3L	---	-1.4	---	---	---	---	---	---
GOLSYN	FLJ20366	---	-1.56	---	---	---	---	---	---
GPKOW	GPKOW	---	1.6	---	---	---	---	---	---
GPR37	GPR37	---	1.41	1.45	-1.62	---	---	---	---
GPR56	GPR56	---	-1.52	---	---	---	---	---	---
GPX2	GPX2	---	1.74	1.52	---	1.47	---	---	3.07

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
GSDMD	GSDMDC1	---	1.48	---	---	---	---	---	---
GSTM3	GSTM3	---	-1.42	---	---	---	---	---	-1.76
H19	---	---	-2.13	-1.64	---	---	---	---	---
H1F0	H1F0	---	1.53	---	---	---	---	---	---
HCP5	HCP5	---	2.75	2.22	---	---	---	---	---
HERC5	HERC5	1.85	2.58	2.02	---	---	---	---	---
HES6	HES6	---	2.49	---	---	---	---	---	-1.46
HIST1H1C	HIST1H1C	---	1.61	---	---	---	---	---	---
HIST1H3D	HIST1H3D	---	1.55	---	---	---	---	---	---
HIST2H2AA3	HIST2H2AA3	---	1.41	---	---	---	---	---	---
HIST2H2BE	HIST2H2BE	---	1.52	---	---	-1.46	---	---	---
HIST2H4A	HIST2H4A	---	1.51	---	---	---	---	---	---
HLA-A	HLA-A	---	1.66	1.84	---	---	---	---	---
HLA-A29.1	HLA-A29.1	---	1.72	1.93	---	---	---	---	---
HLA-B	HLA-B	---	2	2.00	---	---	---	---	---
HLA-DMB	HLA-DMB	---	3.87	---	---	---	---	---	---
HLA-E	HLA-E	---	1.78	---	---	---	---	---	---
HLA-F	HLA-F	---	2.08	1.78	---	---	---	---	---
HLA-G	HLA-G	---	1.53	---	---	---	---	---	---
HLA-H	---	---	2.03	1.94	---	---	---	---	---
HMGCL	HMGCL	---	1.53	---	---	---	---	---	---
HNRPC	HNRPC	---	1.5	---	---	---	---	---	---
HNRPLL	HNRPLL	---	1.41	---	---	---	---	---	---
HOOK1	HOOK1	---	-1.44	---	---	---	---	---	---
HRASLS2	HRASLS2	---	1.82	---	---	---	---	---	---
HS.127310	---	---	-1.48	---	---	---	---	---	---
HS.145049	---	---	-1.45	---	---	---	---	---	---
HS.193557	---	---	-1.51	---	---	---	---	---	---
HS.201441	---	---	-1.45	---	---	---	---	---	2.95
HS.209244	---	---	-1.53	---	---	---	---	---	---
HS.213061	---	---	-1.51	---	---	---	---	---	---
HS.294103	---	---	-1.5	---	---	---	---	---	---
HS.294603	---	---	-1.52	---	---	---	---	---	---
HS.434957	---	---	-2.12	---	---	---	---	---	---
HS.489254	---	---	1.5	---	---	---	---	---	---
HS.513971	---	---	-1.51	---	---	---	---	---	---
HS.526550	---	---	-1.49	---	---	---	---	---	---
HS.545163	---	---	1.55	---	---	---	---	---	---
HS.568329	---	---	1.46	---	---	---	---	---	---
HS.568690	---	---	-1.65	---	---	---	---	---	---
HS.568928	---	---	-1.52	---	---	---	---	---	1.59
HS.579631	---	---	1.98	1.61	---	---	---	---	---
HS.59203	---	---	1.47	---	---	---	---	---	---
HSH2D	HSH2D	---	1.42	---	---	---	---	---	---
HSPA14	HSPA14	---	-1.47	---	---	---	---	---	---
ICAM3	ICAM3	---	-1.44	---	---	---	---	---	-1.71
IDH2	IDH2	---	-1.44	---	---	---	---	---	---
IFI16	IFI16	---	2.93	1.70	---	---	---	---	---
IFI27	IFI27	---	1.48	---	---	---	---	---	---
IFI35	IFI35	---	1.41	---	---	---	---	---	---
IFIH1	IFIH1	---	1.44	---	---	1.59	---	---	---
IFIT2	IFIT2	1.94	1.85	2.25	---	---	---	---	---
IFIT3	IFIT3	1.43	1.41	1.65	---	---	---	---	---
IFNB1	IFNB1	1.78	1.56	1.54	---	---	---	---	---
IFT52	IFT52	---	1.48	---	---	---	---	---	---
IGSF5	LOC150084	---	2.71	---	1.86	---	---	---	---
IL29	IL29	2.19	1.68	1.60	---	---	---	---	---
IL8	IL8	2.91	3.85	3.15	-1.53	---	---	---	1.57
IMAA	---	---	-1.44	---	---	---	---	---	---
IMPA1	IMPA1	---	4.66	---	---	1.54	---	---	---
IMPA2	IMPA2	---	-1.46	---	---	---	---	---	---
INDO	INDO	---	2.35	---	---	---	---	---	---
INPPL1	INPPL1	---	1.41	---	---	---	---	---	---

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
IRF1	IRF1	---	1.41	---	1.61	2.63	---	---	1.98
ISG15	ISG15	---	1.55	---	---	---	---	---	---
ISOC1	ISOC1	---	1.41	---	---	---	---	---	---
ITGA10	ITGA10	---	1.47	---	---	---	---	---	---
ITM2C	ITM2C	---	1.41	---	---	---	---	---	---
KBTBD2	KBTBD2	---	2.06	---	---	---	---	---	---
KLF13	KLF13	---	-1.48	---	---	-1.41	---	---	---
LAMP3	LAMP3	1.5	2.08	1.75	---	---	---	---	---
LANCL1	LANCL1	---	-1.44	---	---	---	---	---	---
LAP3	LAP3	---	1.55	---	---	---	---	---	---
LBA1	---	---	1.56	1.69	---	---	---	---	1.42
LBP	LBP	1.51	1.65	---	---	---	---	---	---
LCN2	LCN2	1.67	1.41	1.83	---	2.23	---	---	3.78
LGMN	LGMN	---	1.58	1.55	---	---	---	---	---
LHFP	LHFP	---	1.48	---	---	---	---	---	---
LMO2	LMO2	---	1.42	---	---	1.54	---	---	---
LOC100008589	---	---	1.52	---	---	---	---	---	---
LOC158160	---	---	1.44	---	---	---	---	---	---
LOC388275	---	---	-1.49	---	---	---	---	---	---
LOC388588	---	---	1.45	---	---	---	---	---	-1.99
LOC389816	LOC389816	-1.4	-1.64	-1.41	---	---	---	---	-1.87
LOC400948	---	---	1.4	---	---	---	---	---	---
LOC401115	---	---	1.64	1.68	---	---	---	---	---
LOC440927	---	---	1.44	---	---	---	---	---	---
LOC441019	---	---	1.55	---	---	---	---	---	---
LOC642989	---	---	1.56	---	---	---	---	---	---
LOC644250	---	---	1.43	---	---	---	---	---	---
LOC644615	---	---	1.4	---	---	---	---	---	---
LOC646817	---	---	-1.65	---	---	---	---	---	---
LOC649150	---	---	-1.43	---	---	---	---	---	---
LOC651202	---	---	1.67	---	---	---	---	---	---
LOC653506	---	---	-1.43	---	---	---	---	---	-1.63
LOC653631	---	---	1.58	---	---	---	---	---	---
LOC727820	---	---	1.96	---	---	---	---	---	---
LOC728216	---	---	1.55	---	---	---	---	---	---
LOC728492	---	---	2.16	---	---	---	---	---	---
LOC728556	---	---	1.54	---	---	---	---	---	---
LOC730256	---	---	2.11	---	---	---	---	---	---
LOC731950	---	---	1.84	---	---	---	---	---	---
LRP10	LRP10	---	1.6	---	---	---	---	---	---
LRPPRC	LRPPRC	---	-1.55	---	---	-1.73	---	---	---
LRRC20	LRRC20	---	1.55	---	---	---	---	---	---
LRRC26	LOC389816	-1.41	-1.73	---	---	---	---	---	-2.01
LRRC41	LRRC41	---	1.58	---	---	---	---	---	---
MAL	MAL	-1.46	-1.78	---	---	---	---	---	-2.02
MAN2B2	MAN2B2	---	1.5	---	---	---	---	---	---
MAP4K2	MAP4K2	---	1.43	---	---	---	---	---	---
MAPKAPK3	MAPKAPK3	---	-1.4	---	---	---	---	---	---
MAT2B	MAT2B	---	1.48	---	---	---	---	---	---
MCM2	MCM2	---	-1.51	---	---	---	---	---	---
MCM4	MCM4	---	-1.4	---	---	---	---	---	---
MCM6	MCM6	---	-1.63	-1.43	---	---	---	---	-1.56
MDK	MDK	---	1.66	---	---	---	---	---	---
MED30	THRAP6	---	1.4	---	---	---	---	---	---
MEMO1	MEMO1	---	1.47	---	---	---	---	---	---
METRNL	METRNL	---	-1.41	---	---	-1.72	---	---	-1.47
MFSD1	MFSD1	---	1.47	---	---	---	---	---	1.42
MGLL	MGLL	---	-1.44	---	---	---	---	1.61	---
MICAL1	MICAL1	---	1.48	---	---	---	---	---	---
MKLN1	MKLN1	---	-1.49	---	---	---	---	---	---
MLLT10	MLLT10	---	1.41	---	---	-1.74	---	---	---
MMEL1	MMEL1	---	-1.48	---	---	---	---	1.55	---
MMP10	MMP10	---	1.54	---	---	---	---	---	---

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
MMP7	MMP7	---	1.51	---	---	---	---	---	---
MNAT1	MNAT1	---	2.46	---	---	---	---	---	---
MOBKL2C	MOBKL2C	---	1.51	---	---	---	---	---	---
MRPL13	MRPL13	---	1.4	---	---	---	---	---	---
MRPL16	MRPL16	---	1.44	---	---	---	---	---	---
MT1A	MT1A	---	2.02	---	---	---	---	---	---
MT2A	MT2A	---	2.08	1.43	---	---	---	---	---
MTE	MTE	---	1.57	---	---	---	---	---	---
MX2	MX2	1.45	1.89	1.89	---	---	---	---	---
MXD4	MXD4	---	1.47	---	---	---	---	---	---
MYLIP	MYLIP	---	1.48	---	---	---	---	---	---
MYO5C	MYO5C	---	-1.41	---	---	---	---	---	---
N4BP3	N4BP3	---	-1.45	---	1.62	1.76	---	---	---
NAPRT1	NAPRT1	---	-1.41	---	---	---	---	---	-1.46
NAV1	NAV1	---	-1.48	---	2.53	2.06	---	---	1.50
NCOA1	NCOA1	---	1.42	---	---	---	---	---	---
NCOA7	NCOA7	1.71	1.64	1.81	---	1.71	---	---	1.63
NECAP2	NECAP2	---	1.5	---	---	---	---	---	---
NFIL3	NFIL3	---	1.54	---	---	---	---	---	---
NFS1	NFS1	1.66	1.66	1.54	---	---	---	---	---
NMD3	NMD3	---	1.47	---	---	---	---	---	---
NUB1	NUB1	---	1.52	1.50	---	---	---	---	---
NUMB	NUMB	---	1.46	---	---	---	---	---	---
NUP62CL	NUP62CL	---	1.57	---	---	---	---	---	---
NUPR1	NUPR1	1.47	1.77	1.88	---	---	---	---	---
NUSAP1	NUSAP1	---	2.09	---	---	---	---	---	---
OASL	OASL	1.78	2.17	1.65	---	---	---	---	---
ODC1	ODC1	---	1.49	---	---	-1.81	---	---	-1.61
OSGIN2	OSGIN2	---	1.42	---	---	---	---	---	---
P4HA1	P4HA1	---	2.44	---	---	---	---	---	---
P8	NUPR1	1.48	2.09	1.82	---	---	---	---	---
PAICS	PAICS	---	-1.43	---	---	---	---	---	---
PARP10	PARP10	---	1.6	---	---	---	---	---	---
PARP12	PARP12	---	1.47	---	---	1.58	---	---	1.40
PBX3	PBX3	---	1.72	---	---	---	---	---	---
PCDH17	PCDH17	---	2.48	1.99	---	---	---	---	---
PCYOX1	PCYOX1	---	-1.41	---	---	---	---	---	---
PDE4D	PDE4D	---	-1.45	---	---	-1.72	---	---	---
PDE6D	PDE6D	---	1.53	---	---	---	---	---	---
PDE8B	PDE8B	---	-1.59	---	---	---	---	---	---
PDGFRL	PDGFRL	---	1.85	1.56	---	---	---	---	---
PDLIM3	PDLIM3	---	1.47	---	---	---	---	---	---
PDSS1	PDSS1	---	-1.47	---	---	---	---	---	-1.52
PDXK	PDXK	---	-1.5	---	---	-1.40	---	---	---
PEBP1	PEBP1	---	1.45	---	---	---	---	---	---
PECI	PECI	---	1.44	---	---	---	---	---	---
PERP	PERP	---	2.13	---	---	---	---	---	---
PEX11A	PEX11A	---	1.57	---	---	---	---	---	---
PFKFB3	PFKFB3	---	-1.45	---	---	2.15	1.97	3.09	2.04
PHACTR2	PHACTR2	---	-1.48	---	---	---	---	---	---
PHIP	PHIP	---	-1.52	---	---	---	---	---	---
PHTF1	PHTF1	---	1.45	---	---	---	---	---	---
PIAS1	PIAS1	---	1.82	---	---	---	---	---	---
PIGM	PIGM	---	1.43	---	---	---	---	---	---
PIP	PIP	---	1.89	---	---	---	---	---	---
PITX1	PITX1	---	-1.42	---	---	---	---	---	---
PLA2G10	PLA2G10	-1.63	1.51	---	2.79	1.87	1.90	2.25	3.13
PLAC2	PLAC2	---	-1.44	---	---	---	---	---	---
PLCXD1	PLCXD1	---	2.26	---	---	---	---	---	-1.40
PLEKHA1	PLEKHA1	---	1.44	---	---	---	---	---	---
PLEKHA4	PLEKHA4	---	1.72	---	---	---	---	---	---
PLEKHG4	PLEKHG4	---	1.55	---	---	---	---	---	---
PMEPA1	TMEPAI	-1.41	-1.9	---	---	---	---	---	---

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
PMS2L2	---	---	1.46	---	---	---	---	---	---
PMS2L5	PMS2L5	---	1.52	---	---	---	---	---	---
PNO1	PNO1	---	1.81	---	---	---	---	---	---
PPAP2A	PPAP2A	---	1.45	---	---	---	---	---	---
PPAPDC1B	PPAPDC1B	---	1.41	---	---	---	---	---	---
PPARBP	PPARBP	---	1.48	---	---	---	---	---	---
PPM1H	---	---	-1.45	---	---	---	---	---	---
PPP1R14C	PPP1R14C	---	-1.41	---	---	---	---	---	---
PPP3CB	PPP3CB	---	1.4	---	---	---	---	---	---
PPP3CC	PPP3CC	---	1.46	---	---	---	---	---	---
PRIC285	PRIC285	---	1.51	1.53	---	---	---	---	---
PRICKLE1	PRICKLE1	---	1.44	---	---	---	---	---	---
PRKD2	PRKD2	---	1.51	---	---	---	---	---	---
PRKDC	PRKDC	---	-1.43	---	---	---	---	---	---
PSMB9	PSMB9	---	1.8	1.45	1.83	3.18	---	---	1.98
PSMC5	PSMC5	---	1.46	---	---	---	---	---	---
PSPC1	PSPC1	---	1.53	---	---	---	---	---	---
PTMA	PTMA	---	1.49	---	---	---	---	---	---
PTPN21	PTPN21	---	1.54	---	---	---	---	---	---
PVR	PVR	---	2.15	---	---	---	---	---	---
PWWP2B	PWWP2	---	-1.48	---	---	-1.80	---	---	-1.60
PXMP4	PXMP4	---	-1.42	---	---	---	---	---	---
QPCT	QPCT	---	1.79	---	---	---	---	---	---
QSOX1	QSOX1	---	1.8	---	---	---	---	---	---
RAB13	RAB13	---	1.44	---	---	---	---	---	---
RAB22A	RAB22A	---	-1.64	---	---	---	---	---	---
RAB2B	RAB2B	---	1.43	---	---	---	---	---	---
RAB32	RAB32	---	1.65	---	---	---	---	---	---
RAI14	RAI14	---	1.83	---	2.07	3.05	---	---	2.96
RALB	RALB	---	-1.47	---	---	---	---	---	---
RAP1GAP	RAP1GAP	-1.42	-1.41	---	1.44	1.72	2.24	---	---
RASD1	RASD1	---	1.45	1.43	---	---	---	---	---
RASGRP3	RASGRP3	1.45	1.84	1.79	---	---	---	---	---
RDH11	RDH11	---	2.07	---	---	---	---	---	---
REEP5	REEP5	---	-1.43	---	---	---	---	---	---
RFX5	RFX5	---	1.45	---	---	---	---	---	---
RGN	RGN	---	2.5	---	---	---	---	---	---
RGS2	RGS2	---	3.34	---	---	---	---	---	1.92
RINL	FLJ45909	---	1.8	---	---	---	---	---	---
RIPK2	RIPK2	1.48	1.52	---	---	---	---	---	---
RN7SK	---	---	2.06	1.53	---	---	---	---	---
RND1	RND1	---	1.43	---	---	---	---	---	---
RNF114	ZNF313	---	1.43	---	---	---	---	---	---
RNF144	RNF144	---	1.45	---	---	---	---	---	---
RPL22	RPL22	---	-1.57	---	---	---	---	---	---
RPL23	RPL23	---	1.64	---	---	---	---	---	---
RPL7A	RPL7A	---	1.71	---	---	---	---	---	---
RPLP0	RPLP0	---	-1.42	---	---	---	---	---	---
RPP40	RPP40	---	-1.41	---	---	---	---	---	---
RPS23	RPS23	---	-1.6	---	---	---	---	---	---
RPS28	RPS28	---	1.53	---	---	---	---	---	---
RPS6KA5	RPS6KA5	---	1.49	---	---	---	---	---	-1.45
RSAD2	RSAD2	1.68	2.43	1.80	---	---	---	---	---
RTP4	RTP4	---	1.91	1.93	---	---	---	---	---
S100A16	S100A16	---	-1.44	---	---	---	---	---	-2.20
S100A4	S100A4	---	-1.5	---	---	---	---	---	---
S100A7	S100A7	1.52	1.43	---	---	---	---	---	---
SAP30L	SAP30L	---	1.74	---	---	---	---	---	---
SAR1A	SAR1A	---	1.81	---	---	---	---	---	---
SASH1	SASH1	---	1.47	---	---	---	---	---	---
SAT1	SAT1	---	1.62	---	---	---	---	1.84	1.50
SCAP	SCAP	---	1.73	---	---	---	---	---	---
SCARB1	SCARB1	---	-1.48	---	---	1.40	---	---	1.71

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
SCD	SCD	---	-1.46	---	---	---	---	---	---
SCIN	SCIN	---	-1.56	---	---	---	---	---	---
SCNN1G	SCNN1G	---	3.46	---	2.58	2.06	---	---	1.44
SDCBP	SDCBP	---	1.83	---	---	---	---	---	1.62
SEC11C	SEC11C	---	1.42	---	---	---	---	---	---
SEMA3F	SEMA3F	---	-1.52	---	---	1.60	---	---	---
SERBP1	SERBP1	---	-1.69	---	---	---	---	---	---
SERF1B	---	---	1.53	---	---	---	---	---	---
SERPINA3	SERPINA3	1.59	1.69	2.25	1.47	1.60	2.83	---	7.66
SERTAD2	SERTAD2	---	1.73	---	---	---	---	---	---
SGK	SGK	1.78	2.88	2.00	---	---	---	---	---
SGMS2	SGMS2	---	1.42	---	---	---	---	---	---
SLC2A5	SLC2A5	---	4.86	---	---	---	---	---	---
SLC31A1	SLC31A1	---	-1.41	---	---	---	---	---	---
SLC36A1	SLC36A1	---	1.43	---	---	---	---	---	---
SLC37A1	SLC37A1	---	1.46	---	---	---	---	---	---
SLC38A1	SLC38A1	---	-1.68	---	---	-1.51	---	---	---
SLITRK5	SLITRK5	---	1.47	---	---	---	---	---	---
SLK	SLK	---	-1.42	---	---	---	---	---	---
SMC4	SMC4	---	-1.41	---	---	---	---	---	---
SNCA	SNCA	---	1.62	1.50	---	---	---	---	---
SNRNP35	U1SNRNPBP	---	1.44	---	---	---	---	---	---
SNX25	SNX25	---	1.43	---	---	---	---	---	---
SOD1	SOD1	---	1.41	---	---	---	---	---	---
SOD2	SOD2	1.52	1.56	1.68	---	---	---	---	---
SOX4	SOX4	---	1.46	---	---	1.47	---	---	2.08
SPRY2	SPRY2	---	1.81	---	---	1.50	---	---	---
SPTLC2	SPTLC2	---	-1.44	---	---	---	---	---	---
SQRDL	SQRDL	---	1.65	1.46	---	---	---	---	---
SQSTM1	SQSTM1	---	1.43	---	---	---	1.72	---	---
SRM	SRM	---	-1.52	---	---	---	---	---	---
SSH2	SSH2	---	1.74	---	---	---	---	---	---
STAT2	STAT2	---	1.47	1.41	---	1.45	---	---	1.63
STRADA	LYK5	---	1.62	---	---	---	---	---	---
STX10	STX10	---	-1.47	---	---	---	---	---	---
SYT11	SYT11	---	1.93	---	---	---	---	---	---
SYT15	SYT15	---	-1.45	---	---	-1.52	---	---	-1.65
TACSTD2	TACSTD2	---	1.45	---	---	1.50	---	---	---
TARP	TARP	---	3.63	---	---	---	---	---	-1.46
TBC1D22B	TBC1D22B	---	1.71	---	---	---	---	---	---
TCEA3	TCEA3	---	1.45	---	---	---	---	---	---
TDRD7	TDRD7	---	1.49	---	---	---	---	---	---
TEX2	TEX2	---	-1.49	---	---	---	---	---	---
TFPI	TFPI	---	2.11	---	---	---	---	---	---
TGFB3	TGFB3	---	-1.52	-1.46	---	---	---	---	-1.92
TGIF2	TGIF2	---	1.62	---	---	---	---	---	---
TH	TH	-1.44	-1.66	-1.40	---	1.58	---	---	1.55
THADA	THADA	---	1.51	---	---	---	---	---	---
THBS1	THBS1	---	-1.4	---	---	-1.97	---	---	---
TK1	TK1	---	-1.5	---	---	---	---	---	---
TKT	TKT	---	-1.45	---	---	---	---	---	---
TLE1	TLE1	---	1.62	---	---	-1.40	---	---	---
TM2D1	TM2D1	---	1.47	---	---	---	---	---	---
TMED1	TMED1	---	1.53	---	---	---	---	---	---
TMEM140	TMEM140	---	2.18	1.76	---	---	---	---	---
TMEM144	TMEM144	---	1.57	---	---	---	---	---	---
TMEM199	C17orf32	---	1.44	---	---	---	---	---	---
TMPRSS2	TMPRSS2	---	1.71	---	2.41	5.18	2.33	3.66	3.31
TNFAIP3	TNFAIP3	1.64	1.61	1.65	---	---	---	---	---
TNFSF10	TNFSF10	2.04	1.85	2.74	---	---	---	---	---
TNFSF13B	TNFSF13B	1.61	1.68	1.59	---	---	---	---	---
TNFSF9	TNFSF9	---	2.22	---	---	---	---	---	---
TOMM20	TOMM20	---	-1.44	---	---	---	---	---	---

Ilm GENE	ENTREZ GENE	fold GATA3	fold FOXA1	fold SOX9	Fold SKBR3 8h RA+CHX	Fold SKBR3 8h RA-CHX	Fold MCF7 8h RA+CHX	Fold MCF7 8h RA-CHX	Fold SKBR3 24h RA
TOP2A	TOP2A	---	-1.69	-1.49	---	---	---	---	---
TOP2B	TOP2B	---	-1.5	---	---	---	---	---	---
TP53I13	TP53I13	---	1.51	---	---	---	---	---	---
TPST1	TPST1	---	1.56	---	---	---	---	---	---
TRAFD1	TRAFD1	---	1.72	---	---	---	---	---	---
TRIB1	TRIB1	---	1.52	1.50	---	1.50	---	1.51	1.41
TRIL	KIAA0644	---	-1.47	---	---	-1.65	---	---	-1.53
TRIM21	TRIM21	---	1.45	1.49	---	---	---	---	---
TRIM5	TRIM5	---	1.52	1.45	---	---	---	---	---
TRIM56	TRIM56	---	1.55	---	---	---	---	---	---
TRMT12	TRMT12	---	1.56	---	---	---	---	---	---
TRPC4AP	TRPC4AP	---	1.82	---	---	---	---	---	---
TRPM4	TRPM4	---	1.44	---	---	---	---	---	1.46
TSEN54	TSEN54	---	-1.42	---	---	---	---	---	---
TSPAN5	TSPAN5	---	-1.46	---	-1.56	-1.41	---	---	---
TTC25	TTC25	---	1.42	---	---	---	---	---	---
TTC32	TTC32	---	1.54	---	---	---	---	---	---
TTC5	TTC5	---	2.31	---	---	---	---	---	---
TUBB2A	TUBB2A	---	1.41	---	---	-1.47	---	---	---
TXNDC12	TXNDC12	---	-1.53	---	---	---	---	---	---
TXNIP	TXNIP	---	1.78	---	---	---	---	---	1.50
UAP1	UAP1	---	1.79	---	---	---	---	---	---
UAP1L1	UAP1L1	---	-1.48	---	---	-2.09	---	---	-1.68
UBD	UBD	1.54	3.14	3.01	3.81	1.93	1.84	---	3.08
UBQLNL	UBQLNL	---	2.02	---	---	---	---	---	---
UCP2	UCP2	---	-1.4	---	---	---	---	---	---
UGDH	UGDH	---	1.53	---	---	---	---	---	---
UHRF1	UHRF1	---	-1.53	-1.47	---	---	---	---	---
UPP1	UPP1	---	1.52	---	---	---	---	---	---
UTRN	UTRN	---	1.56	---	---	-1.43	---	---	---
VNN3	VNN3	---	1.47	---	---	---	---	---	---
VPS37D	VPS37D	---	1.54	---	---	---	---	---	---
WDR34	WDR34	---	-1.63	-1.42	---	---	---	---	---
WDR64	WDR64	---	1.6	---	---	---	---	---	---
WDR67	WDR67	---	1.66	---	---	---	---	---	---
WNT7B	WNT7B	---	-1.44	---	---	---	---	---	---
XPNPEP3	XPNPEP3	---	1.44	---	---	---	---	---	---
ZC3HC1	ZC3HC1	---	1.4	---	---	---	---	---	---
ZCCHC11	ZCCHC11	---	2.3	---	---	---	---	---	---
ZFYVE1	ZFYVE1	---	1.46	---	---	---	---	---	---
ZMIZ1	ZMIZ1	---	-1.49	---	1.80	1.89	---	---	---
ZNF148	ZNF148	---	-1.42	---	---	---	---	---	---
ZNF428	ZNF428	---	1.47	---	---	---	---	---	---
ZSWIM5	---	---	1.7	---	---	---	---	---	---

Chapter 3: Synergistic Growth Inhibition by Retinoic Acid and Herceptin in HER2/RARA-Amplified Breast Cancer Cells

Marieke Rozendaal, Slim Fourati, Martine Bail, David Laperriere and Sylvie Mader

Manuscript in preparation for submission to Cancer Research

As first author, I designed the body of the study, performed cell proliferation and viability assays, prepared cells for FISH, microarray and Q-PCR analyses and performed western analysis. I also wrote the first version of the manuscript and made most figures.

Synergistic growth inhibition by retinoic acid and Herceptin in HER2/RARA-amplified breast cancer cells

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Conflict of interest: the authors have declared that there is no conflict of interest.

List of non-standard abbreviations: ERα, estrogen receptor alpha; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR retinoid X receptor.

RUNNING TITLE:

Synergy of RA and Herceptin in HER2/RARA-amplified breast cancer

ABSTRACT

Success of retinoid treatments for breast malignancies has so far been limited. This is likely due to the high level of heterogeneity of the disease and the lack of good markers to predict response to retinoids. In breast cancer cell lines, antiproliferative response to retinoic acid (RA) is correlated with the expression of the estrogen receptor, ERα, and inversely correlated with the overexpression of the human epidermal growth factor receptor 2 (HER2). Herceptin™ (trastuzumab), a humanized monoclonal antibody that inhibits the tumorigenic effects of HER2, is currently used for treatment of HER2 positive breast cancer. Despite a clear positive impact of adjuvant treatment with Herceptin™ on prognosis, a majority of patients that originally respond will develop resistance within a year. Association of Herceptin™ with other cancer therapeutic drugs may alleviate development of resistance. Here we show that the ER-negative and HER2-amplified SK-BR-3 cells carry a co-amplification of the HER2 and RARA genes and that RA and Herceptin™ synergize to suppress proliferation and viability of these cells, but not of HER2-amplified/RARA-unamplified cells. Thus, lower doses of both drugs suffice to obtain similar anti-tumor activity. Herceptin enhances transcriptional regulation by RA, leading to synergistic regulation of cell cycle regulated genes. At the same time we also observed that RA regulates several genes implicated in resistance to Herceptin, suggesting that RA could be beneficial for preventing the development of resistance. Since the co-

amplification of RARA with HER2 also takes place in human breast cancer samples, we propose that this subgroup of HER2 positive tumors could benefit from co-treatment with RA and Herceptin™.

KEYWORDS:

Retinoic acid, Herceptin, breast cancer, gene amplification, microarray.

INTRODUCTION

Natural retinoids include vitamin A or retinol and its derivatives retinal and retinoic acid (RA). RA functions through binding to retinoic acid receptors (RARs) belonging to the nuclear receptor superfamily of ligand-dependent transcription factors (1; 2). The regulation of RA target genes is mediated by heterodimers between RARs and members of another family of nuclear receptors, retinoid X receptors (RXRs) (3). Three isotypes, α, β and γ, exist for both RARs and RXRs and several N-terminal variants are expressed for each gene (3). Retinoic acid signaling plays important roles in the regulation of cell growth and differentiation and as such is essential for embryonic development and normal tissue homeostasis (4; 5) . In addition, retinoids have been shown to inhibit growth and survival in various tumor models (6). RA can efficiently inhibit the growth of breast cancer cells in vitro as well as in animal models, a process involves cell cycle inhibition and apoptosis (7-10). Sensitivity of breast cancer cell lines to the antiproliferative effects of RA largely correlates with expression of the estrogen receptor ERα. Estrogen-mediated activation of ERα was shown to induce the expression of RARα, the RAR isotype that appears to be mainly responsible for RA signaling in mammary carcinoma cells (11; 12). However, ERα negative cell lines were also shown to respond to the effects of RA treatment (13; 14).

HER2 (ERBB-2, *neu*), a member of the epidermal growth factor (EGF) receptor family, is overexpressed in 25-30% of breast cancers. This overexpression leads to the constitutive activation of the PI3K/Akt signaling pathway, resulting in growth stimulation and survival advantage for such HER2-overexpressing tumors. HER2 overexpression also

correlates with prognosis in breast tumors. In the vast majority of cases, the overexpression is due to the amplification of the HER2-gene located on the long arm of chromosome 17 (17q12) (15; 16). A small amplicon was characterized of 280-750 kb and containing up to little over 20 genes (17; 18) ; in addition, a longer amplicon can contain up to 40 genes (19) and extend as far as the TOP2A gene on 17q21 (20). Variability in co-amplified genes has suggested the existence of biologically diverse subgroups of HER2+ tumors (17; 20). Indeed, overexpression of TOP2A resulting from its co-amplification was correlated with tumor response to anthracycline-based chemotherapy (21-23). The RARA gene, encoding the RARα protein, is located close to the TOP2A gene on the side proximal to the HER2 locus on chromosome 17q21; its amplification was confirmed in some cases of HER2 amplified breast tumors (20) and is likely to be occuring with similar frequencies as that of the TOP2A gene.

HER2 has become an important target in the treatment of breast cancer because of its causative role in breast tumorigenesis and its frequent and strong overexpression. Following demontration that antibodies against the HER2 protein could inhibit tumor cell proliferation *in vitro* (24), the humanized monoclonal antibody Herceptin™ (trastuzumab), targeting the extracellular domain of HER2, has been used as a single agent or in the adjuvant setting in combination with chemotherapy. The mechanisms of action of Herceptin appear to be multiple, involving down regulation of HER2 (25), increased $p27^{Kip1}$ levels (26), decreased Akt phosphorylation and activity (27), inhibition of angiogenesis through down regulation of VEGF (28) and activation of antibody dependent cellular cytotoxicity (29-31). Primary resistance to Herceptin™ mono-therapy varies

between 66% and 88% of cases and response rates can be significantly improved by combining the treatment with chemotherapeutic drugs. However, virtually all patients showing initial response will develop resistance within the first year of treatment (32).

Combination therapies associating retinoids and ERBB- targeting treatments were previously suggested based on the impact of HER2 signaling on RARα expression (13). Here we demonstrate that retinoic acid, as well as the RARα selective ligand Am580, work in a synergistic manner with Herceptin™ in SK-BR-3 breast cancer cells, which carry a co-amplification of RARA with the HER2 gene. This synergy could not be observed in BT-474 cells, which are HER2 amplified and express RARα, but do not carry the co-amplification of the RARA gene. Herceptin™ enhanced the transcriptional response to RA in SK-BR-3 cells. We propose a role for FOXO family transcription factors in mediating the observed synergy. Finally, we show that transcriptional targets of Herceptin™-RA co-treatment can accurately predict overall and distant metastasis free survival in HER2 positive tumor samples. We propose that the subset of HER2 positive breast tumors carrying an RARA co-amplification could benefit from combined treatment regimes with Herceptin™ and RARα-selective retinoids.

MATERIALS AND METHODS

Cell culture

SkBr-3, MCF-7 and BT-474 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Oakville, ON, Canada) at 37°C in humidified air containing 5% CO_2. MDA-MB-361 cells were cultured in L15 medium (Wisent) supplemented with 20% FBS. All-*trans*-retinoic acid (RA; Sigma) stocks and working solutions were prepared in DMSO. Herceptin™ was obtained through the pharmacy of the Maisonneuve-Rosemont Hospital (Montreal, QC, Canada) and stocks were prepared in 1.1% benzyl alcohol. All treatments for proliferation assays were performed in DMEM containing 5% FBS. For gene regulation assays, cells were maintained and treated in DMEM with 10% FBS.

Proliferation and MTS essays

Cells were seeded in 6-well plates at a density of 30,000 cells per well and treated every 2-3 days with vehicle (0), retinoic acid (1μM; RA) or Herceptin™ (10μg/ml; Herc.) or Herceptin+RA in DMEM containing 5% FBS. After 9 days, cells were collected and protein concentrations were measured as described previously (33). For MTS assays, cells were seeded at a density of 6,000 cells per well in 96-well plates. The next day, medium was changes for DMEM with 5% FBS containing retinoids and/or Herceptin at indicated concentrations. Medium and treatments were changed 2 days later and another 3 days later cell viability was measured using CellTiter 96® AQueous Non-Radioactive Cell

Proliferation Assay (Promega, Madison, WI, USA). Briefly, medium was replaced by medium containing 5% FBS and 5% MTS reagent and cells were incubated at 37°C for 2-3 hours. O.D. values were then read at 490nm on a SpectraMax plate reader with SoftMax Pro software and cell viability was expressed as relative values (treatment/vehicle).

Analysis of synergy

The cytotoxic effect obtained with retinoic acid (RA) and Herceptin combinations was analyzed according to the Chou and Talalay method (34). Combination index (CI) values above 1.1 indicate antagonistic, 0.9 to 1.1 additive, 0.7 to 0.9 moderately synergistic, 0.3 to 0.7 synergistic, and < 0.3 strongly synergistic effects. The Chou and Talalay method was implemented in the R language (source code available on request).

Microarray analysis

For microarray of RA and Herceptin regulated genes, SK-BR-3 cells were plated at 1M cells per 10-mm dish. Cells were allowed to adhere to the dishes overnight and were then treated with vehicle, 30 nM RA, 1 µg/ml Herceptin or a combination of the two. 24 hours later, the cells were harvested in 1 ml of Trizol (Invitrogen, Burlington, ON) and total RNA was extracted according to the manufacturers recommendations. Total RNA was then purified using the RNeasy MinElute Cleanup Kit (QIAgen, Mississauga, ON). For analysis of FOXO3A target genes, SK-BR-3 cells were electroporated (5 million cells, 240 V, 950 µF) with 6 µg of pCMV-XL4-FOXO3 (OriGene, Rockville, MD, USA) or empty vector and RNA was extracted 48 hours after transfection as mentioned above. cRNA synthesis from total RNA, labeling and hybridization to Illumina WG-6 v3.0 BeadChips were

performed at the Genome Quebec and McGill University Innovation Center (Montreal, QC).

Un-normalized summary probe profiles were output from BeadStudio and analyzed using the lumi (35) and limma (36) packages of the Bioconductor open-source software project (http://www.bioconductor.org). The raw intensities were transformed using the vst method and normalized with the robust spline normalization scheme.

Genes deemed significantly regulated were those with ≥1.4-fold change, average log2-expression levels greater than 5 across all samples and a BH-corrected p-value (for a moderated t-statistics) smaller than 0.01.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed using the GSEA java application version 2.0.5 developed by Subramanian A. *et al.* (37). For this analysis we compared the two phenotypes being compared (e.g. SKBR3_24h_HERCEPTIN versus SKBR3_24h_DMSO) and looked for enrichment of the C2 curated catalogue of functional genes sets. We also curated from the literature gene sets representing ERBB2/growth factor activation (38), FOXO3 downstream targets genes (this study) RAS, MYC SRC, E2F3 and β-Catenin (39) oncogenic pathway dysregulation. 1000 permutations were used to test significance of enrichment.

FISH

Exponentially growing cells were sent to the cytogenetics platform of the Maisonneuve-Rosemont Hospital for FISH analysis. Used probes were: *RARA dual color*

(Abbott #30-191 011) and *TOP2A/ HER2 /CEP17* (Abbott #30 191 095). Results were analyzed by visual inspection of at least 12 mitoses and 60 interphase nuclei per probe per cell line.

Screening for transcription factor binding sites

Human Genomic sequences +/- 10 Kbp around the transcription start sites (TSS) were extracted for all annotated gene in the RefSeq track (40) from the UCSC Genome Browser Database (hg17, May 2004) (41). Matrices from TRANSFAC 2010.2 (42) were used to screen these sequences for transcription factor binding sites using a base score cut-off of 65% and 5% increments as described previously (43). *Z*-scores and *P*-values from a Fisher exact test were used to evaluate the significance of the observed enrichment in promoters of different sets of regulated genes versus those of all annotated genes. The Z-scores and P-values were calculated with programs adapted from oPOSSUM perl application programming interface (API) using the cut-off (*Z*-scores > 10, *P*-value < 0.01) recommended by the authors (44).

Boxplots

Microarray data from Li *et al.* (45) were MAS5.0 normalized to a scale of 500. Expression levels are presented as log2 mean-centered ratio. One way anova test was used to test for significant ($p \leq 0.05$) differences of mean expression between classes (HER2+/-TOP2A+/-). Tukey HSD and Scheffe a posteriori tests were used to identified between which class difference of expression was significant ($p \leq 0.05$).

Hierarchical clustering

Expression of mRNA transcripts showing significant up- or down-regulation ($|FC| \geq 1.4$ and adj. p.value ≤ 0.01) in at least one of the three SK-BR-3 treatments (relative to vehicle) was examined in HER2+ primary tumors (46). Hierarchical clustering (Euclidean distance, Ward linkage) of 55 HER2+ tumors according to the expression of regulated transcripts was performed and tumors were partitioned in the two major classes identified. For each class of target transcripts (RA, Herceptin-RA up- or down-regulated transcripts), only the 25 transcripts exhibiting the highest differential expression between the two classes of tumors were used for the heatmap representation. Kaplan-Meier analysis was used to assess the differential distant-metastasis and overall survival rates in the two classes of tumors.

RESULTS

RARA and RALDH3 mRNA levels correlate with HER2-amplicon size in HER2-positive tumor samples.

The large HER2 amplicon encompasses several genes that are of potential interest for therapeutic goals, such as TOP2A, GRB7, STARD3 and RARA (16). We investigated whether RARA mRNA levels correlate with amplicon size in HER2+ tumors. While HER2 mRNA levels were as expected high in tumors with both the small amplicon (HER2-amplification only) and the large amplicon (HER2- and TOP2A-amplification), TOP2A levels were significantly higher in HER2-positive tumors with the large amplicon than in those with the small amplicon or in HER2-negative tumors ($p = 1.43 \times 10^{-5}$, Figure 1A-B). Comparing Figure 1B with Figure 1C and E, it is clear that RARA mRNA levels are a better indicator for amplicon size ($p = 1.43 \times 10^{-5}$ vs. $p = 3.97 \times 10^{-8}$ or $p = 3.57 \times 10^{-9}$, respectively). Our group recently observed that suppression of the RA synthesizing enzyme RALDH3 appears to be a prerequisite for proliferation of RARα-expressing luminal cells, due to the antiproliferative effects of endogenous RA production (Parisotto *et al*., manuscript in preparation). We therefore assessed whether the higher RARA mRNA levels found in HER2+ tumors with large amplicons, are associated with decreased RALDH3 mRNA levels. Indeed, tumors with the large amplicon have lower RALDH3 levels than HER2-negative or small amplicon tumors (Figure 1D-F). Therefore loss of RA production appears to be required for tumor progression of RARα-overexpressing cells, which suggests that these tumors could be sensitive to treatment with exogenous retinoids.

Differential response of HER2-amplified cell lines to the anti-proliferative effects of retinoic acid: role of RARA co-amplification with HER2.

Although HER2-positivity of breast tumors and cell lines is generally considered to be negatively correlated with their response to retinoic acid, we and others have observed that the proliferation of the ERα-negative, HER2-positive cell line SK-BR-3 is strongly inhibited by RA. Observations of others (17; 20) show that the large HER2-amplicon can extend as far as the RARA and TOP2A genes. We previously showed that these cells have levels of RARA mRNA and protein that are much higher than those of ERα-negative MDA-MB-231 cells and compare favorably even with ERα-positive MCF7 cells. To test whether the high RARα levels in SK-BR-3 cells result from gene amplification, we performed FISH analysis using probes for HER2, TOP2A and RARA on SK-BR-3 cells and several other HER2-overexpressing cell lines. We also included MCF-7 cells as a control for non-amplified cells. Amplification of HER2 was detected in SK-BR-3, MDA-MB-361 and BT-474 cells as expected (Figure 2A**)**. TOP2A is amplified in BT-474 cells only. In contrast, RARA was found to be amplified in SK-BR-3, in concordance with previously published results (17). BT-474 cells, although expressing RARα at the protein level, do not carry an amplification of the gene. The MDA-MB-361 cell line, which expresses low levels of the RARα protein, has on the other hand lost one copy of the gene. Figure 2B summarizes the FISH results. Corresponding protein levels of HER2 and RARα were analyzed by western blot using specific antibodies (Figure 2C). In concordance with the FISH results, HER2 protein levels were undetectable in MCF-7 cells, moderate in

MDA-MB-361 cells and high in BT-474 and SK-BR-3 cells. RARα protein could be detected in all cell lines except MDA-MB-361. Levels were moderate in BT-474 cells, but high in RARA amplified SK-BR-3 and ERα-positive MCF-7 cells.

To test whether amplification of the RARA gene leads to a good response of cell lines to RA, we performed growth essays with these cell lines, testing their response to the HER2-targeting antibody Herceptin and RA over 9 days. Response to RA correlated with RARA amplification (Figure 2D), as RARA-amplified SK-BR-3 were the only tested HER2-amplified cells that responds to RA, with even greater sensitivity than the ERα+ MCF-7 cells. As expected, all HER2-positive cell lines respond to some extend to Herceptin. Thus, we propose that HER2-RARA co-amplified tumors represent a class of breast tumors that could benefit from treatment with retinoids, potentially in combination with Herceptin.

Herceptin™ and RA synergize in SK-BR-3 cells to reduce cell growth and viability

Since SK-BR-3 cells carry a HER2/RARA co-amplification and are sensitive to the antiproliferative effects of RA, we hypothesized that treatment of these cells with a combination of Herceptin and RA could have synergistic effects. We used an MTS assay to determine cell viability after a 5-day treatment with Herceptin and/or RA. When combining the two drugs together at either a 1:30 or a 1:100 ratio, the effect of the combined treatments exceeded the combined effects of the two individual treatments at the same concentration, suggesting a synergy (Figure 3A). Using the Chou and Talalay method (34), combination index values were determined to be lower than 1 (Figure 3B) for affected

fractions of ≥20%, indicating a synergy between RA and Herceptin at both ratios. A similar synergy was not observed in BT474 cells, which are not RARA-amplified (Suppl. Figure 1). To test whether the observed synergy is due to the specific activation of RARα, we also performed the MTS assays using the RARα selective ligand Am580 and the RARβ/γ selective ligand TTNN (Suppl. Figure 2). A synergy was observed with the Am580 but not with TTNN, indicating the importance of RARα in the synergy between RA and Herceptin.

Herceptin™ enhances transcriptional regulation by RA

Since the anti-proliferative effects of RA are mediated by some of its target genes (Rozendaal *et al*., in prepararation), we hypothesized that part of the observed synergy in antiproliferative effects could be due to an enhancement of RA-mediated transcriptional regulation by Herceptin. We performed gene expression microarray analysis of SK-BR-3 cells treated for 24 hours with either 30 nM RA, 1μg/ml Herceptin or a combination of the two. Using principal components analysis of all 48803 probes, a pair of coordinates was determined for each mRNA profile to construct a two-dimensional view that reflects the relative global similarity or dissimilarity of the profiles to each other. On this two-dimensional view, individual profiles within each experimental group formed distinct clusters from the other groups (Figure 4A), which is a good indication both of reproducibility for replicate profiles within each treatment and widespread differences in gene expression between treatments. Principal component analysis also indicates that the effect of the combined treatment is not solely due to either one of the individual treatments (Herceptin+RA samples fall in a separate quadrant, Figure 4A).

Treatment with Herceptin™ alone does not lead to a big change in gene expression (Figure 4B). Only 82 genes were found to be regulated in a significant way (54 up-regulated and 28 down-regulated genes). In comparison, RA treatment resulted in 621 regulated genes (396 up-regulated and 225 down-regulated genes). Combining the two treatments greatly increases the number of regulated genes in a manner that was more than additive, with a total of 1569 genes regulated (793 up-regulated and 776 down-regulated genes). Strikingly, Herceptin not only increased the number of regulated genes, but also the overall amplitude of the transcriptional regulation (see heatmap in Figure 4C).

Herceptin and RA synergistically regulate genes implicated in cell cycle and cell death

Genes regulated by RA, Herceptin, or the RA and Hercepting combination were analyzed using Ingenuity Pathway Analysis (IPA). As expected, all three groups of genes were strongly enriched in genes regulating cell cycle and cell death related processes (Figure 5A), and this enrichment was much stronger in the co-treatment than in either one of the individual treatments. Next, we performed Q-PCR analysis to validate the regulation of selected genes, as well as the apparent synergistic regulation of some of these genes in a time-course experiment (Figure 5B). The cell cycle regulators E2F2, E2F7, MCM6 and MCM10 are clearly downregulated in a synergistic manner by RA and Herceptin. This synergy is observed at 8 hours of treatment, whereas cell cycle arrest cannot be observed before 24 to 48 hours (data not shown). PIK3R2, the p85 regulatory subunit of the phosphoinositide-3-kinase, important in the HER2 downstream signaling cascade, is also downregulated in a synergistic manner. On the other hand, FOXO3A, a well-know inhibitor

of cell cycle progression and regulator of apoptosis, is upregulated in a synergistic manner by the two treatments at later timepoints.

A possible role for FOXO transcription factors in mediating the synergy between RA and Herceptin

In our search for potential mediators of the synergy between Herceptin and RA, we became particularly interested in the transcription factor FOXO3A. As mentioned above, this gene is a potent regulator of both cell cycle and apoptosis and regulated by both RA and Herceptin. Recently, the FOXO member FOXO1 was shown to be important in the response of SK-BR-3 cells to Herceptin (47). Also, FOXO3A has already been implicated in the response of breast cancer cells to the EGFR-inhibitor Iressa™ (48). Here we find that FOXO3A is synergistically upregulated by Herceptin and RA (Figure 5B). Since FOXO3A is a transcription factor, it could play a role in amplifying the transcriptional synergy of Herceptin and RA. To determine whether RA-Herceptin target genes are potential targets of FOXO3A, we performed a search for FOXO transcription factor binding sites in the promoter regions of regulated genes using the TRANSFAC database. FOXO binding sites (motif presented in Figure 6A) were enriched at ±2.5 kb and ±5 kb around the transcriptional start sites of RA-Herceptin target genes (Figure 6B and C). To more directly identify FOXO3A target genes in SK-BR-3 cells, we transiently overexpressed FOXO3A and performed a gene expression microarray analysis. Next, we used gene set enrichment analysis (GSEA) to investigate the enrichment of these targets with in the RA-Herceptin target genes. The enrichment plot in Figure 6D shows a strong enrichment of FOXO3A

targets in RA-Herceptin regulated genes, substantiating the importance of FOXO3A as a mediator of RA-Herceptin synergy in breast cancer cells.

Genes regulated by Herceptin and RA predict overall- and distant metastasis-free survival.

To investigate whether Herceptin and RA co-treatment may have beneficial effects not only on HER2-RARA co-amplified SK-BR-3 cells, but also on tumors carrying a similar co-ammplification, we investigated whether genes regulated by the co-treatment have a prognostic value in HER2+ tumors. We used the largest publicly available dataset of HER2-positive tumors from Staaf *et al.* (46), including 58 HER2+ tumors. Tumors were collected form Lund University and the Reykjavik University but were all processed together. Adjuvant treatment of these tumors consisted of endorcine therapy, chemotherapy or a combination of those. Information on neo-adjuvant treatment is not available, however communication with one of the authors confirmed that none of the patients had received Herceptin treatment. Tumors were separated into two different classes based on their expression of RA-Herceptin regulated genes (Figure 7A). These two classes differed in a statistically significant manner in distant metastasis-free and overall survival, as illustrated by Kaplan-Meier curves (Figure 7B and C). Higher expression levels of RA-Herceptin upregulated genes and lower expression of downregulated genes correlated with fewer metastases and higher overall survival. Reproducibility of these results was confirmed

using smaller datasets (data not shown). Thus, RA-Herceptin target genes predict outcome in HER2+ tumors and some of them may directly contribute to limiting tumor progression.

DISCUSSION

Anti-proliferative effects of retinoic acid in solid tumors such as breast cancer have been studied for well over a decade, but despite promising results in cell line models, responses in tumors have been less successful. This is possibly due to the fact that only subsets of mammary tumors are responsive to retinoids. Here we have investigated whether gene amplification of the RARA gene in breast tumors defines a class of breast tumors that might benefit from treatments with retinoids.

In most cases HER2 overexpression in breast cancer results from gene amplification, which has been shown to include several other genes, one of which can be the RARA gene, encoding the retinoic acid receptor alpha (19). It was shown that the activation of RARα alone is sufficient for mediating the antiproliferative effects of RA in the SK-BR-3 and T47-D breast cancer cells (7). Regulation of RARα by estrogens also appears to explain the greater sensitivity of ERα positive cell lines to the effects of RA. Thus, amplification of RARA, leading to higher expression of RARα, may characterize a class of mammary tumors that could benefit from treatment with retinoids. We therefore first investigated whether large amplicon size in HER2 amplified tumors correlates with higher RARα mRNA levels. Figure 1C and E show that this is indeed the case. We also observed a negative correlation between large amplicon size and RALDH3 mRNA levels (Figure 1D and F), suggesting that absence of RA production in these cells allows for proliferation even in the presence of high levels of RARα. Those observations are similar to our recent observations that RARα-expressing luminal breast cells require suppression of RALDH3

expression for proliferation (Parisotto *et al*, in preparation). The co-amplification of the RARA and HER2 genes has been described in tumor samples , possibly defining a subclass of RA-sensitive breast tumors.

It has been suggested by Arriola *et al*. that the SK-BR-3 cell lines carries a co-amplification of HER2 and RARA (17). The high RARA (Rozendaal *et al*., in preparation; present study Figure 2C) and low RALDH3 (Parisotto *et al*., in preparation) mRNA and protein levels in these cells are consistent with the high RARA and low RALDH3 mRNA levels in tumors with the large amplicon (Figure 1). We used FISH to confirm amplification of the RARA gene (Figure 2A-B) and then investigated the potential of targeting both HER2 and RARA to inhibit tumor cell growth. RA and Herceptin, at a 1:30 or 1:100 ratio (M:g/ml), were strongly synergistic in reducing cell viability (Figure 3) and proliferation **(**Suppl. Figure 3**)** in SK-BR-3 cells, but not in BT-474 cells (Suppl. Figure 1). The latter express RARα (Figure 2B), but do not carry a co-amplification of RARA and HER2 (Figure 2A-B), suggesting the large amplicon is a requirement for the synergy.

Recently, Koay and colleagues observed a synergy between Herceptin/RA and Herceptin/RA/Tamoxifen in reducing proliferation in BT474 cells but not in SK-BR-3 cells (49). This might in part be explained by differences in culture conditions. In the present study, all proliferation assay were performed in 5% FBS, whereas in their assays Koay *et al*. used 10 or 15% FBS, respectively. This means that different concentrations of growth factors were available, especially for SK-BR-3 cells, a factor that is likely to impact the response to growth inhibitory signals. It will be important to further investigate impact of the local availability of growth factors on the synergy. It is possible that elevated levels of

for example EGF or other ligands for EGFR or IGFR family members could bypass inhibitory effects of Herceptin. In that respect, it could also be of interest to investigate the possible synergy between RA and other HER2 inhibitors, particularly lapatinib, which targets both HER2 and EGFR (50).

The work of several groups, including ours, has described the importance of transcriptionally active RARs, particularly RARα, for the antiproliferative response of breast cancer cells to RA ((7; 11; 51-54); Rozendaal *et al.* in preparation). We report here that Herceptin enhances the transcriptional regulation by RA (Figure 4B, C). This might in part be because of reduced activity of Akt, a key downstream molecule in HER2 signaling. Treatment of SK-BR-3 and BT-474 cells, but not of Herceptin-resistant cell lines, resulted in lower levels of active Akt (27). Akt phosphorylation of RARα was shown to be inactivating it in non-small cell lung carcinoma (NSCLC) cells, thus the inhibition of Akt signaling by Herceptin could lead to more active RARα in the cells (55). Impact of Herceptin on transcriptional regulation by RA has previously been suggested in MDA-MB-453 cells, where pretreatment with Herceptin increased binding of RAR to an RARE *in vitro* (56).

Forkhead O (FOXO) transcription factors are important mediators of cell cycle arrest, DNA repair and apoptosis. They are major targets of Akt and SGK kinases, which inactivate them. Loss of FOXO function can lead to uncontrolled proliferation (reviewed in (57)). FOXO3A has been identified as a critical signaling molecule in the response of breast cancer cells to the EGFR inhibitor Iressa (48). Similarly, FOXO1A was shown to be a target for Herceptin treatment in SK-BR-3 cells (47). Constitutively active FOXO4 was

shown to reduce HER2-mediated tumorigenicity through modulation of Akt activity and $p27^{Kip1}$ stabilization (57). We have looked at the possible implication of FOXO3A in the synergy between RA and Herceptin, because this gene is regulated by both RA and Herceptin and its regulation is enhanced by the cotreatment (Figure 5B). Both FOXO binding sites and FOXO3A targets are enriched in Herceptin-RA target genes (Figure 6B-D). Target genes that are common between Herceptin-RA and FOXO3A play roles in tumorigenesis and proliferation (Suppl. Table I**)**. Together, these observations strongly suggest a role for FOXO3A in mediating at least part of the synergy between RA and Herceptin. Other FOXOs may share a subset of FOXO3A target genes, due to similarity in the DNA binding domain (58). FOXO1 is regulated 1.46 fold by RA in our arrays and this regulation is comparable (1.51 fold) in the Herceptin-RA condition. Herceptin alone does not significantly increase FOXO1 expression (Suppl. Table II**).** FOXO4 regulation is not significant for either one of the individual treatments, but this gene is increased by 1.42 fold after cotreatment. Thus, other FOXOs might also be implicated in the synergy. More studies will be needed to better understand the individual roles of FOXO factors in the observed synergy.

A major problem of Herceptin-based treatments is the intrinsic or acquired resistance of tumors that eventually occurs in virtually all patients. Several molecular mechanisms for this resistance have been proposed. Interestingly, RA and RA-Herceptin treatments regulate some of the genes that have been implicated in this resistance, including multiple components of the IGF signaling pathway, which can be hyperactivated in Herceptin resistance. The best example is IGFBP3, an inhibitor of IGF1 mediated activation of

IGF1R. Addition of IGFBP3 to the culture medium of IGF1R overexpressing SK-BR-3 cells can overcome Herceptin resistance (59). Thus, it is possible that upregulation of this gene by RA will counteract Herceptin resistance in breast cancer cells due to increased IGF1R signaling. On the other hand, IGFL1, belonging to the IGF family (60), is down regulated, which could diminish IGF1R signaling. Although it has not previously been implicated in Herceptin resistance, another gene of interest in this context could be IGFBP5. This gene has been shown to be associated with metastasis and aggressive tumor phenotype in breast cancer (61; 62) and is downregulated by RA, an effect that is enhanced by the co-treatment with Herceptin. In MCF-7 cells, IGFBP5 appears to contribute to the survival effects of IGF (63). On the other hand, IGFBP5 was also shown to have antiproliferative roles in some cell lines (64). Therefore, its role in breast cancer and potentially in the resistance to Herceptin is likely to depend on the cellular context. The downstream effect of increased IGF1R signaling leading to Herceptin resistance is increased levels of the $p27^{Kip1}$ ubiquitin ligase SKP2, and the latter is also downregulated by RA treatment. Decreasing levels of $p27^{Kip1}$ due to the overexpression of SKP2 depend on PI3K/Akt signaling, which is also affected by RA treatment. The p85 regulatory subunit of PI3K is downregulated by RA (PIK3R2; Figure 5B), as well as AKT. On the other hand, PIK3IP1, a negative regulator of PI3K (65; 66) is upregulated by RA. Thus, RA is affecting multiple facets of pathways implicated in HER2 signaling and Herceptin resistance and could be of interest in battling the problem of Herceptin resistance.

Because of the beneficial effects of RA-Herceptin treatment in HER2/RARA SK-BR-3 amplified cells, we hypothesized that expression of its target genes could be correlated with

outcome in HER2 positive tumors. Indeed, as can be seen in Figure 7A, two clusters of HER2 positive tumors can be distinguished according to expression levels of RA-Herceptin target genes, and correlated with lymph node status, metastasis and death. This clustering accurately predicts tumor outcome based on distance metastasis free survival (Figure 7B) and overall survival (Figure 7C). These results suggest that genes targeted by simultaneous activation of RA signaling and suppression of HER2 activity correlate with and may contribute to better prognosis. In the original paper describing the dataset used to produce these results a 158 gene signature was proposed that predicts outcome of HER2+ breast cancer (HER2-derived prognostic predictor: HDPP). Comparing our HRA gene set to this 158 gene HDPP signature shows an overlap of only 15 genes. The Staaf *et al.* study identified 3 clusters, and described cluster 3 as follows: “Tumors in cluster 3 showed better OS and DMFS and had, to some extent, smaller size and less LN involvement but were also highly proliferative (S phase fraction, CSR activated), high-grade tumors with an active PI3K signaling signature." (46). These contradicting observations within the cluster suggest a suboptimal separation of the tumors. Using our HRA profile on cluster 3 of the Staaf study places 10 tumors into the poor outcome group, 6 out of 8 of those relapsed (2 data not available). Out of the 11 tumors that our profile put into the good outcome group only 2 relapsed. Although it is hard to do statistical analyses on these results due to low patient numbers, this suggests that our HRA profile could be used to improve results obtained with the HDPP signature.

Altogether the results presented in this paper suggest that HER2/RARA co-amplification defines a subclass of breast tumors sensitive to RA induced growth arrest.

The synergy observed between Herceptin and RA in a cell lines carrying such a co-amplification suggests the therapeutic benefit of combining HER2-targeting therapies with retinoids to improve response rates and reduce resistance. *In vivo* models for this subtype of HER2-amplified breast tumors will need to be developed to further verify the predicted therapeutic benefit of combined treatment with Herceptin and retinoids, in particular RARα-selective retinoids.

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FIGURE LEGENDS

Figure 1: Boxplot representation of HER2, TOP2A, RARA and ALDH1A3 expression in pretreatment biopsies from 120 patients with ER negative breast cancer treated with Epirubicin monotherapy as neoadjuvant chemotherapy (45). (A-B) High HER2 and TOP2A mRNA levels were observed in HER2+ amplified and TOP2A amplified tumors, respectively. (C-F). Low RALDH3 mRNA levels and high RARA mRNA levels were observed in the HER2/TOP2A co-amplified tumors. (A-D) HER2 status (amplification) and TOP2 status (deletion/amplification) was tested by FISH. TOP2A status was recoded as not amplified (ratio < 2) and amplified (ratio ≥ 2). (E-F) Cutoffs were derived from fitting a mixture of two normal distributions to the observed distribution of HER2 and TOP2A mRNA levels by maximum likelihood optimization. HER2 positivity and TOP2A positivity were defined as an mRNA expression above those specifics cutoffs.

Figure 2: RARA can be coamplified with the HER2 gene. (A) FISH analysis of four breast cancer cell lines, showing SK-BR-3 cells carry an amplification of HER2 (left panel, green), as well as TOP2A (left panel, red) and RARA (right panel, red/green). MCF-7 cells, known not to have an amplification of the HER2 gene, were used as a control. (B) Summary table of performed FISH analysis. -, no amplification; +, amplification detected at this locus; --, deletion detected at this locus. CEP17, chromosome 17 centromere marker used as a control of ploïdy. (C) Western blot showing HER2 and RARα protein levels in the analyzed cell lines. (D) 9 day growth essay showing the response of cells to 1μM RA,

10μg/ml Herceptin or the combination of the two treatments. (C, D) Representative results of two individually performed experiments are shown.

Figure 3: Herceptin and RA synergize to reduce cell growth and viability in SkBr-3 cells. (A) Cells were grown in the presence of RA and/or Herceptin at indicated concentrations. After 5 days medium was replaced by medium containing MTS reagent and incubated for 2-3 hours before colorimetric analysis. Bars represent relative viability compared to vehicle control. Ratios are given as nM RA for ng/ml Herceptin. (B) Combination index plot for the analysis of cooperativity between the RA and Herceptin at 1:30 and 1:100 ratios. CI < 1 indicates synergy. Fa, affected fraction (34). A representative result of 3 separately performed experiments is shown.

Figure 4: Co-treatment with Herceptin and retinoic acid enhances gene expression regulation compared to individual treatments. SkBr-3 cells were treated with RA (30 nM), Herceptin (1 μg/ml), Herceptin and RA or vehicle for 24h. Cells were then harvested and total RNA was extracted for analysis on Illumina WG-6 chips. (A) Principal components analysis plot of global mRNA expression profiles of RA, Herceptin and the co-treatment in SkBr-3 breast cancer cells. Principal components (the first two being shown) were extracted using all 48,803 Illumina WG-6 probes. (B) Venn diagram of genes showing regulation in each treatment relative to vehicle. (C) Supervised clustering of expression values for mRNA transcripts showing significant up- or down-regulation ($|FC| \geq 1.4$ and adj. p.value ≤ 0.01) in at least one of the three SkBr-3 treatments, relative to

vehicle. Genes significantly expressed were ordered by their RA fold induction in SkBr-3 cells.

Figure 5: Synergy of regulation of cell cycle and cell death processes. (A) Top 4 enriched Molecular Functions in target genes of 24h RA and/or Herceptin treatment. Enrichment studies were performed using Ingenuity Pathway Analysis software. (B) Q-PCR kinetics of regulation by RA and Herceptin of selected cell cycle related target genes. Regulation is strongly enhanced with combined treatment compared to the individual treatments. A representative result of 2 separately performed experiments is shown. Error bars represent standard deviations on three replicats within the same experiment.

Figure 6: Regulator of growth arrest and apoptosis FOXO3A as a potential mediator of the Herceptin-RA synergy. (A) Position weight matrix used for identification of putative FOXO sites in the vicinity of target genes. (B) Enrichment of FOXO sites in a window of 2.5 kb from the TSS of upregulated genes. (C) Enrichment of FOXO sites in a window of 5 kb from the TSS of upregulated genes. (D) Enrichment of FOXO3A regulated genes in Herceptin+RA regulated genes. The top portion of the enrichment plot shows the running enrichment score for the FOXO3A-induced genes. The bottom portion of the plot shows the value of the ranking metric.

Figure 7: Herceptin-RA target genes expression in HER2+ human primary breast cancers. (A) Shown are the subsets HER2+ of breast cancer samples (46) with the strongest coordinate induction of the Herceptin-RA targets genes after accounting for multiple hypothesis testing (FDR <0.05). Heatmap colors represent fold change induction

in log-space. Clinical characteristics of each tumor sample are presented in boxes below each sample. (B-C) Induction of Herceptin-RA upregulated genes in HER2+ breast primary tumors predicts reduced probability of distant metastasis (B) and death (C). Kaplan-Meier curves are shown for the complete dataset of 55 HER2+ tumors (46).

SUPPLEMENTARY DATA

Suppl. Figure 1: Herceptin and RA do not synergize in BT474 cells. MTS essays were performed as in **Figure 3**A. A representative result of three individually performed experiments is shown.

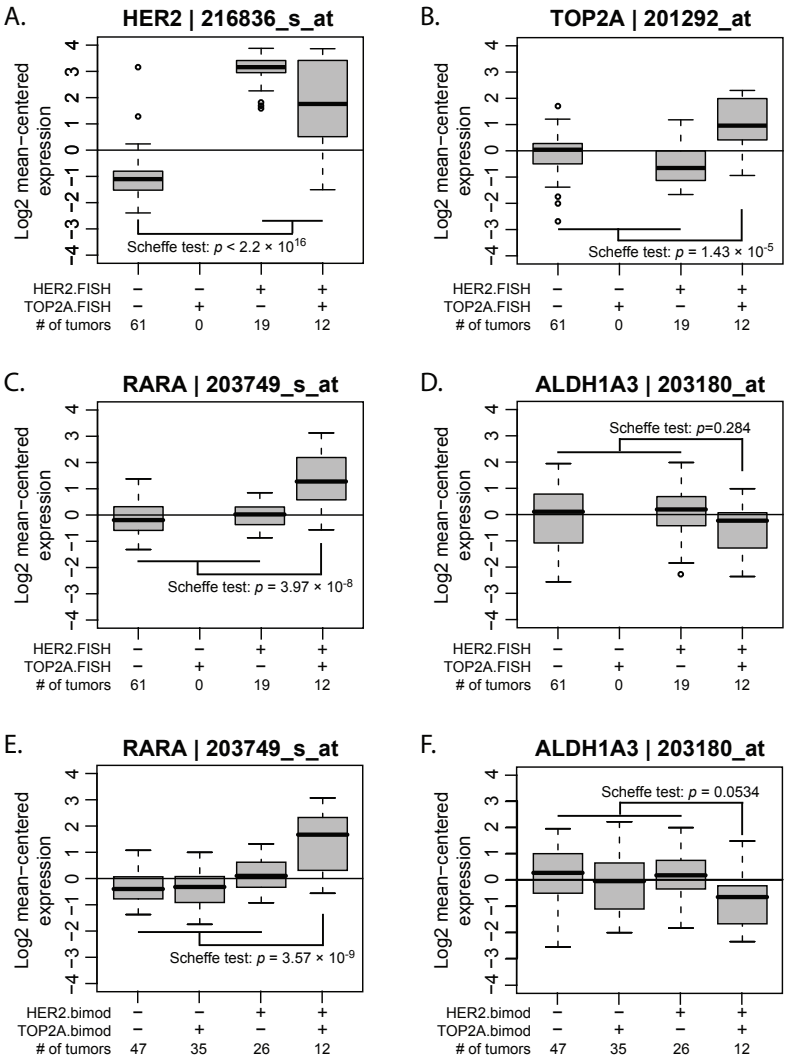
Suppl. Figure 2: Am580, but not TTNN, synergizes with Herceptin. MTS essays with Am580 (A) and TTNN (B) and CI calculations were performed as in **Figure 3**A. A representative result of three individually performed experiments is shown.

Suppl. Figure 3: Herceptin enhances RA mediated growth arrest. SkBr-3 cells were treated every 2 days with vehicle or RA and/or Herceptin at indicated concentrations. After 9 days protein concentrations were analyzed as an indirect measure for cell growth. A representative result of three individually performed experiments is shown.

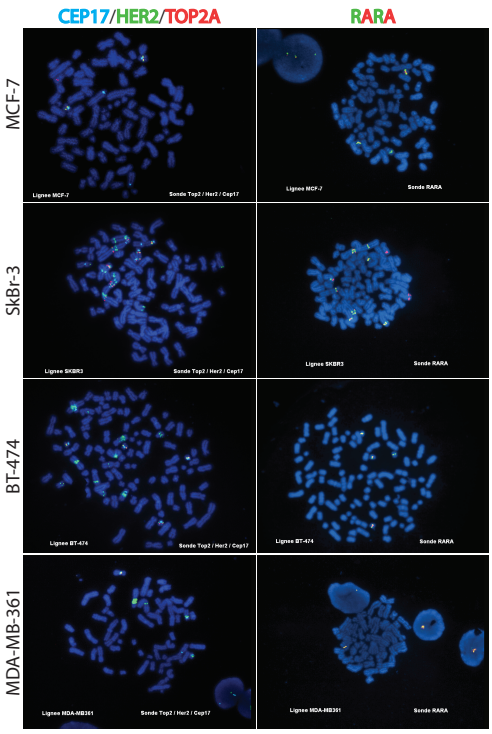
Suppl. Table I: IPA analysis of genes in common between Herceptin-RA treatment and FOXO3A overexpression.

Suppl. Table II: Regulation of FOXO genes by RA and Herceptin.

ROZENDAAL ET AL, FIGURE 1



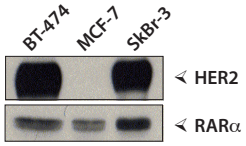
A.



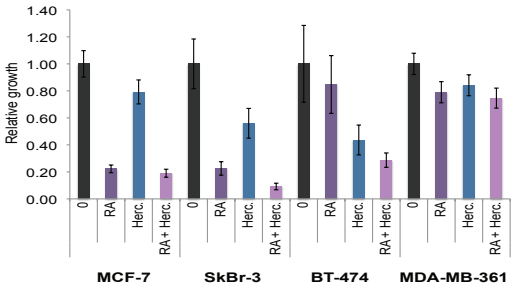
B.

Cell line	HER2	TOP2A	RARA	CEP17
MCF-7	-	-	-	Increased
SkBr-3	+	-	+	Increased
BT-474	+	+	-	Increased
MDA-MB-361	+	-	--	Increased

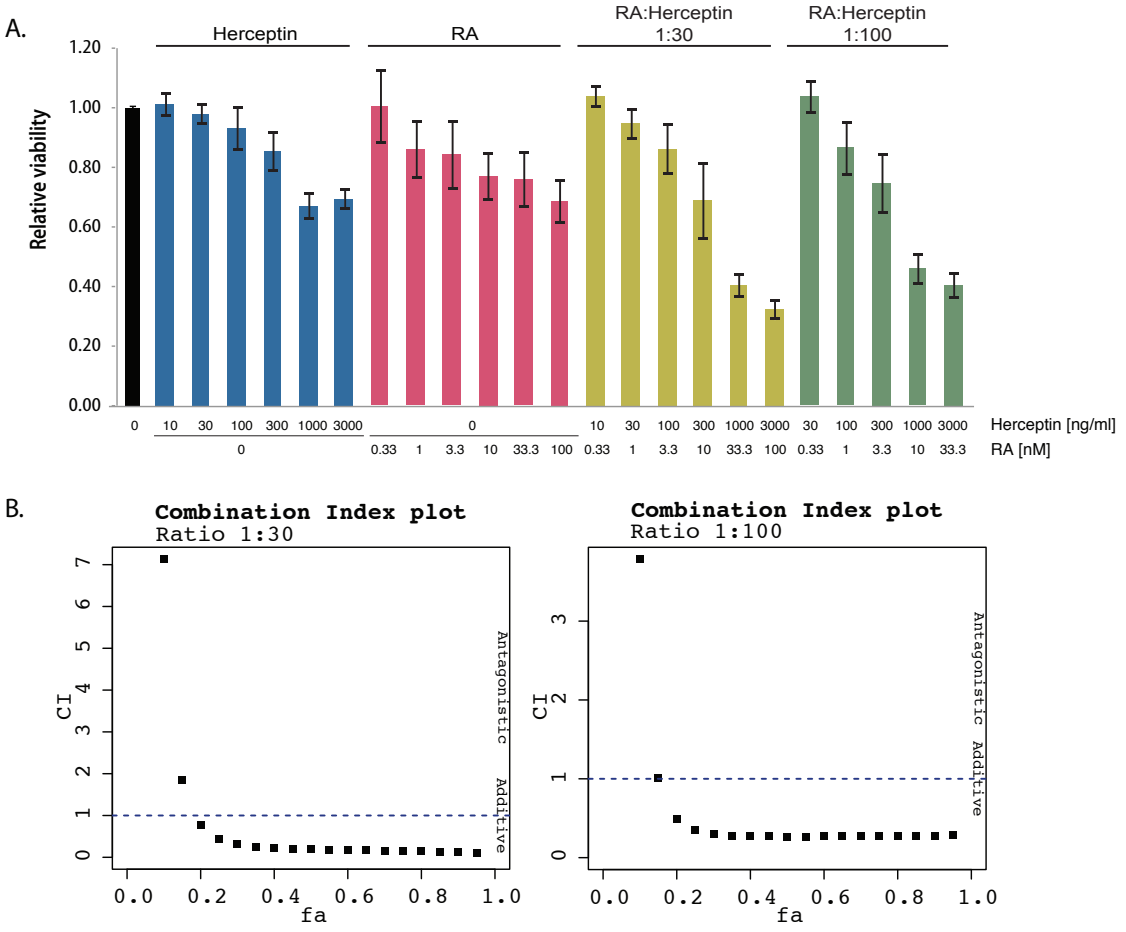
C.



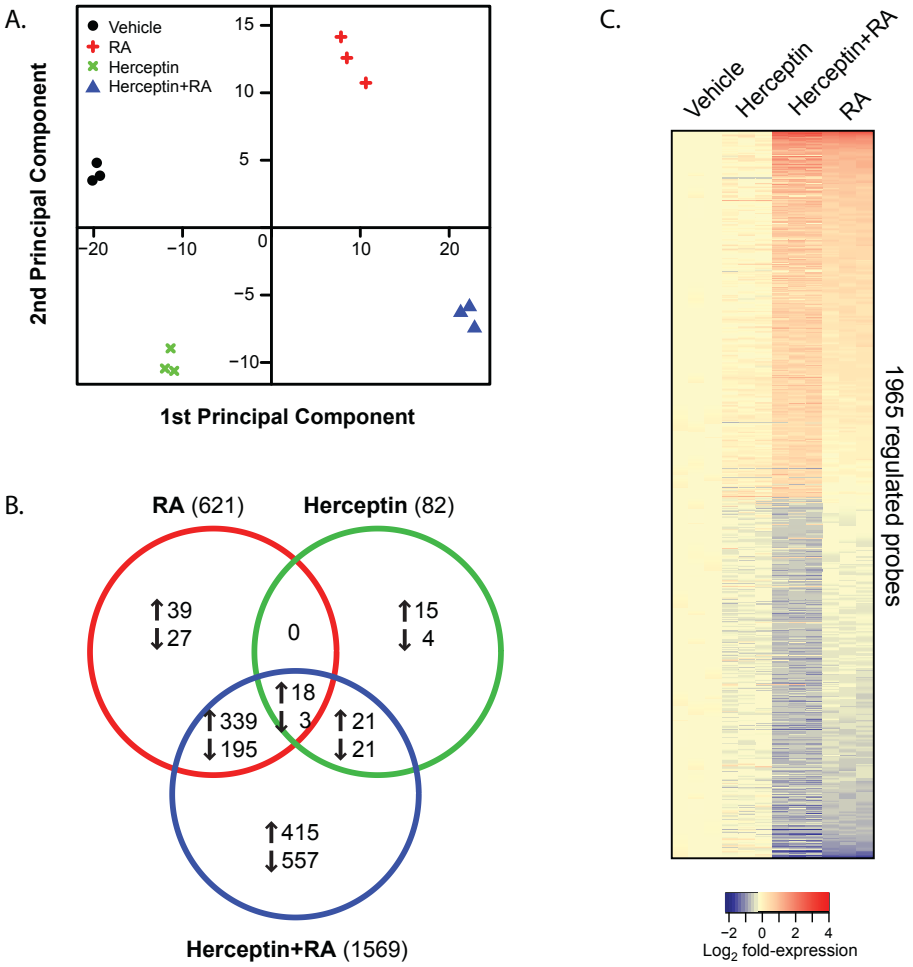
D.



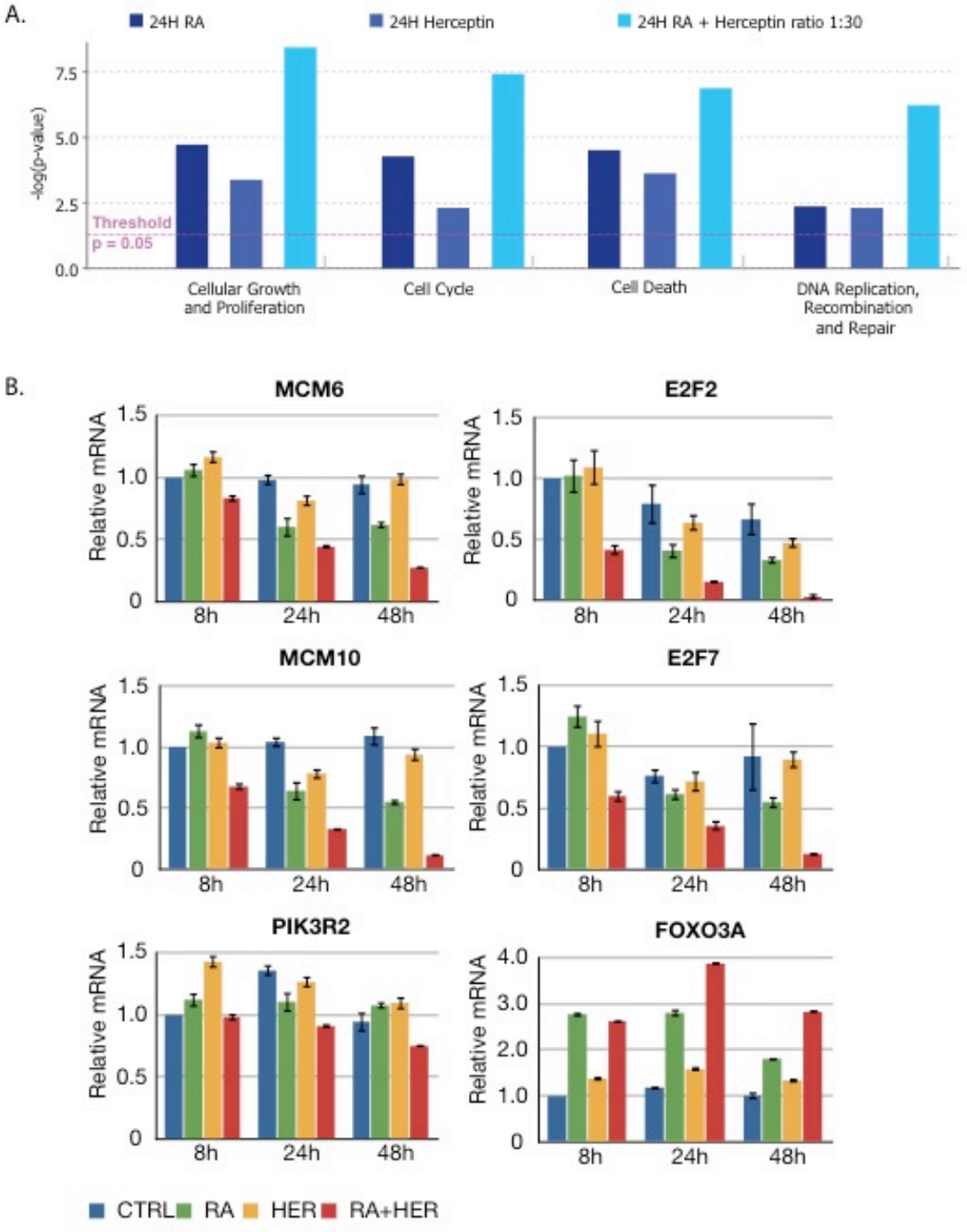
ROZENDAAL ET AL, FIGURE 3



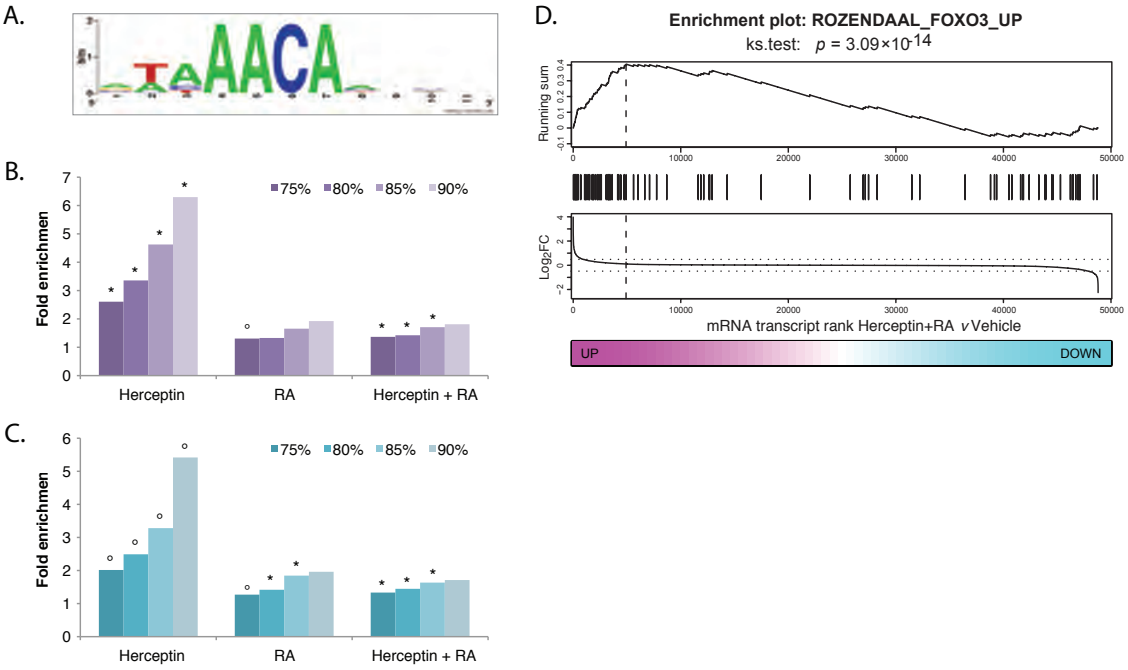
ROZENDAAL ET AL, FIGURE 4



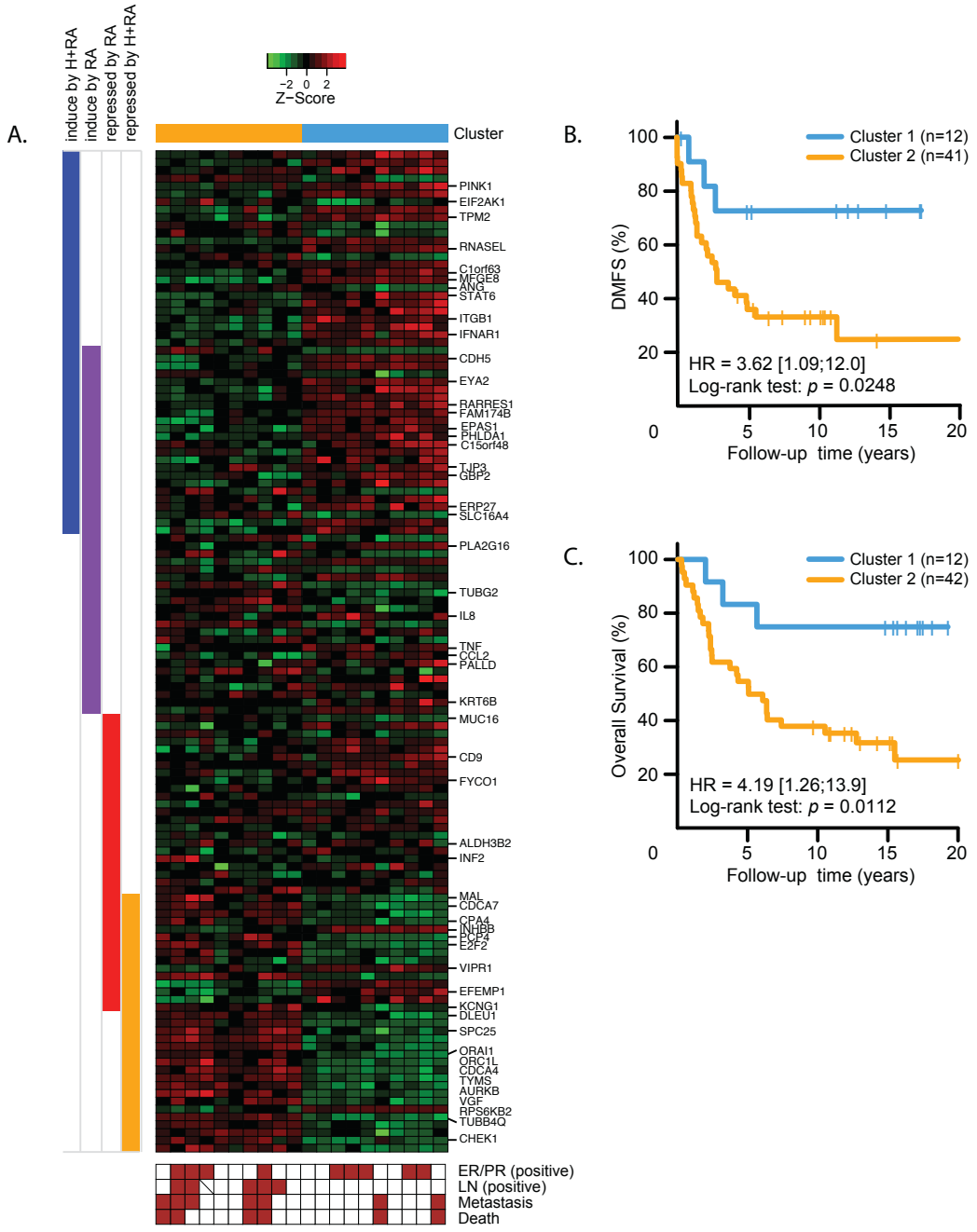
ROZENDAAL ET AL, FIGURE 5



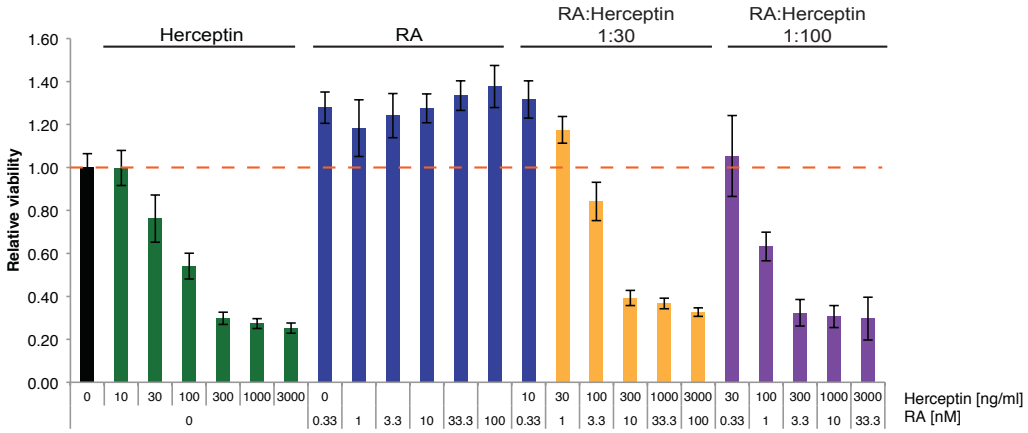
ROZENDAAL ET AL, FIGURE 6



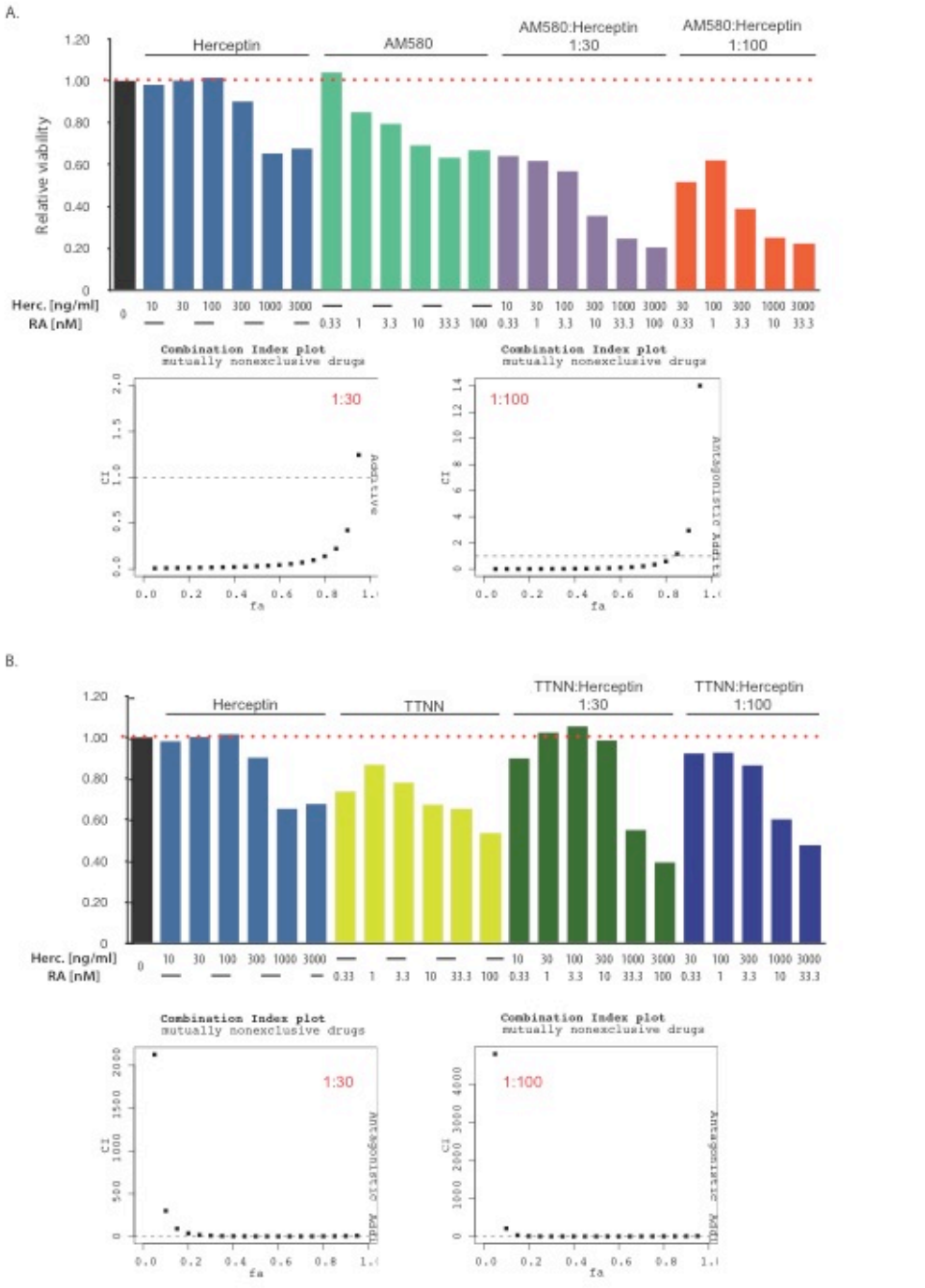
ROZENDAAL ET AL, FIGURE 7



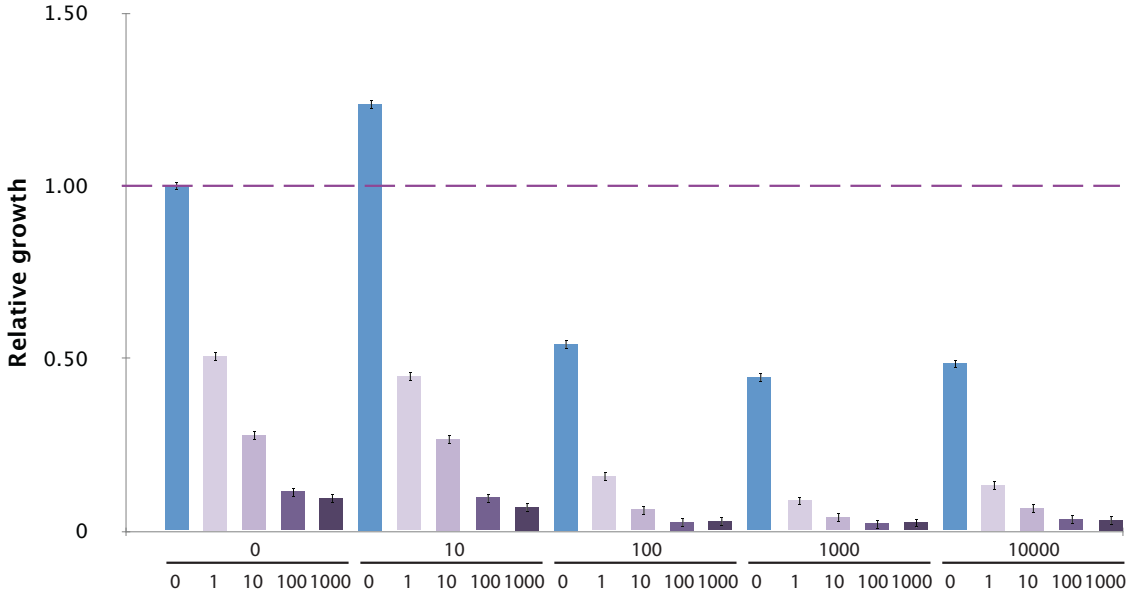
ROZENDAAL ET AL, SUPPL. FIGURE 1



ROZENDAAL ET AL, SUPPL. FIGURE 2



ROZENDAAL ET AL, SUPPL. FIGURE 3



ROZENDAAL ET AL, SUPPL. TABLE I

Category	Functions Annotation	p-Value	Molecules	# Molecules
Cancer	tumorigenesis	6.24E-05	EXOSC9, FOXO3, IFIT2, NUPR1, PDK4, PIK3IP1, RHOB, S100A14, SAT1, TXNIP	10
Cancer	cancer	2.41E-04	EXOSC9, FOXO3, IFIT2, PDK4, PIK3IP1, RHOB, S100A14, SAT1, TXNIP	9
Cellular Growth and Proliferation	proliferation of eukaryotic cells	4.84E-04	FOXO3, NUPR1, PIK3IP1, RHOB, SAT1, TXNIP, YPEL3	7

ROZENDAAL ET AL, SUPPL. TABLE II

Illumina probe ID	Gene	RA		HERCEPTIN		HERCEPTIN+RA	
		Fold	p-value	Fold	p-value	Fold	p-value
ILMN_1681703	FOXO3	2.22	2.25E-06	1.40	7.31E-03	3.57	1.44E-08
ILMN_1738816	FOXO1	1.46	3.80E-07	1.13	1.15E-02	1.51	8.11E-08
ILMN_1712095	FOXO4	1.12	1.02E-01	1.21	1.33E-02	1.42	2.28E-05

Third part:

Chapter 4: :General discussion

Chapter 4: General discussion

4.1 ER-dependent and –independent retinoid signaling in breast cancer cell lines

The role for ER in the signaling by RA has been a subject of discussion, but is still not entirely clear. In Chapter 2:, we used two luminal breast cancer cell lines, the ER-positive MCF-7 cells and the ER-negative SK-BR-3 cells to investigate ER-dependent RA signaling. We show that in ER-negative and ER-positive RA-sensitive cells there is a significant overlap of RA target genes. Several of these common genes were able to mimic RA-induced cell cycle arrest in ER-negative SK-BR-3 cells, indicating that intact ER-signaling is not required for these responses.

Specific targets in either cell line could explain cell line specific responses. In addition to a cell cycle arrest in G0/G1 phase, RA induces the expression of senescence associated β-galactosidase (SA-β-gal) in MCF-7 cells (Figure 8). Increased levels of SA-β-Gal were not detected in SK-BR-3 cells (Figure 8), suggesting that senescence is induced specifically in ER-positive cells and might depend on ER-signaling. It would be interesting to see whether the knockdown of ER in MCF-7 cells results in the loss of several or all of the MCF-7 specific target genes, and in particular of SA-β-Gal.

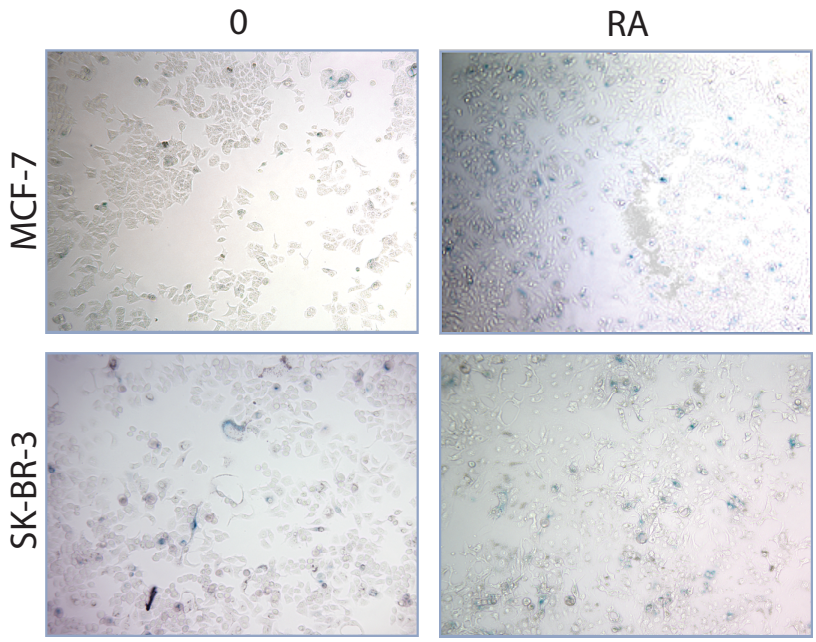


Figure 8 RA induces SA-β-gal expression in MCF-7, but not SK-BR-3 cells.
Cells were treated with 1 μM RA for 72 hours followed by gluteraldehyde fixation and X-gal staining at pH=6 for detection of senesence specific β-galactosidase activity.

On the other hand, in SK-BR-3 cells RA induces the formation of lipid droplets, that can be deteced by Oil Red O (ORO) staining (Figure 9). Lipid droplet formation has been associated with and used as a marker for lactogenic differentiation of mammary cells. In AU-565 cells, derived from the same patient as SK-BR-3 cells, lactogenic differentiation could be induced using a ligand for HER2, gp30, which at high concentrations inhibits proliferation of these cells (431). Although we could not observe the induction of other lactogenic markers such as β-casein expression and phosphorylation of Stat5A to be induced by RA in SK-BR-3 cells (data not shown), it is possible that these markers are induced at later time points of treatment. A differentiation phenotype in these cells correlates well with the observation that the expression of direct RA targets in SK-BR-3 cells is associated with a more luminal phenotype in breast tumor samples (Chapter 2:; Figure 7). The lactogenic phenotype in SK-BR-3 cells might depend at least in part on their

expression of HER2, which appears to be implicated in lactogenesis. In mice expressing a DN form of HER2 in the mammary gland lobuloalveaolar differentiation was disturbed and milk production was reduced (432). Since HER2 is not expressed in MCF-7 cells, this could account for the phenotypical difference between the two cell lines.

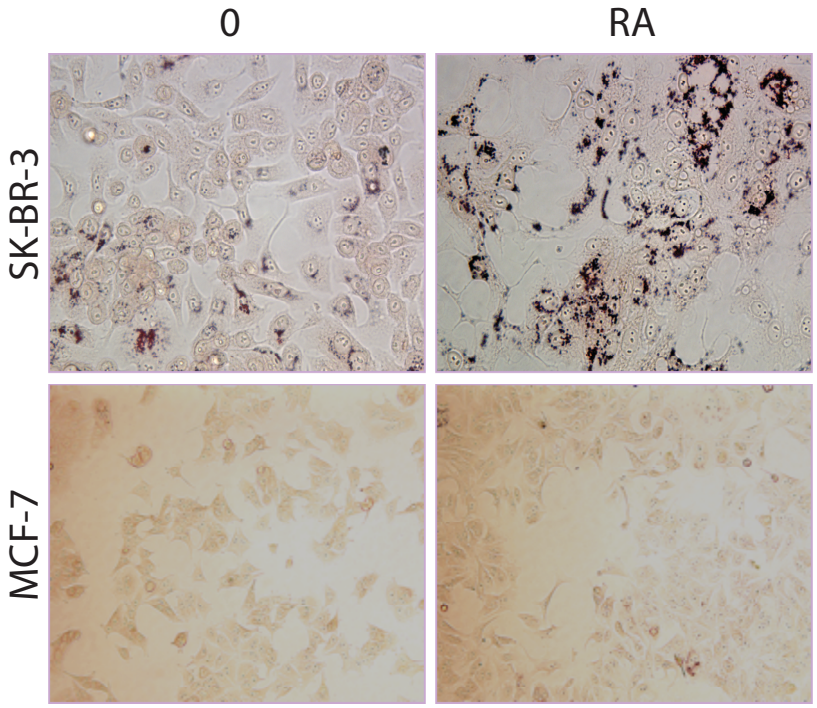


Figure 9 RA induces lipid droplet formation in SK-BR-3, but not MCF-7 cells.

Cells were treated with 1 μM RA for 72 hours, followed by fixation in 10% formol and staining with an oil red O (ORO) solution for the detection of accumulated lipids.

4.2 Modulation of proliferation and differentiation signals by direct retinoic acid target genes

4.2.1 Potential contributions of ER- and p53-dependent signaling in transcriptional regulation by RA and antiproliferative activity of RA target genes.

In Chapter 2, we used ER-negative SK-BR-3 cells and ER-positive MCF-7 cells, both sensitive to the proliferation inhibiting effect of RA, to identify targets genes of RA. Several RA target genes that were identified in this study have been described before to regulate apoptosis and/or proliferation in breast cancer cell lines. The homebox gene HOXA5 was shown to induce apoptosis in breast cancer cell line MCF-7 in a p53 dependent manner (406) as well as by activation of caspases 2 and 8 in p53-mutant cell line Hs578T (408). However, here we have looked only at the effects on G0/G1 phase of cell cycle, an event on which HOXA5 overexpression might not have an impact. Since it has been suggested that the response to RA in breast cancer is biphasic, that is to say, induction of G0/G1 arrest (2 days) followed by induction of apoptosis (5-6 days) (397), the HOXA5 gene might play an important role only in the second part of the response. It should also be noted that the SK-BR-3 cells used in this study carry a mutation in the p53 gene which is not the same as the one in Hs578T (433), and it is therefore well possible that overexpression of HOXA5 does not affect this cell line in the same way as it does p53-wildtype cells. BTG2 is a p53 target gene that is regulated in a primary manner by RA only in MCF-7 cells. In SK-BR-3 cells its regulation can be seen in the absence of CHX only, suggesting that primary regulation of this gene depends on intact ER signaling (Chapter 2; Table II, Suppl. Table I and II). BTG2 contains a functional LXXLL motif, through which it was shown to modulate transciptional activation by ERα (434). In addition to p53-dependent cell cycle arrest and apoptosis, part of its actions might therefore also come from modulation of ERα function. Since SK-BR-3 cells are ER-negative, this could explain the

limited effects observed in overexpression studies. It is worth noting that both HOXA5 and BTG2 seemed to have a very marginal effect in a two week colony formation assay performed in SK-BR-3 cells (data not shown).

Thus, it seems that some of the MCF-7 specific RA target genes might function in a p53 dependent manner. SK-BR-3 cells are not only ER-negative, but also have a p53 mutation. This could explain why those genes, even when expressed in an exogenous manner in this cell line, do not affect proliferation.

4.2.2 Networks of antiproliferative responses mediated by RA target genes

We have been able to identify several ER-independent RA target genes that are responsible for at least part of the antiproliferative response (Chapter 2:; Figure 6). Of the three genes that we found to induce a cell cycle arrest (SOX9, FOXA1, ELF3), two were induced in a synergistic manner by RA+Herceptin (SOX9, ELF3; Chapter 3:).

Analysis of transcriptional targets of SOX9 and FOXA1 strongly suggests their implication in mediating the antiproliferative effects of RA. IPA analysis of their target genes showed a significant enrichment of genes implicated in cellular functions such as cell cycle regulation, cell death and cellular proliferation. Overexpression of FOXO3A allowed to identify several transcriptional targets with roles in cell cycle and apoptosis regulation, and these targets were significantly enriched within the set of Herceptin+RA transcriptional targets. An effect on cell cycle progression could however not be observed upon overexpression of FOXO3A. This might be because in a situation where cells are not treated with RA and/or Herceptin too much active Akt is present and the overexpressed FOXO3A will be phosphorylated and inactivated. Differences between cell cycle analysis and gene expression studies could be explained by the duration of the overexpression : cell cycle analysis was performed after 72 hours of overexpression, whereas for gene expression studies cells were analyzed after 24 hours of overexpression. Therefore it is likely that in the gene expression studies more active FOXO3A was available in the cells. Repeating these experiments with a constitutively active form of FOXO3A (non-

phosphorylatable by Akt : T32A, S253A, and S315A; described in (435)) should allow for identification of more transcriptional targets. Also, such a mutant should be able to induce the expected cell cycle arrest.

4.2.3 Feedback mechanisms through regulation of RA metabolism and signaling

RA is known to regulate several genes that are implicated in its metabolism and signaling. This is also true in both of the studies presented in this thesis, as we identified several of these genes regulated.

RALDH3 was found to be regulated in both SK-BR-3 and MCF-7 cells in an indirect manner (Chapter 2; data not shown). In Chapter 3, RALDH3 was not regulated in a significant manner by RA alone in SK-BR-3 cells, but its regulation by Herceptin is borderline significant and increased in the RA+Herceptin cotreatment. It should be noted that in the Herceptin-RA study we used a three-fold lower concentration of RA, which could account for the discrepancy between the two studies. Our lab has previously shown that RALDH3 expression is suppressed in RA sensitive luminal breast cancer cells (Annex I; Figure 1 and 2), a requirement for proliferation of these cells. The expression of RALDH3 in SK-BR-3 or Zr75 cells led to the induction of a cell cycle arrest that was comparable to that observed upon treatment with RA (Annex I; Figure 5C and Suppl. Fig. 10B). Thus, it appears that this modulation of RA metabolism by RA in these cells may refelct a feed-forward mechanism in the anti-proliferative response.

CYP26A1 is the strongest regulated RA target gene in both cell lines and in both studies. This enzyme is know to be implicated in the transformation of RA into less active catabolites. It has therefore been suggested that this enzyme could be implicated in RA resistance. As mentioned before, breast cancer cells often have an increased capacity to induce the expression of this gene, thus providing them with an efficient way of decreasing local levels of RA that would otherwise inhibit their proliferation. Treatment with retinoic acid metabolism blocking agents (RAMBAs) has been suggested as an alternative route for

retinoid therapies and proven efficient in the treatment of skin conditions such as psoriasis (436). CYP26B1 is also a direct RA target is both cell lines (Chapter 2; Table II).

DHRS3 is regulated in both SK-BR-3 and MCF-7 cells in a CHX-insensitive manner. This gene encodes a short chain dehydrogenase that can mediate the conversion of retinal into retinol, but has also been implicated in increased retinyl ester production. Its upregulation by RA probably has limiting effects on RA signaling on short term periods, but might extend the duration of the signal due to increased retinoid storage. This will however depend on the cellular presence of other components of the RA metabolism pathway.

Other common upregulated RA target genes include the retinol receptor STRA6, the coactivator NCOA3 and the corepressors NCOR2 and NRIP1 (Chapter 2; Table II).

As becomes clear from the examples given above, the influence of RA on its own metabolism and signaling are multiple and variable. These feedback loops need to be investigate in more detail to better understand the mechanisms of RA response and resistance in cell lines and tumors.

4.2.4 The role of downregulated target genes

Although not further investigated in the present work, several of the RA downregulated genes could also be implicated in mediating growth arrest.

The cell cycle and apoptosis regulator KLF4 (Krüppel-like factor 4) is a transcriptional repressor that has previously been described to function as a context dependent oncogene and whose function depends on p21 and cyclin D1. When KLF4 was depleted from overexpressing breast cancer cells, this led to the induction of p53 dependent apoptosis (437). KLF4 has been shown to downregulate RARα expression by direct binding to the promoter and decrease RARα mediated PI3K and MAPK signaling in vascular smooth muscle cells (438). On the other hand, KLF4 was also shown to interact with ERα and reduce its transcriptional activity leading to decreased proliferation of breast cancer cells

(439). Another KLF family member, KLF5, was shown to interact with RARα in vascular smooth muscle cells, and this interaction was inhibited by treatment with the synthetic retinoic Am80 (440). Therefore, KLF4 might also function as a corepressor for RARα and its downregulation such as observed in SK-BR-3 cells in Chapter 2 could lead to enhanced RARα signaling and growth inhibiting effects.

Another interesting gene that was found to be downregulated in SK-BR-3 cells is SATB1. SATB1 is a global chromatin organizer and transcription factor, that is key factor integrating higher-order chromatin architecture with gene regulation. Gene expression is regulated by SATB1 through recruitment of chromatin remodeling enzymes and transcription factors to genomic DNA regions (441). SATB1 expression in breast cancer has been shown to promote tumor growth and is correlated with more aggressive tumor subtypes. Its overexpression in SK-BR-3 cells leads to the induction of a gene program correlated with aggressive tumor phenotypes and metastatic potential. On the other hand, the depletion of this gene from MDA-MB-231 cells made them less aggressive (442).

Both these genes could be implicated in the ER-independent response to RA in SK-BR-3. This would need to be confirmed by gene knockdown (mimic RA effect) and overexpression (reduced response to RA). Downregulated targets that mediate anti-proliferative effects could also be of potential interest as drugable target.

4.2.5 Modulation of TGFβ signaling by RA and its impact on breast cancer progression.

Several RA target genes that are common in SK-BR-3 and MCF-7 cells are part of the TGFβ signaling pathway, which plays important roles in normal mammary development as well as in mammary tumorigenesis. IPA analysis showed a statistically significant enrichment of TGFβ signaling components in RA-regulated genes, and this enrichment was stronger in the Herceptin+RA cotreatment (more genes in the pathway regulated). TGFβ

plays various roles in normal mammary development as well as in mammary tumorigenesis.

In mammary tumorigenesis, the effects of TGFβ as a tumor suppressor or an oncogene are variable and depend on the level of progression of the tumor. In normal epithelium, TGFβ functions as a tumor suppressor, arresting cells in G1 phase of the cell cycle. In early carcinomas the TGFβ pathway often gets altered leading to insensitivity of cells to TGFβ. Then as cells become more aggressive, they start producing excessive amounts of TGFβ, which will act on cells of the surrounding stroma and facilitate invasion and metastasis.

The oncogenic face of TGFβ in mammary cancer

In recent years the concept of the epithelial to mesenchymal transition (EMT) as a mechanism for cancer cells to escape primary tumor sites has become widely accepted. This process takes place in various biological settings such as embryogenesis, tissue morphogenesis and wound healing. It consists of the downregulation of E-cadherin and upregulation of certain integrins by epithelial cells, allowing them to move out of the epithelial cell layer (443). In the context of tumor biology this means the process allows epithelial tumors cells to dissociate from the primary tumor and metastasize to distant sites. Three specialized types of EMT can be distinguished, type 1 occurring during embryogenesis, type 2 during tissue remodeling and healing and type 3 during cancer progression (444). Cancer associated EMT is marked by loss of apicobasal plarity, disintegration of tight junctions and cytoskeletal changes, leading to the acquisition of a motile and invasive phenotype that resembles changes taking place in type 1 EMT. Among numerous inducers of oncogenic EMT that have been identified are Wnt, Snail/Slug, Twist and TGFβ (445). TGFβ has been identified by several studies as an important regulator of EMT in both normal and malignant mammary epithelial cells (446-448). TGFβ induced oncogenic EMT was shown to lead to the acquisition, selection and expansion of breast cancer stem cells (449) and a more metastatic phenotype.

In addition to its effects on EMT initiation, TGFβ that is produced in the tumor cells can act in a paracrine manner on the tumor microenvironment to potentiate tumor progression and invasion/metastasis. TGF-β influences stromal–epithelial interactions during carcinoma initiation and progression, affecting both fibroblast and immune cells present in the stroma. TGF-β enhances the migratory and invasive properties by inducing the synthesis of extracellular matrix proteins such as MMP-2 and MMP-9 and downregulating the protease inhibitor TIMP in tumor cells. Studies have shown that disrupted TGFβ signaling in the stromal compartment can lead to tumorigenesis (450, 451).

RA regulation of TGFβ in breast cancer

RA regulates genes implicated at different levels of the TGFβ signaling pathway (Figure10). This regulation can affect the pathway both positively and negatively, but overall appears to be mainly activating, with induction of positive regulators such as SMAD3, GDF15, SOX9 and TGFBR3. Thus, RA-mediated modulation of the TGFβ signaling pathway can have multiple impacts on breast tumor cells. In the context of the oncogenic face of TGFβ described above, the primarily activating effects of RA on this pathway seem contradicting. With respect to the observation that RA seems to induce a more luminal phenotype in breast cancer cells, generally less aggressive, activation of the TGFβ pathway is likely to turn out to be beneficial for tumor inhibition. However, in more aggressive tumors, TGFβ activation could also provide an explanation for the lack of clear benefits of retinoid treatment in breast tumors and observed adverse effects. This will be particularly true if the activation of TGFβ by RA leads to the activation of EMT, giving rise to a more metastatic phenotype and an increase of the cancer stem cell pool. It will be important to further investigate the role of TGFβ signaling in the response to RA, as it might be important to target this pathway in parallel when trying to use RA in breast cancer treatment.

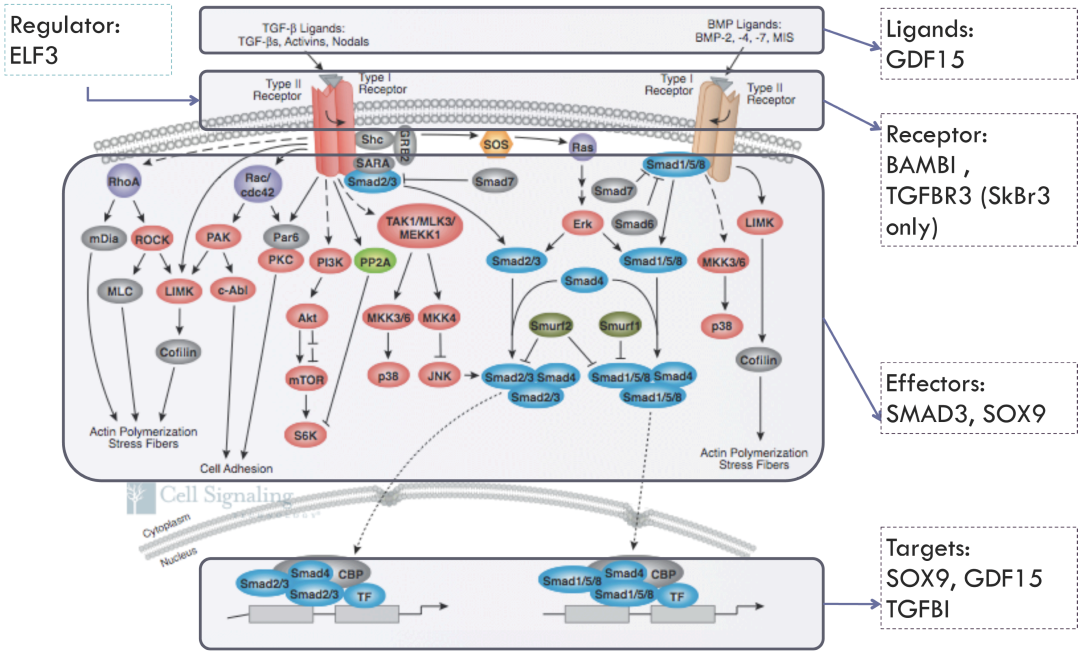


Figure 10 TGFβ signaling pathway
RA regulated components of the pathway are indicated. Figure adapted from www.cellsignal.com/pathways/tgf-beta-smad.jsp.

4.3 The role of other nuclear receptors liganded by RA

It has been described that RA can also function as a ligand for several other nuclear receptors and that in some cases this will lead to pro-proliferative effects rather than anti-proliferative effects, the best known example being PPARβ/δ (166). Even the activation of RARγ has been proposed to be proliferation-promoting in some contexts (421, 452). Stimulation by RA of RARβ expressed in the stroma was recently shown to be tumor promoting (453). It appears therefore that a vital point in the success or failure of retinoids as breast cancer treatments would be to specifically target the 'good' RA responsive receptor(s). It has been described in the literature that RARα appears to be the most important RAR mediating antiproliferative effects of RA in breast cancer cells (370). In

Chapter 3: of this thesis we highlight once more this importance when we show that cells carrying an amplification of the RARA gene are very sensitive to RA. A synergy between RA and Herceptin is observed only in HER2/RARA co-amplified cells and this is specific to RARα activation, since it can be observed also with the RARα selective ligand Am580, but not with the RARβ/γ selective ligand TTNN (Chapter 3:; Figure 3, Suppl.Fig.2), the latter actually appears to have an effect that is more antagonistic with Herceptin.

In Chapter 2: we have identified a large number of genes that have known or proposed inhibitory effects on cell proliferation. We did however also find induction of some genes that have been suggested to be rather pro-proliferative, such as for example ENPP2/autotaxin in SK-BR-3 cells. It would be very interesting to perform chromatin immunoprecipitation (ChIP) assays to determine if these genes are preferentially bound by for example PPARβ/δ rather than RARα. Also, RAR selective agonists and antagonists as well as shRNAs could be used to verify whether the balance between expression of different receptors affects the proliferative vs. antiproliferative outcome.

4.4 RA and luminal differentiation

In Chapter 2 we have observed that expression of direct RA target genes from ER-negative cells in tumor samples correlates with a more luminal phenotype and ER-positivity (Chapter 2; Figure 7). Interestingly, the used cell line SK-BR-3, although ER-negative, is generally classified as luminal and might represent less differentiated luminal cells. We find that several genes induced in an ER-independent manner are markers for the luminal subtypes of breast tumors and have been described to be involved in ER signaling, such as FOXA1, GATA-3 and ESR1 (Chapter 2; Figure 4). GATA3 and FOXA1 have both been shown to regulate the expression of ERα (264, 454). Where GATA-3 and ERα are essential determinants of luminal cell fate in the developing mammary gland and essential for lactation, FOXA1 is necessary for ductal outgrowth during mammary development (264). FOXO3A expression is not correlated with luminal tumor types, but does affect ERα

signaling in several ways, seemingly in a complex feedback system. FOXO3A mediated regulates the expression of ERα through direct binding and activation of the ESR1 promote (435). ERα status correlates with FOXO3A hypophosphorylation and nuclear localization. Contrarily, FOXO3A colocalizes with ERα and represses its transcriptional activity (455), as well as estrogen-dependent breast cancer cell proliferation and tumorigenesis (456). ERα in turn also induces FOXO3A (455).

Taken together these data clearly suggest a role for RA signaling in luminal differentiation in the mammary gland. In the developing mammary gland, RA is synthesized by locally expressed RALDH1 and RALDH3. Vitamin A deficiency in mice and rats was shown to delay mammary gland development, with fewer tubules and lobules being formed and overall smaller mammary glands, suggesting that RA is important in epithelial cell differentiation (457, 458). RA and Am580 have also been shown to be potent inducers (pmolar range) of lumen formation in 3D cultures of J3B1A mouse non-tumorigenic mammary cells. This was shown to depend on mRNA induction and activation of MMP-9 by retinoids (459). In mammary tumors, the expression of RALDH3 is often lost, and this correlates with luminal subtype and ER expression (Annex I; Figure 4). The loss of this enzyme appears to be essential to permit luminal tumor cell proliferation.

4.5 Mechanisms of synergy between Herceptin and RA

In Chapter 3: we have investigated the synergistic antiproliferative effect between Herceptin and RA on HER2-positive tumor cells. Since Herceptin affects the transcriptional activity of RA in ER-negative, HER2/RARA amplified breast cancer cells, we reasoned that mediators of the synergy could be transcriptional regulators that could either interact with and modulate the activity of RARα or function in parallel with it to induce growth arrest and cell death.

4.5.1 Forkhead factors in the transcriptional response to RA and Herceptin

We suggest a role for the FOXO family in mediating the synergistic response mainly because of its known effects on cell cycle and cell death and the fact that its members are synergistically regulated by Herceptin and RA (Chapter 3; Figure 5B and Suppl. Table II) and we find an enrichment of FOXO binding sites (Chapter 3; Figure 6B-C). Knockdown studies will need to be done to firmly show the role of FOXOs in the synergy. Importantly, due to the overlapping target genes of the FOXO family members, it might be necessary to knock down more than one member at a time, because other family members might be able to compensate for the lack of signaling.

Several members of the Forkhead family have been shown to be implicated in NR signaling. Various FOX proteins were found to interact with and function as corepressors of the androgen receptor (460-462). FOXA1 has been described as a pioneer factor for activation of AR and ER signaling (463, 464). FOXA1 can also interact with the progesterone receptor and modulate its activity (465). It is possible that one or more of the FOXOs that are regulated in a synergistic manner by Herceptin and RA could function as cofactors for RARα. In this respect, it would be interesting to see whether our predicted FOXO binding sites coincide with RARα binding sites, particularly ERα-independent sites. The ChIP-chip and ChIP-seq studies that we have analyzed in Chapter 2: could be used for a first analysis. However, since these studies were both performed in the ER-positive MCF-7 cells, and RARα binding is likely to be different in ER-negative SK-BR-3 cells, where only ER-independent RA signaling is present. Therefore, large scale ChIP analysis should be performed in SK-BR-3 cells in order to compare binding of RARα and FOXO3A (and/or other FOXO family members). If FOXOs are implicated in RARα mediated signaling and this is enhanced by the cotreatment with RA and Herceptin due to its synergistic induction and activation, we could expect to see a colocalization of RARα and FOXOs in promoter regions of coregulated genes.

4.5.2 Other possible mechanisms for RA-Herceptin synergy

Modulation of RARα activity by Herceptin treatment

In NSCLC cells it has been shown that Akt can phosphorylate RARα, leading to its inactivation (385). Whether this phosphorylation can also occur in breast cancer is not known. This could provide an additional explanation for the reporteded resistance of HER2-positive breast cancer cells to RA (331). Herceptin treatment, through inactivation of HER2, inactivates the normally constitutively active Akt in these cells. By doing so, it might also increase RARα transcriptional activity. This would be an additional explanation for the observed effect of Herceptin on RA mediated transcriptional regulation (Chapter 3; Figure 4B-C and Figure 5B).

ADCC

One of the mechanisms by with Herceptin suppresses propagation of HER2-positive tumors is by the activation of the immune system in a response that is known as antibody mediated cellular cytotoxicity. This response is mediated by natural killer cells, such as dendritic cells (346). As described in chapter 1.1.2.2, RA has been shown to play important roles in the function of the immune system. Of particular interest in the context of ADCC, RA has been shown to mobilize DCs (83). Although not relevant in *in vitro* cell culture models, this could provide for an even more efficient synergy between Herceptin and RA in an *in vivo* setting. It would be very interesting to study this possibility in a xenograft model of SK-BR-3 cells, quantifying mobilization of NK cells to the tumor site after Herceptin, RA or combination treatments.

4.5.3 Synergy with RARα specific ligands and/or other RTK inhibitors

The work described here has focused specifically on the synergy between RA and Herceptin. However, other combinations of retinoids and HER2 inhibitors or inhibitors of other RTKs and potentially of downstream signaling molecules such as PI3K could also be investigated.

In Chapter 3 we have observed a synergy between Herceptin and RARα ligand Am580, whereas the combined effect with RARβ/γ ligand TTNN is than additive at best, and is even antagonistic in some cases (Suppl. Figure 2). Since RA can activate all three RAR isotypes, the effect of co-treatment with Herceptin is likely to be variable according to the levels of different receptors expressed in a given cell type. RARγ was shown to have pro-tumorigenic effects in hepatocarcinoma cells. Those effects were found to be mediated by interaction of RARγ with the p85 subunit of PI3K and subsequent activation of Akt-dependent survival pathways (452). RARγ also increased the proliferation rate of immortalized mammary cell line MCF10A cell when overexpressed, rendering them insensitive to pRb and p27 dependent growth arrest (421). The existence of similar mechanism in breast cancer cells would explain the effects observed with TTNN. Although this retinoid has a higher affinity for RARβ, in our experimental setting the levels of RARβ are neglectable, whereas substantial levels of RARγ could be detected in SK-BR-3 cells by western blot (Chapter 2; Figure 1D). Particularly in an *in vivo* context, it is important to consider unwanted effects coming from stimulation of different RAR isotypes.

In addition to the effects of RARγ expression and stimulation within the tumor, RA stimulation of the surrounding stromal tissue might stimulate tumorigenesis. A recent study has shown that expression of RARβ in stromal cells leads to RA-induced mammary tumor formation (453). It will be of interest to assess the effect of knock-down of RARα compared to RARβ in stromal cells versus epithelial cells in order to test the hypothesis that RARα specific ligands may be advantageous for the treatment of breast cancer.

The major problem with Herceptin treatment is the high rate of intrinsic or acquired resistance. Although some of the gene programs that are activated by RA suggest that RA could prevent or delay resistance in a combination treatment setting, this is a hypothesis that remains to be confirmed. In the mean time, it could be worth looking into potential synergies with other molecules that target HER2. Pertuzumab, currently in phase II clinical trials for breast cancer, is another HER2 inhibitor that inhibits heterodimerization and

marks cells for immune attack. Lapatinib is a tyrosine kinase inhibitor that has been approved for the treatment of HER2-positive breast cancer (466). Both these molecules would be interesting to test in combination with RARα specific ligands. Because resistance to Herceptin often involves altered expression of and signaling by downstream pathways, notably PI3K/Akt signaling (360), inhibitors of downstream effectors such as PI3K could also be tested in combination with retinoids.

4.6 Retinoids in the treatment of breast cancer

4.6.1 HER2 positive RARA amplified breast cancer

The co-amplification of RARA with HER2 appears to be a fairly rare event. 33-50% of the HER2 positive tumors (~25% of total mammary tumors) were found to be RAR amplified, making for a total of around 10% of all breast tumors that would carry a HER2/RARA coamplification. To our knowledge, the only other cell line that has been described to carry this co-amplification is the UACC-812 cell line (318). We confirmed this co-amplification by FISH, however since this cell line has an extremely low proliferation rate, it is not a good model for *in vitro* proliferation assays.

To mimic the HER2/RARA amplification in a mouse mammary tumorigenesis model, one could consider creating mice that have mammary-specific overexpression of Her2 and Rara, using for example the MMTV promoter. However, it is possible that other genes that are present in the long amplicon contribute for the observed synergistic effect, and in such a model these genes would be expressed at normal levels. Therefore an *in vivo* beneficial effect of the retinoid treatment might be missed in such a model. SK-BR-3 cells form mammary tumors when injected in the fat pad of nude mice. This model could be used to study the effect of RA and Herceptin on HER2/RARA amplified tumors in the tumor microenvironment. Such *in vivo* studies are absolutely necessary to investigate the potential of retinoid treatment, particularly in HER2+ tumors, because a recent study has suggested

that RA could have tumor-promoting effects through activation of RARβ that is expressed in the stroma (453).

4.6.2 ER-positive breast cancer

ER-positive cells that carry a HER2 amplification are often resistant to anti-estrogen treatments. Generally, these cells express low levels of ERα. Also, HER2 signaling has been shown to play a role in the resistance to AEs. We have shown here that RA induces a more luminal phenotype in breast cancer cells. RA induces several components of the ER-signaling pathway, such as GATA-3, FOXA1, FOXO3A and ESR1 itself. Although the induction of the latter in SK-BR-3 cells could only be detected at the levels of the mRNA, we have not investigated the effects of sustained RA treatment.

Low levels of ER in HER2+/ER+ cells have been associated with the inactivity of FOXO3A, which was shown to bind the ESR1 promoter and regulate its transcription (435). This could explain differences observed in ESR1 regulation in HER2-positive and HER2-negative cells (Chapter 2:; Figure 4). Subsequently, it suggests that ESR1 expression is likely to be regulated in HER2+/ER+ breast cancer cells. If this is indeed the case, it would open ways to combined treatments of retinoids and antiestrogens. The combined treatments of RA and tamoxifen or RA and tamoxifen and Herceptin were recently shown to have synergistic effects on cell proliferation and apoptosis in HER2+/ER+ in BT474 cells (467).

4.6.3 RA targets as predictive markers

We have shown that expression of ER-independent RA target genes correlates with a luminal phenotype and lower grade of breast tumor samples (Chapter 2:; Figure 7). In addition to this observation, we further show that expression of Herceptin+RA transcriptional targets defines two classes of tumors within HER2+ tumor samples, that significantly correlate with tumor outcome (Chapter 3: Figure 7). This indicates the importance of RA target genes in inhibiting tumor survival. Thus, a subset of RA target

genes could be developed as predictive markers for response to treatment with Herceptin, alone or in combination with chemotherapy. Using hierarchical clustering methods according to RA target gene expression and subsequent Kaplan-Meier analyses, the predictive value of RA targets could be tested on datasets available in the literature (although it has to be noted that reports are scarce on Herceptin monotherapy). RA target genes could thus be used not only as prognostic markers for overall survival (independent of treatment, such as presented in Chapter 3), but potentially also to predict the outcome of treatment in patients.

4.6.4 Clinical application of retinoids for breast cancer treatment

The work presented in this thesis has focused on understanding the mechanisms of RA antiproliferative effects and identifying a sensitive subtype of tumors. However, when considering treatment with RA, another problem needs to be considered besides resistance, namely the occurrence of side effects. Administration of RA, typically in the case of APL treatment, may lead to the development of the retinoic acid syndrome. This syndrome occurs in 14-16% of APL patients treated with RA, and 2% will die because of it. Most common symptoms are respiratory distress and fever, but a variety of other symptoms, including high blood pressure and acute renal failure may also occur (468).

If subsequent studies based on the work presented here, notably using animal models such as discussed in paragraph 4.6.1, confirm the interest of RA for treatment of a subclass of breast tumors, it would be important to consider ways to limit possible side effects. One way to do this is by targeting a specific receptor subtype with a selective ligand, thereby limiting the effects that could be mediated by the other two subtypes. In the case of breast cancer RARα selective ligands seem appealing. Still, there are risks to giving systemic retinoid treatment. The best example is the case of RARβ, widely accepted as a tumor suppressor, that appears to have tumor promoting effects when expressed in the stroma (453). This indicates that it is critical not only to target the right receptor, but also to target it in the right place.

In the case of the HER2/RARA co-amplification, it could be possible to use the overexpression of the two proteins to the advantage of the treatment. It has already been shown that it is possible to encapsulate RA into liposomes, allowing for better targeting to tumor sites. These RA liposomes were shown to prevent relapse in breast cancer due to cancer stem cell outgrowth (469). It has also been shown that Herceptin can be conjugated to liposomes (470), allowing for targeting of the liposome specifically to HER2 overexpressing cells. Combining this knowledge suggest that it could be possible to use liposomes to target RA or an RARα selective ligand directly to the HER2/RARA co-amplified tumor cells.

4.7 Limitations of used techniques

4.7.1 Cell lines in cancer research

In the studies presented in this thesis, we have made extensive use of cell lines as a model for tumorigenesis. Tumor cell lines are easy tools for cancer research since they are fairly easy to manipulate, and provide with an almost infinite supply of material to study. Although an excellent tool to study aspects of tumor cell proliferation, differentiation and the influence of drug treatments, it is important to be aware of the limitations of these models. Tumors might not be realistically represented by the cell lines due to several factors. First, cell lines are most of the times not derived from primary tumors, but rather from more aggressive metastases or pleural effusions. Also, cultured cell lines are prone to accumulate genotypical and phenotypical changes throughout their time in culture. This way tumor cell lines diverge from the original tumors. Various subpopulations of a cell line can occur due to longtime culture of a cell line in different laboratories, this phenomenon has been described by two separate groups for MCF-7 cells (471, 472). Finally, tumor cell lines are generally very homogeneous populations, which makes them easy to work with on one hand, but hard to compare to generally very heterogeneous tumor samples on the other. In addition to these factors, it is obvious that the environment in which tumor cell lines are

being grown cannot be compared to the *in vivo* situation of a tumor. The tumor microenvironment is an important factor for the growth and potential treatment of a tumor, and cannot be accurately mimicked in a traditional *in vitro* culture system.

For the reasons mentioned above, it is clear that however valuable tumor cell lines are for cancer research, it is of importance to be cautious when interpreting results and translating them to tumor biology. Reproduction of results in other systems, such as primary tumor cultures and animal tumor models is essential for connecting cell line research to *in vivo* tumor situations.

4.7.2 Genome-wide expresssion profiling techniques

In order to try to understand the biological relevance of the regulated genes indentified in the two studies here presented, we have taken advantage of available datasets of genome-wide expression profiling of tumor samples. Although this type of profiling data can be very valuable to better understand tumor biology and response to treatments, a certain level of caution should be used when interpreting such data. Because of the very heterogeneous composition of tumors, a biopsy used for analysis on arrays may not accurately represent the original tumor. Furthermore, correlations with treatment outcome based on the expression of certain genes will be biased towards the most present cell population in the sample. Notably, tumors will contain a small proportion of cancer stem cells. This population will, if not affected by the treatment, allow for regrowth of the tumor after the treament is finished.

4.8 Conclusion

In conclusion, we have shown here that, contrary to previous reports in the literature, a large part of RA signaling in breast cancer cells is independent of ER signaling. ER-independent RA targets are at least in part responsible for the RA-induced antiproliferative effects.

Our results present new insights in both the mechanisms of regulation of the antiproliferative response to RA, as well as tumor subclasses that could benefit from retinoid treatment. Particularly the observation that retinoids can synergize with HER2-targetting molecules will be of interest to find new treatment strategies for some otherwise hard to treat subtypes of breast cancer.

Although preliminary from a drug-development point of view, the data presented in this thesis provide substantial information about the mechanisms of action of retinoids in both ER-positive and ER-negative breast cancer. These data can be used as a basis for *in vivo* studies to further examine the potential of retinoids, either alone or in combination with HER2- and/or ER-targeting molecules, in breast cancer treatment.

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Annexes

Annex I : Low RALDH3 Activity in Luminal Breast Tumors is a Prerequisite for Proliferation of Retinoic Acid-sensitive Cancer Cells.

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As second author, I performed RALDH3 overexpression FACS experiments and did several RNA extractions and Q-PCR analyses. I also helped making figures and correcting the manuscript.

Low RALDH3 activity in luminal breast tumors is a prerequisite for proliferation of retinoic acid-sensitive cancer cells

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List of non-standard abbreviations: ALDH, aldehyde dehydrogenase; EdU, 5-ethynyl-2′-deoxyuridine; ERα, estrogen receptor alpha; HMEC, human mammary epithelial cell; IHC, immunohistochemistry; ICI, ICI182,780; mAU, milli Absorbance Unit; PR, progesterone receptor; RA, retinoic acid; RALDH, retinal dehydrogenase; RAR, retinoic

acid receptor; RARE, retinoic acid response element; RXR retinoid X receptor; Tam, tamoxifen.

RUNNING TITLE:

RALDH3 controls RA synthesis in mammary epithelial tumors

ABSTRACT:

Although retinoic acid (RA) is antiproliferative in some breast cancer cell lines, its synthesis in mammary tissue and role in breast tumorigenesis remain poorly characterized. We show that RALDH3 is expressed predominantly in luminal ductal cells in normal human and mouse mammary glands and drives RA synthesis in normal and transformed breast epithelial cells. However, RALDH3 expression and retinal oxidation are weak in RA-sensitive human luminal tumor cells, resulting in a strong negative correlation between RALDH3 expression and RA sensitivity in breast cancer cells. Functional but not catalytically inactive RALDH3 expressed in luminal breast cancer cells induces RA signaling and decreases proliferation, consistent with low RALDH3 levels being a prerequisite for proliferation in the presence of retinol. Endogenous RALDH3 expression is induced by RA in a positive feed-back regulatory loop that is suppressed by estrogens, in agreement with low RALDH3 levels in ERα-positive luminal tumors, but restored by estrogen blockade or withdrawal. Accordingly, higher RALDH3 levels correlate with enhanced distant metastasis-free survival in ERα-positive tumors, consistent with RALDH3 contributing to therapeutic success in estrogenic blockade therapies.

KEYWORDS: retinoic acid/retinal dehydrogenase/breast cancer/luminal tumor

INTRODUCTION

Retinoids, the natural or synthetic derivatives of vitamin A, play important roles in embryonic development, cell differentiation and cancer prevention (Mark et al 2009, Wald et al 1980). Vitamin A is important for the maintenance of the differentiation state of epithelial tissues and its deficiency has been linked with a higher incidence of cancer and increased susceptibility to chemical carcinogens in the mammary gland (Freemantle et al 2003). Retinoids can prevent carcinogen-induced tumorigenesis in various tissues including breast (Lacroix et al 1990, Lotan 1996), and inhibit proliferation of some breast cancer cell lines *in vitro* (Raffo et al 2000, Seewaldt et al 1997) and in animal xenograft models (Appleyard et al 2004, Wetherall et al 1984). Several reports provide evidence for morphogenetic roles of RA in the mammary gland. For example, ductal morphogenesis is reduced in rats treated with the synthetic retinoid N-(4-hydroxyphenyl)-retinamide (Moon et al 1985), and increased branching morphogenesis is observed in mouse models with suppressed retinoid signaling (Cohn et al 2010, Wang et al 2005). Retinoids also induce *in vitro* lumen morphogenesis in cultured cell lines (Montesano and Soulie 2002).

The principal natural forms of retinoids associated with cell differentiation and tumor prevention, the all-*trans* and 9-*cis* isomers of retinoic acid (RA), are ligands for two families of nuclear receptors. The RARs interact with the two RA isomers, while the RXRs interact specifically with 9-*cis* RA (Leid et al 1992, Mangelsdorf et al 1992). RA receptors act as transcription factors, binding to specific DNA sequences and exchanging

transcriptional corepressors for coactivators upon ligand binding (Napoli 1999). RARs and RXRs are expressed in normal and tumorigenic human breast cells, with a reported decrease in RARβ expression during tumorigenesis ((Yang et al 1999) and refs therein).

Although cell-specific receptor expression patterns play an important role in determining sets of target genes, expression of RA-synthesizing enzymes is required for tissular response to RA (Napoli 1999, Petkovich 2001). All-*trans* RA is synthesized in target tissues by a two-step oxidation of serum retinol. The second step of synthesis is controlled by retinal dehydrogenases RALDH1-3, corresponding to ALDH1A1, ALDH1A2, ALDH1A3, respectively (Duester et al 2003, Vasiliou et al 1999). Genetic deletion of *Aldh1A2* or *Aldh1A3* in mice results in lethality in the early embryo or at birth, respectively, due to developmental defects that can be rescued to some extent by maternal RA administration (Dupe et al 2003, Mic et al 2002). *Aldh1a1* is dispensable for development and viability, but plays a role in RA synthesis in the developing eye and in clearance of excess retinol in the liver (Fan et al 2003, Matt et al 2005).

While suppression of receptor expression represents a mechanism of escape from the differentiating effects of RA (Berard et al 1996, Freemantle et al 2003), accumulating evidence also points to alterations in RA biosynthesis during tumorigenesis. For instance, RA concentrations and RALDH2 expression levels are lower in prostate tumor tissues than in corresponding normal tissues, correlating with shorter remissions (Kim et al 2005, Miller 1998). In an N-methyl-N-nitrosourea induced mammary carcinoma model, retinal oxidase activity was reduced compared to normal rat tissues (Bhat and Lacroix 1989). Other studies

described a reduction in RA synthesis from retinol in specific human breast cancer cell lines compared to normal or immortalized cells (Hayden and Satre 2002, Mira et al 2000, Rexer et al 2001). However, it remains unclear whether breast tumorigenesis is significantly associated with decreased RALDH expression and whether RALDH expression plays a role in the control of breast tumor cell proliferation.

Here we provide evidence that RALDH3 can drive RA synthesis in normal breast epithelial cells, but that its expression is weak in luminal tumors. Transient expression of RALDH3 in luminal tumor cells cultured in retinol-containing medium mimics the effects of added RA and decreases cell proliferation, consistent with a low expression of RALDH3 being a prerequisite for optimal growth of luminal tumors.

RESULTS

Retinal oxidation is deficient in tumor cell lines:

Synthesis of RA from retinol (2 μM) was previously found to be impaired in 5 out of 6 tested cancer cell lines compared to primary or immortalized cells (Mira et al 2000). Defects in RA synthesis may result from loss of enzymes converting either retinol to retinal, or retinal to RA. In order to assess whether breast cancer cell lines have a lower capacity to synthesize RA from retinal, protein extracts from primary mammary epithelial cells (HMEC), from 2 immortalized cell lines and from 9 transformed cell lines were tested for all-*trans* retinal oxidation *in vitro* as previously described (Sima et al 2009). RA production was detected in HMECs and in immortalized 184B5 and MCF-10F cells (Figure 1A), but was very low in luminal (MCF-7, T47D, ZR-75, BT-474, SKBR3, MDA-MB-361) or basal B (MDA-MB-231) breast tumor cell lines (Neve et al 2006). Significant activity was only observed in two basal A cell lines, BT-20 and MDA-MB-468 (Figure 1A). To verify these findings under cell culture conditions, we treated the cell lines of our panel with a dose of retinol (2 μM) for 18 h. Production of RA in cells was then monitored by reverse-phase HPLC analysis of cell culture media. Identification of the peak of RA was performed with a standard of pure RA (Figure 1B), and by UV spectrum analysis of the peak (Supplemental Figure 1). RA formation was observed for MDA-MB-468, BT-20, but not for BT-474, MDA-MB-231, SKBR3 or MCF-7 cells (Figure 1B, and data not shown), in agreement with results observed *in vitro*.

RALDH3 expression correlates with RA synthesizing activity in breast cancer cell lines:

To identify the specific RALDH(s) responsible for RA production in breast epithelial cells, we compared RALDH expression profiles in breast cell lines both at the mRNA and protein levels. While expression of RALDH1 and RALDH2 was extremely low in normal human mammary epithelial cells (HMECs) and in immortalized cells 184B5 and MCF-10F (Figures 2 A, B and D), high-level expression of RALDH3 was observed in these cells (Figures 2 C and D).

In tumor cell lines, RALDH1 expression was high in the basal A MDA-MB-468 and lower, but detectable both at the mRNA and protein levels, in HER2-expressing luminal BT-474, SKBR3 and MDA-MB-361 cells (Figures 2A and D). RALDH2 was detected at the RNA level in MDA-MB-468 cells only (Figure 2B). On the other hand, RALDH3 expression was high in transformed basal A MDA-MB-468 and BT-20 cells, but very low in basal B MDA-MB-231 and in all luminal cells both at the mRNA and protein levels (Figures 2C and D), correlating with *in vitro* and *in vivo* RALDH activity (Figures 1A, B). RALDH1 and/or RALDH2 expression may contribute to RA synthesis in cells such as MDA-MB-468, which co-express RALDH3. However, RALDH1 expression in BT-474 cells is not sufficient to yield significantly higher *in vitro* or *in vivo* RA synthesis than in other luminal cell lines (Figure 1A and data not shown).

RALDH3 expression is comparatively low in luminal tumors:

To assess RALDH3 expression in the human and mouse mammary gland, we stained normal tissues with an RALDH3 antibody (see supplemental Figure 3 for isotype control of RALDH3 staining in mouse and human mammary gland sections). In mouse, RALDH3 expression was observed in luminal epithelial cells (supplemental Figure 4A). Co-staining with the basal cell-restricted marker SMA revealed that RALDH3 expression occurred predominantly in the luminal compartment of mouse mammary epithelium (supplemental Figure 4B). In human tissue, RALDH3 expression was observed predominantly in ductal cells in samples from two different individuals (Figure 3). In ducts, RALDH3 expression was localized to luminal cells, suggesting that its expression patterns in mouse and human are similar. Finally, staining of formalin-fixed paraffin-embedded pellets of Hela cells transiently expressing RALDH1, 2 or 3 provided no evidence for cross reactivity of the anti-RALDH3 antibody with RALDH1 or RALDH2 (Supplemental Figure 5).

As expression of RALDH3 was observed in luminal cells both in mouse and in human mammary glands, but its levels were low in all luminal tumor cell lines compared to normal or immortalized breast epithelial cells, we examined its expression in an ERα-positive tumor. Transformed, ERα-positive cells did not stain detectably for RALDH3 whereas ERα-negative duct-like structures included in the tumor displayed RALDH3 expression (supplemental Figures 6A,B), suggesting lower RALDH3 expression in the tumor cells (see also Figure 3A for normal adjacent tissue of the same tumor). In contrast, expression of RALDH3 was detected in epithelial cells in an ERα-negative tumor (supplemental Figure 6C).

To examine whether low RALDH3 expression is associated specifically with ERα-positive (luminal) tumors, we first analyzed RALDH3 mRNA expression in different published tumor datasets with associated microarray analyses (Chang et al 2005, Chin et al 2006, Loi et al 2008, U.N.C). Tumor types were determined using the PAM50 classifier (Parker et al 2009). In all four datasets, RALDH3 expression was lower in luminal than in HER2-positive (HER2+) or basal tumors (Scheffe test: $p<0.0005$ in all four graphs, Figure 4A). We then analyzed a tumor microarray comprising 140 invasive carcinomas classified in ERα-positive, HER2-positive and triple-negative types (triplicate cores were arrayed for each tumor). Scores were attributed to each core based on intensity of staining (Figure 4B), and distribution of average scores of triplicates were analyzed for each tumor type (Figure 4C). Luminal tumors were associated with lower scores (<2) with a p value of 0.000216 in the $\chi 2$ contingency test. Histogram and pie chart score distributions illustrate the lower representation of high scores in luminal tumors (Figures 4D and E), consistent with the low expression levels observed above in luminal cell lines. These results, together with those described above (Figures 1-4), suggest that RALDH3 is under-expressed in luminal tumors versus normal luminal cells.

RA-sensitive tumor cell lines express low levels of RALDH3.

In agreement with reports that RA has antiproliferative activity mostly in luminal cell lines (Raffo et al 2000, van der Burg et al 1993, van der Leede et al 1995), all RA-sensitive lines in our panel were luminal breast cancer cell lines (SKBR3, BT-474, ZR-75, MCF-7

and T47D, Figure 1C). Importantly, all RA-sensitive cell lines had low RALDH activity and low RALDH3 expression (Figures 1A, and B, and 2C and D). Conversely, lines that expressed significant levels of RALDH3 protein and have RALDH activity *in vitro* were not growth-inhibited by incubation with RA (MDA-MB-468 and BT-20, Figure 1C). In addition, two cell lines were both RA-insensitive and RALDH3-low (MDA-MB-231 and MDA-MB-361, Figure 1C).

In contrast to cancer cells, normal or immortalized cells, which can synthesize RA from retinol, were sensitive to RA. Note however that HMEC cells are routinely cultured in serum-free MEGM medium, and that their growth in medium with serum is markedly less efficient, consistent with inhibitory effects of intra- or autocrine RA synthesis. In addition, sensitivity of immortalized 184B5 cells to RA was observed only in the absence of EGF supplementation, consistent with a role of EGF in suppressing RA signaling (Grunt et al 2005). Together, these results suggest that the capacity of RA-sensitive breast cancer cells to proliferate in the presence of retinol (~100-200 nM in medium with 10% serum as assessed by HPLC; data not shown) may depend on low RALDH3 expression.

Expression of RALDH3, but not its catalytically inactive mutant, mimics the antiproliferative effects of RA on luminal cell lines.

Since expression of RALDH3 is low in RA-sensitive luminal cells, we transiently expressed in these cells an HA-tagged RALDH3 or a mutant carrying a C314A mutation, which abrogates *in vitro* enzymatic activity (data not shown), together with a membrane-

targeted EGFP expression vector, in order to test the impact on cell cycle distribution. Western analysis of transfected ER-negative luminal SKBR3 cells indicated that expression levels of RALDH3 and of its mutant were comparable to each other (Figure 5A) and to those in RALDH3-expressing 184B5 immortalized cells (Supplemental Figure 8). HPLC analysis of the culture media of transfected cells revealed that expression of RALDH3, but not of its catalytically inactive mutant, led to synthesis of RA from retinol present in the culture medium (Figure 5B). To verify that RA synthesis had a functional impact on gene expression, we assessed expression of SOX9, a well-characterized RA target gene with antiproliferative properties in breast cancer cell lines (Afonja et al 2002). Expression of SOX9 was not altered by expression of the catalytically inactive mutant, but was markedly enhanced by expression of RALDH3 (Figure 5A), suggesting that RA signaling takes place. Moreover, mRNA levels of several RA target genes were up-regulated as soon as 24 h after RALDH3 transfection (e.g. see CYP26A1 and RARB2 in Supplemental Figure 9). Finally, transfected EGFP-positive cells expressing WT RALDH3 displayed about 10% increase in the G0/G1 content ($p<0.01$ in Student's t test) in FACS analysis, while no change was observed in cells expressing the catalytically inactive mutant (Figure 5C). These effects mimic those of RA treatment (1 μM), which led to a 15% increase in G0/G1 content (or almost a 2.5-fold reduction in cells in any other phase of the cell cycle; Figure 5C). RA treatment or expression of RALDH3, but not of its catalytically inactive mutant, also led to significant decreases in the incorporation of nucleoside analogue 5-ethynyl-2'-deoxyuridine **(**EdU) in SKBR3 cells (Figures 5D, E).

Similar results were obtained using ERα-positive ZR-75 cells (Supplemental Figure 10). Furthermore, stable expression of WT, but not of mutant RALDH3 in MCF7 cells adapted to culture in serum-free medium led to a reduction in the number and size of colonies in a colony formation assay in medium containing 100 nM retinol, indicating that RALDH3 also plays an antiproliferative role in MCF7 cells (Supplemental Figure 11). Finally, contrary to results obtained in luminal cells, expression of RALDHs in RA-insensitive basal MDA-MB-231 cells did not affect the cell cycle in a significant manner (data not shown). Together, these results indicate that over-expression of RALDH3 in RALDH-low luminal cells sensitizes these cells to serum levels of retinol through RA synthesis, induction of RA target genes and growth arrest.

RALDH3 expression is inhibited by 17β-estradiol but restored upon suppression of estrogen receptor signaling in luminal breast cancer cell lines.

As RALDH3 expression is low in all RA-sensitive ERα-positive breast cancer cell lines, we tested whether it is modulated by RA and/or E2 treatment. Unexpectedly, RA treatment of MCF7 cells maintained in estrogen-depleted medium induced RALDH3 expression 2 to 3-fold in (Figure 6A). Similar results were obtained in T47D (Supplemental Figure 13A) and SKBR3 cells (data not shown). These results suggest the existence of a positive feed-back loop of RALDH3 expression through RA signaling. Consistent with this hypothesis, expression of endogenous RALDH3 and of RA-target gene SOX9 was increased by long-term (6 d) treatment of MCF7 cells with retinol in a dose-dependent

manner (Supplemental Figure 13B), suggesting that even the low levels of RALDH3 present in these cells are sufficient for sensitivity to retinol (Supplemental Fig. 13B).

The above-described results indicate that RALDH3 expression is not permanently abolished in luminal cell lines, but is actively repressed. Strikingly, treatment of MCF-7 cells with 17β-estradiol (E2) suppressed basal RALDH3 mRNA levels 2-3 fold after 8, 24, 48 or 72 h treatments (Figures 6A and B). In addition, E2 suppressed RALDH3 induction by RA in a time-dependent manner (Figure 6A and B). Western analysis after 72 h treatment confirmed these observations at the protein level (Figure 6C). E2 also significantly suppressed RA-induced levels in T47D cells (Supplemental Figure 13A). These results suggest that estrogen signaling protects ERα-positive cells from potential auto- or paracrine effects of RA on RALDH3 expression. Thus, estrogenic blockade therapies should result in increased intratumoral RALDH3 levels. We compared the effect on RALDH3 levels of treatment with the selective estrogen receptor modulator tamoxifen or with the full antiestrogen ICI182,780 (fulvestrant) with those of estrogen deprivation, mimicking aromatase treatment (Figure 6B). The antiestrogens had little effect by themselves in the absence of estrogen, but reversed partially (tamoxifen) or fully (ICI182,780) the suppression of RALDH3 expression by estradiol. On the other hand, tamoxifen unexpectedly had a partial agonist effect in the presence of RA in MCF7 cells, inhibiting RA-induced expression by 2-fold (Figure 6B). As a result, saturating concentrations of tamoxifen led to a lower level of RALDH3 expression in the presence of

E2 and RA compared to estrogen withdrawal. In contrast, ICI182,780 potentiated the effects of RA in the absence or presence of E2 (Figure 6B).

RALDH3 is a predictor of recurrence-free survival in breast cancer patients.

Low expression of RALDH3 in RA-sensitive breast luminal tumor cells could provide a selective advantage in a physiological environment containing retinol, while increased RALDH3 expression upon inhibition of estrogen signaling or synthesis could contribute to inhibition of tumor growth. To determine if RALDH3 expression levels represent a positive prognostic marker in patients with luminal tumors, we classified patients from 3 independent large-scale clinical studies (Chang et al 2005, Chin et al 2006, Loi et al 2008) in two groups according to RALDH3 mRNA levels. In the three studies, patients with ERα-positive tumors associated with higher expression of RALDH3 (above the median, Figures 7A-C, blue lines) had a lower risk of distant metastasis compared to patients with lower expression of RALDH3 (Figures 7A-C, red lines). This correlation did not hold for patients with ERα-negative tumors in the same studies (Figures 7A and B), suggesting that RALDH3 represents a marker for good prognosis specifically in ERα-positive tumors.

DISCUSSION

Results presented herein indicate that RALDH3 expression correlates with RA synthesis capacity in in normal HMECs. Furthermore, some transformed breast epithelial cells (basal A type) maintain both RALDH3 expression and RA synthesis capacity. On the other hand, no detectable RA synthesizing activity was observed in cell lines expressing RALDH1 but with low RALDH3 activity (BT-474, SKBR3, MDA-MB-361). This may result either from RALDH1 protein levels insufficient for detectable RA synthesis and/or from a catalytic efficiency with retinal substrates lower than that of RALDH3, as previously reported for murine enzymes in *in vitro* enzymatic assays (Gagnon et al 2002, Gagnon et al 2003, Sima et al 2009).

While RALDH3 is expressed in the luminal epithelium of the adult mammary gland, its expression is low in luminal breast tumors. As sensitivity to RA in breast cancer cell lines also correlates with the luminal phenotype, this suggests that low RALDH3 expression is needed for tumor cell proliferation in the presence of retinol. This hypothesis is strongly supported by the observation that overexpression of RALDH3 in sensitive SKBR3 and ZR-75 cells to levels found in normal and immortalized cells led to a marked increase in G0/G1 content and reduced DNA replication in the absence of added retinoids, as assessed by FACS analysis and EdU incorporation. These effects coincide with RA synthesis and activation of several RA target genes, including SOX9, a gene with demonstrated antiproliferative properties in mammary epithelial cells. Conversely, basal A tumor cells that express RALDH3 and are capable of RA synthesis are not growth-arrested

by RA (BT-20 and MDA-MB-468). These tumor cells may correspond to luminal progenitors (Visvader 2009), which have been reported to express RALDH3 (Raouf et al 2008). The role of RALDH3 expression in basal A tumor cells remains to be investigated, but is not expected to lead to growth suppression.

RALDH3 expression is actively repressed in luminal tumors through estrogen signaling. Thus, the overexpression of the aromatase and ERα genes in luminal tumor cells is a probable mechanism of escape from the antiproliferative effects of RALDH3 expression. The combined suppression of RALDH3 and up-regulation of RARα (Laganiere et al 2005, Raffo et al 2000, van der Burg et al 1993, van der Leede et al 1995) by estrogen signaling in luminal tumors should lead to the accumulation of unliganded RARα, and thus to active suppression of antiproliferative RA target genes.

Taken together, our results indicate a role of RALDH3 expression in RA signaling in the luminal breast epithelial cell lineage and in the control of ERα-positive cell proliferation through autocrine and/or paracrine mechanisms. This proposed role is compatible with the observation that the RALDH3 gene is found within a mouse breast tumorigenesis modifier locus, its expression levels correlating with lower susceptibility in p53 heterozygote mouse strains (Koch et al 2007), and in an overlapping rat modifier of sensitivity to DMBA-induced mammary carcinoma, *Mcs3* (Shepel et al 1998). Low levels of RALDH3 expression and thus of intratumoral RA synthesis may account for luminal tumor progression in spite of sensitivity to the antiproliferative effects of RA. Restoring RALDH3 expression in tumor cells during therapeutic treatments based on estrogenic

blockade may thus contribute to therapeutic success due to RA synthesis from circulating retinol. In this respect, we observed that prolonged exposure to low levels of retinol in the absence of E2 signaling is sufficient to induce endogenous RALDH3 expression and RA signaling in MCF7 cells. Importantly, our observations indicate that RALDH3 expression levels predict longer distant metastasis-free survival in breast cancer patients with ERα-positive, but not ERα-negative tumors.

MATERIALS AND METHODS

Recombinant plasmids

The mouse RALDH3 cDNA was inserted in the pCDNA3.1-neo vector (Invitrogen, Carlsbad, CA, USA) and fused with an adaptor encoding an N-terminal hemagglutinin (HA) tag sequence. The catalytically dead RALDH3 mutant was generated by site directed mutagenesis of the catalytic cysteine (C314A) using a Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

Cell culture

Primary human mammary epithelial cells (HMECs, Lonza, Basel, Switzerland) were maintained in MEGM serum-free medium (Lonza). All cell lines were purchased from American Type Culture Collection ATCC (Manassas, VA, USA). Tissue culture conditions for all cell lines used are listed in Supplemental Figure 14.

Preparation of protein extracts

For *in vitro* enzymatic assays and Western analysis of untreated cells, cells were maintained in their respective media until near-confluence and switched to DMEM supplemented with 10% FBS and 1% penicillin/streptomycin 24 h before harvest. For analysis of hormonal regulation of RALDH3 expression, cells were switched to DMEM

supplemented with 10% charcoal-treated FBS 3 days before treatment with E2, RA, Tam or ICI182,780 (Sigma-Aldrich). Cells washed twice with ice-cold phosphate buffer saline (PBS) were collected in ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% NP-40, 5% glycerol) supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Sigma-Aldrich). Cells were sonicated 3 min (maximal intensity, three on/off cycles) on a Bioruptor (Diagenode, Sparta, USA) followed by centrifugation (16,200 g, 15 min). Protein concentrations in supernatants were quantified following the Bradford method (Bio-Rad Laboratories, Hercules, CA).

***In vitro* enzymatic assays.**

Enzymatic assays were performed with 50 µg of whole cell protein extracts in 250 µl reaction buffer (100 mM Tris-HCl pH 8.5, 0.02% Tween 20, 600 µM NAD^+, 1 mM dithiothreitol) containing all-*trans* retinal (10 µM, Sigma-Aldrich). All assays were performed for 30 min at 37°C in triplicates. The reaction was stopped by freezing in an ethanol/dry ice bath. Retinoids were extracted by addition of butanol/acetonitrile (50:50 mix, 400 µl) to frozen reactions. Tubes were incubated at room temperature until complete thawing and centrifuged (3,500 g, 15 min). Supernatants (20 µl) were analyzed by reverse phase HPLC for RA quantification as described below.

Western blot analysis

Protein extracts were separated on SDS-polyacrylamide gels (10%) and transferred onto polyvinylidene difluoride membranes (Millipore Corporate, Billerica, MA, USA). Membranes were blotted with antibodies directed against RALDH1 (ALDH, BD Biosciences, Mississauga, ON, Canada), RALDH3 (ALDH1A3, C13, Santa Cruz Biotechnology, Santa Cruz, CA, USA), SOX9 (AB5535, Millipore, Temecula, CA, USA), the HA tag (12CA5, Santa Cruz Biotechnology) or β-Actin (A-5441, Sigma-Aldrich). Secondary antibodies (anti mouse-HRP, anti-goat-HRP and anti rabbit-HRP) were purchased from Cedarlane (Burlington, ON, Canada).

***In vivo* metabolism assays**

Cells were plated and maintained in their respective media until near-confluence, and then switched 24 h before treatment to DMEM 10% FBS 1% penicillin/streptomycin. Cells were treated with 2 μM retinol (Sigma-Aldrich) or vehicle (DMSO, final dilution 1:1000) for 18 h. At the end of the incubation period, aliquots (400 μl) of media were collected from each treated plate and frozen at -80°C in borosilicate tubes (Fisher Scientific, Ottawa, ON, Canada). To extract retinoids, frozen aliquots were incubated with 400 μl of butanol/acetonitrile (50:50 mix, Sigma-Aldrich) until totally thawed and quickly vortexed. After addition of 40 μl of 10M K_2HPO_4 and centrifugation (3,500 g, 4°C, 15 min) supernatants (100 μl) were analyzed by reverse phase HPLC using a Shimadzu LC10-ADVP equipped with a SIL-HTC autosampler and cooling system (Man-Tech, Guelph, ON, Canada) and a 10-ODS (250x4.5 mm) analytical column (Phenomenex Inc., Torrance,

CA, USA). Retinoids were eluted with a mobile phase (acetonitrile 65%/water 35%, 10 mM ammonium acetate) at a flow rate of 1.2 ml/min. Retinoids were detected with a photodiode array detector (Shimadzu model SPD-M10 AVP).

RNA extraction, reverse transcription and quantitative PCR

Cells plated and treated as described for preparation of protein extracts were harvested after removal of the culture medium in 1 ml of TRI-Reagent (Sigma-Aldrich). Total RNAs were extracted and 2 μg were reverse-transcribed using the RevertAid H first minus strand cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada). Reverse transcription products were diluted 10 times in water prior to real-time quantitative PCR. Gene expression levels were determined using primer and probe sets from the Universal Probe Library (Roche Diagnostic, Laval, Que, Canada).

Gene expression profiling analysis

For analysis of Agilent arrays from the UNC microarray database, Loess normalization was performed on background-subtracted intensities. Flagged values were removed, and data were log2 transformed and median-centered for each gene across samples. For Affymetrix arrays (Acc. numbers ArrayExpress E-TABM-157 and GEO database GSE16795, (Chin et al 2006, Loi et al 2008)), RMA normalization and median-centering for each gene were performed. Breast tumor subtypes (luminal A, luminal B, HER2+, basal-like or normal-like) were determined using the nearest centroid method applied to the

PAM50 gene set (Parker et al 2009) using Spearman correlation as distance. Luminal A/B tumors were grouped and normal-like tumors and metastases excluded for better comparison with results from the tissue microarrays.

Tissue staining and immuno-histochemistry

All cell samples, mouse and human tissue samples (obtained in accordance with ethics protocols approved by the Comité de Déontologie de l'Expérimentation sur les Animaux de l'Université de Montréal and the Comité d'Ethique de la Recherche du Centre Hospitalier de l'Université de Montréal) were formalin-fixed and paraffin-embedded at the IRIC histology core facility (IRIC, Montreal, QC, Canada). Primary antibodies used were directed against RALDH3 (C-13, Santa Cruz Technologies), ERα (SP1, Ventana Medical Systems). Normal rabbit IgG (Santa Cruz Technologies) were used as isotype control for RALDH3 staining. Primary antibody incubation was followed by incubation with appropriate biotin conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). For staining, streptavidin-horseradish peroxidase, and 3,3 diaminobenzidine were used (DABmap detection Kit, Ventana Medical Systems). For double staining, the first staining was performed using the RALDH3 antibody as for single staining but was followed by a denaturation step (90°C, 4 min) before ERα staining with the REDmap detection Kit (Ventana Medical Systems). Counterstained sections were scanned at X40 magnification using the C9600 NanoZoomer System (Hamamatsu

Corporation, Bridgewater, NJ, USA). NDP Scan software (version 2.2.17) was used to visualize virtual slides and extract images.

Tissue microarrays

Tissue samples from 140 patients with invasive breast carcinoma were classified as ERα-positive (ERα+), HER2-positive (HER2+) or triple-negative (Triple neg.) according to ER/PR and HER2 status. Tissue microarrays prepared from these tumors include three samples from each donor tissue block to mitigate tumor heterogeneity. All immunohistochemically-stained samples (see above) were scored independently by two pathologists. RALDH3 staining was interpreted using a grading scale of 0-4 established by initial classification of degrees of expression in different tumors. Scoring was performed on each of the triplicate samples and averages were calculated for each tumor and each class of tumors.

Growth assays

Cells were seeded at low density in their respective media supplemented with charcoal-treated FBS. In addition, EGF supplementation was omitted for 184B5 and MCF-10F cell lines. Cells were treated at days 0, 2, 4, 6 with vehicle (DMSO, 1:1000 dilution), retinol (100 nM) or all-*trans* RA (100 nM). After 9 days cells were collected and protein

concentrations were quantified by DC protein assay (Life Science, Mississauga, ON, Canada).

Cell cycle analysis of RALDH3-expressing cells

SKBR3, ZR-75 and MDA-MB-231 cells were electroporated (5 million cells, 240 V, 950 μF for SKBR3, 1000 μF for ZR-75 and 975 μF for MDA-MB-231) with either the parental pCDNA3.1-neo vector or vectors expressing RALDH3 or its catalytically inactive mutant, together with a ten-fold lower amount of an expression vector for membrane-targeted EGFP (pEGFP-spectrin, (Kalejta et al 1997)). Cells were seeded in 10 cm plates in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and left untreated or treated with retinol or RA at indicated concentrations. After 72 h cells were trypsinized and a fraction of transfected cells were collected in lysis buffer for western analysis using a monoclonal antibody against the HA tag. The remaining transfected cells were fixed in 70% ethanol, stained with propidium iodine (Sigma-Aldrich) and analyzed on a Canto flow cytometer (BD Biosciences). At least 20,000 GFP-positive cells were used for each condition for cell cycle profile analysis with ModFit LT 3.2 software (Verity Software House, Topsham, ME, USA).

5-ethynyl-2'-deoxyuridine (EdU) incorporation assays

SKBR3 cells were transfected by electroporation as described above. Cells were seeded in 96-well plates (20,000 cells/well) and treated or not for 48 h with all-*trans* RA (1

μM). Proliferation assays were performed using a Click-iT™ EdU Alexa Fluor High-Throughput Imaging (HCS) Assay (Invitrogen) according to the supplier's protocols after incubating cells with EdU (10 μM) for 2 h. Images were captured using ImageXpressMicro and analyzed with MetaXpress (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

For FACS analysis and EdU assays, a two sample t test was used to assess difference in number of cells in G0/G1 phase and EdU-positive cells. For RNA expression profile analysis, an ANOVA test was performed to determine whether RALDH3 mRNA expression was significantly different between class of tumors (Luminal, HER2+, Basal) and a Scheffe test to investigate the statistical significance of individual comparisons. A P-value < 0.05 was considered significant. For tissue microarray analysis, a $\chi 2$ contingency test with a Yates correction was used to determine the association between tumor types and patient IHC scores. A P-value < 0.05 was considered significant. All statistical procedures (two-sample t test, $\chi 2$ test, ANOVA test and Scheffe test) were performed in the open-source R statistical environment (http://www.r-project.org).

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FIGURE LEGENDS:

Figure 1. Sensitivity to the antiproliferative effects of RA correlates with low retinal dehydrogenase activity in breast tumor cells. **(A)** *In vitro* RA synthesis from retinal directed by protein extracts from normal, immortalized and transformed breast cell lines. RA concentrations were measured after *in vitro* incubation of equal amounts of protein extracts from the indicated cell lines with all-trans retinal (10 μM) at 37^{o}C. Results of one of two experiments are shown. Error bars indicate the standard error of the mean (SEM) of triplicate measurements. **(B)** Synthesis of RA from retinol in cultured cells. RA concentrations were measured by HPLC in the culture medium of cells incubated with retinol (2 μM) for 18 hours. RA peaks (validated by spectrum analysis) are indicated on the HPLC profiles. Results of one of two experiments are shown. **(C)** Effect of RA treatment (100 nM) on proliferation of breast cell lines maintained in their respective media over 9 days. Protein concentrations in whole cell extracts were measured by Lowry assay. EGF supplementation was omitted from the media of immortalized cells to reveal RA sensitivity. Results are expressed as percentage of growth in the vehicle-treated cells for each line. Error bars indicate the SEM of triplicate measurements.

Figure 2. Expression of RALDH3 correlates with retinal dehydrogenase activity. **(A-C)** Quantitative PCR of reverse transcribed mRNAs from normal, immortalized or transformed mammary epithelial cells. Primer pairs specific to human RALDH1 **(A)**, RALDH2 **(B)** or RALDH3 **(C)** were used and signals were normalized to expression of the

house-keeping gene β-Actin. Error bars indicate the SEM of triplicate measurements in one of two experiments. **(D)** Western analysis of expression of RALDH1 and 3 using monoclonal antibodies directed against human RALDH1 or RALDH3. Equal amounts of whole cell extracts (20 μg) were loaded on a 10% polyacrylamide-SDS gel and levels of β-Actin were monitored as internal reference (HMEC P1 and HMEC P2: HMEC cells at passage 1 and at passage 2). Similar results were obtained in two experiments. Full-length blots are presented in Supplemental Figure 2.

Figure 3. RALDH3 is expressed predominantly in human ductal luminal breast epithelium. **(A-B)** Expression of RALDH3 in human mammary tissue was determined by IHC using an anti-RALDH3 mAb (brown stain, see arrows) in sections of normal tissue adjacent to an ERα-positive tumor (A) and of 2 different regions of a breast reduction mammoplasty sample **(B).** Scale bars represent 100 μm **(A)** and 200 μM **(B)**.

Figure 4. RALDH3 is under-expressed in ERα-positive tumors. **(A)** RALDH3 (ALDH1A3) mRNA levels are low in luminal breast tumors. Box-plot representation of ALDH1A3 levels in 4 breast tumor transcriptional profiling studies. RALDH3 levels were lower in luminal versus HER2+ and/or basal tumors (Scheffe test: $p<0.0005$ in all graphs). **(B-E)** Paraffin-embedded formalin-fixed sections of breast tissue arrays containing triplicate samples from 140 patients were stained with an anti-RALDH3 mAb. Results were scored from 0 to 4 according to intensity of staining using a scale established by initial

classification of expression in all tumors **(B).** The average scores in the three types of breast tumors are shown **(C)**. The p-value of the Chi-squared test for the association of luminal subtype with low RALDH3 (score<2) is 0.000216. Histogram and pie chart representations of the distributions of scores by tumor type are also shown **(D-E)**. The rounded values of the median scores of the three core biopsies were plotted for each sample.

Figure 5. Active RALDH3 restores RA synthesis and prevents proliferation of SKBR3 cells more efficiently than active RALDH1. **(A)** Western analysis of HA-tagged wild type or inactive RALDH3 mutant, SOX9 or β-Actin levels in transiently transfected SKBR3 cells using indicated antibodies. Full-length blots are presented in Supplemental Figure 7. **(B)** HPLC analysis of RA production from retinol in the culture medium of SKBR3 cells transiently expressing RALDH or inactive mutants**. (C)** G0/G1 fraction in transiently transfected SKBR3 cells expressing RALDH or inactive mutants with or without RA treatment, as determined by FACS analysis. Error bars indicate the SEM of measurements in 3 independent experiments. *p<0.01, **p<0.001, two-sample t-test. **(D)** SKBR3 cells were transfected with the parental vector or with the same vector expressing HA-tagged RALDH3 or its inactive mutant. Cells were seeded in 96-well plates and treated or not for 48 h with RA (1 μM), and then with EdU (10 μM) for 2 h. Cells were fixed, permeabilized, and incorporated EdU was detected by a fluorescent-azide coupling reaction. Cells were counterstained with Hoechst. EdU/Hoechst staining ratios are shown; error bars indicate the SEM of measurements in two independent experiments. *p<0.05, two-sample t-test. (E)

Representative pictures of the different transfection/treatment conditions. Scale bars represent 50 μm.

Figure 6. RALDH3 expression is induced by RA and repressed by estradiol in MCF-7 cells. **(A-B)** Quantitative PCR analysis of RALDH3 expression in MCF-7 cells. Cells maintained 4 days before treatment in phenol-red free DMEM medium supplemented with 10% charcoal-treated FBS were treated for indicated time periods **(A)** or for 72 h **(B)** with estradiol (E2 25 nM in A, 5 nM in B), retinoic acid (RA, 100 nM), antiestrogens tamoxifen (Tam) and ICI 182,780 (ICI) or combinations thereof. For cells treated for 48 and 72 h, treatments and medium were replaced every day. Error bars represent the SEM of triplicates in one of two experiments. **(C)** Representative immunoblot demonstrating regulation of RALDH3 expression by RA and E2 in MCF-7 cells after 72 h treatment with (RA 100 nM, E2 25 nM). Three experiments were performed with similar results. Full-length blots are presented in Supplemental Figure 12.

Figure 7. Expression level of RALDH3 in tumors predicts distant metastasis-free survival in breast cancer patients. Patients from 3 independent large-scale clinical studies (A, B, C, see text for references) containing a large number of ERα-positive tumors with associated transcriptional profiles were divided into 2 groups based on median expression of RALDH3. Kaplan-Meier curves for emergence of distant metastases were computed based on provided patient history in these two groups (high expression of RALDH3: blue

lines; low expression: red lines). One-sided p-values and Cox hazard ratios (HR) between the 2 groups are shown.

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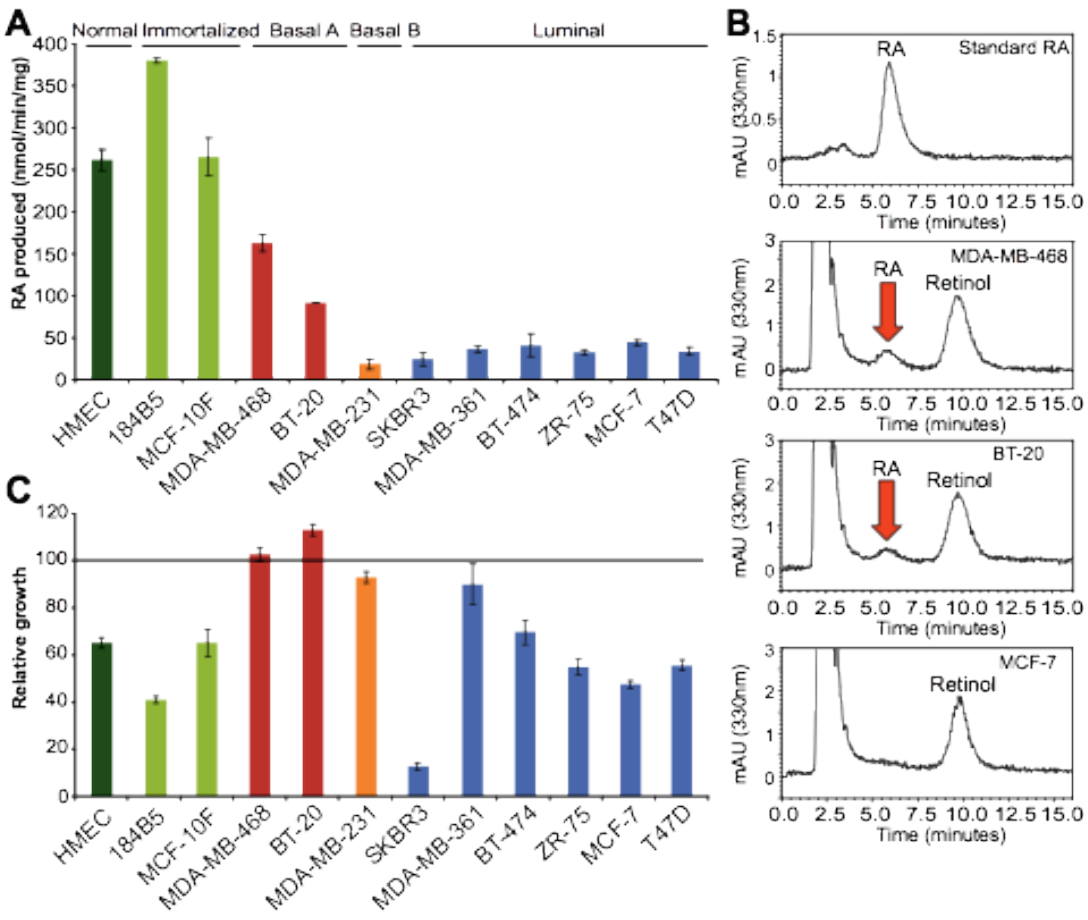

A
Normal
Immortalized
Basal A
Basal B
Luminal
RA produced (nmol/min/mg)
HMEC
184B5
MCF-10F
MDA-MB-468
BT-20
MDA-MB-231
SKBR3
MDA-MB-361
BT-474
ZR-75
MCF-7
T47D
B
Standard RA
RA
mAU (330nm)
Time (minutes)
MDA-MB-468
Retinol
BT-20
MCF-7
C
Relative growth

Figure 1

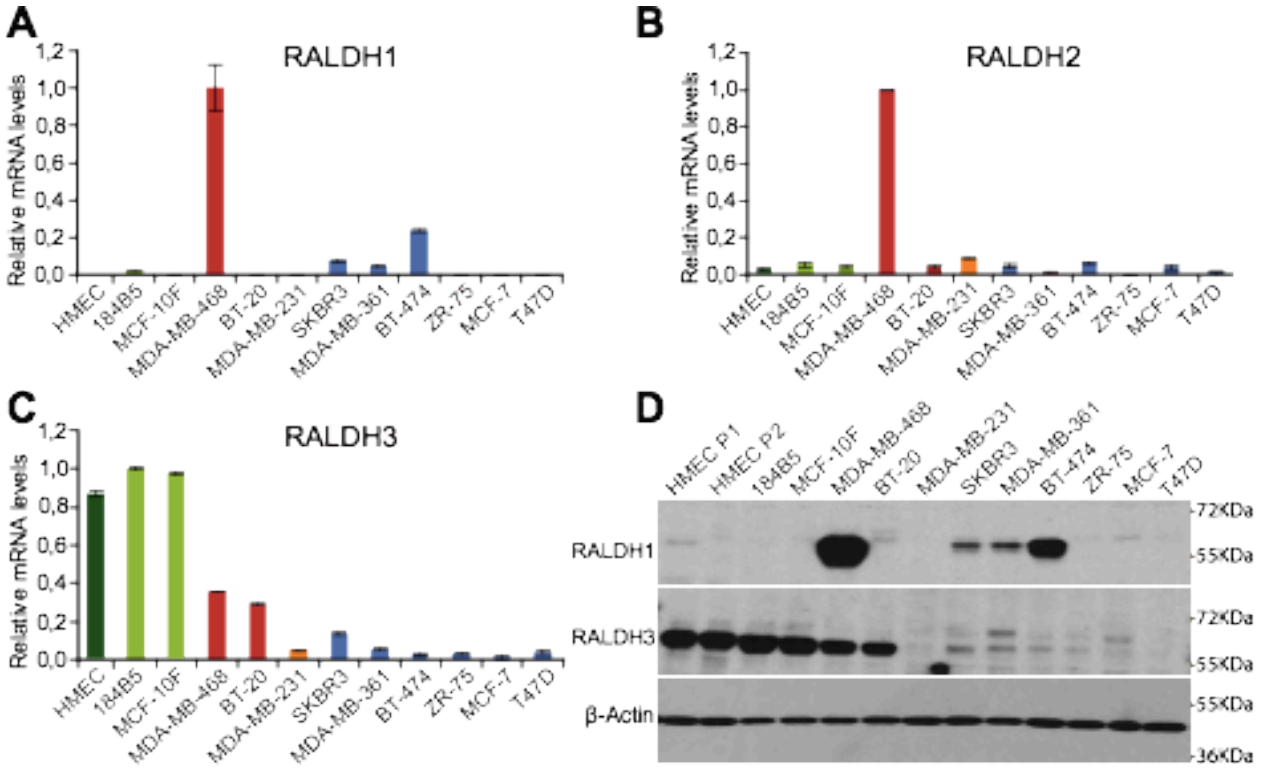

A
RALDH1
Relative mRNA levels
1,2
1,0
0,8
0,6
0,4
0,2
0,0
HMEC
184B5
MCF-10F
MDA-MB-468
BT-20
MDA-MB-231
SKBR3
MDA-MB-361
BT-474
ZR-75
MCF-7
T47D
B
RALDH2
C
RALDH3
D
HMEC P1
HMEC P2
RALDH1
RALDH3
β-Actin
72KDa
55KDa
36KDa

Figure 2

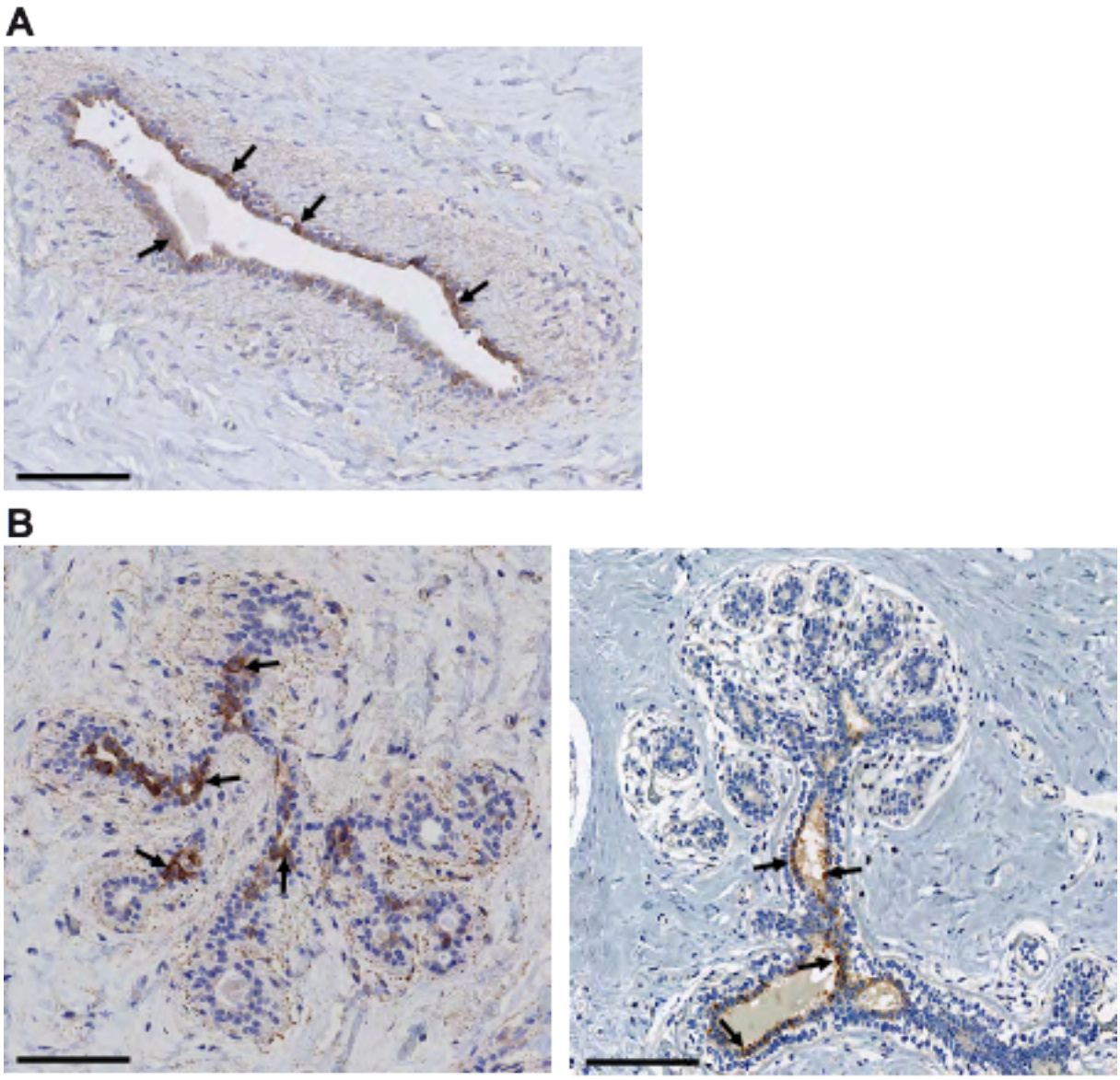
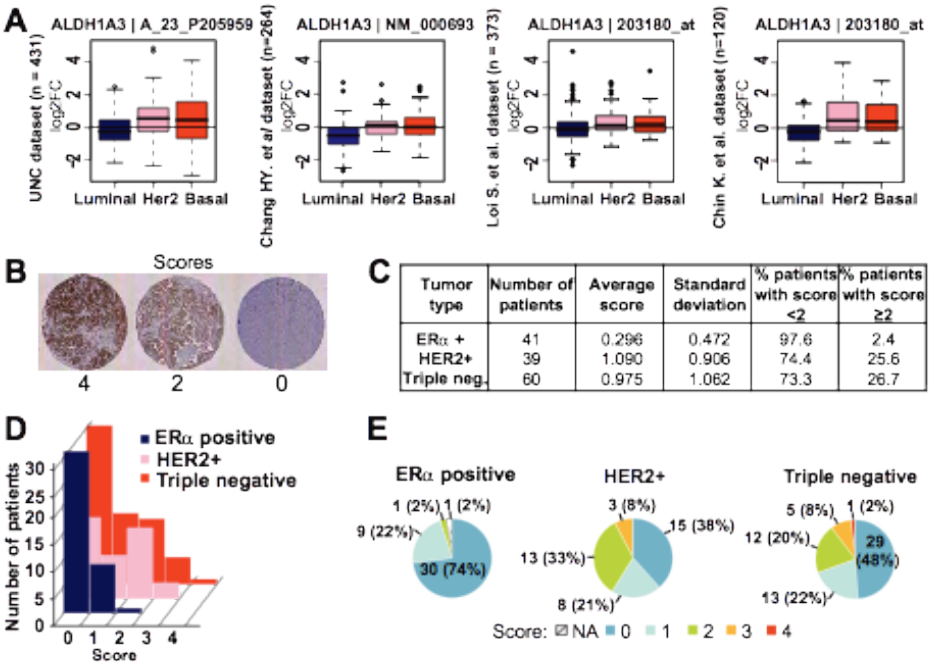


Figure 3



Tumor type	Number of patients	Average score	Standard deviation	% patients with score <2	% patients with score ≥2
ERα +	41	0.296	0.472	97.6	2.4
HER2+	39	1.090	0.906	74.4	25.6
Triple neg.	60	0.975	1.062	73.3	26.7

Figure 4

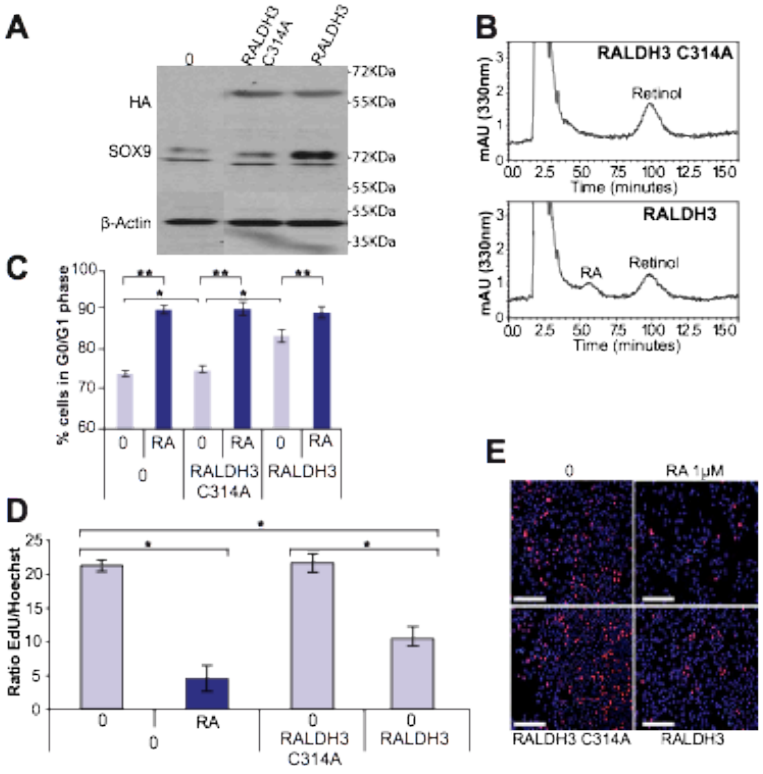

A
0
RALDH3 C314A
RALDH3
HA
SOX9
β-Actin
72KDa
55KDa
72KDa
55KDa
55KDa
35KDa
B
RALDH3 C314A
Retinol
mAU (330nm)
Time (minutes)
RALDH3
RA
Retinol
C
% cells in G0/G1 phase
0
RA
RALDH3 C314A
RALDH3
D
Ratio EdU/Hoechst
E
0
RA 1μM
RALDH3 C314A
RALDH3

Figure 5

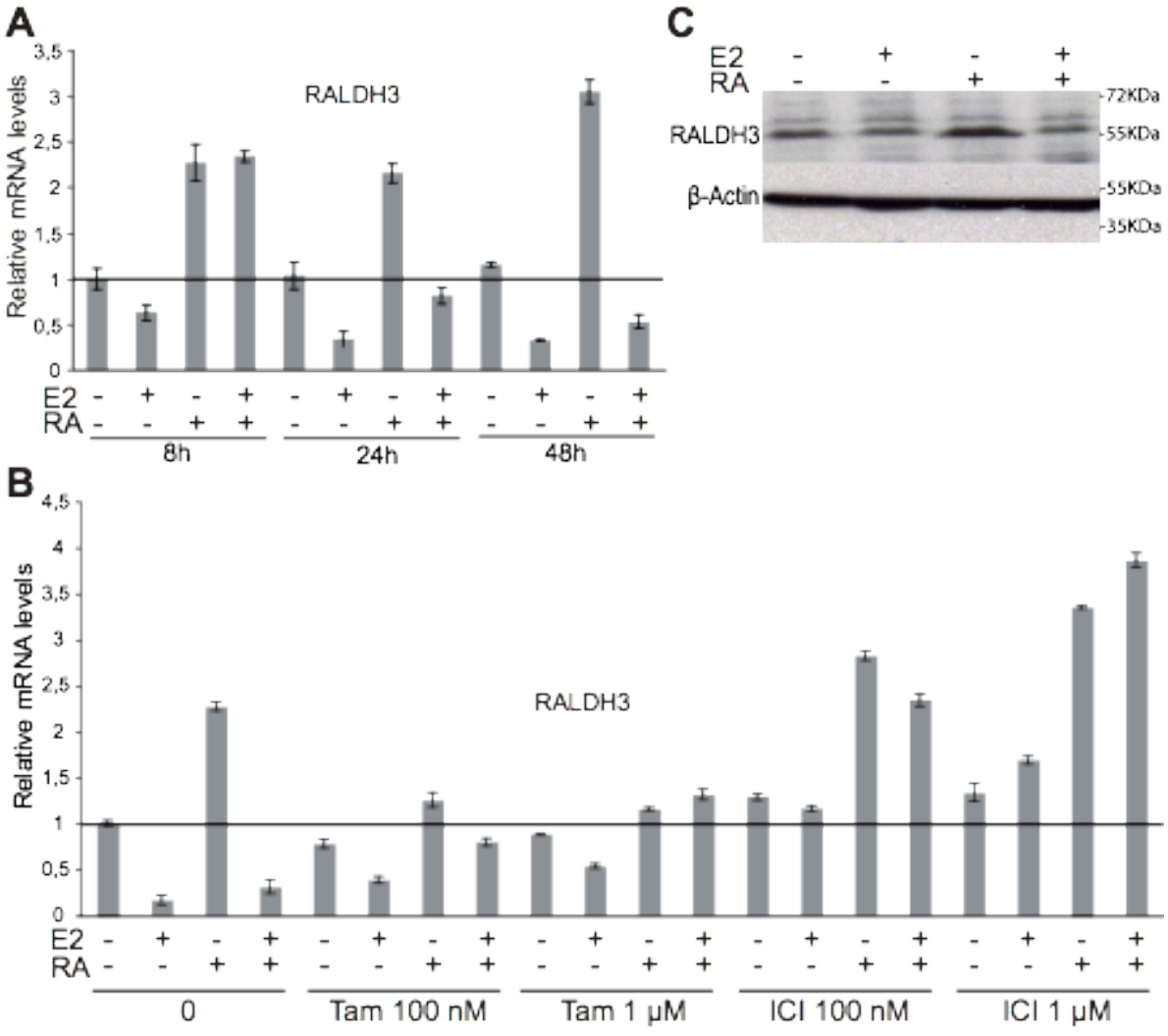

A
RALDH3
Relative mRNA levels
3,5
3
2,5
2
1,5
1
0,5
0
E2 - + - + - + - + - + - +
RA - - + + - - + + - - + +
8h
24h
48h
C
E2 - + - +
RA - - + +
RALDH3
β-Actin
72KDa
55KDa
55KDa
35KDa
B
RALDH3
Relative mRNA levels
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
E2 - + - + - + - + - + - + - + - + - + - + - + - +
RA - - + + - - + + - - + + - - + + - - + + - - + +
0
Tam 100 nM
Tam 1 μM
ICI 100 nM
ICI 1 μM

Figure 6

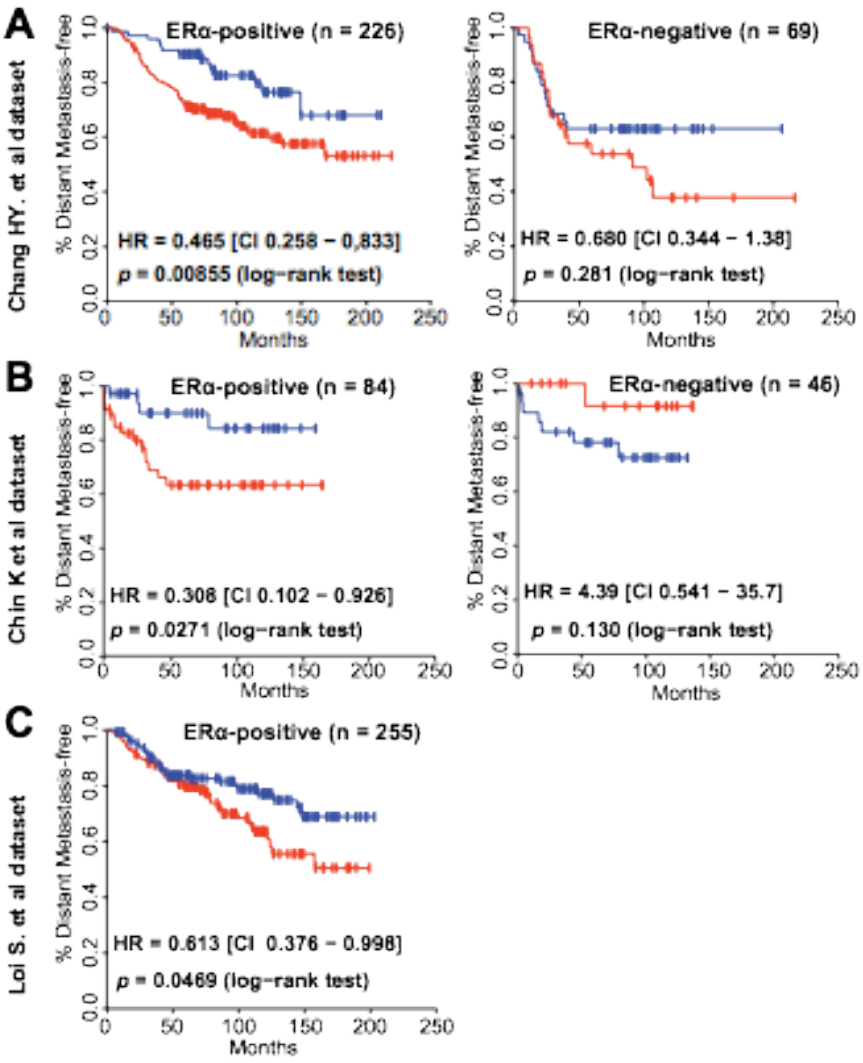

A
Chang HY. et al dataset
ERα-positive (n = 226)
HR = 0.465 [CI 0.258 – 0.833]
p = 0.00855 (log-rank test)
ERα-negative (n = 69)
HR = 0.680 [CI 0.344 – 1.38]
p = 0.281 (log-rank test)
% Distant Metastasis-free
Months
B
Chin K et al dataset
ERα-positive (n = 84)
HR = 0.308 [CI 0.102 – 0.926]
p = 0.0271 (log-rank test)
ERα-negative (n = 46)
HR = 4.39 [CI 0.541 – 35.7]
p = 0.130 (log-rank test)
C
Loi S. et al dataset
ERα-positive (n = 255)
HR = 0.613 [CI 0.376 – 0.998]
p = 0.0469 (log-rank test)

Figure 7

SUPPLEMENTAL FIGURES LEGENDS

Supplemental Figure 1. Detection of RA in medium of cultured cell lines. The retention time and absorbance spectrum in UV of a standard of pure RA was used as a reference **(A)** to identify the RA peak (red line) in medium from cell lines by reverse-phase HPLC **(B).** The peak identified by the red line presents the same retention time and has the same absorbance spectrum as the peak of pure RA.

Supplemental Figure 2. Full-length blots of figure 2

Supplemental Figure 3. Isotype controls of the antibody directed against RALDH3. Successive sections of the same region of formalin-fixed paraffin-embedded mouse **(A)** and human **(B)** mammary glands stained either with the antibody directed against RALDH3 or with the corresponding isotype (goat IgG). The brown staining (black arrows) is observed only with the antibody directed against RALDH3 but not with the isotype control. Scale bars represent 100 μm**.**

Supplemental Figure 4. RALDH3 is expressed in normal mouse luminal breast epithelium. **(A)** Expression of RALDH3 was determined by IHC using an anti-RALDH3 (as in Fig. 2) in a section mouse mammary tissue (brown stain, see arrows). Scale bars

represent 200 μm. **(B)** Expression of smooth muscle actin (SMA, red stain, see red arrows) was detected by co-staining with RALDH3 in the mammary tissue of mice (RALDH3: brown stain, see black arrows). Scale bars represent 100 μm.

Supplemental Figure 5. Selectivity of antibodies against RALDH3 under IHC conditions. HeLa cells were transfected with plasmids expressing human RALDH1, RALDH2, RALDH3 or with the parental vector (0). Formalin-fixed paraffin-embedded pellets of transfected cells were stained with the anti-RALDH1 or anti-RALDH3 Ab as in figure 3, demonstrating the specificity (stained cells appear in dark brown, see arrows for examples). Scale bars represent 100 μm.

Supplemental Figure 6. Absence of RALDH3 expression in an ERα-positive but not ERα-negative tumor. **(A)** RALDH3 and ERα levels were detected by IHC in 2 consecutive sections of an ERα-positive tumor sample using an anti-RALDH3 Ab (brown stain, right panel, see black arrows for examples) or an anti-ERα mAb (brown stain, left panel, see red arrows for regions of RALDH3 expression). Two different regions are shown in a synchronized view of the 2 stained sections. **(B)** Expression of RALDH3 and ERα were detected by costaining of a single section of an ERα-positive tumor sample using an anti-RALDH3 mAb (brown stain, see arrows for examples) or an anti-ERα mAb (red stain). **(C)** Expression of RALDH3 was detected by IHC in an ERα-negative tumor sample using an

anti-RALDH3 mAb (brown stain, see arrows for examples). Scale bars represent 100 μm **(A-B)** and 200 μm **(C).**

Supplemental Figure 7. Full-length blots of figure 5

Supplemental figure 8. RALDH3 expression levels in transfected SKBR3 and ZR-75 cells are comparable to endogenous levels in immortalized non-tumorigenic 184B5 cells. SKBR3 and ZR-75 cells were transfected as for FACS analysis. RALDH3 expression in transiently transfected SKBR3, ZR-75 and in non-tumorigenic 184B5 cells was compared by western blot analysis using the anti-RALDH3 mAb as in Figure 2D. Note the presence of a faster migrating band in SKBR3 cells transfected with WT RALDH3, corresponding to endogenous RALDH3.

Supplemental Figure 9. RALDH3 expression induces the expression of known RA target genes. **(A)** Q-PCR analysis of 4 known RA target genes (SOX9, CYP26A1, FOXA1 and RARβ2) in SKBR3 cells transiently expressing similar levels of HA-tagged RALDH3 or its catalytically-inactive mutant. **(B)** Western blot analysis of the expression of HA-tagged RALDHs and of the RA target gene SOX9 in transfected SKBR3 cells.

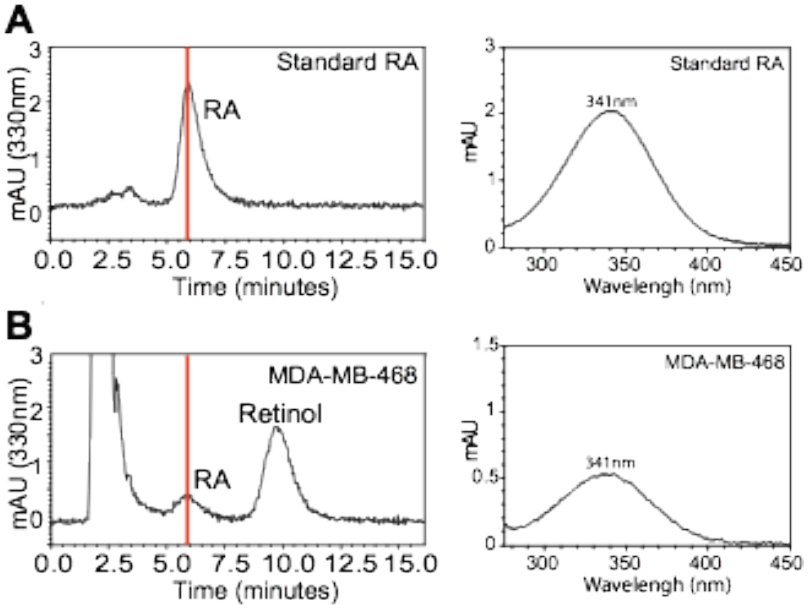
Supplemental Figure 10. RALDH3 restores RA synthesis and increases the G0/G1 fraction in ZR-75 cells. **(A)** Western analysis of HA-tagged RALDH3 and SOX9 expression in transiently transfected ZR-75 cells, in the absence or presence of added RA (1 μM), Retinol (Rol 100 nM) or vehicle. SOX9 levels are induced by RA or RALDH3 expression, while the catalytically inactive mutant does not significantly modulate SOX9 expression. **(B)** G0/G1 fraction in parental or transiently transfected ZR-75 cells expressing RALDH3 or its catalytically inactive mutant in the absence or presence of retinol or RA, as determined by FACS analysis. Error bars represent the SEM of measurment on three independent experiments. (*p ≤0.05, **p≤0.005, ***p≤0.001, two-sample t-test).

Supplemental figure 11. Expression of RALDH3, but not of the catalytically inactive RALDH3 mutant C314A, suppresses colony formation in MCF-7. **(A)** Colony formation assay with MCF-7. Cells were infected with a retroviral vector (pMSCV-CMV) expressing RALDH3, RALDH3 C314A or the parental vector and were selected with puromycin in serum-free medium. Cells were seeded at low density (2,000 cells per well) and colonies were grown with Retinol (Rol, 100 nM) or RA (100 nM) and stained with crystal violet after 10 days. Treated media were renewed every 2 d. **(B)** Representative immunoblot showing expression levels of WT and mutant RALDH3 in transduced MCF-7 cells.

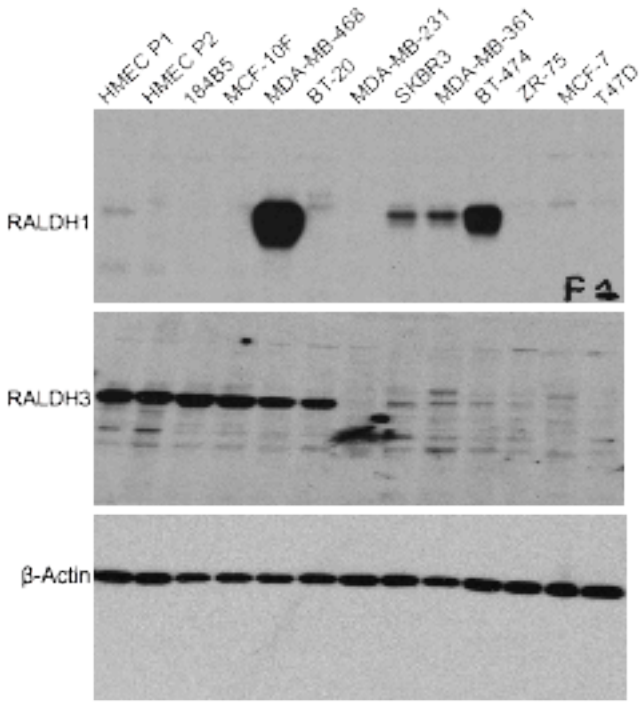
Supplemental figure 12. Full-length blots of figure 6

Supplemental Figure 13. RALDH3 expression is induced by retinoids and repressed by E2 in luminal cells. **(A)** Quantitative PCR analysis of RALDH3 expression in MCF-7 and T47D luminal cells treated for 72h with retinoic acid (RA, 100 nM), estradiol (E2, 25 nM), or both. Treatments and medium were replaced every day. **(B)** Quantitative PCR analysis of RALDH3 and SOX9 expression in MCF-7 treated for 6 days with increasing concentrations of retinol. Error bars represent the SEM of triplicate measurements.

Supplemental Figure 14. Tissue culture conditions of cell lines used in this study.



Supplemental Figure 1



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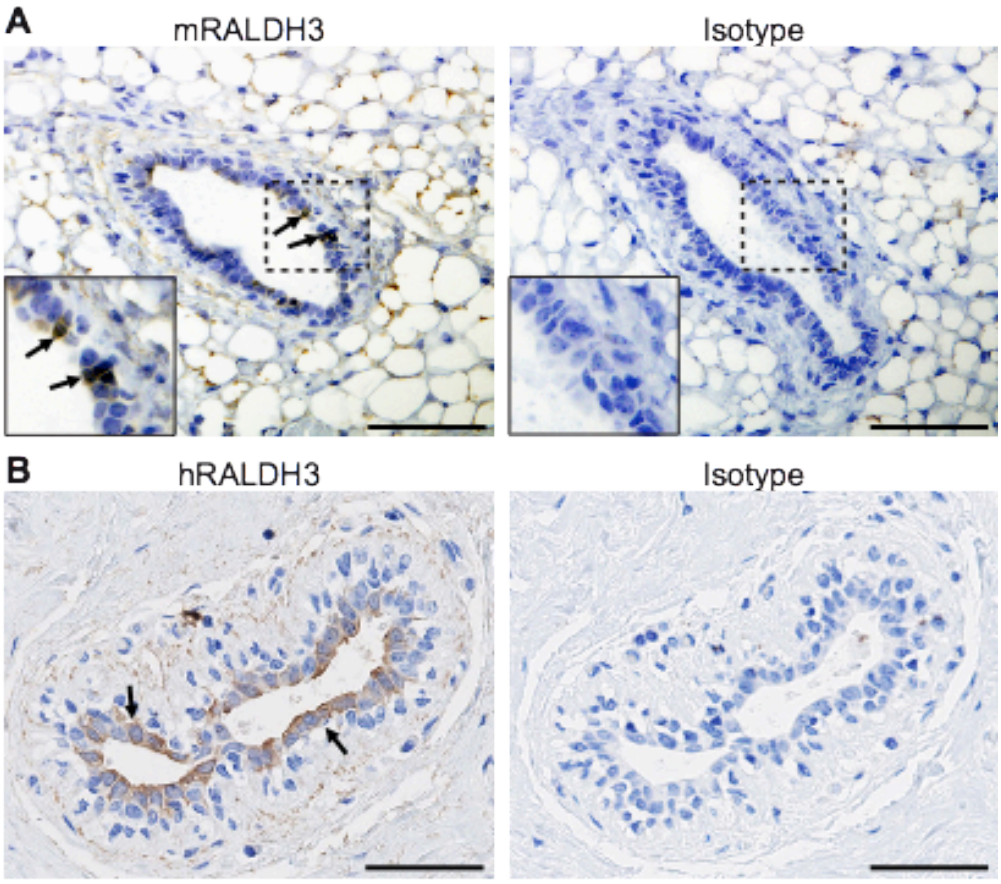

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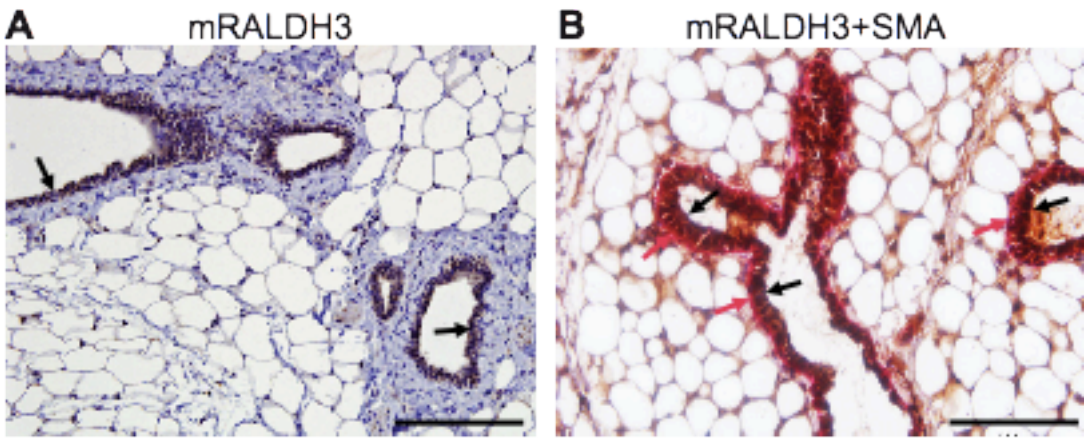

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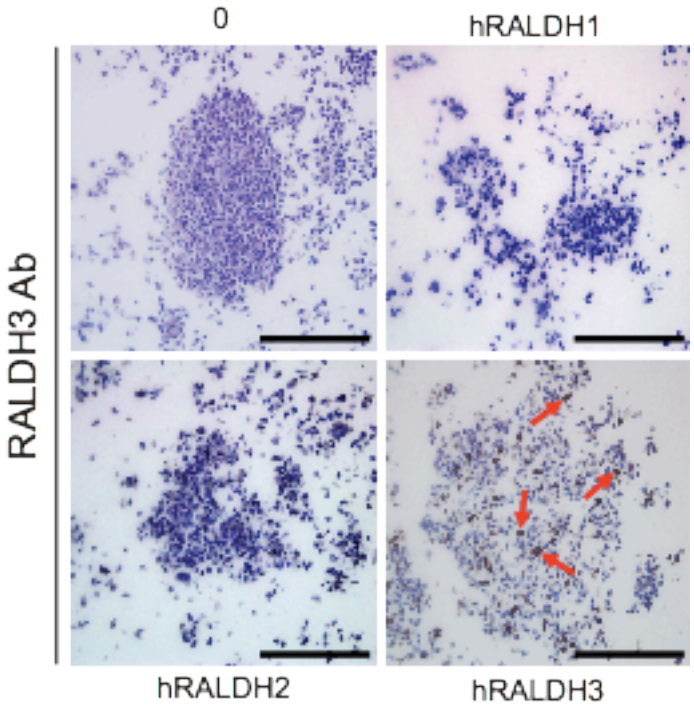
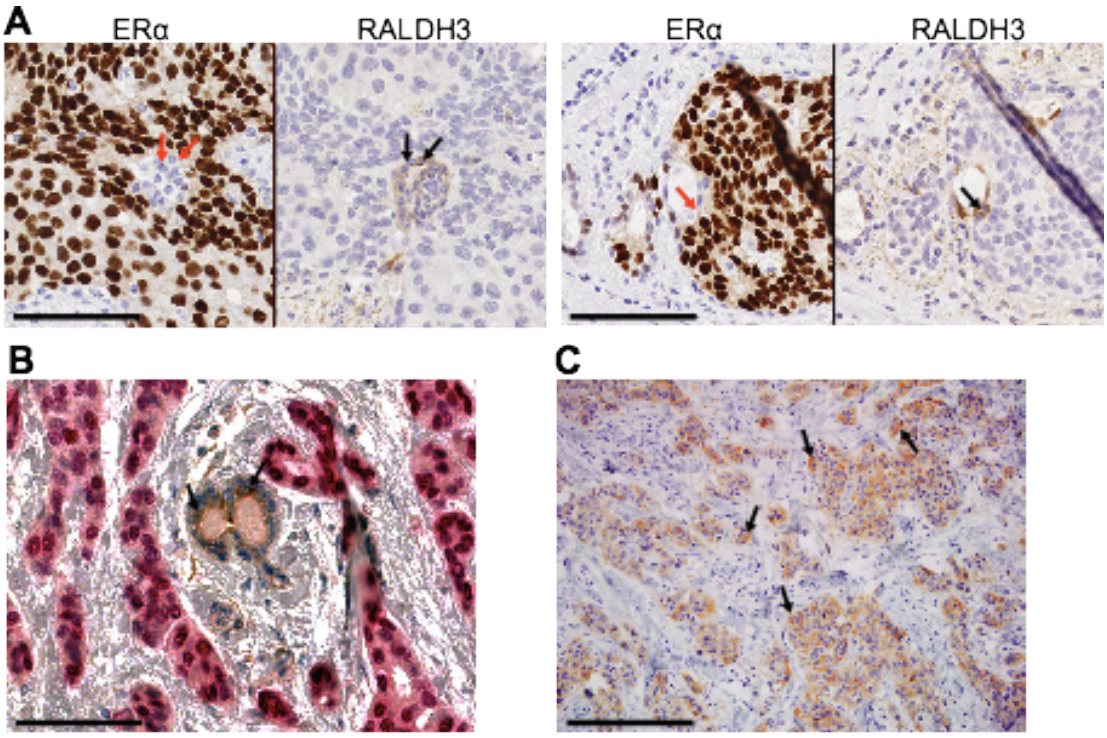
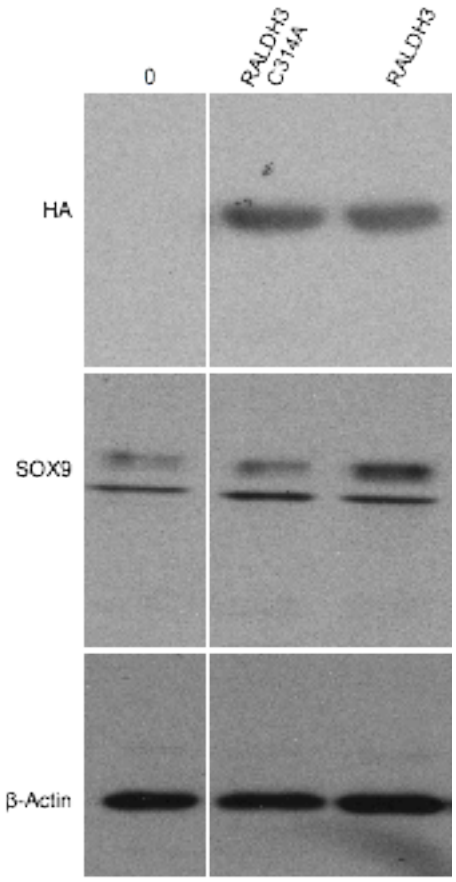


Supplemental Figure 5



Supplemental Figure 6



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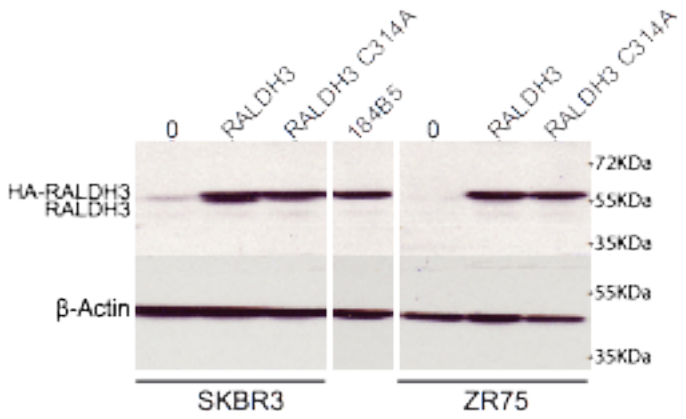

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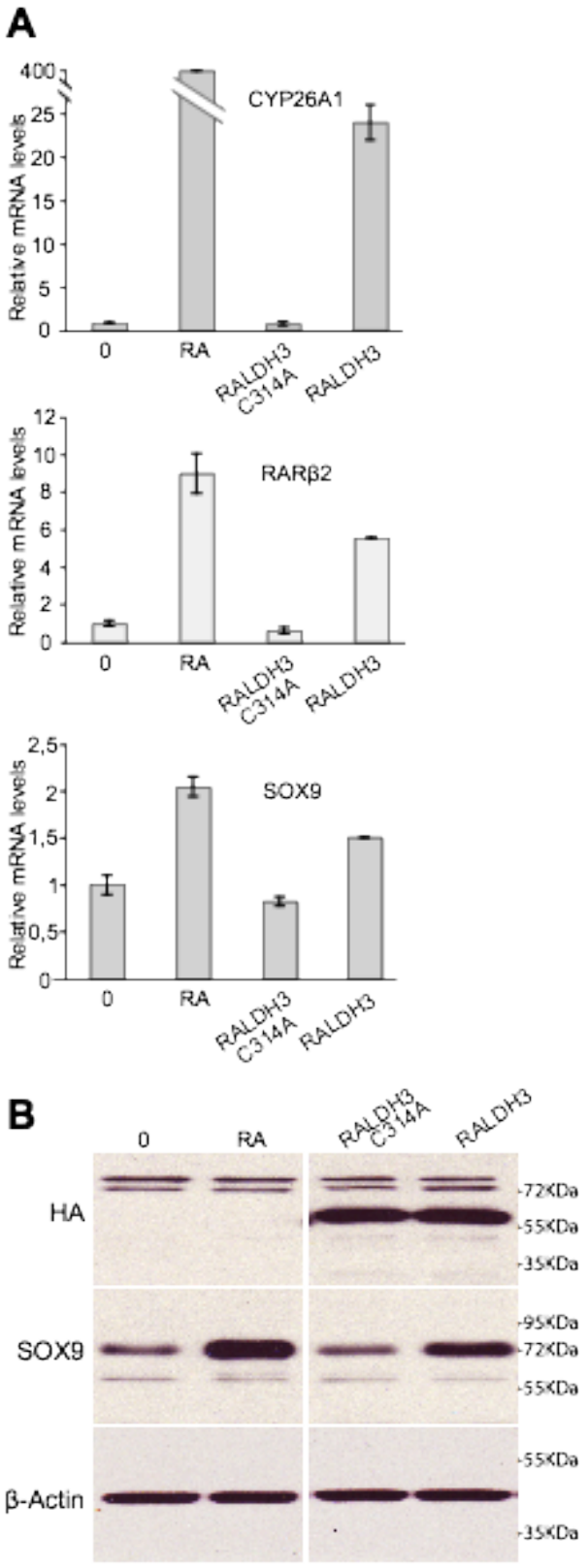

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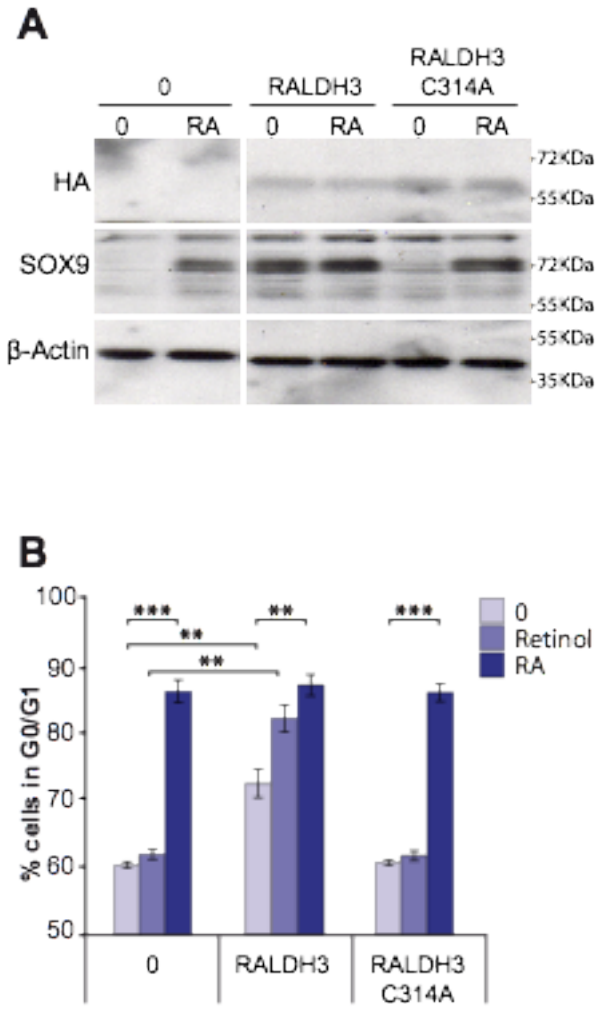

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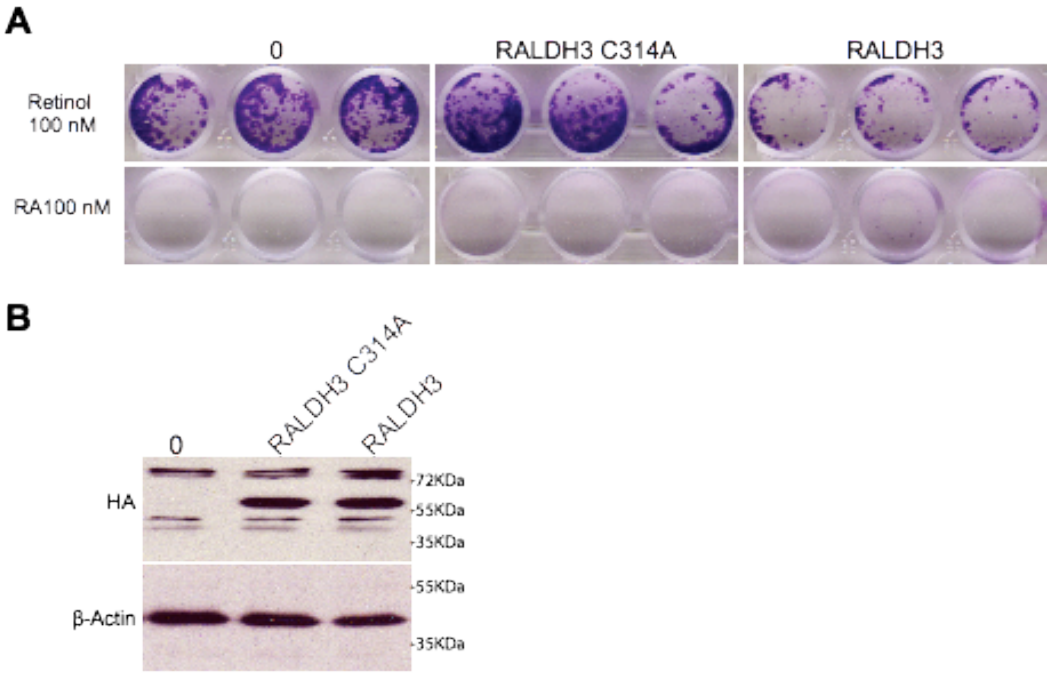
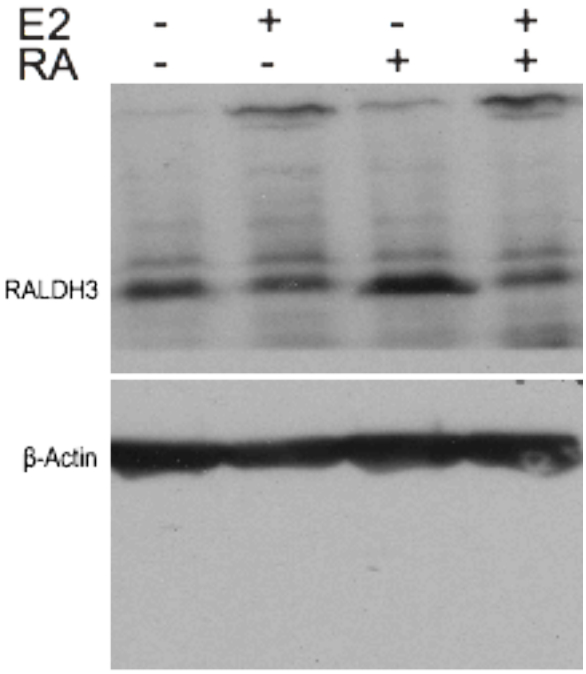
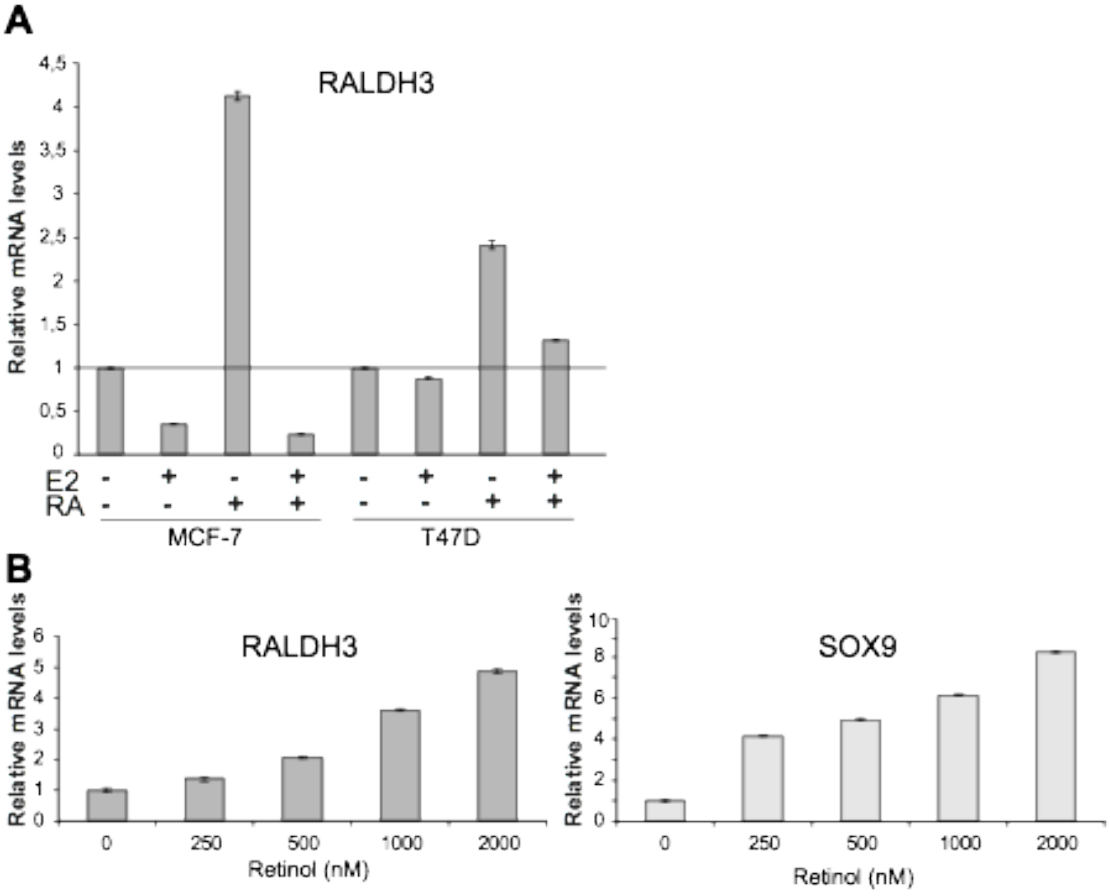


Supplemental Figure 11



Supplemental Figure 12



Supplemental Figure 13

Cell line	Medium	Serum (FBS) concentration	Pen/strep concentration	Supplements
HMEC	MEGM	/	/	
184B5	DMEM/F12	10 %	1 %	0.5 µg/ml hydrocortisone 10 µg/ml insulin 1 ng/ml Cholera toxin
MCF-10F	DMEM/F12	10 %	1 %	0.5 µg/ml hydrocortisone 10 µg/ml insulin 100 ng/ml Cholera toxin
MDA-MB-468	L-15	10 %	1 %	/
BT-20	α-MEM	10 %	1 %	1% L-glutamine
MDA-MB-231	DMEM	5 %	1 %	/
SKBR3	DMEM	10 %	1 %	/
MDA-MB-361	L-15	20 %	1 %	/
BT-474	DMEM	10 %	1 %	/
ZR-75	DMEM	10 %	1 %	/
MCF-7	DMEM	10 %	1 %	/
T47D	DMEM	10 %	1 %	/